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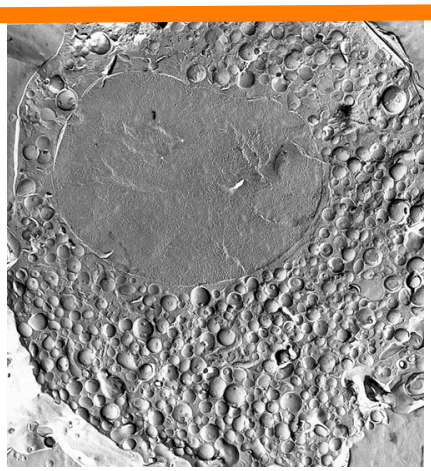
Cell Biology of the Chromaffin Cell



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Cell Biology of the Chromaffin Cell

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Preface.

In 1982, a Spanish Mediterranean island, Ibiza, hosted the First International Symposium on Chromaffin Cell Biology (ISCCB-1; organizers: A.G. García and V. Ceña). This meeting was followed by others in Colmar, France (D. Aunis), Coolfont, USA (H. Pollard and P.F. Fleming), Alice Spring, Australia (B. Livett and P. Marley), Jerusalem, Israel (K. Rosenheck and O. Zinder), Marburg, Germany (K. Usinker and M. Gratzl), Ottawa, Canada (J.M. Trifaró), Eddinburg, UK (J. Phillips and J.P. Apps), Sapporo, Japan (T. Kanno, Y, Nakazato and K. Kumakura), Bergen, Norway (K.B. Helle, T. Flatmark and G. Serk-Hanssen), San Diego, USA (D. O'Connor and S. Mahata). We closed this long tour in another Spanish island, this time on the Atlantic Ocean, La Palma. Ricardo Borges and his co-workers organised the ISCCB-12 in a magnificent atmosphere, both from the scientific as well as the social points of view.

The philosophy of these meetings is to facilitate the exchange and dissemination of ideas in the field of calcium signalling, exocytosis and cell communication, to favour friendship and to promote collaborations between different laboratories. Although it was agreed that books would not arise from these meetings, two books were edited from the meetings of Sapporo and San Diego. The third book comes to light now from the meeting held in La Palma, on September 2003.

This book contains many of the communications presented at the ISCCB-12 on calcium signalling, chromogranins, chromaffin vesicles, vesicle movements, the proteins of exocytosis, the fusion pore, novel methodologies and techniques. Ricardo Borges has managed to assemble this frontier information in a book that will interest not only the 150 participants at La Palma meeting, but also to many scientist devoted to the field of stimulus secretion coupling processes. Enjoy it.

Antonio G. García, Universidad Autónoma de Madrid, Spain

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Modulation of exocytosis by exogenous and endogenous factors.

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Intercellular communication is initiated by the release of neurotransmitters from the presynaptic nerve cell, i.e. exocytosis. Therefore, the strength of the presynaptic signal is determined by the type of neurotransmitter released, the number of neurotransmitter-containing vesicles that fuse and the amount of neurotransmitter released per vesicle. Consequently, modulation of the presynaptic signal plays a crucial role in neurotransmission and, therefore, is of vital importance.

Exocytosis is part of a complex process, in which Ca^{2+} plays a key role, consisting of vesicle docking and priming, formation of a fusion pore, fusion of vesicle and plasma membrane, and subsequent release of vesicle contents. As a result, a variety of cytoplasmic- and membrane-bound proteins is involved in the regulation of specific steps of the vesicle cycle. Thus, modulation of the presynaptic signal may occur at several levels. In the Exocytosis W&BC, held on September 21, 2003, several of these levels were a matter of debate. This summing-up is intended to briefly review these levels of modulation, i.e. proteins involved in the modulation of exocytosis, the regulation of Ca^{2+}-signals in the modulation of exocytosis and some more general matters, regarding contaminations, constructs of fluorescent proteins, analysis of amperometric signals, and different modes of exocytosis.

Proteins involved in the modulation of exocytosis. Due to the sophisticated nature of exocytosis, a great number of proteins is involved in its regulation. However, the basic fusion event itself is based on the interaction between three highly-conserved proteins as discussed by Colin Rickman (*Target SNARE dimers in chromaffin cells* by *C Rickman, F Meunier and B Davletov;* crickman@mrc-lmb.cam.ac.uk). Catecholamine release from chromaffin cells requires the regulated fusion of cargo containing granules with the plasma membrane. This exocytotic process is driven by three SNARE proteins; syntaxin and SNAP-25 on the plasma membrane and synaptobrevin on the vesicular membrane. These three proteins form an extremely stable trimeric complex composed of four α-helices. The formation of this stable complex is believed to provide the energy to drive merger of the two membranes.

The molecular assembly pathway that leads to formation of this fusogenic complex and the distribution of these SNAREs in resting chromaffin cells have been examined biochemically. Using brain-purified full length SNAREs it was found that, of all theoretically possible binary SNARE combinations, only the syntaxin/SNAP-25 heterodimer (target SNARE dimer) was able to form. The complex produced by these two SNAREs is equimolar in stoichiometry and forms with a high affinity. Given that these two proteins are present on the same cellular membrane, this affinity would suggest they could readily associate in the cell.

Using immunocytochemistry it was shown that, in bovine chromaffin cells, endogenous syntaxin and SNAP-25 exhibit punctate staining on the plasma membrane in equatorial confocal sections. In polar sections these clusters are defined, show almost perfect coincidence of the two SNAREs and occupy approximately 10% of the total plasma membrane surface area. Staining for the vesicular SNARE, synaptobrevin, demonstrated only a very small degree of colocalisation with the syntaxin/SNAP-25 clusters. Therefore, it was concluded that the plasma membrane SNARE clusters represent areas of pre-assembled syntaxin/SNAP-25 heterodimers and likely define sites of release.

Besides the basis of exocytosis, which is formed by the SNARE proteins, numerous other proteins are involved in exocytosis and preceding steps. The exocytosis of a single vesicle is thought to be mediated by a sequence of interactions between cytosolic, vesicular and plasma membrane proteins. The availability of an accurate kinetic model would be advantageous for investigating the dynamics of protein-protein interactions in relation to exocytosis, as discussed by Uri Ashery (*The molecules that drive vesicle fusion* by *A Mezer, E Nachliel, M Gutman and U Ashery;* uria@post.tau.ac.il). In the last decades, the functions of specific proteins in this process have been intensively studied. However, the precise sequence of protein-protein interactions that drive the exocytotic process is still a matter of debate and a comprehensive description of protein-protein interaction, based on chemical kinetics had not been attempted.

To pinpoint this issue, the reactions between the synaptic proteins were transformed into a set of coupled, non-linear ordinary

differential equations where the rate constants and some of the proteins concentrations are adjustable parameters. Recent studies provided some of these parameters, while a search in the parameters space for the others led to accurate reconstruction of the basic Ca^{2+}-driven vesicular fusion. In addition, using the model it was possible to reconstruct complex experimental protocols as a sequence of stimulations and munc13 overexpression. Moreover, the model can also give an indication of the dynamic changes of different intermediate complexes during exocytosis.

For example, it allows for identification of the empirically defined 'Readily Releasable' and the 'Slowly Releasable' pools of vesicles with precise synaptic protein complexes formed during the maturation process. The model allows postulation and prediction of different scenarios regarding deletion and manipulation of some proteins involved in the exocytosis process. The strength of the presented model is that it can easily implement another intermediate step according to newly available data about the concentration of specific protein or information about binding kinetics. Thus, this is the first time a comprehensive kinetic model is able to describe the dynamics of interaction between key synaptic proteins that are associated with exocytosis.

One of many classes of proteins involved in the regulation of neurotransmitter release is formed by Rho proteins (Rho, Rac, Cdc42). Rho proteins are widely expressed monomeric GTPases, which cycle between a soluble, GDP-bound inactive state and a membrane-associated GTP-bound state that stimulates downstream effectors. Glucosylation of Rac GTPase by Lethal Toxin (LT) from *Clostridium sordellii* inhibits neurotransmitter release (Doussau et al., J. Biol. Chem. (2000) 275:7764-70), possibly by decreasing the number of release sites competent for fusion (Humeau et al., J. Neurosci. (2002) 22:7968-81). Furthermore, phosphatidic acid produced by phospholipase D (PLD), as a result of signaling activity, is thought to play a role in membrane vesicle trafficking. PLD is one of the multiple Rac effectors that might be silenced following LT action. This possibility, including the role of PLD in neurotransmitter release, was discussed by Frédéric Doussau *(A role for phospholipase D1 in neurotransmitter release* by *F Doussau, Y Humeau, N Vitale, S*

Chasserot-Golaz, J-L Dupont, G Du, MA Frohman, M-F Bader and B Poulain; doussau@neurochem.u-strasbg.fr).

Using purified rat brain synaptosomes, it was shown that PLD1 is associated with the particulate fraction containing the plasma membrane. Additional immunostaining of cultured rat cerebellar granule cells showed localization of PLD1 at the neuronal plasma membrane in zones specialized for neurotransmitter release and exocytosis (axonal neurites, varicosities, growth cone-like structures). To determine the potential involvement of PLD1 in neurotransmitter release, recombinant catalytically-inactive PLD1 (K898R; 50 nM, intrasomatic) was microinjected into *Aplysia* neurons. Using conventional electrophysiological recordings to study the effects of this manipulation on evoked acetylcholine release, it was shown that PLD1 (K898R) produced a fast and potent dose-dependent inhibition of ACh release, whereas paired-pulse facilitation and the time course of postsynaptic responses evoked by high frequency stimulations was not altered. This finding suggests that the inhibition of exocytosis caused by PLD1 (K898R) is not the result of an alteration in stimulus-secretion coupling or in vesicular trafficking. Analysis of the fluctuations in amplitude of the postsynaptic responses revealed that the PLD1 (K898R) blocked ACh release by reducing the number of active presynaptic releasing sites without effects on quantal size or output probability.

Thus, these results are the first demonstration of a role of PLD1 in a late stage of Ca^{2+}-dependent synaptic vesicle exocytosis at nerve terminals (Humeau et al., (2001) Proc. Natl. Acad. Sci. USA 98:15300-05). This extends the idea that membrane lipid constituents are essential partners for proteins in the control of vesicular membrane trafficking events. Additionally, these findings reinforce the concept that lipid cones such as phosphatidic acid, by locally remodeling membrane curvature and fluidity, mark sites of fusion.

The phosphorylation state of exocytotic proteins, which is modulated by a tight interplay of kinases and phosphatases, modulates exocytosis to a large extent. The mechanisms used by receptors which couple to PKC to facilitate exocytosis has recently become an issue of debate following the discovery of another diacylglycerol-regulated

protein, Munc13, as discussed by Elizabeth Seward (*Munc-ing around with PKC* by *Elizabeth Seward*; e.p.seward@sheffield.ac.uk).

Previous studies have suggested that PKC may facilitate exocytosis by (1) facilitating transfer of vesicles from the reserve pool to the readily releasable pool through phosphorylation of MARCKS and changes in the cytoskeleton, (2) it may speed up vesicle recruitment by phosphorylation of SNAP-25, and/or (3) it may promote vesicle docking by phosphorylation of Munc-18. In all three scenarios, an increase in vesicle dynamics is predicted, if it is assumed that the reserve pool and releasable pool(s) are morphologically distinct. Munc13 on the other hand, is thought to act by priming already docked vesicles, and therefore no such increase in vesicle dynamics is expected. To examine in more detail the molecular mechanisms used by agonists at Gq-protein coupled receptors to facilitate exocytosis, a combination of membrane capacitance measurements to measure pool size and total internal reflection fluorescence microscopy to monitor vesicle dynamics in patch-clamped chromaffin cells has been used.

Preliminary data discussed at the meeting showed that while agonists at these receptors clearly increased the size of the RRP, this did not appear to be accompanied by an increase in the number of morphologically docked vesicles, at least under the recording conditions used. Clearly this favours the view that agonist activation of the diacylglycerol-regulated signalling cascade increases vesicle priming rather than docking, more experiments are however, required to identify the proteins involved.

The regulation of Ca^{2+}-signals in the modulation of exocytosis. Calcium plays a key role in exocytosis. Calcium not only triggers the actual exocytotic event, it is involved a large number of regulatory cascades as well. Therefore, the influx of Ca^{2+} through a variety of calcium channels is of great importance for the modulation of exocytosis. The significance of L, N, and P/Q-type Ca^{2+}-channels is well recognized. However, T-type Ca^{2+}-channels are involved in the modulation of exocytosis as well, as discussed by Emilio Carbone (*T-type Ca_V3 channel coupling to exocytosis in rat chromaffin cells* by *A*

Giancippoli, V Carabelli, M Novara, P Baldelli, D Cavallari and E Carbone; emilio.carbone@unito.it).

Rat chromaffin cells (RCCs) express marked densities of low-voltage-activated Ca^{2+} channels when incubated for 3-4 days in solutions containing the membrane permeable cAMP-analogue, pCPT-cAMP (200 μM). The cAMP-recruited channels are readily identifiable as T-type on the basis of their biophysical and pharmacological characteristics: 1) availability from –40mV in 5mM Ca^{2+}, 2) steep voltage-dependent activation, 3) slow deactivation but fast and complete inactivation, 4) full inactivation following short conditioning pre-pulses to –30mV, 4) large block of Ca^{2+}-influx with 50 μM Ni^{2+}, 5) comparable permeability to Ca^{2+} and Ba^{2+}, 6) sensitivity to high doses of nifedipine (≥ 10 μM) but insensitivity to ω-CTx-GVIA (3.2 μM), ω-Aga-IVA (2 μM) and SNX-482 (1 μM).

The extent to which these cAMP-recruited T-channels contribute to the secretory response of RCCs was investigated. Exocytosis has been measured as cell capacitance increases (ΔC) during 100 ms pulses depolarizations (–50 to +40mV) (Carabelli et al., (2003) Biophys. J. 85:1326-37), in the presence of nifedipine (1-5 μM), ω-CTx-GVIA (3.2 μM), ω-Aga-IVA (2 μM) and in Na^{+}-free external solutions in order to minimize the contribution of L, N, P/Q Ca^{2+} channels and Na^{+} channels, respectively. It was shown that compared with control RCCs in which secretion was uniquely ascribed to R-channels, the prolonged exposure to cAMP potentiated the exocytosis by 110%. Furthermore, measuring the ratio between mean ΔC (≈10fF) and mean charge density (≈0.15pC/pF) at –20 mV, it was found that cAMP-recruited T-channels contributed to secretion with a 3-fold higher degree of efficacy compared with the high-threshold ones expressed in control RCCs (50fF for a charge density of 2pC/pF at +10mV). This implies that low-threshold channels may play a critical role to the secretory activity of RCCs during sustained sympathetic stimulation, which is capable of inducing high levels of cAMP through the autocrine activation of β-adrenergic receptors (Cesetti et al., (2003) J. Neurosci. 23:73-83).

Influx of Ca^{2+} through calcium channels is only one of several processes shaping the intracellular calcium signal. Since intracellular

calcium signals are responsible for initiating a spectrum of physiological responses, calcium signals have to be tightly regulated, both temporal and spatial. Calcium binding proteins (CaBPs) are critically involved in this regulation as discussed for caldendrins by Robert Burgoyne (*Calcium Binding Protein 1 is an inhibitor of agonist-evoked, inositol 1,4,5-trisphophate-mediated calcium signalling* by *LP Haynes, AV Tepikin and RD Burgoyne;* burgoyne@liverpool.ac.uk). The caldendrins represent mammalian-specific members of the CaM superfamily. CaBPs display a restricted pattern of expression in neuronal/retinal tissues suggesting a specialised role in Ca^{2+} signalling in these cell types. Recently it was reported that a splice variant of CaBP1 functionally interacts with inositol 1, 4, 5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) to elicit channel activation in the absence of $InsP_3$ (Yang et al., (2002) Proc. Natl. Acad. Sci. USA 99:7711-16). These data indicate a new mode of $InsP_3R$ modulation and hence control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neuronal tissues.

Burgoyne et al analysed the biochemistry of the long form splice variant of CaBP1 (L-CaBP1) and show that, *in vitro*, a recombinant form of the protein is able to bind Ca^{2+} with high affinity and undergoes a conformational change. Additionally, the localisation of endogenous and overexpressed L-CaBP1 in the model neuroendocrine, PC12, cell system was described. In PC12 cells, L-CaBP1 was associated with the plasma membrane and Golgi complex in a myristoylation-dependent manner. Furthermore, it was shown that overexpressed L-CaBP1 is able to substantially suppress rises in $[Ca^{2+}]_i$ in response to physiological agonists acting on purinergic receptors and that this inhibition is due in large part to blockade of release from intracellular Ca^{2+} stores. The related protein Neuronal Calcium Sensor-1 was without effect on the $[Ca^{2+}]_i$ responses to agonist stimulation. Measurement of $[Ca^{2+}]$ within the ER of permeabilised PC12 cells demonstrated that L-CaBP1 directly inhibited $InsP_3$-mediated Ca^{2+} release. Expression of L-CaBP1 also inhibited histamine-induced $[Ca^{2+}]_i$ oscillations in HeLa cells. Together these data suggest that L-CaBP1 is able to specifically regulate IP_3R-mediated alterations in $[Ca^{2+}]_i$ during agonist stimulation, which could greatly alter exocytosis and its time-course.

Additional factors affecting exocytosis: fluctuating fusion pores, contaminations, analysis of amperometric signals and new journals. The major determinants of presynaptic neurotransmission are the number of exocytotic events and the amount of neurotransmitter released per exocytotic event. Both could be affected by a single process, i.e. fluctuation of the fusion pore, as discussed by Roland Staal (*Dopamine neurons release transmitter via a fluctuating fusion pore* by *RGW Staal, E Mosharov and D Sulzer;* rs1215@columbia.edu). Vesicles in neuronal and neuroendocrine cells can release neurotransmitter via full fusion, which starts with the formation of a fusion pore that ultimately undergoes radial expansion until the vesicle collapses into the plasma membrane. Additionally, it has been demonstrated that vesicles in neuroendocrine cells can undergo transient fusion or kiss-and-run exocytosis. Transient fusion events occur when the fusion pore closes instead of expanding and collapsing into the plasma membrane, thereby potentially limiting neurotransmitter release. Based on this, two key goals for understanding neurotransmission were suggested, i.e. determination of whether neuronal small synaptic vesicles (SSVs) can release neurotransmitter via transient fusion events in addition to full fusion and determination of how these modes of exocytosis are regulated.

This was examined using carbon fiber amperometry to measure neurotransmitter release from dopamine neurons. It was found that ~20% of quantal events recorded from dopamine neurons did not possess the "simple" amperometric spike shape usually seen with single vesicle exocytosis in other cell types. Rather, a "complex" spike shape with multiple peaks was regularly observed. These multiple peaks sequentially decreased in amplitude, suggesting that neurotransmitter was being released from SSVs via the high frequency (~4 kHz) flickering of a small diameter (~2 nm) fusion pore. The quantal sizes of simple events were significantly smaller than those of complex events, suggesting that simple events resulted from transient fusion events. The incidence of complex amperometric spikes was enhanced by staurosporine and reduced by phorbol-12,13-dibutyrate (PDBU), demonstrating that this mechanism might be regulated by a protein kinase.

Although full fusion is believed to be the dominant form of exocytosis in neuroendocrine chromaffin and PC12 cells, SSVs in dopamine neurons appear to be able to release neurotransmitter through a rapidly flickering fusion pore. Neurotransmitter release through this rapidly flickering fusion pore might represent a novel mechanism distinct from both kiss and run exocytosis and full fusion.

Besides modulation of exocytosis at the level of the fusion pore, exocytotic proteins or by regulation of Ca^{2+}-signals, exocytosis can be, unintentionally, modulated by the experimental conditions. This sounds not completely surprising and it is well known that results obtained from fresh cells can differ from results obtained from cells already in culture for several days, and that results obtained from dissociated chromaffin cells could differ from results obtained from chromaffin cells in adrenal slices. What is less known, is that the cell culture and saline solutions surrounding the experimental objects may unintentionally confound the results.

In the example presented by Remco Westerink (*Pb^{2+} contamination and neurotransmitter release?* by *RHS Westerink;* rwesteri@science.uva.nl) it was shown that very low concentrations (30 nM) of the heavy metal Pb^{2+} induce vesicular neurotransmitter release from ionomycin-permeabilized PC12 cells, even in the absence of Ca^{2+}. This finding becomes alarming if one takes into account the composition of "standard" extracellular saline. On average, standard saline contains 30 g of salt/l. According to the "certificates of analysis", maximum Pb^{2+} contamination amounts to 0.0005%, which equals 150 mg/l standard extracellular saline, i.e. 720 nM Pb^{2+}! Of course, this does not necessarily means that standard extracellular saline indeed contains these tremendous amounts of heavy metal contamination, but it could be.. One of the questions that then arises is: can we trust our control conditions, and are we sure that our control conditions do not saturate the endogenous buffer capacity of the cell? Related to this basic question: are contaminations affecting normal onset and progression of exocytosis?

Since the frequency of spontaneous release events from intact and permeabilized cells is rather low, even after prolonged exposure to external saline solutions, the amount of Pb^{2+}-contamination appears too low to drastically alter onset and progression of exocytosis.

Nonetheless, it is wise to use ultra-pure chemicals, especially when using permeabilized cells, to minimize the risks of contaminations. Additionally, one should keep an open mind to these kinds of problems, since they are applicable not only to external saline, but to cell culture and dissociation media as well. Furthermore, this possible hazard is not restricted to Pb^{2+}. Our media may contain a several other contaminations, each with a potential effect on cellular processes, including onset and progression of exocytosis. Finally, exocytosis kinetics reported by fluorescent proteins depends on the specific protein construct used and on the cell in which the fluorescent protein is expressed (*Kinetics of exocytosis differ markedly in chromaffin and insulin granules expressing the same lumenal fluorescent protein* by *DJ Michael, M Edwardson, CJ Rhodes and RH Chow;* rchow@email.usc.edu), underlining the notion that care should be taken when interpreting experimental results and that experimental conditions may, unintentionally, modulate exocytosis.

In addition to his presentation on kinetics of exocytosis, Robert Chow presented some general matters concerning exocytosis related experiments. One major point of concern in amperometric experiments is the issue of analysis (*Complexities in analysis of amperometric signals* by *RH Chow*; rchow@email.usc.edu).

Electrochemical methods enable us to make highly sensitive and high-time-resolution studies of individual exocytotic events in cells secreting oxidizable transmitters. An important problem is that, until now, there has been no consensus on how to analyze the individual events, much less on what the meaning is of the overall shape of the individual events. The "foot signal" has been attributed by some investigators to molecules tricking through a narrow "fusion pore" that connects the vesicle to the plasma membrane at an early stage of exocytosis. However, not all investigators see foot events. And some feel that the criteria for determining the beginning and end of the foot signal are not clear. What factors determine the shape of the "spike phase" of amperometric events is another area of controversy. Some feel that it reflects fusion pore dynamics, while others feel that the fusion pore does not contribute. Another area of uncertainty has been how to do analysis of the many events that one records during a typical experiment. What statistical approaches are valid? Probably,

these issues can only be solved if all investigators agree on using some sort of universal criteria to analyse amperometric signals.

As a final point in this summing-up, Robert Chow brought a new journal to attention (*Public Library of Science Biology - a new journal* by *RH Chow*; rchow@email.usc.edu). Many scientists have expressed outrage over the lack of free access to publicly funded research. Most of our research is paid for by national agencies, which commonly stipulate that research results and other products of research should become freely available in the public domain once the results have been published. Yet, access to such materials often requires payment of hefty journal subscriptions or computer down-loading fees. In addition, copyright to the published material is often owned by the journals. In reaction to what is now widely seen as an unfair system controlled by these journals, a number of biologists, including Nobel Prize winner Harold Varmus, have banded together to create a new journal. This journal is called the Public Library of Science Biology, and its first issue will appeared October 13, 2003. The journal will make available all articles for free by computer access. Furthermore, copyright will belong to the authors. Since there will be no revenue from journal subscriptions, authors will be charged $1500 to cover the costs of publication - not unreasonable, if you consider that one color plate in a journal like Nature will cost the authors $1000. The list of editors of PLoS reads like a Who's Who of modern biology, which will ensure that only articles of the highest quality will be published. When you are considering where to submit your next "hot" paper, why not consider PloS (http://www.plosbiology.org)?

CONCLUSIONS

Although exocytosis is a common process, founded on the interaction of highly-conserved proteins, its modulation will probably puzzle us for a few more years. At the protein level, intensive studies, using a variety of techniques, have shed some light on the molecular interactions underlying exocytosis and preceding steps. However, considering the tremendous amount of proteins involved in exocytosis it is likely that additional protein-protein interactions, capable of modulating exocytosis, will be revealed. Furthermore, every year the list of proteins involved in exocytosis increases. Consequently, the list

of possible protein-protein interactions is still growing. The same holds for regulation of intracellular Ca^{2+}-signals. The importance of intracellular Ca^{2+}-signals in the modulation of presynaptic neurotransmission is no matter of debate. However, intracellular Ca^{2+}-signals are regulated by a tight interplay of Ca^{2+}-influx, Ca^{2+}-buffering and Ca^{2+}-extrusion and Ca^{2+}-sequestration, which in turn are regulated by a large number of effectors in a manner that is still not completely understood. Furthermore, exocytosis can be modulated at the biophysical level of the fusion pore. Opening and successive closing, flickering or expansion of the fusion pore is of importance for the kinetics and the number of exocytotic events as well as for the amount of neurotransmitter released per exocytotic event. The occurrence of these different modes of exocytosis is a matter of debate, not to mention the possible mechanisms underlying and modulating these different modes of exocytosis. Finally, experimental conditions may unintentionally modulate exocytosis, confounding interpretation of the results. Therefore, the final and conclusive picture of exocytosis and its regulation is still shaded and it probably takes a few more W&BCs before we are at the long-searched for final stage of understanding exocytosis and its modulation.

Second messengers control the kinetics of exocytosis by modifying the intravesicular pH.

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Exocytosis constitutes the main cellular mechanism for secreting neurotransmitters, which gave support to the classical quantal theory. It was inferred that the unique way to increase a postsynaptic response is by increasing the number of vesicles released by nerve terminals. However, recent observations carried out analyzing the kinetics of exocytosis, at the single event level, finds that the concentration of neurotransmitter reaching the postsynaptic cell can be altered without change the number of vesicles released[1]. The mechanisms that underlay these changes in the kinetics of exocytosis remain obscure, although it might imply: i) the expansion of fusion pore[2], ii) water secretion associated with exocytosis[3] and iii) changes in the aggregation of intravesicular components[4].

Vesicular ATPase is a proton pump that maintains a pH of about 5.5 and a membrane potential of +80 mV with respect to cytosol. This H^+ gradient is also the driving force for the uptake of catecholamines, Ca^{2+} and ATP. If there is a candidate to serve as the sensitive mediator for intracellular signals that modulate the aggregation of intravesicular components it should be the vesicular Ph (pHi). Here, we postulate a correlation between the degree of pHi and the kinetics of exocytosis at single event level. We also show that several second messengers could modify this kinetics through this novel mechanism.

RESULTS AND DISCUSSION

It is well reported that fluorescent weak bases like acridine orange (AO) are accumulated into acidic cellular compartments, thus increasing the fluorescent signal as pHi falls. Both dyes seem to accumulate within the same cell structures since these probes exhibit the typical 'granular' pattern and because this accumulation is inhibited by bafilomycin A_1. However, the participation of early endosomes or Golgi in the fluorescent signal cannot be excluded.

Figure 1a shows the effect of several drugs the time-course of the accumulation of AO in single chromaffin cells and NH_4Cl. Ammonia accumulation inside vesicles causes their alkalinization thus decreasing the fluorescence signal of AO. This effect is mimicked also by the incubation with the NO donor sodium nitroprusside (SNP) whereas the NO scavenger C-PTIO causes the opposite. Figure 1b shows the average of several drugs on the pHi.

We have shown that NO promotes rapid changes in the kinetics of exocytosis acting through the classical cGMP route (Machado et al, 2000), Conversely, the NO scavenger carboxyl-PTIO (C-PTIO), which removes the NO present inside and outside the cell, produces an acceleration of the kinetics of exocytosis. To test whether these action mechanisms of NO might involve changes in the pHi, we conduct experiments monitoring the AO accumulation in the presence and in the absence of NO. SNP (10 μM) yields a free NO concentration of about 10 nM that strongly slow down the kinetics of exocytosis. Figure 1a & b show the effects of SNP and C-PTIO on the time course of AO accumulation. These experiments indicate that NO reduces the H^+ gradient across the vesicular membrane thus promoting its alkalinization, they also evidence the presence of a basal level of NO in the bathing media that is revealed after its removal with the scavenger. The rapid NO withdrawal produces an acidification of secretory vesicles.

Acute incubation of cells with the vesicular H^+-pump inhibitor bafilomycin A_1 (0.1-100 nM) results in a drastic slow down of the kinetics of exocytosis (figure 1c) thus resembling to that observed with NO and cAMP[5,6], suggesting that the action mechanism of these kinases might be mediated by changes in pHi. However, higher concentration of bafilomycin also causes the reduction of vesicular content. Drugs known to alter the pHi also modify the kinetics of exocytosis. Conversely, drug treatments that affect the kinetics of exocytosis also produce changes in the pHi. Alkalinization is associated with a slow-down of exocytosis.

Some authors suggest that pHi and intravesicular potential would contribute to the fusion process[7]; however, after experiments on permeabilized cells[8] this controversy seemed to be over. Nevertheless, all of these studies are carried out by measuring the secretion of catecholamines from a population of millions of chromaffin cells. In the present work, we have tried to get an approach to the role of the pHi in the kinetics of exocytosis at single event level and to establish a relation between pHi and the modulation of quantal release of catecholamines by cell signalling.

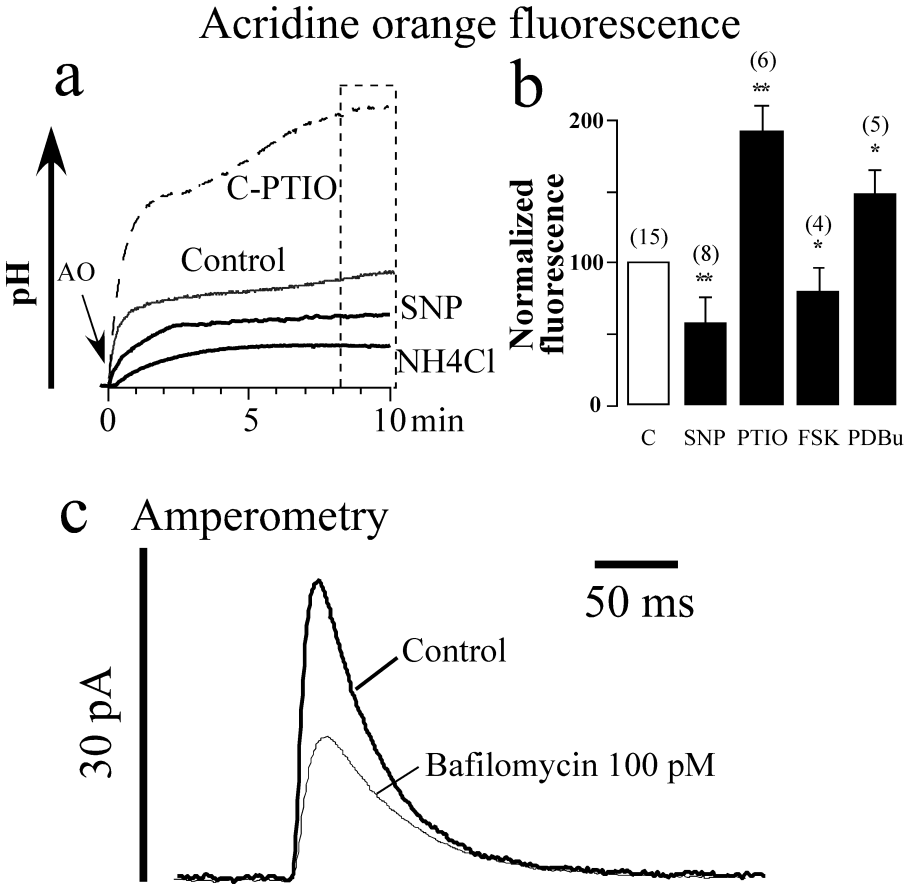

Figure 1. The effects of several drugs in the accumulation of acridine orange and in the kinetics of exocytosis in single chromaffin cell. a) Typical traces showing the cellular accumulation of acridine orange (AO, arrow; 10 nM, 30 s) measured using an inverted fluorescent microscope. Chromaffin cells are incubated with Krebs solution in the absence and in the presence of: NH_4Cl 10 mM, the NO donor SNP or the NO scavenger carboxy-PTIO during 10 min. **b)** Pooled data from experiments done as described in a, measures are done at the end of traces by averaging the values within the dashed rectangles. C, saline; PTIO, carboxy-PTIO 10 nM; FSK, forskolin 100 nM; SNP, sodium nitroprusside 10 μM; PDBu, phorbol dibutirate ester 100 nM. *p<0.05, **p<0.01 student t-test. **c)** Bafilomycin A_1 reduces the kinetics and the quantal size of exocytosis. Cells are treated with bafilomycin 100 pM for 10 min. Exocytosis is triggered by 5 s pulse of 5 mM $BaCl_2$ in the vicinity of the cell and secretory spikes measured by amperometry and quantified as described [9], Traces are reconstructed with the averaged data obtained from more than 400 spikes from each group obtained the same day with the same electrode.

There are experimental data indicating that fusion pore dynamic is not implicated in the regulation of exocytotic kinetics. For instance, NO still modulating the kinetics of exocytosis even in pre-fused vesicles from cells secreting under hypertonic solutions. In addition, the duration of the pre-spike phenomenon (foot) does not change in response to NO[9]. In addition, NO modified the kinetics of exocytosis in rat mast cells without changes in the fusion pore kinetics (Guillermo Alvarez de Toledo, personal communication). The major structure implicated in the maintenance of pHi is the V-ATPase because it possesses several loci suitable for phosphorylation/de-phosphorilation whereas the later is selectively blocked by bafilomycin A_1[10].

The control of the kinetics of exocytosis by second messengers has been demonstrated in chromaffin[9,11,12], PC12 (Borges, unpublished observations) and mast cells; conversely, it has not demonstrated in neurons. It is however likely that this effect could be a general and fine mechanism for the regulation of synaptic transmission. To our known, this is the first experimental evidence that unveils the mechanism implicated in the regulation of the kinetics of exocytosis by intracellullar signals.

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Myosin II roles in vesicle transport and fusion in Chromaffin Cells

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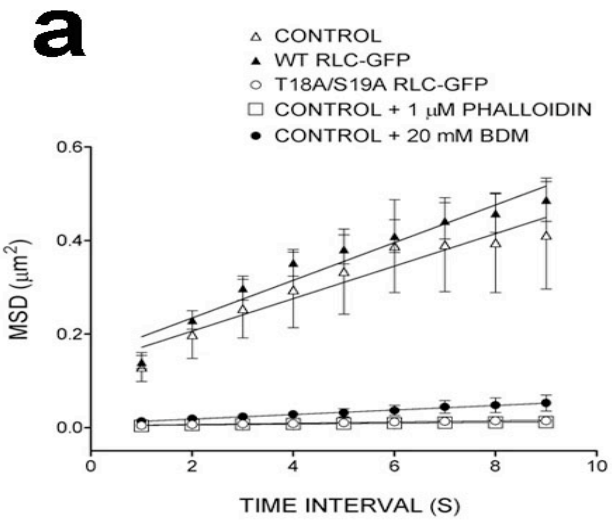
Cytoskeletal proteins play an essential role among the cytosolic elements organizing the transport of vesicles from the internal regions where chromaffin granule biogenesis takes place towards the subcortical region where they are stored to form a reserve pool to sustain continuous stimulation[1,2]. In these sense different myosins have been suggested to play a motor role in such transport and therefore they could modulate vesicle supply during secretion[3,4,5]. Here we studied whether the overexpression of myosin II regulatory light chain (RLC) and an inactive form affect different stages of the secretory process. Our results suggest the implication of myosin II in the transport of vesicles, and, surprisingly, in the final phases of exocytosis involving transitions affecting the activity of docked granules, and therefore uncovering a new role for this cytoskeletal element.

RESULTS AND DISCUSSION

Dynamic confocal microscopy has been used to study vesicle movements in chromaffin cells overexpressing wild type RLC-GFP or its inactive unphosphorylatable form (T18A/S19A RLC-GFP) and loaded with quinacrine. Using this technique we have observed a marked decrease in vesicle mobility in cells expressing such unphosphorylatable form when compared with control non-treated cells of cells expressing the wild type form. In effect, calculation of the coefficient of diffusion through mean square displacements (MSD) vs time plots (Figure 1a) yielded values of $10.1\pm1 \times 10^{-3}$ $\mu m^2/s$ for RLC-GFP moving granules and $0.32\pm0.02 \times 10^{-3}$ $\mu m^2/s$ for vesicles in cells expressing such unphosphorylatable form. This reduction in vesicle mobility was similar to the caused by agents affecting F-actin (phalloidin) or myosin ATPase activity (BDM) as shown in Figure 1a. The study of individual fusion kinetics is potentially useful when looking for the mechanisms underlying the different effects of RLC-GFP constructs and can be achieved by analyzing the shape of single amperometric events[6] using carbon fiber electrodes. The analysis was performed in $BaCl_2$ stimulated cells, in order

to search for well-separated spikes with amplitudes over 2.5 pA. We measured spike amplitude (Imax), event charge (Q), half-width ($t_{1/2}$) and time-to-the-peak (Tp). Non-infected cells or cells expressing wild-type RLC-GFP were characterized by a very similar pattern of distribution, showing mean amplitudes of 68 and 73 pA respectively. However, the mean amplitude obtained with mutants T18A/S19A RLC-GFP (Figure 1b) was clearly reduced to half that of the control value (32±5 pA). The observed alteration in mean amplitude did not represent a subsequent change in the amount of catecholamines released per event, since charge remained relatively unaltered. It is clear from the modifications in single vesicle fusion parameters, that the secretory events found in cells expressing the T18A/S19A RLC-GFP construct were considerably slower than control or wild type RLC-GFP expressing cells, whilst maintaining the charge released per event (Figure 1 b). These data demonstrate that myosin II activity influences the very final events linked to the exocytotic fusion of membranes and the release of catecholamines in this neuroendocrine cell model.

This might imply that myosin II activity influenced either the kinetics of exocytotic pore expansion or the degree of neurotransmitter dissociation from the vesicular matrix. It is unlikely that myosin II could influence processes taking place in the interior of the vesicular matrix, therefore we favor the first possibility. In conclusion, the present work demonstrates that cytoskeletal elements such as conventional myosin II affect different stages of the exocytotic process and not merely the transport of vesicles. Orienting research toward these newly proposed interactions may help to clarify and unravel some of the complex aspects of the exocytotic cycle.


a
CONTROL
WT RLC-GFP
T18A/S19A RLC-GFP
CONTROL + 1 μM PHALLOIDIN
CONTROL + 20 mM BDM
MSD (μm²)
0.6
0.4
0.2
0.0
0
2
4
6
8
10
TIME INTERVAL (S)

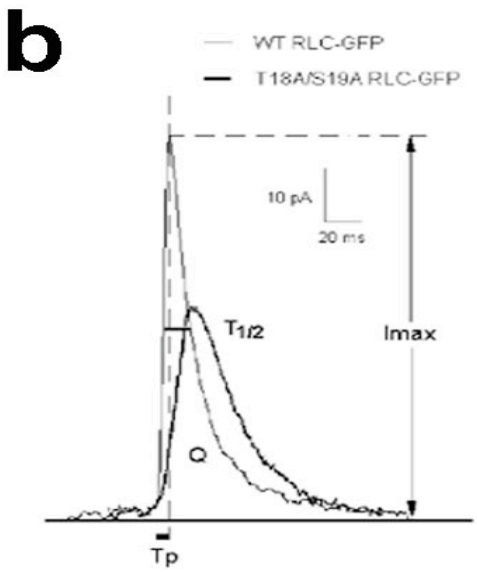

b
WT RLC-GFP
T18A/S19A RLC-GFP
10 pA
20 ms
T1/2
Imax
Q
Tp

Figure 1. a. Average of square of the mean distance moved (MSD) during the time intervals expressed in the abscissa, for vesicles in wild type RLC-GFP expressing cells, and cells expressing T18A/S19A RLC-GFP. In addition, were given MSD vs time plots for control cells treated with 1 μM phalloidin or 20 mM BDM. Also plotted were the best linear fits for the different curves, used to estimate the diffusion coefficient. **b.** This panel shows spikes representing the average properties of events in cells expressing wild type RLC-GFP and T18A/S19A RLC-GFP constructs, in addition to describe the parameters analysed.

A long version of this work can be found in reference 7.

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cAMP and β-adrenergic stimulation regulate L-type channel gating and exocytosis in rat chromaffin cells.

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In rat chromaffin cells (RCCs), L-channels carry ~50% of the total Ca^{2+} current and are effectively inhibited by the neurotransmitters released by their own secretory granules. This inhibition is membrane-delimited and mediated by G protein-coupled receptors co-localized with L-channels in membrane microdomains. The inhibition is reversed when intracellular cAMP is raised or after exposure to isoprenaline (β-AR-stimulation), suggesting the existence of parallel and opposite effects on L-channel gating by distinctly activated membrane autoreceptors. Rising intracellular cAMP by either applying the membrane permeable analogue pCPT-cAMP or through β-AR stimulation induces multiple effects on RCCs: *i)* a 20% increase of L-currents, *ii)* a 100% potentiation of the depolarization-evoked secretion occurring downstream of Ca^{2+}-entry and *iii)* a slow recruitment of T-type channels following long-term exposures of permeable cAMP or isoprenaline. This multiform action of pCPT-cAMP appears to nicely counterbalance the inhibitory effect on L-channels and argues in favor of an effective increase of catecholamine secretion and cell excitability under conditions of sustained cell stimulation with minor increases of intracellular Ca^{2+}.

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Modulation of Ca^{2+}-entry through voltage-gated L-channels may occur in various ways: by down regulating or recruiting newly available channels or by inhibiting or facilitating the channel gating[1-3]. L-channel gating modulation has received great attention in the last two decades and appears an effective system for controlling Ca^{2+} ions entering the cell. Among the many modulatory pathways, two appear of particular interest because of their autocrine nature: the G protein-dependent inhibition and the cAMP/PKA-mediated potentiation[1-3]. In chromaffin cells, both pathways are activated by autoreleased neurotransmitter molecules and produce opposing effects of comparable entity[4]. The inhibition is complete within few seconds and is mediated by PTX-sensitive G proteins coupled to P_{2y}-purinergic, δ/κ-opioidergic, α_2- and β_2-adrenergic receptors[5-10]. In contrast, the potentiation is selectively triggered by β_1-ARs and occurs slowly through the activation of a cAMP/PKA pathway, which may act at distant sites from receptors[11].

An interesting issue is whether Ca^{2+} changes associated to L-channel modulation play a critical role in the control of exocytosis. Recent observations suggest that down- or up-modulation of L-currents do not always produce proportional effects on exocytosis indicating that intracellular [Ca^{2+}] increases following Ca^{2+} channels activation are preliminary to secretion but may be either amplified[12] or depressed[13] by a downstream action on the secretory machinery. Here, besides discussing the molecular features of cAMP-mediated potentiation and G protein–induced inhibition of L-channels via β_1-ARs and β_2-ARs stimulation, we will focus on some recent findings concerning L-channel modulation and its coupling to secretion.

The β1- and β_2-ARs modulation of L-channels: an example of direct and remote signaling pathways in chromaffin cells. As recently shown, the $G_{i,o}$ protein-dependent inhibition of L-channels in RCCs is not limited to opioidergic, purinergic and α_2-adrenergic autoreceptors[3]. There is in fact evidence also for the involvement of β-ARs[9]. Thus, the question is whether there is a rationale for the presence of β-ARs, which can up- or down-modulate the major Ca^{2+} current component controlling neurotransmitter release in RCCs. The answer comes from recent findings in which RCCs are shown to

express two distinct β_1- and β_2-AR activated signaling pathways[4]. The β_1-AR cascade acts by selectively up-regulating the L-channel through a PKA-mediated pathway and develops slowly due to its diffusive characteristics. On the contrary, the β_2-AR signaling is fast and primarily coupled to PTX-sensitive G proteins. Fig.1A shows an example in which a RCC responds to ISO stimulation with rapid inhibition of Ca^{2+} currents followed by a slow potentiation. The final balance is a slight increase of Ca^{2+} currents, which allows the cell to elevate cAMP concentration, with little increases of Ca^{2+} fluxes. This is different from the β-AR stimulation of cardiac cells in which the cAMP/PKA stimulation is accompanied by a marked amplification of Ca^{2+} fluxes required for increasing the strength of cardiac contraction during sympathetic discharges[14].

An interesting aspect of the β-ARs modulation in RCCs is the peculiar role of β_2-ARs, which are directly coupled to an inhibitory PTX-sensitive G protein pathway and are unable to produce L-currents potentiation through the activation of adenylate cyclase, as in cardiac myocytes[15]. Fig.1B shows that zinterol (a β_2-AR selective agonist) does not produce the slow stimulatory effect of isoprenaline, but causes fast inhibition of Ca^{2+} currents. Sequential application of zinterol and isoprenaline nicely mimics the effects of isoprenaline alone; indicating that complete activation of β_2-ARs produces an inhibition (direct action) followed by a slow potentiation mediated by β_1-ARs (remote action) (see Fig.1C).

L-channel modulation and exocytosis. An interesting issue concerning L-channel modulation is how these mechanisms interfere with the exocytotic cell activity. Excitation-secretion coupling in chromaffin cells is triggered by elevations of cytosolic [Ca^{2+}] mainly associated to Ca^{2+} influx through voltage-gated Ca^{2+} channels. Thus, modulation of L-channel gating by neurotransmitters may represent an effective mechanism for regulating secretory responses. This is particularly critical in rat, mouse and human chromaffin cells, which express high densities of L-channels[16]. However, modulation by neurotransmitters may occur also directly on the secretory machinery (downstream of Ca^{2+}-entry), thus introducing a further degree of variability to the phenomenon.

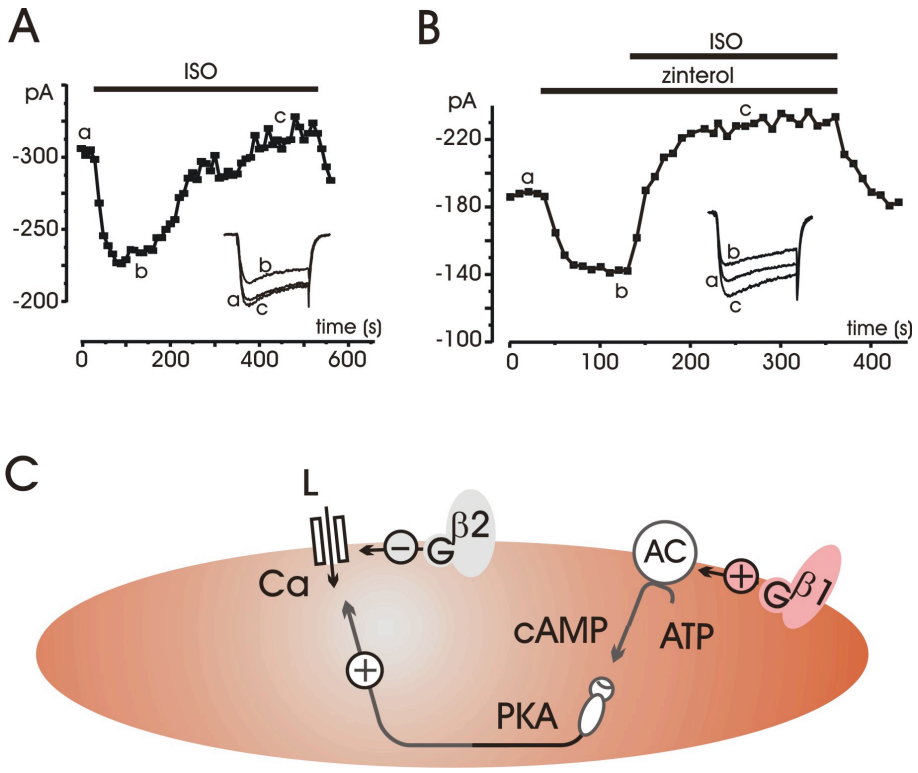

Fig. 1. Sequential inhibition and potentiation of L-type Ca^{2+} currents during β_2- and β_1-ARs stimulation in RCCs. **A)** Isoprenaline alone (ISO, 1 μM) causes a rapid inhibition and a slow recovery of L-current amplitude. **B)** Addition of isoprenaline following the fast inhibition induced by zinterol (selective β_2-ARs agonist; 1 μM) induces a marked potentiation of L-currents. The symbols are peak current amplitudes measured during a 25 ms step depolarization to +10 mV repeated every 10 sec (holding potential –40 mV). The insets show the original recordings taken at the time indicated. Modified from ref [4]. **C)** Schematic drawing of the signaling pathways associated to β_1- and β_2-ARs stimulation on a RCC.

The effects of neurotransmitters on Ca^{2+} currents and secretion have been studied in bovine chromaffin cells (BCCs) and RCCs by combining membrane capacitance measurements and whole-cell current recordings[13,17,18]. Most of the works point to a marked inhibition of ATP on L- N- and P/Q-type currents and a proportional inhibition of exocytosis. In BCCs, ATP neither alters the Ca^{2+}-

dependent fusion of vesicles to the plasma membrane nor the vesicle supply to release sites, thus confirming that the ATP-induced inhibition of exocytosis is primarily associated to its action on Ca^{2+} channels[17,18]. The action of ATP appears more complex in RCCs[13]. ATP inhibits exocytosis by either depressing Ca^{2+} currents (L, N and P/Q) or by directly acting on the secretory machinery through a Ca^{2+}-independent pathway. The latter occurs independently of Ca^{2+} channels and accounts for most of the inhibitory effect on exocytosis induced by ATP.

The cAMP/PKA-mediated potentiation of secretion and L-channel gating. The effects of cAMP on secretion in chromaffin cells are quite heterogeneous. Some reports point to a marked increase of basal and stimulus-evoked secretion from adrenal chromaffin cells together with an increased Ca^{2+}-entry through L-channels induced by cAMP, PACAP and forskolin[19-22]. On the contrary, other data support the existence of an inhibitory action of cAMP on nicotine-induced release and Ca^{2+} currents in BCCs[23,24]. In some works, L-channels and membrane voltage are shown to play an exclusive role in increasing the stimulus-induced secretion by cAMP[25], while in others the role of these components appears more limited or unnecessary[20,26]. Among this complex pattern of responses, the contribution of Ca^{2+} channels and their modulation by cAMP in the control of exocytosis has been recently investigated in RCCs[12]. The cAMP permeant analog pCPT-cAMP is found to potentiate both the L-currents and the depolarization-evoked secretion, but the current increase accounts for only 20% of the total secretory response. cAMP doubles the size of the readily-releasable pool (RRP) of vesicles by almost doubling the mean size of unitary exocytic events (from 1.1 to 2.1 fF), without affecting the probability of release. cAMP potentiates the secretion independently of the activated Ca^{2+} channel type and the same effects are induced by β_1-AR stimulation through a PKA-mediated pathway.

CONCLUSIONS

Given the critical role of L-type Ca^{2+} channels in controlling cell excitability and neurotransmitter release, it is not surprising that this class of channels is extensively regulated by a number of signaling

pathways. The work of the last five years has shown that neuroendocrine L-channels undergo a marked autocrine modulation induced by the material released during cell activity, causing either inhibition or potentiation of the Ca^{2+} current controlling exocytosis. The most original aspect of this action is the existence of a direct G protein-mediated inhibition of L-channels, which co-exists with the classical up regulation mediated by the cAMP/PKA-signaling cascade. In chromaffin cells, these opposing modulations of L-channels have the advantage of allowing the increase of intracellular cAMP by means of β-ARs stimulation, with consequent increased exocytosis and recruitment of newly synthesized T-type channels[27], without significantly altering the intracellular Ca^{2+} levels. The overall effect of L-channels auto-regulation by released neurotransmitters (adrenaline, noradrenaline, ATP and opioids) is therefore a remodeling of cell excitability and an enhancement of neurotransmitter release with little changes to intracellular [Ca^{2+}], which may result deleterious for cell survival during maximal sympathetic stimulation and sustained catecholamine release.

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The modulation of vesicular volume and its effects on neurotransmitter secretion through the fusion pore in PC12 Cells.

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Cell Biology of the Chromaffin Cell
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Understanding vesicular exocytosis is fundamental to developing insight into the biology, chemistry and physics of neurotransmission. Through the direct 'presynaptic' observation of quantal release using amperometry it becomes strikingly evident that the exocytotic release event is regulated by multiple mechanisms[1]. For instance, many transients in amperometric records of exocytosis events exhibit a pre-spike feature, or foot, which represents a steady-state flux of neurotransmitter through a stable fusion pore connecting the vesicle lumen to the extracellular fluid[2]. This flux is thought to be driven by diffusion along a gradient of chemical potential. Recent work in our laboratory indicates that vesicular volume prior to secretion is strongly correlated with the characteristics of amperometric foot events, and furthermore, suggests that the protein dense core in many vesicles acts to pressurize the inner solution and drive catecholamine through the fusion pore[3].

RESULTS AND DISCUSSION

In order to alter vesicular volume, we have used pharmacological manipulations that directly affect the VMAT-mediated transport of catecholamines into PC12 cell vesicles. Cells were incubated in 100 μM L-3,4-dihydroxyphenylalanine or 100 nM reserpine for 90 minutes in order to increase and decrease, respectively, the volume of single pheochromocytoma (PC12) cell vesicles[4]. Amperometry experiments utilizing a same-cell paradigm were used in conjunction with transmission electron microscopy to determine that as vesicle size is decreased the frequency with which foot events are observed increases. Consistent with this, fewer events with feet are observed as vesicle size is increased. Additionally, both the amount and duration of neurotransmitter released in the foot portion of the event decrease as vesicle size decreases, whereas both of these characteristics increase as vesicle size increases. Finally, smaller vesicles release a greater percentage of their total contents in the foot portion of the event. These trends provide evidence for a nanotube-like intermediate structure for the exocytotic fusion pore. This is shown in Figure 1B, where a model is presented to describe the effect of vesicle volume on membrane tension and fusion pore

dynamics. A brief description of the mechanisms regulating neurotransmitter release via the fusion pore is presented in the figure legend.

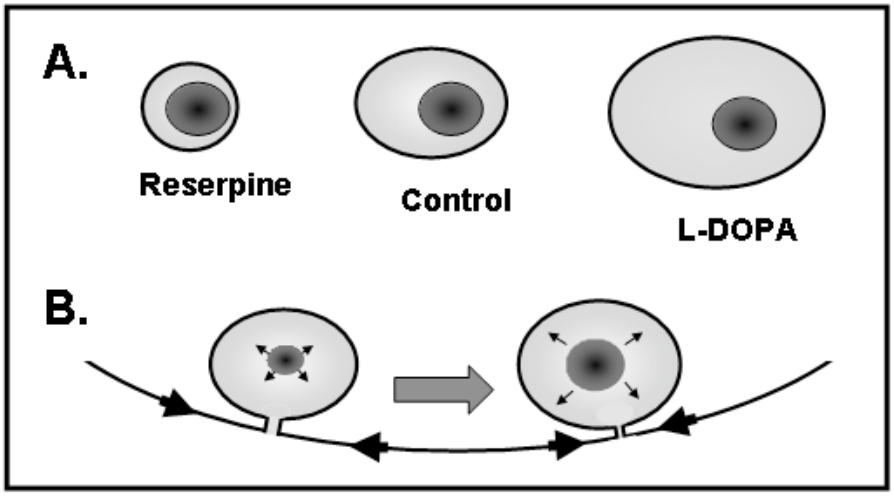

Figure 1: A) Pharmacological manipulation of vesicle volume alters the dense core to vesicular halo volume ratio. Treatment with reserpine diminishes the vesicular halo; however the volume of the dense core changes relatively little. In contrast, treatment with L-DOPA swells the vesicular halo, and again, the dense core volume remains relatively constant. **B)** Upon opening of the fusion pore, the dense core expands resulting in enhanced pressure-driven mass transport through the fusion pore and fluidic pressure on the interior vesicle walls. As the dense core to vesicular halo volume ratio is greatest for reserpine treated vesicles, it appears that the intravesicular pressure is greatest for these cells. An increase in intravesicular pressure increases vesicular membrane tension. If the fusion pore is of a nanotube geometry, this will lead to an immediate constriction of the fusion pore, as shown[3]. Subsequently, the increase in the difference in membrane tension from the cell to the vesicle should result in increased lipid flow through the fusion pore, decreasing its overall stability and resulting in an abrupt opening of the pore.

These studies are noteworthy because the dynamics of the exocytotic fusion pore have recently been proposed to play a role in synaptic plasticity[5]. Additionally, physiological[6-8], genetic[8] and pharmacological[3,10] conditions can affect vesicle size, and in several instances vesicle size has been found to be inversely related to levels of synaptic activity[9]. This work provides new insight into the biological consequences of such vesicular volume modification. It

provides evidence that cells may modulate the rate of neurotransmitter release, or the duration of that release, in the synaptic cleft by manipulating vesicle volume. This appears to be true in the case of release via the fusion pore that is formed in the early stages of exocytosis from PC12 cells.

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Modulation of Ca^{2+}-independent, Pb^{2+}-induced exocytosis from rat PC12 cells by CaMK II.

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Exocytosis is a complex, Ca^{2+}-dependent process involving docking and priming of vesicles, the formation of a fusion pore and fusion of vesicle and plasma membrane and the release of vesicle contents. Apart from Ca^{2+} ions, which are required to trigger the actual exocytotic event, a variety of cytoplasmic proteins is involved in the regulation of specific steps of the vesicle cycle[1-3]. Several Ca^{2+}-dependent proteins associated with exocytosis are activated by Pb^{2+} as well. Pb^{2+} is a potent activator of calmodulin[4], PKC[5], calcineurin[6], and synaptotagmin[7]. Additionally, Pb^{2+} has been reported to cause an enhancement of spontaneous neurotransmitter release, which occurs irrespective of the presence of extracellular Ca^{2+} and has been attributed to intracellular effects of Pb^{2+} [8-9]. Previous reports demonstrate that addition of >5 μM Pb^{2+} to external saline is required to induce neurotransmitter release from undifferentiated PC12 cell populations[8]. The aim of this study was to determine the origin of Pb^{2+}-induced neurotransmitter release, to investigate whether Pb^{2+} stimulates release directly or indirectly, and to investigate the intracellular signaling pathways involved in Pb^{2+}-induced exocytosis.

MATERIALS AND METHODS

PC12 cells (ATCC CRL-1721) were grown essentially as described previously[10]. For all experiments cells were differentiated in culture medium (RPMI 1640, Gibco, Grand Island NY, USA) supplemented with 5 μM dexamethasone (Genfarma, Zaandam, The Netherlands) starting 2 days after subculturing. Culture dishes and coverslips were coated with 5 μg/cm^2 poly-L-lysine (Sigma, St. Louis MO, USA). Experiments were performed 5-8 days after initiating differentiation. Carbon fiber microelectrode (∅10 μm) fabrication and data recording and analysis were as described previously[10-11]. Exocytosis was evoked by superfusion with nominal Ca^{2+}-free saline containing > 30 nM Pb^{2+}. Experiments were performed at room temperature (21-23 °C).

RESULTS AND DISCUSSION

To investigate the direct intracellular action of Pb^{2+}, cells were permeabilized by superfusion with saline containing 5 µM ionomycin Ca^{2+}-salt. Pores formed by ionomycin have been shown to be highly permeable to Pb^{2+} as well as to Ca^{2+} [12]. Permeabilized cells were superfused with saline containing various concentrations of Pb^{2+}. Superfusion with 30 nM Pb^{2+} resulted in detectable vesicular catecholamine release after a delay of several minutes (Fig. 1A), whereas 30 min of superfusion with nominal Pb^{2+}-free saline or with 10 nM Pb^{2+} was without effect (data not shown). At increased concentrations of 100 nM or 1 µM Pb^{2+} the delay to the onset of release decreased (Fig. 1A) from 4.8 ± 4.2 to 3.8 ± 1.3 and 0.9 ± 0.8 min, respectively. In addition, the maximum frequency of vesicles released increased with increasing Pb^{2+} concentration with a maximum at 1 µM Pb^{2+}. Switching back to nominal Pb^{2+}-free saline reversed the Pb^{2+}-induced release only slowly (Fig. 1A). The concentration-dependent effects demonstrate that the threshold concentration of extracellular Pb^{2+} to induce vesicular neurotransmitter release in permeabilized PC12 cells is close to 30 nM.

Using calcium and heavy metal chelators it was shown that 1 mM Ca^{2+}-evoked release is readily reversed by superfusion with nominal Ca^{2+}-free saline containing the membrane-impermeable chelator EGTA (500 µM; not shown), demonstrating that Ca^{2+} rapidly diffuses out of the permeabilized cell. Pb^{2+}-induced release is reversed only slowly by superfusion with saline containing 500 µM EGTA (Fig. 1B), demonstrating that the effect of Pb^{2+} is independent of intracellular Ca^{2+}. Conversely, 10 µM of the membrane-permeable heavy metal chelator TPEN quickly reversed the Pb^{2+}-induced release (Fig. 1B), whereas TPEN did not affect basal, spontaneous release or 1 mM Ca^{2+}-evoked exocytosis (not shown). These findings demonstrate that exocytosis is caused by binding of Pb^{2+} to an intracellular binding site and that Pb^{2+}-induced exocytosis continues in the absence of Ca^{2+}.

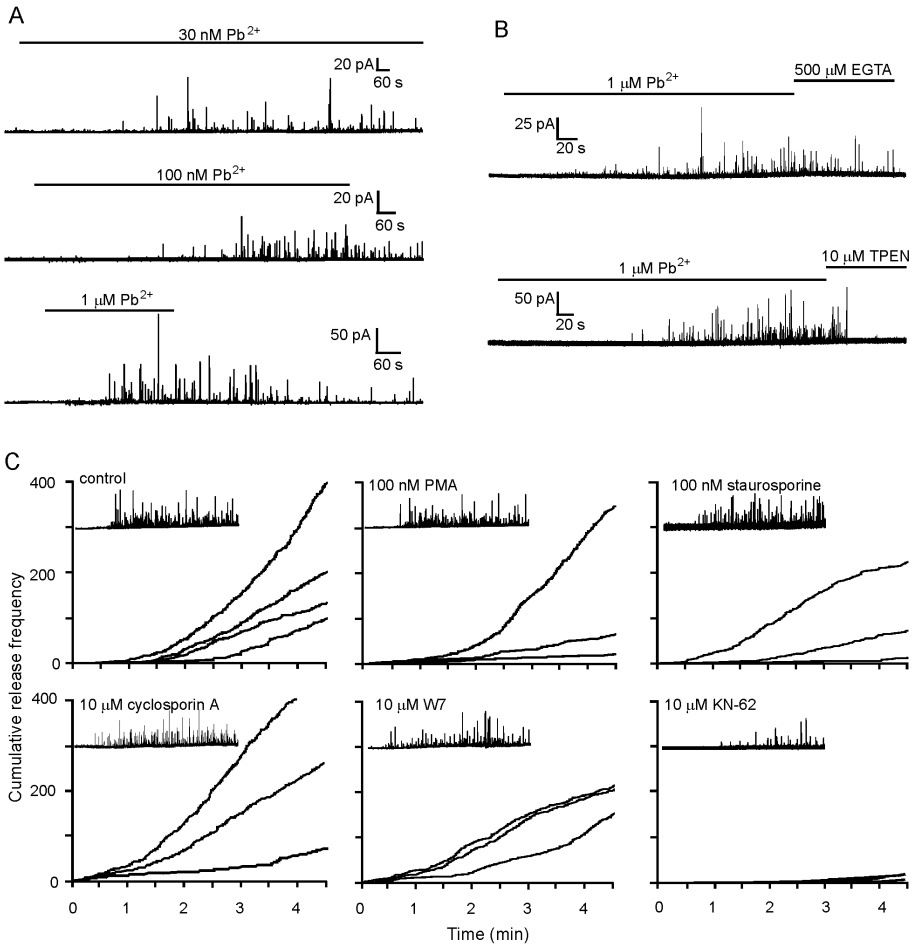

Figure 1 A) Concentration-dependence of Pb^{2+}-evoked exocytosis in permeabilized PC12 cells. Amperometric recordings from permeabilized PC12 cells superfused with Ca^{2+}-free saline containing 5 μM ionomycin Ca^{2+}-salt and 0.03, 0.1 and 1 μM Pb^{2+}, as indicated by the bars on top of the recordings. **B)** Ca^{2+}-independence of Pb^{2+}-evoked exocytosis in permeabilized PC12 cells. Pb^{2+}-induced (1 μM) exocytosis is not reversed by the membrane-impermeable chelator EGTA at concentrations sufficient to rapidly reduce the intracellular Ca^{2+} concentration below the threshold for release (upper trace). Conversely, the membrane-permeable heavy metal chelator TPEN rapidly reduces the

intracellular Pb^{2+} concentration below the threshold for release (lower trace). Superfusion periods are indicated by the bars on top of each recording. **C)** Effects of drugs modulating PKC (PMA and staurosporine), calcineurin (cyclosporin A), calmodulin (W7) and CaMK II (KN-62) activity on the frequency of Pb^{2+}-induced exocytosis in ionomycin-permeabilized PC12 cells. Drug application started 15 min before superfusion with saline containing 1 □M Pb^{2+} and the same concentrations of the drug. The panels show cumulative frequencies of exocytotic events recorded from different cells following the start of superfusion with Pb^{2+}-containing saline (t = 0) and the drugs indicated; insets show representative examples of amperometric recordings. The curves show that the frequency of exocytosis varies between cells. However, the delay to the onset of Pb^{2+}-evoked release, which amounts 1 - 2 min in control cells, is reduced to < 30 s by W7 and is increased by KN-62 to > 2 min. In addition, the number of events recorded from KN-62-treated cells was strongly reduced.

Activation of PKC by PMA or inhibition of calcineurin by cyclosporin causes a significant increase of the basal release frequency in permeabilized cells. Conversely, inhibition of PKC, calmodulin and CaMK II by staurosporine, W7 and KN-62, respectively, did not affect basal exocytosis. These results indicate that enhancement of the phosphorylation state of intracellular proteins by PKC and calcineurin directly increased the basal frequency of exocytosis[13]. In case of Pb^{2+}-induced exocytosis, systematic effects of these drug treatments on the frequency of Pb^{2+}-induced events were only observed for the inhibition of CaMK II by KN-62 and for inhibition of calmodulin by W7. Inhibition of CaMK II caused a consistent and strong decrease in the frequency of exocytosis (Fig. 1C). Consequently, KN-62 caused a significant decrease in the total number of vesicles released during the first 270 s of Pb^{2+} exposure, as well as an increase in the delay to the onset of Pb^{2+}-induced exocytosis (Fig. 1C). Conversely, exocytosis in W7-treated cells was consistently evident already within the first 30 s of superfusion of the cells with Pb^{2+}-containing saline (Fig. 1C). Exposure of the cells to PMA, to staurosporine, and to cyclosporin caused neither systematic nor differential effects. An early apparent effect in cyclosporin-treated cells is due to the enhancement of basal release by cyclosporin before the onset of Pb^{2+}-evoked release, which was not observed with W7. The results

presented in Figure 1C show that Pb^{2+}-induced exocytosis is modulated by calmodulin and CaMK II activity**.**

The results from this study provide a direct demonstration that Pb^{2+}-induced catecholamine release has a vesicular origin and that the threshold concentration to evoke exocytosis in permeabilized PC12 cells amounts to ~30 nM (Fig. 1A). The direct effects of Pb^{2+} on exocytosis are mediated by intracellular mechanisms. This is demonstrated by the fact that the extracellular application of membrane-impermeable chelators EGTA rapidly reduces the intracellular Ca^{2+} concentration below threshold levels and thereby reverse Ca^{2+}-evoked exocytosis, whereas Pb^{2+}-induced exocytosis is reversed only very slowly (Fig.1B). Conversely, the membrane-permeable heavy-metal chelator TPEN rapidly reverses Pb^{2+}-induced exocytosis (Fig. 1B), indicating that Pb^{2+} is tightly associated with an intracellular binding site. The results show that Pb^{2+}-induced exocytosis involves a direct interaction with an intracellular, high-affinity site.

The extracellular threshold concentration of ~30 nM Pb^{2+} to evoke exocytosis is slightly higher than the concentrations of Pb^{2+} found in blood after Pb^{2+}-poisoning. However, this does not distract from the toxicological hazard of this heavy metal. Since high-affinity buffering facilitates the intracellular accumulation of Pb^{2+}, much depends on the availability of extracellular Pb^{2+}, i.e., free Pb^{2+} concentration as well as the amount of remaining extracellular Pb^{2+} and the affinity by which it is bound. If the conditions are such that a critical amount of Pb^{2+}, estimated to be 2-3 attomol for PC12 cells[10], accumulates intracellularly, sustained exocytosis may be triggered and cause the adverse effect.

In ionomycin-permeabilized PC12 cells modulation of the phosphorylation state of the exocytotic machinery by PKC/calcineurin results in modulation of exocytotic function[13]. Systematic effects of pretreatment with PMA, staurosporine, and cyclosporin A on Pb^{2+}-induced release from ionomycin-permeabilized PC12 cells are not observed. This is a surprising result, since Pb^{2+} has been reported to be a potent activator of PKC[5], and activation of PKC enhances basal

exocytosis in permeabilized PC12 cells[13]. The calmodulin inhibitor W7, which does not cause significant effects on basal release in permeabilized PC12 cells[13], clearly reduced the delay to onset of Pb^{2+}-induced exocytosis (Fig. 1C). Since the delay to onset of release is reduced with increasing Pb^{2+} concentration, the effect of W7 is equivalent to an apparent enhancement of the sensitivity to Pb^{2+} (cf. Fig. 1C and Fig. 1A). Inhibition of CaMK II activity by KN-62 leads to a strong reduction of the number of vesicles released and to an apparent increase in the delay to onset of Pb^{2+}-evoked exocytosis (Fig. 1C), which appears to be independent of calmodulin. These results indicate that CaMK II is required for Pb^{2+}-induced vesicular catecholamine release.

Although a direct demonstration of activation of CaMK II by Pb^{2+} is lacking, CaMK II is known to phosphorylate a range of intracellular proteins, including synapsin I[14], synaptotagmin[15], and the t- and v-SNAREs[16]. Furthermore, CaMK II-induced phosphorylation is associated with an increase in the number of releasable vesicles[17]. Therefore, CaMK II provides a novel and plausible target for the direct intracellular action of Pb^{2+} leading to neurotransmitter release.

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Cdc42 and N-WASP regulate actin filament organization during exocytosis in PC12 cells.

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Cell Biology of the Chromaffin Cell
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A series of recent studies has strengthened the link between the actin cytoskeleton and intracellular membrane trafficking[1]. In particular, attention has been paid to the mechanisms regulating actin filament organization and progress has been made in identifying specific actin binding proteins and actin signalling molecules. Interestingly, among them, several appear to stimulate the actin nucleation activity of the Arp2/3 complex. One of the best characterized example is the Wiskott-Aldrich syndrome protein (WASP) family of proteins that can be regulated by the Rho GTPase Cdc42[2].

In chromaffin cells, like in many secretory cells, the cortical network of actin filaments forms a physical barrier to exocytosis for the majority of secretory granules since they are excluded from the subplasmalemmal zone[3]. On the other hand, evidence is emerging that actin filaments may as well control and/or modulate an active step in exocytosis. We previously proposed that Cdc42 regulates an actin-dependent function in chromaffin cell secretion[4]. The aim of our recent work was to dissect the role of Cdc42 on the actin network re-organization process during exocytosis in PC12 cells. We will briefly review here our findings that led us to propose N-WASP and Arp2/3 as molecules that bridge Cdc42 signaling to the actin cytoskeleton and the exocytotic machinery[5].

RESULTS AND DISCUSSION

Using a growth hormones (GH) release assay[6], we established that a constitutively active mutant of Cdc42 ($Cdc42^{L61}$; defective in GTP hydrolysis) stimulates the secretory activity of PC12 cells (Figure 1A) whereas its corresponding dominant inactive mutant preferentially binding GDP ($Cdc42^{N17}$) slightly decreases it, thus demonstrating the active participation of Cdc42 in large dense-core granule exocytosis. Interestingly, exogenous $Cdc42^{L61}$ localized to the plasma membrane and triggered the formation of actin filament in stimulated PC12 cells (Figure 1B). This observation led us to the idea that stimulation of exocytosis induced by Cdc42 might be related to its ability to promote actin polymerization in the cell periphery, hence suggesting N-WASP as a potential effector for Cdc42.

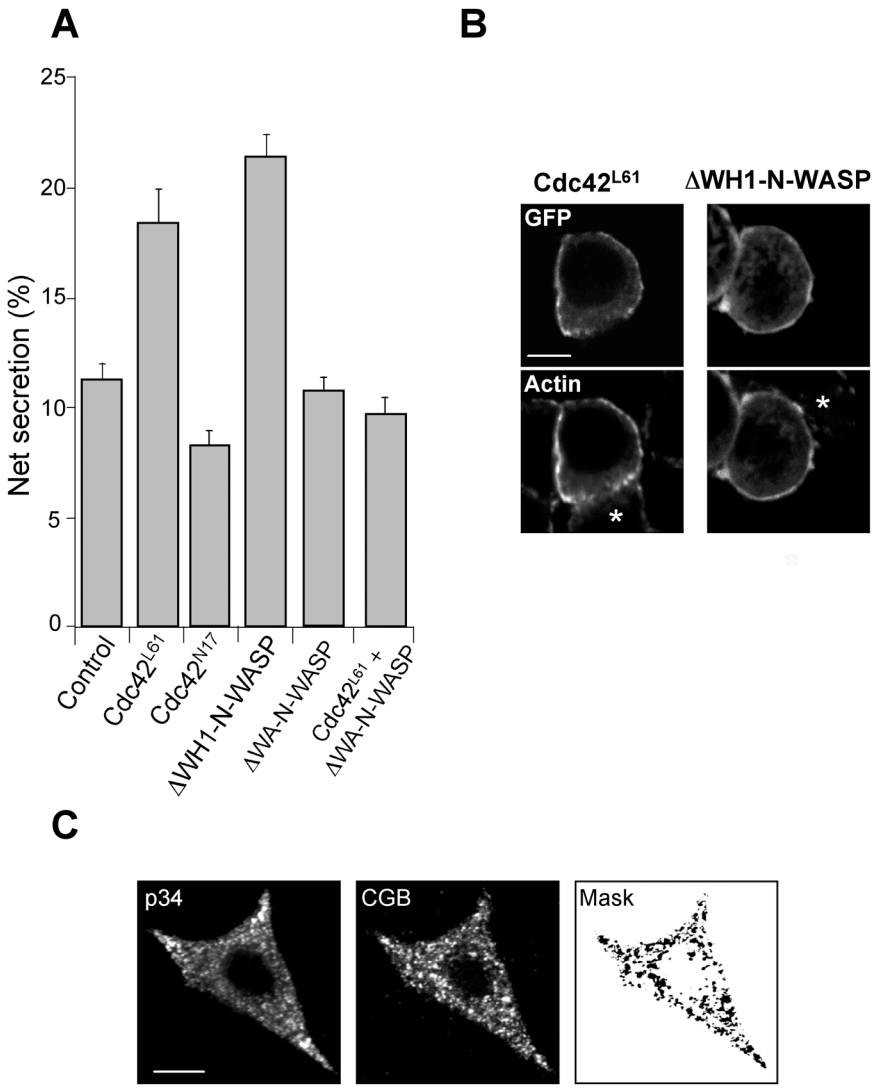

Figure 1. A) Cdc42 and N-WASP enhance the exocytotic release of GH from PC12 cells*:* PC12 cells co-expressing GH and the indicated proteins were incubated for 10 min in calcium-free Locke's solution or stimulated for 10 min with 59 mM K^+. Control cells were transfected with the empty pBR6 vector. Basal release (~ 8%) was unchanged and was subtracted from the release evoked by 59 mM K^+ to obtain the net secretory response. Data are given as the mean values ± SEM (n=3). **B) Active Cdc42 and N-WASP localized in the cell periphery and stimulated actin formation.** PC12 cells expressing GFP-$Cdc42^{L61}$ or GFP-ΔWH1-

N-WASP (GFP) were stimulated for 10 min with 59 mM K^+ and subsequently fixed and stained with rhodamine-conjugated phalloidin to visualize actin filaments (Actin). The asterisks indicate non-transfected cells displaying a classical disruption of the cortical actin network in response to K^+-stimulation. Scale bar represents 5 μm. **C) Intracellular distribution of Arp2/3 in PC12 cells:** PC12 cells were fixed and stained with anti-p34-Arc (Arp2/3 subunit) and anti-chromogranin B (CGB; a marker for large dense core secretory granules) antibodies. Masks representing the region of co-localization are obtained by selecting the pixels double-labeled. Scale bar represents 5 μm.

Accordingly, expression of a dominant active mutant of N-WASP (ΔWH1-N-WASP) stimulated GH release in PC12 cells and enhanced the formation of actin filaments in the subplasmalemmal region to a similar extent as the dominant active GTP-bound $Cdc42^{L61}$ (Figure 1A and B). Moreover, a dominant negative N-WASP mutant unable to induce actin polymerisation (ΔWA-N-WASP) had no effect on GH release (Figure 1A) and did not promote actin filament formation (not shown). Finally, co-expression of ΔWA-N-WASP completely abolished the stimulatory effect of $Cdc42^{L61}$ on GH release (Figure 1A). Taken together, these results elect N-WASP as one of the downstream effector by which Cdc42 organizes the actin architecture required for exocytosis.

The carboxy terminus of WASP family members initiates the growth of new actin filaments by bringing together actin monomers and the Arp2/3 complex[2]. In PC12 cells, Arp2/3 complexes are associated to large dense-core granules as revealed by double labeling experiments with chromogranin B and p34 antibodies (Figure 1C). According to this differential localization, it is tempting to postulate that the interaction between Cdc42, N-WASP, Arp2/3 and the actin monomers takes place only at the interface between granules and the plasma membrane in stimulated cells, providing a way to specifically target local actin filament polymerization to the granule docking and fusion sites.

To conclude, we propose that activation of secretion in neuroendocrine cells does not simply trigger the disassembly of the cortical actin barrier but rather induces a fine remodeling of the peripheral actin network into structures required for exocytosis. Further studies are now required to investigate whether the local

production of actin filaments during exocytosis would serve for docking, scaffolding, fusion and/or membrane retrieval.

ACKNOWLEDGEMENTS

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Quantal release of catecholamines. A new target for β-adrenergic antagonists.

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The use of β-adrenergic blockers (β-B) in the management of hypertension has been lasted for decades. However, its precise mechanisms of action remain obscure because their putative targets (cardiac output, renin secretion or CNS) are not common to all β-B and because the onset of hypotensive effect is delayed several days upon the initiation of therapy.

One possible alternative target is the interaction with the quantal release of catecholamines that sustain the arterial tone. It is known that β-B progressively reduce the arterial tone, which is difficult to understand because β_2 receptor activation relaxes muscular arterioles.

Our hypothesis points towards to a β-B accumulation into secretory vesicles thus displacing catecholamines and the co-release of these drugs together with their agonist thus reducing its action on the postsynaptic cell. We have use amperometry on single chromaffin cell to measure the characteristics of the quantal release of catecholamines and a flow injection system to quantify the release of catecholamines and β-B from stimulated cells.

RESULTS AND DISCUSSION

In order to test the hypothesis of an intravesicular accumulation of the drug in chromaffin granules and its possible secretion by exocytosis, we take the advantage of the fluorescent properties of both β-B. Particularly, labetalol exhibits fluorescence within a light spectrum of wavelengths slightly larger ($\lambda_{335/420}$ nm) than propranolol ($\lambda_{305/354}$ nm) being more suitable for recording. Figure 1a shows the effect of repetitive pulses of 100 µM of the nicotinic agonist dimethyl-phenyl-piperazinium applied with a rotary valve with a 100 µl loop. Cells (2 million) are packaged in a perfusion chamber of a very low dead volume and perfused at 2 ml/min with a HPLC pump [6]. The effluent is passed successively through a fluorimetric detector and an amperometric detector to detect labetalol and catecholamines. Control and cells acutely incubated with labetalol release catecholamines as detected by amperometry but only very small signals come from the fluorescence detector. The origin of these signals could be due to the release of weakly fluorescent compounds released by vesicles like catecholamines. However, cells chronically incubated with labetalol

release the drug together with catecholamines thus increasing the ratio fluorescence/amperometry (figure 1b).

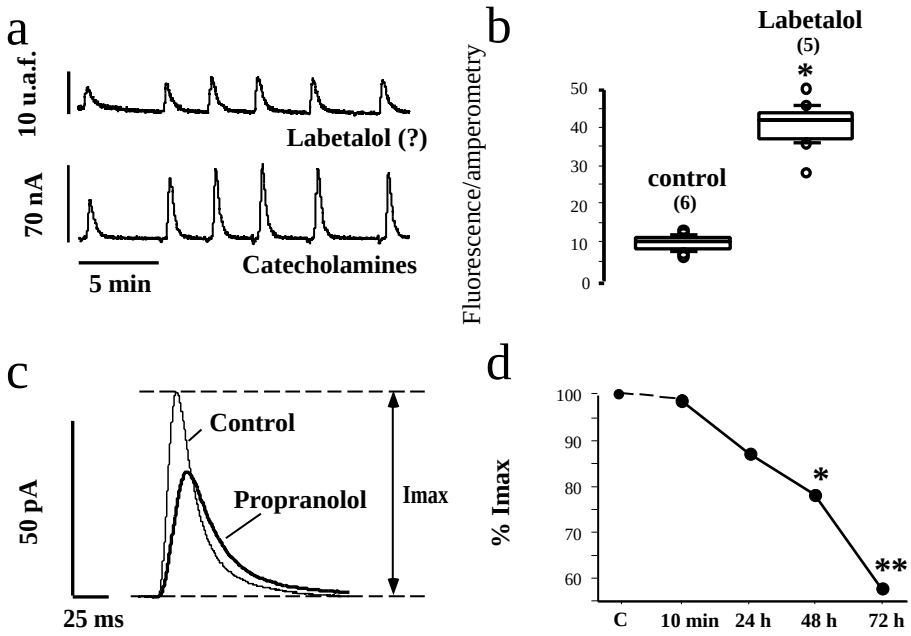

Figure 1. Beta blockers interact with the storage and release of catecholamines. a) Labetalol is released from chromaffin cells stimulated with pulses of DMPP 100 µM. Upper trace show the fluorescent signals whereas the lower show the amperometrical recording from chromaffin cells incubated 30 h with 10 µM of labetalol. **b)** Average from the ratios between fluorescent and amperometry (median ± percentile) from untreated cells (control) and treated (Labetalol). **c)** Representative spikes constructed with the average of kinetics parameters obtained from cells treated with 1 µM propranolol for 72 h. **d)** Normalized data showing the effect of incubation time with propranolol 1 µM on the Imax of secretory spikes. **p*<0.05, ***p*<0.01 student *t* test.

If β-B are entering into chromaffin granules it is likely that they compete with catecholamines interfering their storage. Amperometrical recordings do not exhibit any difference between control and cells that receive acute incubation with 0.1-10 µM of labetalol or propranolol. However, both compounds reduce slow down the exocytosis process and reduce the quantal size when are incubated for

1-3 days (figure 1c). These effects are evident even with low β-B concentrations (100 nM) and are more pronounced with propranolol than with labetalol probably due to the higher liposolubility of the former. The kinetics of exocytosis is more sensitive to β-B treatment than the quantal size. Both are time and concentration dependent (figure 1d).

Many β-Bs are weak bases that progressively accumulate into acidic compartments like chromaffin granules (pH 5.5)[5]. This process seems to be relatively slow when compared with other drugs like amphetamine[2-3] or hydralazine[9], which have rapid uptake. However, this slow time course is compatible with that observed in the clinical practice where several days are required to promote relevant reduction on blood pressure. To our knowledge this is the first time that these presynaptic mechanisms are proposed to explain the action of β-B on hypertension.

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Use of phospho-specific antibodies to demonstrate phosphorylation of Munc18/nSec1 in chromaffin cells.

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In most regulated secretory cells, including chromaffin cells, the signal for exocytosis is an increase in the cytosolic free Ca^{2+} concentration[1]. Another general characteristic of regulated exocytosis is its acute regulation by protein phosphorylation. Many studies over the past 20 years have shown that exocytosis is modulated by protein kinases (PKs) in almost all regulated secretory cell types, including neurons and chromaffin cells[2-4]. Pharmacological approaches using cell permeable activators or inhibitors have implicated a wide range of serine/threonine and tyrosine kinases, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), casein kinase II (CK2), mitogen activated protein kinase, myosin light chain kinase and src family kinases[5]. However, one shortfall of this approach is that the modulation of exocytosis may be indirect. Thus, application of pharmacological modulators of kinase activity (or better still, purified kinases themselves) to permeabilized cells, where receptors and ion channels are bypassed, is a much more rigorous demonstration of a role for kinases in the direct modulation of the exocytotic machinery. A review of the literature reveals that only PKA (cAMP-dependent protein kinase) and PKC (phospholipid/diacylglycerol-dependent protein kinase), or their pharmacological effectors, modulate exocytosis in almost all permeabilized regulatory secretory cell types examined, including exocrine, endocrine and neuronal cells. The primary action of both PKA and PKC in neurons has been shown to be downstream of Ca^{2+} entry, and indeed purified PKA and PKC enzymes enhance exocytosis in permeabilised chromaffin cells[6,7]. Abundant evidence therefore suggests that the effects of PKA and PKC on regulated secretion are due to phosphorylation of components of the exocytotic machinery.

In order to identify the functionally important phosphoproteins that mediate these effects of protein kinases on exocytosis, various labs (including ours) have screened exocytotic proteins for in vitro phosphorylation by purified kinases[4,5]. Although this has revealed many in vitro substrates, relatively few of these have been shown to be phosphorylated in cellular preparations and to be functionally altered in terms of their biochemical characteristics and effect on exocytosis in cells. One such protein is Munc18/nSec1, which is a member of the Sec1 protein family that has been implicated in most membrane fusion events. Indeed Munc18 is essential for synaptic vesicle exocytosis, as indicated

by the complete absence of neurotransmitter release in Munc18-1 knockout mice[8]. As these animals contain a normal complement of docked synaptic vesicles, this suggests a post-docking role for Munc18 in the late stages of exocytosis. Such a role is consistent with the observed effects of Munc18 mutants on the kinetics of catecholamine release in chromaffin cells[9]. However, in chromaffin cells from the above knockout mice, there is a profound impairment of vesicle docking[10], suggesting an additional, early role for Munc18 upstream of tethering. Clearly, the functional role(s) of Munc18 remains elusive, although it is generally agreed that its high-affinity interaction with syntaxin is important for at least one function. Munc18 is phosphorylated *in vitro* by PKC on Ser-306 and Ser-313, and this phosphorylation reduces the affinity of Munc18 for its binding partner, syntaxin, in *in vitro* binding assays[11]. A recent study has demonstrated that mutation of Ser-306 and Ser-313 to glutamate (to mimic the negative charge imposed by phosphorylation of these residues) causes a similar reduction in affinity for syntaxin[12]. Importantly, overexpression of this phosphomimetic mutant in chromaffin cells had the same effect on catecholamine release kinetics as the PKC activator, PMA: decreasing the half-width, rise-time and quantal size of amperometric spikes[12]. Furthermore, as PMA was unable to elicit further changes to spike kinetics upon expression of the phosphomimetic mutant, this strongly suggests that the mechanism by which PKC activation alters exocytotic release kinetics is via phosphorylation of Munc18 on Ser-306 and/or Ser-313.

In light of these recent data, we investigated whether the phosphorylation of Munc18 on Ser-306 and Ser-313 seen *in vitro* also occurred *in vivo* in response to physiological stimulation. To do this, antisera were raised against phosphorylated peptides surrounding these phosphorylation sites. Phospho-specific antisera to both sites were generated, as defined by their ability to detect Munc18 that had been pre-phosphorylated in vitro by PKC but not unphosphorylated protein. Specificity of the antisera was further shown by the fact that neither antiserum detected recombinant proteins mutated at Ser-306 and Ser-313 after the proteins had been incubated with PKC. No specific signal could be detected in secretagogue- or PMA-treated chromaffin cells or synaptosomes using the phospho-Ser306-specific

antibody. This suggests either that the antibody cannot detect endogenous Ser-306-phosphorylated Munc18 or that this site is not phosphorylated *in vivo* under the conditions examined here. In contrast, Munc18 phosphorylation at Ser-313 was readily observed in PMA-treated chromaffin cells and synaptosomes using the phospho-Ser313-specific antibody. In addition, Ser-313 phosphorylation also occurred in permeabilized chromaffin cells in response to free Ca^{2+} concentrations that trigger exocytosis. Maximal phosphorylation was observed at 10 μM Ca^{2+}, the optimum for exocytosis. This phosphorylation is likely caused by PKC, as the PKC inhibitor, bisindolylmaleimide, inhibited the Ca^{2+}-induced increase in phosphorylation observed in permeabilized cells. Furthermore, Ser-313 phosphorylation was also induced in intact cells by histamine, which causes Ca^{2+}-dependent exocytosis in chromaffin cells. Therefore, Munc18 is phosphorylated in adrenal chromaffin cells on secretagogue stimulation, and not only in response to PMA treatment. Interestingly, the phosphorylation of Ser-313 observed with combined Ca^{2+} and PMA treatment was greater than that seen with either treatment individually. It may be that Ca^{2+}-induced membrane fusion frees Munc18 from syntaxin, as when these two proteins are bound Munc18 cannot be phosphorylated by PKC [11].

In summary, we have shown that Ser-313 of Munc18 is phosphorylated in chromaffin cells response to secretagogue stimulation. As we have also found this to be true for rat brain synaptosomes [13], this suggests that activity-dependent phosphorylation of this residue may be a general phenomenon. Intriguingly, both presynaptic protein phosphorylation and modulation of neurotransmitter release kinetics have been implicated in synaptic plasticity. It is conceivable that phosphorylation of Munc18 at Ser-313 - a residue essential for the modulation of catecholamine release kinetics in chromaffin cells - could be a mechanism linking these processes.

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Chromaffin granules as target for the antihypertensive drug hydralazine.

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Several drugs are known to modify catecholamine release from adrenal chromaffin cells with regards to the kinetics and quantal size[1 3]. Here we asked whether the beneficial effects of hydralazine (HYD), a drug used in the treatment of certain forms of drug-resistant hypertension, might be explained by its interference with catecholamine storage, or exocytosis. Our results demonstrate that, even at nanomolar concentrations, HYD accumulates rapidly into secretory granules, thereby causing dramatic changes in the kinetics and the quantal size of individual release events.

RESULTS AND DISCUSSION

Carbon fiber amperometry is a powerful tool for the study exocytosis. It allows measurements of release kinetics at the level of individual exocytotic events and of the apparent quantal size[4]. Using this technique we found that acute application of HYD (10 nM) slowed the rate of transmitter release (Imax from 22.9±2.3 to 13.9±1.9 pA), which was accompanied by a 35 % decrease in quantal size (Q) (Figure 1a). Notably, spike firing frequency was not significantly altered by the drug.

HYD is a weakly fluorescent compound that emits light at 405 nm when is excited at 483 nm. Confocal microscopy of chromaffin cells that were preincubated with HYD revealed that the drug rapidly accumulates into vesicular structures (not shown). The pattern of distribution within the cell was similar to that of the dye acridine orange (AO), which is known to accumulate in secretory granules. We studied the time course of HYD accumulation by standard epi-fluorescence microscopy (Figure 1B). In control conditions, uptake of HYD (10mM) into chromaffin granules occurred with a half-time of 300 s. Interfering with granular acidification by preincubation with 100 nM bafilomycin A_1 prevented accumulation of the drug. This suggests that HYD is mostly compartmentalized by protonation within chromaffin granules, although accumulation in other acidic organelles may occur as well.

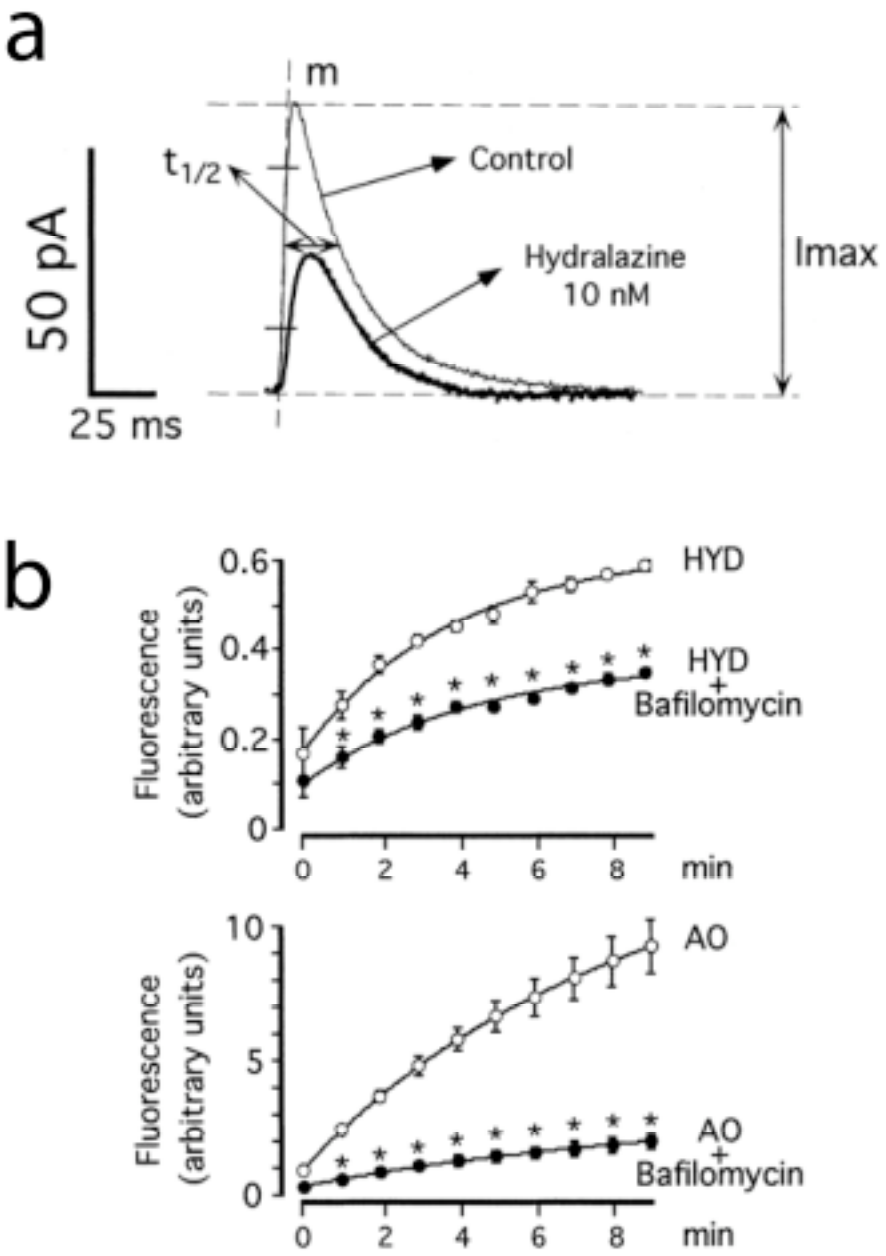

Figure 1. a) Representative spike traces were plotted using the kinetic parameters from averaged values. Hydralazine was applied as a brief 5-second

pulse together with $BaCl_2$ (5 mM). Vertical and horizontal bars are the calibration for the oxidation current and time. **b)** Time course of hydralazine and acridine orange accumulation in single bovine adrenal chromaffin cells. Hydralazine was applied for 10 seconds by pressure at 10 μM. Data also show the effects of incubation with 100 nM bafilomycin. Acridine orange was applied for 10 seconds at 10 nM in the presence or in the absence of 10 nM bafilomycin.

The decreased quantal size in presence of HYD, combined with its granular accumulation suggests the drug may act by displacing the catecholamines to the cytosol. To address this possibility we measured cytosolic catecholamine contents by permeabilizing cells with a 10-second pulse of digitonin (20 μM) in the absence of extracellular Ca^{2+} [5]. Under these conditions digitonin application did not evoke secretory spikes. Any current evoked at the carbon fiber electrode should therefore reflect release of cytosolic catecholamine. When digitonin was applied on a HYD-treated cell, this elevation was significantly increased (2.5 times). This effect is similar to that recently described in chromaffin cells for other weak bases like tyramine and amphetamine[6,7]. In summary, our data suggest that HYD acts by displacing catecholamines from the granule, thus causing a reduced quantal size slowed kinetics of transmitter release. On the systemic level, similar changes in sympathetic neurons would be expected to cause a significant drop in catecholamine release and this could explain many of the therapeutic and side effects of this drug.

A long version of this work you can find in reference 8.

ACKNOWLEDGMENTS

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Differing mechanisms of exocytosis for large dense core vesicles in chromaffin cells and small synaptic vesicles in dopamine neurons.

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Synaptic neurotransmission depends on the exocytosis of neurotransmitter (NT) during the fusion of vesicles with the plasma membrane. A key goal for understanding the mechanism of exocytosis is to determine whether vesicles undergo full fusion or kiss and run exocytosis and how this process may be regulated. Studies using carbon fiber amperometry and capacitance measurements have demonstrated that the predominant mechanism of exocytosis for large dense core vesicles (LDCVs) in chromaffin cells is full fusion, although kiss and run exocytosis can also occur[1-4]. Several studies have demonstrated that phorbol esters and kinase inhibitors can modulate the mode of exocytosis in chromaffin cells[5-9]. The authors generally conclude that the target of these drugs is protein kinase C (PKC) although many of the drugs used are not selective for this kinase. To our knowledge, no studies to date have identified which of the 12 known PKC isoforms is responsible for the modulation of exocytosis. We used a variety of small inhibitory molecules and peptides to further investigate the role of PKC in chromaffin cell exocytosis and to identify the PKC isoform(s) responsible. Calphostine (100 nM), chelerythrine (2 □M), rottlerin (10 □M) and the myristoylated PKC□-pseudosubstrate peptide (10nM), but not Gö6976 (20 nM) or the myristoylated PKC□-pseudosubstrate peptide, 10 nM) markedly slowed the rising and falling phases of quantal events, increased the $t_{1/2}$ while decreasing the maximal peak height (Table 1, all drug concentrations are 2-3 fold their IC_{50} of PKC inhibition). These data suggest that PKC can regulate the formation and size of the fusion pore as well as shifting the mode of exocytosis from full fusion to kiss and run exocytosis. Rottlerin's (but not Gö6976) ability to alter the kinetics of neurotransmitter release suggest that PKC□ and/or PKC_ (but not PKC□ and PKC□) are the isozymes responsible. The presence of PKC□ and □ have been confirmed by western blotting of proteins from chromaffin cell cultures (which also contain fibroblasts) as well as immunohistochemistry (data not shown).

Our data in chromaffin cells suggest that PKC□ and/or PKC□ can switch the mechanism of exocytosis in chromaffin cells from the full fusion to kiss-and-run exocytosis. This is consistent with many previous reports implicating PKC in regulating the mechanism of exocytosis[5-9]. Interestingly, PKC□ and PKC□ are both Ca^{2+} independent

isoforms of PKC. Utilizing Ca^{2+}-independent isoforms of PKC may be an important attribute for a pathway regulating exocytosis, a process that is triggered by Ca^{2+}.

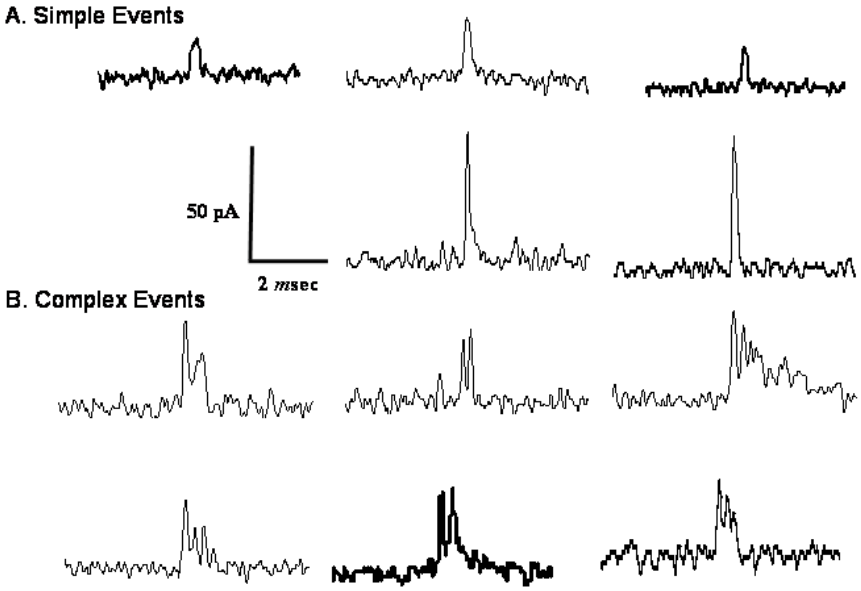

Figure 1. Representative traces quantal dopamine release from rat ventral midbrain dopamine neurons stimulated with K^+. To be considered an *event*, the derivative dI/dt must cross a 4.5 x RMS threshold. The event begins and ends when dI/dt = 0. **A)** Events with derivatives that cross the 3.0 x RMS threshold once in a rising trajectory are *simple*. **B)** Events that cross the 3.0 x RMS threshold multiple times are *complex*.

Since the exocytic machinery is believed to be similar in chromaffin cells and neurons, we examined the kinetics of NT release from ventral tegmental area dopamine neurons in culture. Using carbon fiber amperometry to measure NT release, we found that ~20% of quantal events recorded from neurons did not possess the "simple" (Fig 1a) amperometric spike shape usually seen with single vesicle exocytosis in other cell types. Rather, we observed a "complex" spike shape with multiple peaks (Fig 1b), usually with sequentially decreasing amplitudes, consistent with the release of NT via the high frequency (~4 kHz) flickering of a small diameter (~2 nm) fusion

pore. Complex events had significantly larger quantal sizes than simple events, suggesting that the latter arose from the partial release of transmitter via a single flicker of the fusion pore. The incidence of complex amperometric spikes was enhanced by staurosporine and reduced by phorbol-12,13-dibutyrate (PDBU), demonstrating that exocytosis in neurons can also be regulated by second messenger systems, possibly PKC, although other systems cannot be ruled out at this point.

TABLE 1. Effect of PKC Inhibition on Kinetics of Exocytosis.

Cells were stimulated with 40 mM K^+ administered by puff pipette (2 μm diameter, 10 psi, 3 sec). Catecholamine release was detected amperometrically from at least three cells under control conditions before inhibitors were added to the media.

Treatment	# of Cells	$t_{1/2}$ (ms) (n)	Peak Height (pA)	Quantal Size ‡ (molecules)	Rising Slope ($^{pA}/_{ms}$)	Decay
Control	13	4.9±0.1 (1359)	105± 8.1	2,197,000 ± 90,000	83 ± 3	6 ± 0
Calphostine (10 nM)	22	15.5±0.9* (226)	16.3±1.5*	1,139,00±150,000*	9 ± 1*	20 ± 1*
Chelerythrine (2 μM)	13	14.7±0.4* (718)	16.1±0.7*	1,237,000 ± 68,000*	6 ± 0*	25 ± 2*
Gö6976 (20 nM) Inhibits α and β	11	6.5±0.3 (693)	95.5±5.8	1,954,000 ± 127,000	67 ± 4	8 ± 1
Rottlerin (10 μM) Inhibits δ and ε	10	10.5±0.5* (315)	22±2.7*	847,000± 80,000*	14 ± 2*	15 ± 1*
PKC ζ Pseudo-substrate	24	17.1±0.6* (635)	24.6±1.5*	1,437,00± 70,000*	13 ± 1*	23 ± 1*
PKC ι Pseudo-substrate	8	7.9±0.2 (594)	65.7±3.4	2,295,00± 164,000	43 ± 3	10 ± 0*

‡ Data are the average ± SEM from all cells.

* $P < 0.05$, compared to control using ANOVA.

While the dimensions of the initial fusion pore of LDCVs and SSVs appear to be similar, exocytosis of NT by SSVs appears to be

accomplished by the rapid flickering (opening and closing) of the fusion pore, whereas exocytosis of LDCVs is primarily accomplished by full fusion. Although the fusion pore of LDCVs can flicker, this most likely reflects the failure of the fusion pore to radially expand into full fusion[1-2] which is in contrast to SSVs where the fusion pore opens and closes (or rhythmically fluctuates in size) once or several times without undergoing full fusion. Furthermore, the flickers in SSVs were ~1000 fold shorter than those observed in LDCV foot events and the SSV flickering frequency was ~25 fold faster. Our results suggest that although full fusion is the dominant form of exocytosis for LDCVs, dopaminergic SSVs release NT through a rapidly flickering fusion pore, a novel mechanism that is distinct from kiss and run exocytosis as well as full fusion.

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Amperometric secretory spikes in mouse adrenal slices.

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Most studies to analyse the kinetic of the exocytotic catecholamine release responses have been performed in primary cultures of chromaffin cells dissociated from adrenal medullary tissues, mostly from bovine and rats, using non-oxygenated Krebs-HEPES (KH) solutions lacking bicarbonate and phosphate anions. In this work, we have used single chromaffin cells in acute fresh mouse adrenal slices, bathed in Krebs-bicarbonate solutions equilibrated with 95% O_2/5% CO_2 (BBS), a preparation closer to the physiological situation.

With the use of carbon fiber microelectrodes, we have compared the kinetic properties of the amperometric exocytotic spikes obtained in cells in slices, with those obtained in mouse chromaffin cells in culture (2-4 days), as well as the kinetic of the spikes obtained in slices bathed in Krebs-HEPES solution, instead of BBS. To get the amperometric secretory spikes, 5 s pulses of solutions of BBS or KH containing acetylcholine (ACh) or high K^+ were applied.

Here, we show that the kinetic of the amperometric secretory spikes in culture cells differ not very much from those obtained from slices. Besides, we also demonstrate that the components of both types of the bath solution used are not of critical importance to modulate the kinetic of the exocytosis.

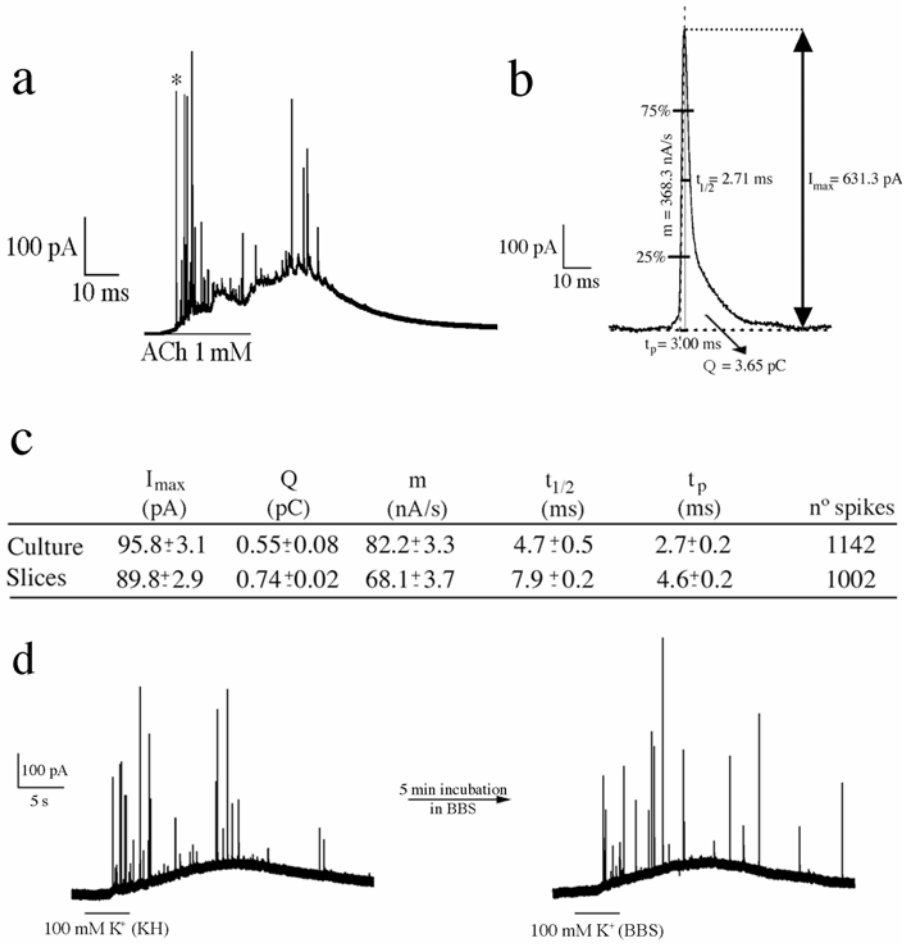
RESULTS AND DISCUSSION

Most of the experiments in the literature have been mainly performed in isolated and cultured cells, using prolonged (minutes) depolarizing pulses of high K^+ or action potentials, probably due to technical complexity of using slices of tissue, bathed with oxygenated bicarbonate-based solutions.

To characterize the amperometric exocytotic signals in chromaffin cells of mouse adrenal slices, exogenous application of 1 mM ACh or 100 mM K^+ in BBS-based solutions was first tried (figure 1a). A burst of amperometric spikes was recorded after 5 s of stimulation. Typical single fast amperometric event evoked by ACh, with a detailed analysis of its kinetic parameters, is shown in figure 1b. The following kinetic parameters were measured: I_{max} (peak amplitude); Q (charge); m (ascending slope, calculated from the linear part of the trace located between 25% and 75% of the I_{max}); $t_{1/2}$ (half-

width or duration of the amperometric signal at 50% of its peak amplitude); and t_p (time-to-peak, determined between the point at which the back-extrapolation of the slope line crossed the base line at the point of I_{max}). The average values of the spike kinetic parameters obtained (808 spikes when the stimulus was ACh, and 756 spikes when the stimulus was K^+, from 7 cells) are: 114±4.10 pA; 1.00±0.03 pC; 73.5±3.47 nA/s; 7.9±0.20 ms; and 4.6±0.17 ms for ACh and 99.1±3.45 pA; 0.82±0.03 pC; 68.9±3.92 nA/s; 7.0±0.16 ms; and 4.4±0.33 ms for K^+.

These data exhibit drastic and important differences with those published by Herrero and coworkers[1] in cultured mouse chromaffin cells. These authors perfused 1 mM ACh in HEPES-based solutions for 5 s, obtaining amperometric spikes with the following parameters: I_{max}=22.8±1.8 pA; Q=0.48±0.02 pC; m=5.2±0.6 nA/s; $t_{1/2}$=19.9±6.1 ms; t_p=22.5±1.5 ms (n=250 spikes). To be able to elucidate if differences were due to the physiological preparation (culture vs slices) or to the perfusion solution (BBS- vs HEPES-based solutions), additional experiments in chromaffin cells in culture, using BBS as the perfusion solution, were performed (figure 1c). Statistical analysis, carried out by the non-parametric Mann-Whitney U test, revealed that spikes from culture cells have shorter times of release ($t_{1/2}$: 4.7±0.5 vs 7.9±0.2 ms; t_p: 2.7±0.2 vs 4.6±0.2 ms). We think these are very surprising results since cells in slices should show a higher quality characteristics than cells in culture. We believe that this kind of stimulus application, by means of a multibarrelled glass pipette positioned closed to the cell under study, also stimulate a great number of neighbouring cells. Catecholamines secreted by them could as well be detected for the carbon fiber electrode, which would reach it with a very different kinetic. Probably, this is the reason why we have found extremely fast and large single events accompanied by other of slower and smaller amplitude in the same recording in slices. So, we guess that average kinetic parameters of pooled results shown in figure 1c are distorted by those amperometric events coming from neighbouring cells to the one being recorded.



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	I_{max} (pA)	Q (pC)	m (nA/s)	$t_{1/2}$ (ms)	t_p (ms)	nº spikes
Culture	95.8±3.1	0.55±0.08	82.2±3.3	4.7±0.5	2.7±0.2	1142
Slices	89.8±2.9	0.74±0.02	68.1±3.7	7.9±0.2	4.6±0.2	1002

Figure 1. Amperometric exocytotic events obtained in different experimental conditions, recorded from a single cell of mouse adrenal medulla. a) Original recording obtained with 5 s stimulus of 1 mM ACh. **b)** The amperometric spike marked with an asterisk in a) is represented in an expanded time scale with its kinetic parameters indicated. **c)** Detailed analysis from individual spikes in cells isolated and maintained in culture (n=20, n=1142 spikes), and in cells in slices (n=14, n=1002 spikes), following the protocol in a) using 100 mM K^+ as a stimulus. **d)** Typical amperometric recordings of a cell in slice of tissue superfused with KH and BBS, following the protocol in a).

Afterwards, we explored the effect of the buffer solutions (BBS vs KH) on catecholamine secretion. To elucidate this point, we performed experiments stimulating the slice while bathing in BBS or

in KH, alternatively. After several stimulations in one of the buffers, the slice was then superfused during 5 min with the other, before applying a new stimulation (figure 1d). This sequence was repeated one more time (not shown) in order to be sure that the level of secretion hadn't decreased during the experiment. Then, using different slices, we performed the same protocol but changing the buffers order, and similar results were found. Analysis of amperometric spikes from mouse adrenal slices after superfusion with KH and BBS was, respectively: I_{max}: 98.2±2.88 vs 96.6±3.35 pA; Q: 0.72±0.02 vs 0.67±0.02 pC; m: 77.9±5.76 vs 76.0±4.15 nA/s; $t_{1/2}$: 6.3±0.11 vs 6.0±0.14 ms; t_p: 3.85±0.02 vs 3.80±0.01 ms. Data are pooled from 944 (KH) and 616 (BBS) individual secretory spikes, from 10 and 8 cells, respectively.

These results imply the following conclusions between single secretory amperometric events between mouse adrenal chromaffin cells in situ (fresh slices) and cultured cells: (1) ACh and high K^+ produced similar secretory responses; (2) no differences were observed between cells in culture and in slices, when the following kinetic parameters are compared: I_{max}, Q and m. However, amperometric spikes from cells in culture exhibit smaller $t_{1/2}$ and t_p than those in slices, probably due to the contamination with secretory vesicles coming from neighbour cells; (3) no differences were seen in the kinetic parameters of spikes when cultured cells or slices were superfused with oxygenated BBS or with non-oxygenated KH solutions and (4) amperometric secretory events described here are much faster than those previously reported[1]. This suggests that mouse chromaffin cells undergo exocytosis following a fast kinetic pattern, which approaches that of neurons[2].

In conclusion, we have recorded extremely fast spikes in chromaffin cells in situ ($t_{1/2}$=1.66±0.05 ms, m=234.0±4.1 nA/s), much faster than that previously reported using ACh as a stimulus in cultured mouse chromaffin cells ($t_{1/2}$=19.9±6.1 ms, m=5.2±0.6 nA/s)[1]. We do not know the exact reasons for it, but it is plausible that the different experimental conditions used such as slice versus cultured cells, or even the different sampling rate, 14.5 kHz versus 1kHz, explain these different results.

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Lipids at exocytotic sites: How and why?

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To date, many proteins that catalyze the formation, targeting and fusion of secretory vesicles with the plasma membrane have been identified. However, the lipid composition of vesicles and their target membrane is also critical and lipid modifications may be required in several stages of the exocytotic pathway. Phospholipase D (PLD) generates phosphatidic acid (PA), a multifunctional lipid that can activate selected enzymes, serve as protein attachment site or alter membrane curvature. Expression of the catalytically inactive $PLD1_{K898R}$ mutant in PC12 cells or microinjection of $PLD1_{K898R}$ into chromaffin cells strongly inhibits secretion, supporting the notion that PLD1 plays a major role in the exocytotic pathway. Using deletion and mutagenesis analysis, we found that the PX and $PI4,5P_2$-binding domains are critical for the association of PLD1 to the plasma membrane in resting PC12 cells. However, in secretagogue-activated cells, PLD1 lacking palmitoylation falls into the cytoplasm and fails to activate exocytosis, suggesting that a translocation step into lipid rafts is required for PLD1 to function in exocytosis. We propose that exocytotic sites are determined by the local formation of lipid microdomains, which are potentially important to allow structural and spatial organization of the exocytotic machinery. Among them, PLD1 seems to play a decisive role in the late stages of exocytosis, most likely by adding specific lipid modifications that may be required to allow the membrane fusion machinery to function.

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Membrane fusion is the final step of all vesicular trafficking reactions in eukaryotic cells including exocytosis of hormones and neurotransmitters. In the case of biological membranes, fusion reactions require specific proteins that pull membranes close together to destabilize the lipid/water interface and initiate mixing of the lipids. Several models for protein-mediated fusion reactions have been proposed that differ in the interactions of the fusion proteins with membrane lipids and in the transition states of the lipid bilayers, but they share as common feature the participation of lipids in the late stages of the fusion reaction[1]. Hence, fusion pores in exocytosis have been described as aqueous pores of irregular size that open and close rapidly before irreversibly expanding, supporting the notion that even if fusion reactions are mediated by proteins, fusion pores themselves are essentially lipidic[2]. In other words, the lipid composition of vesicles and their target membrane is likely to be critical and lipid modifications at the site of fusion may be required to allow the membrane fusion machinery to function. We have concentrated our attention on PLD that brakes phosphatidylcholine to produce PA and choline. Two mammalian isoforms, PLD1 and PLD2, being differently regulated by small GTPases, have been identified. Here, we will review our results that led us to propose PLD1 as a key factor for the late stages of exocytosis and discuss the mechanisms that specifically recruit activated PLD1 to the granule docking sites in stimulated chromaffin cells.

RESULTS AND DISCUSSION

In the presence of a primary alcohol, PLD is able to catalyze a specific reaction, the transphophatidylation, to produce the corresponding phosphatidyl-alcohol instead of phosphatidic acid. This unique reaction has been used for many years to reveal the activation of PLD in cells and tissues. To probe the idea that PLD plays a role in the exocytotic reaction, we first compared the PLD activity in subcellular fractions prepared from resting and stimulated chromaffin cells. We found very little PLD activity in the fractions prepared from resting cells but detected a peak of PLD activity in the plasma membrane-containing fractions obtained from stimulated cells[3]. Thus, secretagogue-evoked stimulation and exocytosis is

accompanied by the activation of PLD at the plasma membrane. To establish more directly the role of PLD in exocytosis, we examined the effect of overexpressing in PC12 cells wild type PLD1 or PLD2 or the corresponding catalytically inactive mutants. Co-transfection with a plasmid encoding for growth hormone GH was used to monitor exocytosis in transfected cells[4].

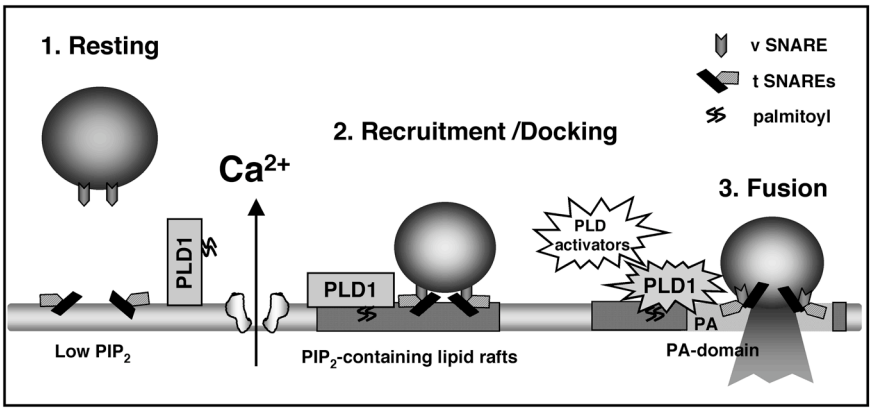

Figure 1. Model for the involvement of PLD1 in regulated exocytosis. In resting cells, PLD1 is associated to the plasma membrane through its N-terminal PX domain. Stimulation and calcium entry triggers the formation of PIP_2-containing micro-domains in the plasma membrane that cluster SNARE complexes and recruit PLD1 through its palmitoylated PH domain. Following granule docking, activated PLD1 locally elevates PA, which promotes the late fusion process.

We found that wild type PLD1 strongly enhanced the exocytotic response, whereas wild type PLD2 had no effect[3]. Moreover, the catalytically inactive $PLD1_{K898R}$ mutant abolished almost completely the exocytotic response evoked by high potassium, whereas the inactive PLD2 mutant had no effect[3]. In line with these results, microinjection of $PLD1_{K898R}$ into chromaffin cells strongly reduced the exocytotic response in a dose-dependent manner[3]. Thus, PLD1 but not PLD2 plays a role in the exocytotic pathway in chromaffin and PC12 cells.

Amperometry provides a number of additional information including the number of granules that have been released given by the number of spikes, the average content of the granules, and the

kinetics of the pore formation, expansion and closure reflected by the rising and decay phases of each spike. We investigated further the step of the exocytotic pathway that requires PLD1 by analyzing the residual spikes in cells microinjected with the inactive PLD1 mutant. $PLD1_{K898R}$ not only inhibited the extent of secretion but it also affected the characteristics of the individual spikes by significantly increasing the spike rise time[3]. Since the spike rise time is thought to reflect the kinetics of the fusion pore opening or expansion, we concluded from these results that PLD1 is probably not involved in the recruitment or movement of the granules to the exocytotic sites, but most likely in the very late fusion step of the granule membrane with the plasma membrane.

The possible involvement of PLD1 in the fusion process implies a tight temporal and spatial regulation of its enzymatic activity. In vitro, PLD1 has a low basal activity and requires activation in a synergistic manner by protein kinase C, Rho and ARF family members[5]. Hence, we found that ARF6 and PLD1 directly interact at sites of exocytosis in PC12 cells, and we could demonstrate that ARF6-stimulated PLD1 activation at the plasma membrane is critical for exocytosis[6]. PLD1 has many membrane-interacting domains including a PX domain at the N-terminus known to interact with various phosphoinositides, a PH domain containing two palmitoylated cysteines, and a central PIP_2 interacting domain[5]. We expressed in PC12 cells several PLD1 proteins mutated in these domains, namely a Δ-PX mutant lacking the PX domain, a protein mutated at the cysteine residues 240 and 241 that cannot be palmitoylated[7] and a PLD1 mutated at the PIP_2-binding site that lost its binding capacity to phosphoinositides[8]. We investigated in parallel the distribution of these proteins in resting and stimulated PC12 cells and their effect on GH secretion. In contrast to the wild type protein found at the plasma membrane in resting and stimulated cells, PLD1 mutated in the PIP_2-binding site was cytosolic both in resting and stimulated cells[9]. Conversely, this mutant was unable to stimulate secretion[9]. Δ-PX-PLD1 was also cytosolic in resting cells but this mutant was recruited to the plasma membrane upon cell stimulation[9]. Interestingly, Δ-PX-PLD1 stimulated secretion to a similar extent that the wild type PLD1[9]. This suggests that the PX

domain is required to maintain PLD1 at the plasma membrane in resting cells but it is not sufficient to recruit PLD1 to the granule docking sites for its functional involvement in the exocytotic machinery. In contrast, PLD1 that is not palmitoylated is still at the plasma membrane in resting cells, most likely through its PX domain, but it falls in the cytosol upon cell stimulation and it is unable to stimulate secretion[9]. Thus, the palmitoylated PH domain is necessary for the recruitment of PLD1 to the exocytotic sites.

Taken together, these observations suggest that the association of PLD1 to the plasma membrane occurs through distinct mechanism in resting and stimulated cells, implying to some extent that secretagogue-evoked stimulation induces changes in the lipid composition or organization in the plasma membrane to prepare the docking sites for granules (see Figure 1). Accordingly, the appearance of PIP_2-binding sites in the plasma membrane that are required for exocytosis have been described[10], and cholesterol- and PIP_2-enriched rafts seem to be critical to the clustering and function of SNARE proteins[11,12]. Once recruited at the granule docking site, how may PLD1 play a role in the late fusion step? PLD1 produces PA, a cone-shaped lipid that has intrinsic negative monolayer curvature propensity. Fusion of liposomes or viral fusion is facilitated by adding compounds that promote negative curvature. Thus, when granules are recruited and docked at the plasma membrane through the formation of the SNARE complexes, the local increase of PA might induce deformations at the point of contact between the two membranes, thereby favoring the formation or the expansion of the fusion pore. Alternatively, PA may recruit specific proteins involved in the late fusion step or be rapidly transformed into other signaling lipids. Additional experimental evidence is now required to prove or refute these possibilities.

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Current Understanding of the O_2-Signalling Mechanism of Adrenal Chromaffin Cells.

- The Peter Baker's Lecture-

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Activation of the splanchnic nerve during stress stimulates the chromaffin cells of the adult adrenal gland to release catecholamines into the circulation. This vital component of the fight-or-flight response is characterized by several physiological changes that allow the animal to combat or flee the stressor. In some animals however, splanchnic innervation is immature at birth, yet adrenal catecholamine secretion has been shown to occur during physiological stresses, such as hypoxia. In this paper, I will review the current understanding of the mechanism of this non-neurogenic, hypoxia-induced secretion of catecholamines from neonatal chromaffin cells. Hypoxia induced catecholamine secretion is mediated through an O_2-signalling pathway that appears to be preferentially expressed in neonatal chromaffin cells, insofar as direct responses of the chromaffin cells to hypoxia are lost along a time course similar to the maturation of the splanchnic innervation. The O_2-sensing mechanism appears to involve a mitochondrial based O_2-sensor and reactive oxygen species intermediates that regulate the activity of several K^+ channels. This in turn, is thought to depolarize the chromaffin cell and broaden action potentials, which increases Ca^{2+} in the cytoplasm and evokes catecholamine secretion.

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Cell Biology of the Chromaffin Cell
R. Borges & L. Gandía Eds.
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In adult animals, physiological or metabolic stress increases activity of the sympathetic nervous system, leading to acetylcholine release from splanchnic nerve endings that innervate the adrenal chromaffin cells (AMC). Released acetylcholine activates nicotinic receptors in the plasma membrane of AMC, which ultimately causes catecholamine exocytosis into the blood. The principle role of circulating catecholamines is to ensure that adequate blood flow to vital organs is maintained so that the animal may combat the stress. Interestingly, in species that are relatively immature at birth, such as rat and man, sympathetic innervation to target organs, including the adrenal is not functional[1]. However, despite this lack of neurogenic control of catecholamine exocytosis in neonatal animals, hypoxia is a potent physiological stress that evokes catecholamine release from AMC[2, 3, 4].

The hypoxic stimulus for neonatal animals appears to arise from two sources. The first is due to the lowered O_2 and elevated CO_2 experienced during birth, resulting from intermittent occlusions of the umbilical cord[5]. The second source of hypoxia arises from intermittent apneas due to interruption in the regular pattern of respiration; a process that is a normal component of the maturation of breathing. Both of these stimuli are thought to contribute to the transition from fetal to air breathing life because catecholamines, released from the chromaffin cells, play pivotal roles in the clearing of lung fluid and in the secretion of surfactant.

Indeed, Seidler and Slotkin[1] demonstrated that blocking □-adrenergic receptors during exposure of newborn rats to hypoxia severely compromised the animal's survivability. Adrenalectomy dramatically reduced neonatal rat survival during hypoxia, but blocking catecholamine release from sympathetic nerve endings did not[1]. In contrast, removing the adrenals from adult rats, did not compromise survival of these mature animals during hypoxia[1] and low O_2 also failed to initiate catecholamine secretion from adrenals that had received a more mature sympathetic innervation[7]. A final indication that sympathetic innervation plays an important role in regulating the direct

sensitivity of neonatal AMC to hypoxia comes from experiments were unilateral denervation of adult adrenals resulted in return of the non-neurogenic, hypoxia-induced catecholamine secretion from the denervated, but not the innervated gland[7].

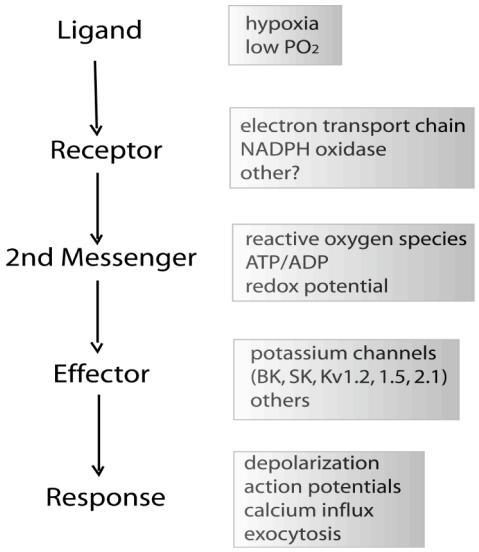

Figure 1. A general model of the O_2-signalling pathway. O_2-sensing occurs via a pathway that can be considered analogous to any ligand-receptor pathway. This model, and the proposed components (boxes), were developed from work on the carotid body glomus cell and pulmonary arteriole myocyte.

There also appears to be a role for adrenal-derived catecholamines in the activation of cardiac β-adrenergic receptors, which promotes survival of neonates during hypoxia. Administration of the β-blocker, phenoxybenzamine concomitantly with hypoxia resulted in a significant alteration of cardiac function. This was characterized by a decline in heart rate, slowing of sinus rhythm, and cardiac failure[6].

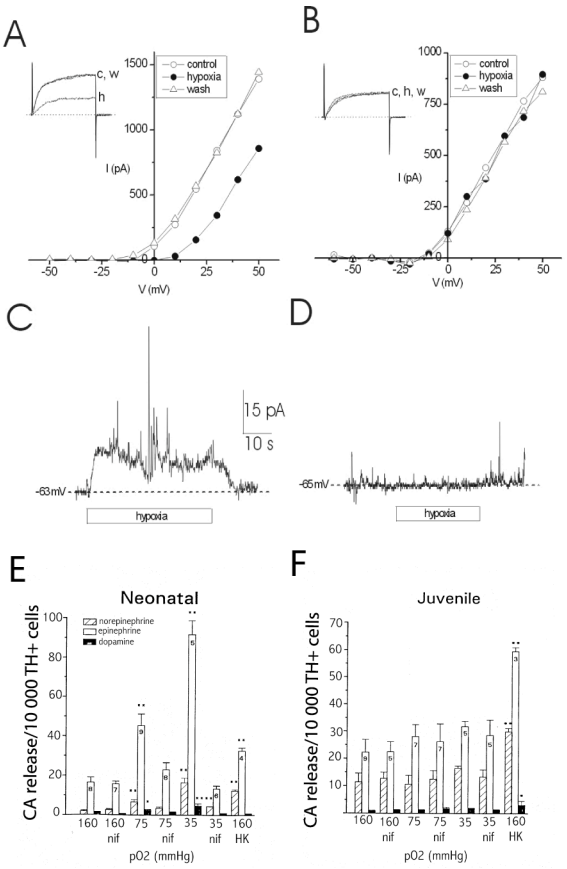

Figure 2. N**eonatal but not juvenile adrenal chromaffin cells are hypoxia-sensitive. A.** I/V plot showing the reversible suppression of outward currents by hypoxia, in neonatal AMC. Hypoxia (h) inhibits currents from the normoxic control (c) level, and the effects are reversible upon wash (w). **B.** hypoxia failed to suppress outward currents in juvenile AMC. The insets in A and B are current traces at a potential of +30 mV from the holding potential of –60 mV. **C**. hypoxia induces a receptor potential of ~ 15 mV in singly isolated neonatal, but not juvenile AMC (**D**). **E**.**&F.** hypoxia induces catecholamine secretion in cultures of neonatal but not juvenile AMC, which was inhibited by the L-type Ca^{2+} channel blocker, nifedipine (nif). Note that 30 mM K^{+} (HK) induced secretion from cells of both age groups, and indicates that juvenile AMC respond have a functional exocytosis mechanism. **C-F**

reproduced with permission from ref 3. These data demonstrate that O_2-sensing by AMC is developmentally regulated.

Taken together, these data suggest that AMC from neonatal animals express a mechanism for directly sensing blood PO_2, which can be considered an adaptation of the fight-or-flight response that is designed to promote survival at, or shortly following birth.

A general model of the O_2-signalling pathway. The work described above lead us to hypothesize that AMC express a developmentally regulated O_2-signalling pathway that mediates catecholamine secretion during hypoxia. O_2-sensing has been well characterized for a number of cell types. These include the prototypical O_2 chemoreceptor, the glomus cell of the carotid body[8], and the pulmonary arteriole smooth muscle cell, which is involved in hypoxic pulmonary vasoconstriction[9]. These specialized, or professional O_2-sensitive cells express an O_2-signalling pathway that can be considered analogous to any ligand-receptor pathway (Figure 1).

In the O_2-signalling pathway, the molecular identification of the O_2-receptor (colloquially known as the O_2-sensor) is a highly contentious issue. As indicated in Figure 1, the O_2-sensor has been proposed to constitute part of the mitochondrial electron transport chain[11-13], as a protein complex similar to the neutrophil NADPH oxidase[14,15], or as an unknown plasma membrane protein directly associated with ion channels[16]. Regardless of the identity of the O_2-sensor, which appears to be dependent upon the cell type being investigated, hypoxia (the ligand) alters that activity of the O_2-sensor, leading to alterations in a 2nd messenger. Several potential 2nd messengers have been proposed. These include reactive oxygen species (ROS) [11,12], cellular redox status[9], and a change in ATP concentration[13, 17].

The activation, or inhibition, of the 2nd messenger component of the O_2-signalling pathway during hypoxia leads to modulation of the effectors, which in turn evoke a physiological response. The effector molecules that ultimately lead to activation of the response have been

well characterized in numerous professional O_2-sensors, and are thought to be voltage-dependent K^+ channels. The types of K^+ channels known to play roles as the effectors in the O_2-signalling pathway are the large, and small conductance Ca^{2+}-dependent K^+ channels (BK and SK, respectively)[9,10,19] and delayed rectifier K^+ channels (K_v 1.2/1.5 and 2.1)[20]. Although other types of ion channels have been implicated[18], this paper will focus on K^+ channels. For AMC, the response is catecholamine secretion (see above). Whereas in glomus cells, it is the release of neurotransmitters to activate the respiratory reflex[10,18].

The O_2-signalling pathway of neonatal AMC. We initially tested the hypothesis that neonatal (postnatal day 1-2; non-innervated), but not juvenile (postnatal day 14-21; innervated) AMC express the O_2-signalling pathway. This was tested by preparing cultures of AMC from each age group and assaying them for hypoxia sensitivity using whole-cell patch clamp recording and HPLC to detect catecholamine secretion[3,13,21]. Figure 2 summarizes the effects of hypoxia on outward currents (Figure 2A and B), membrane potential (C and D) and catecholamine release (E and F) from neonatal and juvenile AMC, respectively.

Cultured AMC were initially exposed to normoxic conditions (PO_2 ~ 150 mmHg), followed by acute hypoxia (~5 mmHg for 2 min in patch clamp experiments and 1 hr for catecholamine release measurements), and then returned to normoxia. It can be seen in Figure 1A, C and E that neonatal AMC responded to hypoxia with a reversible inhibition of outward currents, membrane depolarization that was often associated with action potentials, broadening of action potential duration in spontaneously active AMC (data not shown; see reference 3), and catecholamine secretion[3, 21]. Note that in Figure 1 E, hypoxia-induced catecholamine release from neonatal AMC was blocked by 10 μM nifidepine, suggesting that Ca^{2+} influx through L-type Ca^{2+} channels may be an important step the O_2-signalling pathway. Juvenile AMC exposed to the same protocol failed to respond to hypoxia in any

of the parameters tested (Figures 1B, D and F), supporting the hypothesis that the O_2-sensitivity of AMC is regulated in some way by sympathetic innervation.

What types of K^+ channels are involved in mediating the acute O_2-sensitivity of AMC? Are the same channels involved in the inhibition of outward currents and initiation of the membrane depolarization? We used perforated patch whole-cell recording to address these questions[22]. Exposure of neonatal AMC to inhibitors of various classes of K^+ channels was used to pharmacologically identify the types of K^+ channels involved. We observed that ~ 60% of the hypoxia-sensitive outward current, IKO_2, was blocked by iberiotoxin[22], a potent and specific inhibitor of BK channels. The remaining ~ 40% of IKO_2 was blocked by TEA, indicating that it is likely composed of delayed rectifier channels. Interestingly, in the adrenal-derived MAH cell line, this delayed rectifier component appears to be comprised of 4-AP sensitive K_v 1.2/1.5 heteromultimeric K^+ channels[24]. It is still not known if K_v 1.2/1.5 channels are involved in the O_2 sensitivity of neonatal AMC. A third O_2-sensitive component of IKo_2 was found to be activated by hypoxia. This current was sensitive to glibenclamide and activated by pinacidil[22], or chromanklin[23], which tentatively identifies it as an ATP-dependent K^+ current (K_{ATP}). The molecular identity of these K_{ATP} channels is still unknown.

It was very interesting to us that none of the three components of IKO_2 appeared to contribute to the membrane depolarization (receptor potential) observed upon exposure of neonatal AMC to hypoxia[22]. Evidence for this came from current-clamp experiments in the presence of inhibitors of the various components of IKO_2. Neither iberiotoxin nor TEA blocked the receptor potential. Glibenclamide however, augmented the magnitude of the receptor potential, suggesting that K_{ATP} channels may function to limit the size of the depolarization during hypoxia, perhaps to reduce ATP consumption during this physiological stress[22, 25]. Evidence from two other labs suggests that hypoxia inhibits apamin-sensitive SK channels to initiate the receptor potential[19, 26], and

we observed that SK channels might be involved in generation of the receptor potential in MAH cells[24].

What is the O_2-sensor and 2nd messenger pathway that leads to K^+ channel modulation in neonatal AMC? To answer this, we again employed whole-cell recording, and monitored reactive oxygen species (ROS) levels in neonatal AMC with fluorometric and chemiluminescent indicators. Several lines of evidence suggest that the O_2-sensor is a component of the mitochondrial electron transport chain and the 2nd messenger is ROS. Firstly, the hypoxic inhibition of outward currents was reversed by exogenous application of H_2O_2, and blocked / mimicked by the ROS scavenger, N-acetyl-l-cysteine, which suggests that ROS are decreased during hypoxia. Second, by directly measuring ROS with 2, 7-dichlorofluorescine and luminol chemiluminescence we confirmed a role for ROS as the 2nd messenger (R.J. Thompson, J.A. Buttigieg and C.A Nurse, unpublished). Interestingly, hypoxia-induced changes in ROS were not observed in juvenile AMC. These observations, taken together with the well described modulation of K^+ channels by ROS suggests that H_2O_2 may be the important 2nd messenger in the O_2-signalling pathway of neonatal AMC (see Figure 3).

In parallel studies to the ones described in the preceding paragraph, we found that inhibitors of the mitochondrial electron transport chain that block electron flow at sites upstream (i.e. proximal) of the classical O_2 binding site in cytochrome C oxidase (complex IV) both mimic and attenuate responses of neonatal AMC to hypoxia. The proximal ETC inhibitors, rotenone, antimycin A and myxothiaxol were all found to suppress outward currents in neonatal AMC. Application of these ETC inhibitors concomitantly with hypoxia resulted in no further suppression of outward currents. Furthermore, the ETC inhibitors decreased ROS levels in neonatal AMC in a manner that was not additive with hypoxia, suggesting convergence of the two stimuli (R.J. Thompson, J.A. Buttigieg and C.A Nurse, unpublished). In contrast,

application of 5 mM cyanide, a competitive inhibitor for the O_2 binding site in complex IV, failed to mimic or block the effects of hypoxia.

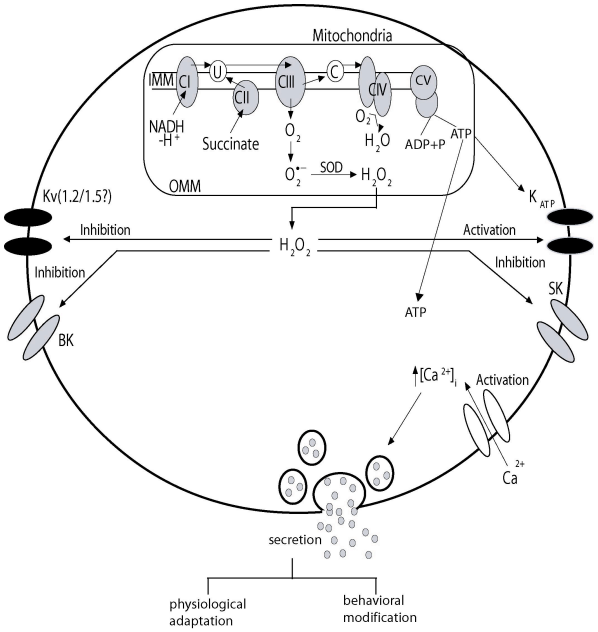

Figure 3. A working model of the O_2-sensing mechanism of neonatal adrenal chromaffin cells. Hypoxia is detected by the electron transport chain, a component of the inner mitochondrial membrane (IMM), reducing the production of superoxide radical (O_2^-). Although the image depicts ROS generation from complex III (CIII) a role of complexes I (CI) or II (CII) cannot be ruled out. O_2^- is rapidly dismutated to H_2O_2 by the mitochondrial enzyme, superoxide dismutase (SOD) and crosses the outer mitochondrial membrane (OMM). The overall decrease in cytoplamic H_2O_2 modulates plasma membrane ion channels. It is proposed that SK channels are inhibited and K_{ATP} channels activated, resulting in a receptor potential. Additionally, in spontaneously active AMC, H_2O_2 is hypothesized to inhibit large conductance Ca^{2+}-dependent (BK) and delayed rectifier (K_v 1.2/1.5) channels, which broadens action potentials. The combined receptor potential and modulation of the action potential waveform opens L-type Ca^{2+} channels, causing Ca^{2+} influx and catecholamine exocytosis into the blood.

A model of the O_2-signalling pathway of neonatal AMC, which was developed from our work and that of several other labs, is presented

in Figure 3. It can be seen that the hypoxic stimulus, occurring at birth or during apnea, is 'sensed' by the proximal mitochondrial electron transport chain. This evokes a decrease in mitochondrial ROS production and corresponding drop in ROS in the cytoplasm. It is then thought that decreased ROS differentially modulates at least four K^+ channels, resulting in a membrane depolarization, action potential generation / broadening, Ca^{2+} influx though L-type channels, and catecholamine exocytosis.

FUTURE DIRECTIONS

Some components of the O_2-signalling pathway require experimental confirmation, and several interesting questions remain unanswered. Despite an extensive understanding of the pathway at the whole-cell level, it is clear that a more molecular approach is needed to confirm the identity of the players. Future approaches will need to determine the molecular identity of the K^+ channels involved, the mechanism of how they are regulated by ROS, and if their expression is changed during the maturation of innervation.

Current evidence suggests that the loss of O_2-sensitivity during development is due to the inability of juvenile AMC to either detect hypoxia, or alter ROS levels during hypoxia. However, the identity of the O_2-sensor is not known and has only been tentatively located to a region of the electron transport chain that contains more than 50 proteins. These candidate O_2-sensors may be narrowed down to those that contain active groups such as Fe-S clusters or copper-containing proteins. This however, still leaves ~ 10 candidate proteins, and does not take into account the possibility that the loss of O_2-sensing in juvenile AMC is due to a multifaceted change in the relative abundance of several proteins and their isoforms.

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Effect of somatostatin on the release of adrenaline and noradrenaline from bovine adrenal chromaffin cells.

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Bovine chromaffin cells are innervated by the splanchnic nerve, which, upon stimulation, releases acetylcholine (ACh), which, in turn, triggers catecholamine (CA) secretion. It has become clear that, besides the cholinergic regulation, adrenal chromaffin cells are under the control of a large number of noncholinergic factors that can evoke or modulate CA secretion.

There is strong evidence that adrenaline (AD) and noradrenaline (NA) are stored in two different types of cells[1] and that these two cell subpopulations may be selectively activated[2, 3].

Somatostatin (SS) acts as a neurotransmitter and neuro-modulador in the central nervous system and in the periphery, producing multiple effects, through interactions with five specific membrane receptors[4]. SS effects on the adrenal medulla secretion are largely unknown.

The aim of our work was to test the hypothesis of SS exerting a modulatory effect over cholinergic-evoked CA release from bovine adrenal chromaffin cells.

The chromaffin cells were isolated by digestion with collagenase A, cultured in DMEM/F12 Ham supplemented with 10% foetal calf serum, and plated in collagen-coated 24-well plastic culture dishes at a density of 4-5 x 10^5-10^6 cells/well. For the experiments, cells were used after 4 days in culture. For studies on CA release, cells were preincubated for 10 min, to measure basal secretion, and incubated for 15 min under basal conditions or under different experimental conditions. CA in the cells and liquids were quantified by means of high pressure liquid chromatography with electrochemical detection (HPLC-ED). The cellular content ratios of AD/NA differed among different cultures, which were grouped in: AD-rich cell cultures (when AD/NA = 2.4-2.5) and AD-poorer cell cultures (when AD/NA = 1.2-1.7).

RESULTS AND DISCUSSION

Adrenal chromaffin cells synthesised and accumulated large amounts of AD and NA. Acetylcholine (ACh), 50 μM-10 mM, increased, in a concentration-dependent manner, the release of CA from both cell cultures. At concentrations of 500 μM and 1 mM, ACh released CA preferentially from AD-poorer cultures.

Similarly, nicotine (5-100 μM) and dimethyl-phenyl-piperazinium (DMPP) (10-100 μM), two selective nicotinic agonists, caused a predominant CA release from AD-poorer cultures.

The release of both CA, elicited by ACh, was significantly reduced by hexamethonium (100 μM), a selective nicotinic antagonist, only from the AD-poorer cultures.

The muscarinic receptor antagonist, atropine (100 μM), had no significant effect on the ACh-evoked CA release from AD-poorer cultures, whereas it inhibited the release of both CA from AD-rich cultures.

Somatostatin was found to increase the ACh-evoked CA release from the AD-rich cultures but was unable to significantly affect the release of CA from AD-poorer cultures. On the other hand, it attenuated the CA secretion from the AD-poorer cultures induced either by nicotine or DMPP.

Taken together these results suggest that: 1) AD-rich and AD-poorer cell cultures respond differentially to muscarinic and nicotinic stimulation; 2) Somatostatin increases the AD/NA ratio in chromaffin cell secretion through a differential effect on AD-rich cultures (increase), probably by interaction with muscarinic receptors, and on AD-poorer cultures (decrease), probably by interaction with nicotinic receptors.

It is generally accepted that cholinergic agents evoke endogenous CA release, from adrenal chromaffin cells, through nicotinic receptors. However, there are some reports, either from isolated perfused adrenal gland[5], or from primary chromaffin cell cultures[6], showing that muscarinic receptors may also be involved in CA secretion. Our results strongly indicate that a muscarinic response comes mainly from AD-rich cells, whereas the other cell type is mainly responsible for the cholinergic evoked CA release. This may explain some difficulty in visualising the muscarinic component in acetylcholine evoked CA release from a mixture of adrenal chromaffin cells.

Although SS is a well-known inhibitory peptide, there are a few reports of this peptide acting as a positive modulator[7,8]. Interestingly, recent works[9,10] have described, in other experimental cell models, a cross talk between SS and other peptides, which also

signal via G_i/G_o-coupled receptors, and agents signalling via G_q-coupled receptors (like muscarinic agonists) leading to the potentiation of the release of several hormones. In addition, similarly to our findings, SS by itself did not affect secretion. The experiments described here have provided, to our knowledge, the first evidence for a positive role of somatostatin on adrenal chromaffin cells. This positive effect involves a specific increase in AD secretion.

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Chromogranin A-derived peptides: functional aspects of vasostatins, pancreastatin, catestatin and parastatin.

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The occurrence of chromogranins and their derived peptides in the extracellular space is a prerequisite for their function as prohormones for regulatory peptides. The effects may be multifaceted, autocrine, paracrine and/or endocrine, acting at concentrations that may reflect the distance from the site of release to their target site. Local concentrations of the chromogranins and their processed products may reflect autocrine and paracrine effects while transport across the vascular endothelium into the circulation would be required for endocrine effects. The first reported target for a CgA-derived peptide was the porcine pancreatic beta cells and accordingly the peptide was named pancreastatin.[1] During the last decade the number of targets for CgA-derived peptides have increased substantially.[2] The aim of this "Wine & Beer" session was to focus on recent findings of effectors for the main CgA-derived peptides vasostatins, pancreatatin, catestatin and parastatin (Fig. 1), to gather insight into mechanisms activated by the multitude of activities currently assigned to these peptides.

The modulatory activity of CgA peptides in different models of hormone secretion was discussed by Maurizio Mandalà, reporting that catestatin, inhibiting catecholamine release in adrenomedullary cells[3] while stimulating histamine release in rat mast cells[4], was completely without modulating effects on the secretory process in the human blood platelets. He also reported on a selective interaction between the smooth muscle layer of the rat posterior cerebral artery and the cationic bovine $CgA_{47\text{-}70}$ and chromofungin ($CgA_{47\text{-}66}$).

Vasostatins (VSs) as inhibitors of myocardial inotropy in vertebrate hearts. Bruno Tota, reported data consistent with a role for VS-1 as cardiosuppressive, counter-adrenergic peptides in vertebrates, extending the inhibitory role of VSs in the vascular system[5] to that of the heart[6]. In the eel (*Anguilla anguilla*) the VS-mediated negative inotropism required an intact endocardial endothelium and involved Gi/o proteins, muscarinic and adrenergic receptors, calcium channels and the NO-cGMP-PKG pathway[7]. VS-1 also counteracted the classical inotropic response to adrenergic stimulation[7]. In the frog (*Rana esculenta*) heart the negative inotropism of VS involved neither the Gi/o proteins nor the NO-cGMP system while required the activation of K^+ channels.[8] In the Langendorff preparation of the rat heart, VS-1, but not VS-2, decreased the inotropism without changing

coronary pressure. Both peptides counteracted the positive inotropism mediated by adrenergic stimulation.[9]

Pancreastatin (PST) as a regulator of energy metabolism. Víctor Sánchez-Margalet reported that in rat adipocytes and hepatocytes the metabolic actions of PST are mediated by specific receptors, resulting in activation of the effector system $G_{q/11}$-PLC-β-PKC-MAPK. [10, 11] In these cells PST has a counter-regulatory effect on insulin signalling and action, inhibiting glucose uptake, glycogen and lipid synthesis as well as promoting lipolysis and also a negative cross-talk with insulin receptor signalling.[12] More recently PST inhibited leptin secretion by decreasing leptin expression in isolated rat adipocytes whereas UCP-2 expression was upregulated. This suggested that PST may modulate energy metabolism by both direct and indirect mechanisms.

Catestatin as a multifunctional peptide with antimicrobial activity. Marie-Hélène Metz-Boutigue reported for the first time that the arginine rich N-terminus of catestatin ($CgA_{344-358}$) was a potent inhibitor of microbial growth, not only of Gram-positive bacteria such as *Micrococcus luteus* and *Bacillus megaterium* (MIC 0.8 μM), but also of pathogenic Gram-negative bacteria such as *Escherichia coli D22* (MIC 8 μM), a range of filamentous fungi and several candida yeast cells (MIC 0.2–10 μM). In contrast, the N-terminal core of catestatin was unable to kill erythrocytes at the concentration range effectively inhibiting microbial growth. These findings were consistent with the hypothesis that the cationic and hydrophobic domains of CgA, chromofungin[13] and catestatin[4], may interact with biological membranes in a receptor-independent manner, analogous to that of other cationic and amphipathic peptides with antimicrobial potencies[14].

The N-terminus of parastatin (PARA) and para-related peptides. The role of these peptides, acting as autocrine inhibitors of porcine parathyroid secretion, was discussed by Brigitte H. Fasciotto Dunn. In the parathyroid cells CgA and parathormone are co-stored and co-secreted upon stimulation by hypocalcemia, yet subject to autoinhibition[15], possibly by three naturally occurring PARA

peptides[16]. Blocking of furin-mediated CgA processing[17] extends the functional concept of CgA-derived PARA peptides in autocrine inhibition of parathormone secretion that could account for the pattern of pulsatile parathormone release *in vivo*.

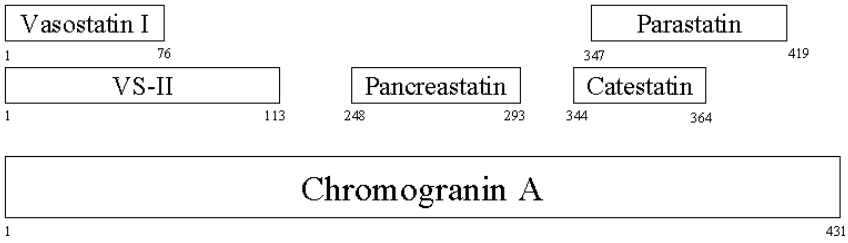

Figure. 1. Schematic presentation of the primary structure of the bovine CgA and main peptides.

Catestatin mutants and hypertension Sushil K. Mahata reported on catestatin's antagonism of nicotinic-cholinergic stimulation of catecholamine secretion and CgA gene transcription in mice, establishing catestatin's autocrine/paracrine effects *in vivo*[18]. He also presented novel data on the discovery of naturally occurring human ($CgA_{352-372}$) catestatin variants ($Gly_{364}Ser$, $Pro_{370}Leu$, $Arg_{374}Gln$) and their differential effects on nicotine evoked catecholamine secretion[19]. $Gly_{364}Ser$ represents a change to an amino acid (Ser) not previously seen at this sequence position in any mammal; $Pro_{370}Leu$ is a reversion of the wild type human amino acid (Pro) to the amino acid (Leu) seen in all non-primate mammals; and$Arg_{374}Gln$ disrupts the usual dibasic processing site ($Arg_{373}Arg_{374}$) flanking the carboxy-terminus of catestatin[19]. In addition, he briefly discussed severe alterations in chromaffin cell physiology in CgA knockout mice implicating that catestatin plays a crucial role in the pathogenesis of hypertension.

SUMMING UP

The most recent findings strengthen the concept of CgA as a prohormone for at least four peptides with modulating potencies in a wide range of target systems. However, apart from the non-competitive inhibition of the nicotinic acetylcholine receptor by catestatin in

chromaffin cells, classical surface receptors for CgA peptides in the other target systems are still elusive. Nonetheless, involvement of G-protein subunits has been implicated in a wide range of cells and tissues, for vasostatins in mammalian blood vessels and fish heart, for pancreastatin in the rat adipocytes and hepatocytes and for catestatin in the rat mast cells. The potent antimicrobial activities of the cationic and amphipathic domains inherent in vasostatin I (chromofungin) and in catestatin, point to receptor-independent activation of intracellular signalling mediated via membrane interaction and penetration. The possibility of similar receptor-independent activations elicited by the vasostatins and by catestatin in target systems of mammalian origin provides a challenge for future approaches.

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Chromogranin A in Tumor and Vascular Biology.

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Chromogranin A (CgA) is a glycoprotein stored in the dense core granules of the adrenal medulla and of many neuroendocrine cells and neurons. This protein is believed to play an intracellular role as a key regulator of secretory granules biogenesis and an extracellular function as a precursor of several regulatory peptides for the endocrine and the metabolic systems. In addition, CgA has been recognized as a useful tissue and serum marker of neuroendocrine tumors and a prognostic indicator in heart failure. A growing body of evidence suggests that CgA is not only an important diagnostic and prognostic marker, but that it could also play important functions in tumor biology and cardiovascular physiology that deserve to be investigated.

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Cell Biology of the Chromaffin Cell
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Chromogranin A (CgA) is an acidic glycoprotein belonging to a family of regulated secretory proteins stored in the dense core granules of the adrenal medulla and of many neuroendocrine cells and neurons[1]. This protein was originally identified as the major soluble protein of the secretory granules of chromaffin cells, co-released with catecholamines from stimulated adrenal medulla[2]. CgA was found later to be a member of a larger family of soluble acidic secretory proteins that includes, besides CgA, also CgB, secretogranin (Sg) II (CgC), Sg III (1B1075), Sg IV (HISL-19), Sg V (7B2) and Sg VI (NESP55)[3]. It has been proposed that CgA is a precursor of several biologically active peptides with important roles in the regulation of the endocrine, metabolic and immune systems[4]. In this chapter I will discuss the evidence that suggest that CgA, besides regulating these systems, could also affect the cardiovascular physiology and the tumor biology.

Biochemical properties and detection of cga in tissues and biological fluids. cDNA and protein characterization studies have shown that human CgA is an O-glycosylated, sulphated and phosphorylated protein of 439 residues[4-7]. CgA may undergo pH- and Ca^{2+}-dependent conformational changes causing exposure of hydrophobic residues and formation of dimers or tetramers[8-10]. The C-terminal region is important for dimer-tetramer equilibrium[11,12]. N-terminal fragments (residues 1-78) may also form dimers, at micromolar concentrations, which rapidly dissociate upon dilution[13].

CgA contains a high number of dibasic sites thought to be important for tissue specific proteolytic processing. Moreover, multiple forms having different hydrodynamic sizes of 600 kDa, 100 kDa and 55 kDa have been detected in the serum of cancer patients[14]. The levels of post-translational modification and proteolytic processing may differ from tissue to tissue[15-18]. Thus, from an analytical point of view, CgA is a highly heterogeneous antigen. Since CgA is usually detected in biological fluids and in tissues using different immunological probes (by RIA, ELISA, western blotting, immunofluorescence microscopy, immunohistochemistry, etc.), it is not surprising that different antibodies can detect CgA with different

efficiency. This is an important point to keep in mind when comparing the results obtained with different assays.

Besides chromaffin cells of the adrenal medulla, other cells of the diffuse neuroendocrine system express CgA. For instance, CgA is co-stored with various hormones in the secretory vesicles of cells of the gastrointestinal tract[19], the adeno- and neuro-hypophysis[20], the parathyroid[21], the endocrine pancreas[22], the thyroid C-cells[23], the immune system[24], and the atrial myocardium[25]. In addition, it is a component of dense-core synaptic granules in many areas of the central nervous system[26,27].

CgA is expressed also by many endocrine and neuroendocrine tumors including pheochromocytomas, various carcinoid tumors of the stomach, lung, intestine, prostate and liver, parathyroid carcinoma, medullary thyroid carcinoma, anterior pituitary tumors, pancreaticoduodenal tumors, neural tumors, small cell lung cancer (SCLC) and many others[3,28]. Interestingly, also certain non-neuroendocrine tumors, such as non small cell lung cancer (NSCLC), prostate cancer and breast cancer may undergo neuroendocrine differentiaton and present focal expression of CgA[3].

CgA is exocytotically released in the extracellular environment, and then in circulation, together with co-resident hormones[29]. In normal subjects the circulating levels of CgA are 0.5-2 nM, depending on the immunoassay used. Circulating CgA can increase several folds in patients with pheochromocytomas and up 0.1-1 μM in patients with carcinoid tumors[30,31]. Many other neuroendocrine tumors can release CgA in circulation, with important diagnostic and prognostic implications[3]. However, CgA levels can increase also in patients with renal failure, hepatic failure, cardiac arrest, heart failure or essential hypertension[3].

Biological activities of CgA. It has been proposed that CgA plays an important role in secretory vesicle biogenesis and hormone packaging[32]. Besides these intracellular functions it is believed that CgA can also play many extracellular functions. The presence of several dibasic sites potentially cleaved by proteases and the observation of tissue-specific proteolytic processing led to the hypothesis that CgA is a precursor of various biologically active

peptides[4,33,34] with endocrine, paracrine and autocrine functions. For instance, CgA residues 248-293 were found to be homologous to pancreastatin, a pancreatic peptide that regulates glucose and lipid metabolism[34], whereas catestatin, a peptide corresponding to residues 344-364 of bovine CgA, inhibits secretion of catecholamines from catecholaminergic cells[35]. Fragments corresponding to aminoacids 1-76 and 1-113, named vasostatin-1 and vasostatin-2, suppress vasoconstriction in isolated blood vessels[36,38]. Vasostatin-1 can also inhibit parathyroid hormone secretion[39], is neurotoxic in neuronal/microglial cell cultures[40], and induces antibacterial and anti-fungal effects[41]. The structural determinants of these activities are located in different regions of the N-terminal domain. For example, peptide 1-40, containing the Cys_{17}-Cys_{38} disulfide bridge, induces vasodilator effects and inhibits parathormone secretion, whereas peptide 47-60 can kill a variety of filamentous fungi[41,42].

In the last years we have found that CgA and vasostatin-1 can modulate, in an indirect manner, fibroblast- and smooth muscle cell-adhesion[43,44]. More recently, we have found that CgA can also affect cell-cell adhesion and permeability of endothelial monolayers[45]. Other studies showed that CgA, at nanomolar concentration, may increase deposition of basement membrane components, such as collagen type IV, laminin and perlecan by mammary epithelial cells, and alter ductal morphogenesis *in vitro*[46], suggesting a role of CgA in cell adhesion and tissue morphogenesis.

CgA and cell adhesion. Solid-phase bound CgA exerts anti-adhesive effects in fibroblast adhesion assays, whereas the N-terminal fragment 1-78 exerts pro-adhesive effects[43]. Proteolytic processing of natural CgA with plasmin decreases its anti-adhesive activity and induces pro-adhesive effects in fibronectin or serum dependent fibroblast adhesion assays[47]. It would appear, therefore, that this protein might work on one hand as a negative modulator of fibroblast adhesion and on the other hand as a precursor of positive modulators. Given the well-recognized importance of fibroblasts and plasminogen activation in tissue invasion, remodeling and repair[53-56], the interplay between CgA and plasminogen/plasmin system could provide a novel mechanism for regulating fibroblast adhesion and function in tumors.

CgA is present in neuroendocrine secretory vesicles at very high concentrations, approaching millimolar levels[50]. Elevated levels of CgA (up to 100-1000 nM) have been detected in the blood of patients with different neuroendocrine tumors[51,52]. Given that CgA and CgA1-78 affect fibroblast adhesion at 7-70 nM and 30-300 nM, respectively[47], it is possible that CgA reaches sufficient levels in tumors to affect stromal fibroblasts. Further work is necessary to assess how CgA is processed within the tumor, e.g. by plasmin, as this could have important effects on its positive or negative effect on stromal fibroblasts.

The results of structure-function studies suggest that the region 47-64 (RILSILRHQNLLKELQDL) is critical for the pro-adhesive activity[44]. This region is 100%-conserved in human, porcine, bovine, equine, and mouse CgA and is 89%-conserved in frog CgA[5,48], pointing to a functional importance. NMR analysis of peptide 47-66[42] suggests that the region 47-51 forms a short hydrophobic helix, followed by an amphipathic helix (residues 53-66). Interestingly, the 47-64 region, besides containing a pro-adhesive site also contains a Ca^{2+}-dependent calmodulin binding site[49].

The receptors or the molecular targets of CgA in cell adhesion are unknown. Analysis of the primary structure revealed that an RGD integrin binding motifs, often present in proteins of the extracellular matrix, is present at residues 43-45. However, a recombinant RGE-CgA(7-439) mutant induced anti-adhesive and pro-adhesive effects after tryptic digestion, as the wild type recombinant RGD-CgA(7-439) [43]. Thus, molecular targets different from RGD-binding receptors must be sought.

CgA and the cardiovascular system. A growing body of evidence suggests that CgA could play a role in the regulation of the cardiovascular system. For instance, the inhibition of catecholamines secretion from catecholaminergic cells by catestatin[35], the vasoinhibitory activity of vasostatins on isolated vessels[38,57], and their negative inotropic activity on isolated working heart[58] suggest that CgA is a precursor of cardiovascular regulatory peptides. Moreover, we have recently reported that CgA can regulate the endothelial cell shape and barrier function[59]. Using mice bearing subcutaneous tumors

genetically engineered to secrete CgA in circulation we have found that increased blood levels of this protein prevent vascular leakage induced by TNF in the liver venous system[45]. Structure-activity studies, carried out with CgA fragments administered to normal mice, showed that an active site is located within the vasostatin-1 domain. Studies of the mechanism of action showed that CgA inhibits TNF-induced VE-cadherin down-regulation and barrier alteration of cultured endothelial cells. These findings suggest that circulating CgA could contribute to regulate the endothelial barrier function and to protect vessels against TNF-induced plasma leakage in pathological conditions characterized by increased production of TNF and CgA.

Interestingly, circulating CgA is increased in patients with chronic heart failure (CHF) depending on the clinical severity of the syndrome[60]. Circulating CgA level is an independent predictor for mortality in these patients. While little or no correlation was observed with adrenaline, noradrenaline, atrial natriuretic factor, aldosterone and plasma renin activity, a good correlation was found between CgA and soluble TNF p55 and p75 receptors[61]. These results point to a regulatory link between cells that secrete CgA and cells that release sTNF-Rs. Although the clinical significance of TNF production in heart failure patients remains uncertain, its ability to induce cachexia, left ventricular dysfunction and pulmonary edema, suggests that TNF, in concert with other inflammatory cytokines, could play a pathogenetic role[62-66]. Considering the inhibitory effect of CgA on TNF-induced vascular leakage observed in mice[45] it is possible that regulated secretion of CgA, in concert with TNF soluble receptors, contributes to reduce the potentially dangerous effects of pathological levels of TNF on the vascular function.

Persistent activation of the neuroendocrine system, particularly the adrenergic and the renin-angiotensin systems, is maladaptive in heart failure[67]. Given that catestatin is a potent non-competitive inhibitor of nicotinic cholinergic receptor mediated catecholamine release[35] and that vasostatin-1 can inhibit noradrenaline-induced vascular tone[38] as well as the positive inotropism induced by isoproterenol in experimental models[58], it is possible that secretion of CgA contributes to counteract the excessive activation of the neuroendocrine system. However, further work is necessary to assess

whether proteolytic processing of CgA occurs in heart failure, and to measure local and systemic concentration of CgA peptides.

CgA in tumor biology. CgA is abnormally expressed by various neuroendocrine tumors and is released in high amounts in the blood stream[28,30,68-71]. Detection of CgA in biological fluids and in tumor tissues has proven useful for diagnosis and for monitoring tumor progression/regression after therapy[3]. However, little is known on the effect of excessive production of CgA on the tumor growth and behavior.

To investigate the effect of CgA secretion on tumor growth we have transfected mouse RMA lymphoma and TS/A adenocarcinoma cells (CgA negative) with the cDNA encoding CgA and studied their proliferation and tumorigenicity *in vitro* and *in vivo*. The most striking observation was that CgA expression was associated with a decreased tumorigenicity in mice. Moreover, CgA production was associated with increased tumor necrosis and multi-nodular growth pattern. Studies on the mechanisms of action showed that CgA expression does not affect the *in vitro* proliferation index of tumor cells, whereas it affects the *in vivo* growth[72]. This suggests that the effect is indirect and host-mediated. One possibility is that CgA affects the complex interplay between neoplastic cells and tumor stroma, which is critical for tumor growth. Given that CgA and its N-terminal fragments can inhibit vascular permeability[45], it is possible that CgA inhibits tumor growth by affecting the vascular compartment of the tumor, for instance, by decreasing the transport of macromolecules critical for tumor cell proliferation across the endothelial barrier. In addition, considering the effect of CgA on fibroblast adhesion, it is also possible that CgA affects the tumor architecture by modulating the physiology of stromal fibroblasts within the tumor, which in turn are important for the production of extracellular matrix proteins and stroma formation. These mechanisms are not mutually exclusive.

CONCLUSIONS

Although the extracellular role of CgA remains rather obscure, a growing body of evidence suggests that abnormal secretion of CgA, e.g. by neuroendocrine tumors or by the (neuro)endocrine system in

heart failure or other pathological conditions, is not simply an epiphenomenon of cell secretory activity, but that it could play important functions in tumor and vascular biology that deserve to be investigated.

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Study of new antimicrobial peptides in chromaffin granules from bovine adrenal medulla: new aspects of innate immunity.

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Secretory granules of chromaffin cells contain with catecholamines several antimicrobial peptides, which are secreted in the extracellular medium following exocytosis. In this report we decided to focus on three active peptides Chromofungin (chromogranin A 47-66), Enkelytin (proenkephalin-A 209-237 and Ubifungin (ubiquitin 65-76). Using confocal laser microscopy we have shown that Chromofungin and Ubifungin are able to cross the cell membranes and penetrate into fungi and yeasts, whereas the N-terminal fragment ubiquitin 1-34 was stopped at the fungal cell wall level. At the intracellular level Chromofungin and Ubifungin are able to inhibit calmodulin-dependent calcineurin, a crucial enzyme for fungal growth. Finally, all together these peptides constitute a mixture of potent antimicrobial peptides, which might represent molecules useful for the development of new therapeutic agents.

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Antimicrobial peptides are present in the hemolymph of insects and are also stored in the secretory granules of immune cells found within mammals and birds[1-3]. The importance of these molecules is clearly established in the innate immunity of invertebrates by the microorganism-induced antibacterial activity, that can be detected in the hemolymph[4]. In vertebrates, these peptides complete adaptive immunity by acting as a first line of defense against pathogens and by controlling natural flora[5]. Similarities have been highlighted between pathogen recognition, signalling pathways and effector mechanisms of innate immunity in Drosophila and mammals[6].

Numerous natural antimicrobial peptides from mammals have been previously characterized in several tissues[5]. In addition to antibacterial peptides, some antifungal molecules were characterized. A first group acts by lysis that occurs after destabilization of the membrane, formation of aqueous pores and intracellular mechanism, whereas the second group interferes with cell-wall synthesis or the biosynthesis of glucan or chitin [6,7].

Secretory granules from adrenal medullary chromaffin cells contain with catecholamines a complex mixture of peptides derived from chromogranins (CGs), proenkephalin-A (PEA) and others precursors. During the past decade, our laboratory has characterized the processing of chromogranin A (CGA)[8], chromogranin B (CGB)[9] and proenkephalin-A (PEA)[10] in chromaffin granules from bovine adrenal medulla and we have identified other unexpected peptides derived from ubiquitin (Ub)[11], that are secreted with catecholamines during stimulation of the chromaffin cells and possess antimicrobial activity[9,11-17]. Interestingly, they are recovered in biological fluids implicated in defense mechanisms, for example, abcess fluids and secretions of stimulated polymorphonuclear neutrophils (PMNs)[13, 14]. These peptides have been hypothesized to play a role in stress situations, acting as an immediate protective shield against pathogens[12].

In this review we focus on three natural antimicrobial peptides derived from CGA, PEA and Ub and their synthetic active domains. They display common structural features that are believed to be important for the molecular mechanisms implicated in their antimicrobial activities.

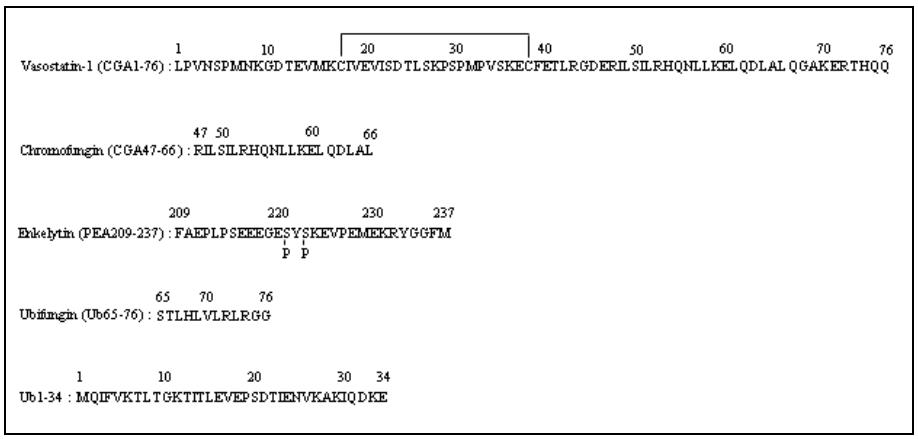

Figure 1: Antimicrobial peptides isolated from chromaffin secretory granules of bovine adrenal medulla. The disulfide bridge and the phosphorylated residues are indicated.

Vasostatin-I and Chromofungin: potent inhibitors of fungi and yeast. Following separation of the soluble material present in chromaffin granules by HPLC, antibacterial activity against *M. luteus* can be detected in several fractions. One of these fractions has been identified as Vasostatin-I (CGA1-76), the highly conserved N-terminal domain in which the disulfide bridge and the sequence 50-62 (SILRHQNLLKELQ) are strictly unchanged[14, 15] (Figure 1). Natural bovine Vasostatin-I is selectively active against *M. luteus* and *B. megaterium* at micromolar range[14], but activity is not detectable against pathogenic Gram-positive and Gram negative bacteria. In contrast, Vasostatin-I was found to be strongly active against a large variety of filamentous fungi, including pathogenic strains and yeasts[14]. Interestingly, this peptide is inactive against erythrocytes and others mammalian cell-types[14]. These experiments were extended by analysis of the recombinant-derived fragments of human Vasostatin-I, that are able to display antifungal activity. An active peptide corresponding to the fragment CGA47-60 was identified by sequencing and MALDI-TOF mass spectrometry. The shortest active peptide with maximun global hydrophobicity and amphipathic features corresponded to CGA47-66 (Figure 1) and was named Chromofungin[15]. The three

dimensional structure of Chromofungin has been determined in water-trifluoroethanol (50:50) using ^{1}H NMR spectroscopy. This analysis revealed the amphipathic helical character of the C-terminal part of the sequence 53-66.

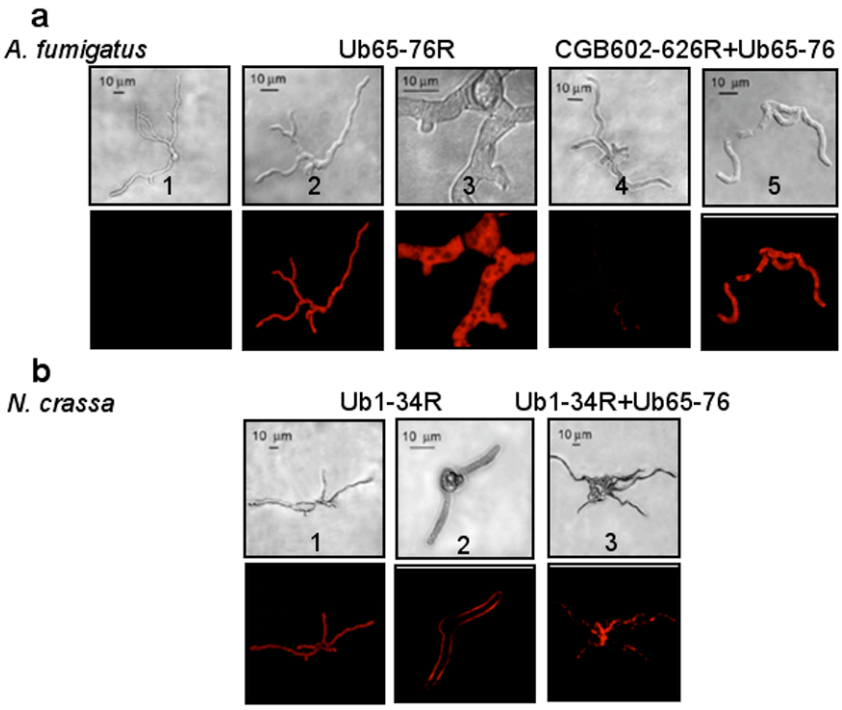

Figure 2: Phase contrast and fluorescence confocal laser micrographs of *Aspergillus fumigatus*, *Neurospora crassa* and *Candida albicans* with rhodamine-labeled synthetic peptides Chromofungin, Ubifungin and Ub1-34. a) 1, *A. fumigatus* in the absence of peptide; 2, *A. fumigatus* after 60 min incubation with Chromofungin (10 µM); 3, *A. fumigatus* after 60 min incubation with rhodminated inactive peptide CGB602-626 (10 µM). **b)** 1, *A. fumigatus* after 2 min incubation with Ubifungin (2 µM); 2, at higher magnification, the core of fungi and unlabeled vacuoles and septum are clearly visible; 3, *N. crassa* was incubated with 5 µM Ub1-34 for 15 min: note the labelling along the cell membrane; 4, *A. fumigatus* was first incubated with 20 µM unlabeled Ubifungin for 45 min before a final incubation with 5 µM rhodaminated Ub1-34 for 15 min. Note the fluorescence within cells revealing Ub1-34 penetration due to membrane destabilization evoked by Ubifungin.

The importance of the amphipathic sequence for antifungal activity was demonstrated by the loss of such activity when two proline residues were substituted for Leu61 and Leu64, disrupting the helical structure and amphipathic character of the peptide[15]. Thus, Chromofungin is a cationic amphipathic molecule with a helical structure and is able to interact with inner and outer membranes to reach intracellular targets, as shown by confocal laser microscopy (Figure 2).

Enkelytin potent inhibitor of Gram-positive bacteria. The processing of PEA has been extensively studied in chromaffin cells obtained from bovine adrenal medulla[10] and proceeds through an orderly series of steps, starting with the removal of the C-terminal domain (PEA209-239). The bisphosphorylated form of PEA209-237 (Figure 1) displays a potent antibacterial activity against Gram positive bacteria including the pathogenic *S. aureus* in the 0.2-4.5 micromolar range. In contrast, to the bisphosphorylated form, the non-modified peptide displays a low antibacterial activity, indicating that the two phosphorylated residues S221 and S223 play an important conformational role[10, 13]. Furthermore, the synthetic modified peptide PEA209-237 with three E residues in place of S215, S221 and S23 conserves antibacterial activity, suggesting the importance of negative charges in the expression of such activity. In contrast to cationic antimicrobial peptides, Enkelytin possess a negative charge (-7) and because of this might be compared with polyaspartic acid peptides identified in secretions from the lung[18]. To characterize the biological function of Enkelytin several fluids and PMN secretions from injured animals with infection have been analyzed[13]. Following secretion at the inflammatory area, Enkelytin was quantified by sequencing and its concentration in bovine periarthritis abscess fluid was estimated from 0.5 to 1 micromolar. As a continuation of these studies, its presence in secretions of PMNs has been demonstrated[13].

PEA has been reported to be significantly expressed in the immune system and might provide a basis for neuroimmune interactions[19]. The local inflammatory response initiates the synthesis and secretion of opioid peptides by immune cells. Therefore, Enkelytin degradation by neuropeptide degrading endopeptidase

(NEP) and angiotensin converting enzyme (ACE) present in granulocytes generate Met-enkephalin and its derived peptides[10]. Met-enkephalin enhances the immune reaction and this pentapepide can bind opiod receptors present in peripheral tissues with inflammation to mediate an analgesic effect[20]. Taken together the major bisphosphorylated form of PEA209-237 and Met-enkephalin would provide a highly beneficial survival strategy for the proinflammatory process.

Ubiquitin, its N- and C-terminal fragments. Ubiquitin is a peptide of 76 residues (Figure 1) found in all eukatyotic cells that display well-conserved sequences from protozoa to vertebrates[21]. Recently, we have reported the subcellular localization of free Ub in bovine adrenal chromaffin cells[11]. Ub is present in secretory granules and secreted with catecholamines after chromaffin cells stimulation. In addition, we have shown that free Ub displays *in vitro* antimicrobial activities and inhibits the growth of *M. luteus* and *B. megaterium* at a MIC of 60 micromolar. At a concentration of 100 micromolar, Ub completely inhibits the growth of *N. Crassa*[11]. Then, we have shown that the C-terminal fragment Ub65-76 is crucial for the expression of the antifungal activity. This peptide named Ubifungin is active against Gram positive, pathogenic *E. coli* and various fimalentous fungi and yeasts[11]. Interestingly, it is inactive against erythrocytes[11]. Because microorganism growth has not resumed after 48 hours, this peptide is believed to exert a lytic mechanism.

The N-terminal fragment Ub1-34 adopts a beta-hairpin (residues 1-17) followed by an alpha helix (residues 18-34)[11] the latter being important for membrane interactions. Antimicrobial assays of Ub1-34 have indicated that this peptide displays weak activity against *N. crassa* at a concentration of 100 micromolar. Interestingly, when Ub1-34 was added to Ub65-76 the two peptides acted synergistically to inhibit the growth of several filamentous fungi[11].

Confocal laser microscopy has allowed analysis of the interaction of Chromofungin, Ubifungin and Ub1-34 with fungal membranes of pathogenic strains of *A. fumigatus* and *C. albicans* (Figure 2). Labeled Ubifungin was visible at the level of the cell wall and in the inner part of the fungi after two minutes of incubation. In addition, Ub1-34 was visible at the level of cell wall but not within

cells. However, when the fungi was treated with unlabeled Ubifungin before incubation with rhodaminated Ub1-34, an intense fluorescence was observed, indicating that Ubifungin destabilizes the cell wall, allowing the peptide Ub1-34 to actively penetrate the fungi.

Chromofungin and Ubifungin: inhibition of the phosphatase activity of calcineurin. In addition, to destabilizing the cell wall, Chromofungin and Ubifungin might also exert activity on intracellular targets. Indeed we have shown that these two peptides are able to inhibit the phosphatase activity of calcineurin a calmodulin dependent enzyme[11,15] crucial for the fungal growth.

CONCLUSIONS

Chromofungin, Enkelytin, Ubifungin and Ub1-34 correspond to highly conserved peptides and their antimicrobial activities probably occurred early in evolution. They are widely distributed in nervous, endocrine, neuroendocrine and immune cells. Their liberation from cells indicates that they probably play a role in inflammatory processes. Therefore, we suggest that in stress situation these peptides might provide a highly beneficial strategy against pathogenic invasion. They might be used together or in combination with classical antifungal molecules to increase their efficiency.

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Agonist-induced chromogranin A secretion coincides with redistribution of IP_3 receptor and compound exocytosis in granular duct cell of rat submandibular gland.

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The granular duct cells of the rodent submandibular gland have unique functions: the cells synthesize transforming growth factor a (TGF-□), hepatocyte growth factor, erythroid differentiation factor, endothelin, insulin-like growth factor including nerve growth factor, and kallikreins[1]. In the granular the duct cells, we have shown that chromogranin A-like immunoreactivity (CgA-like IR) is stored as well, and that highly concentrated CgA-like IR (approximately 1 mM) is secreted by compound exocytosis into saliva in response to 1 μM noradrenaline (NAd)[2]. The secretory response to 3 mM phenylephrine, an □-adrenergic agonist, was hardly inhibited in Ca-deficient environment, and was almost completely inhibited by 100 μM 2-aminoethoxydiphenyl borate (2APB), an inhibitory modulator of inositol 1,4,5 trisphosphate (IP_3)-mediated Ca release from intracellular stores[3]. These results are compatible with a view that mobilization of Ca from IP_3-sensitive pool may preferentially be involved in the secretory response to □-adrenergic agonist. The present study was thus carried out to obtain direct evidence that the duct cells exhibiting the strongest IP_3 receptor type 2 immunoreactivity (IP_3R2 IR) are the granular duct cells, which contain CgA-like IR, that NAd causes compound exocytosis of the secretory granules, and that the compound exocytosis may result in dynamic subcellular redistribution of localized IP_3R2 IR[4].

METHODS

Isolated and perfused preparation of the rat submandibular gland was used. Male Wistar rats were anesthetized and the submandibular gland was isolated and vascularly perfused. The secreted saliva was collected at an interval of 2 min. CgA-like IR was measured by a region-specific enzyme immunoassay (EIA) for rat CgA using anti-rat $CgA_{(359-389)}$ developed in our institute. Cryostat sections, 12 μm thick, were incubated with anti-rat $CgA_{(359-389)}$ serum followed by Cy3-labeled donkey anti-rabbit IgG, and then incubated with mAb against IP_3R2, followed by FITC-labeled donkey anti-mouse IgG (Jackson ImmunoResearch). The immunostained sections were obtained under laser scanning microscope.

RESULTS AND DISCUSSION

The 1 μM NAd-induced secretion of highly concentrated CgA-like IR (~1 mM) was diluted and facilitated by adding 0.1 μM acetylcholine (ACh), which has been known to cause substantial increase in flow rate with indiscernible secretion of CgA-like IR and protein. The first 4 min stimulation induced significant maximum increases in secretory responses (CgA-like IR secretion, protein secretion and flow; $p<0.01$ respectively) within 2 min followed by steep decays during the continuous stimulation. The secretory responses to the second stimulation were smaller than the corresponding first responses: the rate of CgA-like IR secretion, protein secretion and flow were 54%, 44%, and 33% of the corresponding first responses, respectively. The secretory responses to the third stimulation were much smaller than the corresponding responses to the first stimulation. The decay in the responsiveness during the continuous stimulation is, by definition, desensitization. The progressive reduction in the second and third response may also be due to continuous desensitization. In the control sections prepared from resting state, an intense IP_3R2 IR was detected at the apical pole of granulated duct cell containing IP_3R2 IR.

Ultrastructure of the granular cell in the resting state showed that the cells stored numerous membrane-bound granules in the apical cytoplasm. The combined stimulation with 1 μM NAd and 0.1 μM acetylcholine caused immediate maximum increase in secretory responses. When the sections were prepared from the gland at the peak of secretory responses, the apically converged IP_3R2 IR became indistinguishable. Ultrastructure of the maximally stimulated cells exhibited extensive compound exocytosis in the apical half of the granular duct cells. The secretory responses to the stimulation were significantly inhibited by 100 μM-2APB, and the intense IP_3R2 IR was well preserved at the apical pole of granular duct cells containing CgA-like IR.

The NAd-induced extensive compound exocytosis at the apical pole may be initiated by marked increase in local concentration of Ca^{2+} ion, which may be released from the intracellular Ca^{2+} store during activation of IP_3R2. It is recently shown that the activation of IP_3R may drastically be potentiated by the Ca^{2+} storage protein CgA[5],

and the extensive compound exocytosis in the granulated duct cells may be induced by the synergic interaction of IP_3R2 in the membrane of Ca^{2+} store and CgA in the granule.

The divergence of apically localized IP_3R coincides with the development of compound exocytosis may result in desensitization, and thus provides a novel paradigm for the desensitization of G protein-coupled signaling system[4].

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Chromogranin A-derived parastatin peptides as autocrine inhibitors of endocrine secretion.

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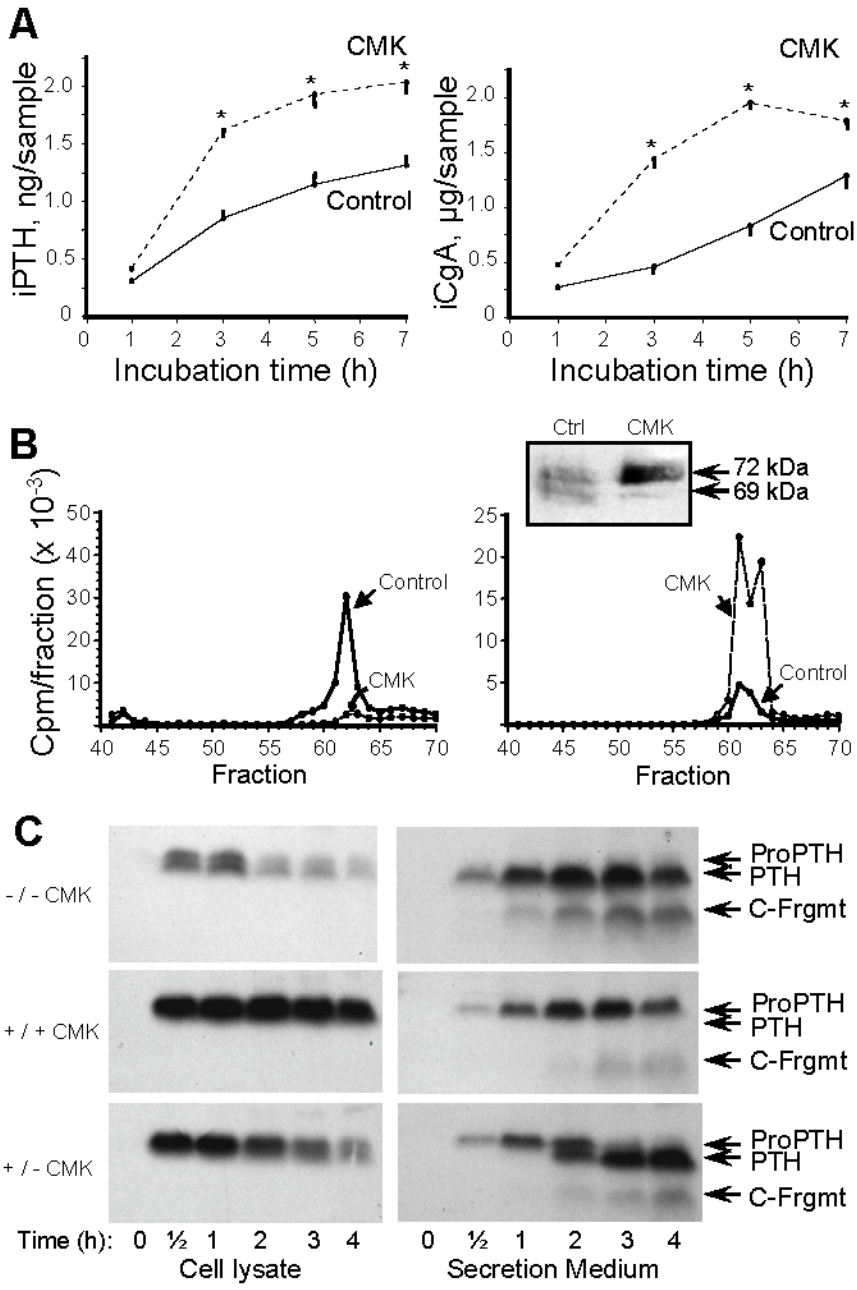
Parathyroid hormone (PTH) and chromogranin A (CgA) represent the two major calcium-regulated secretory proteins of the parathyroid gland. Secretion of PTH and CgA in the parathyroid occurs from a "storage/mature granule" pool and from a "new/immature granule" pool[1]. Upon stimulation by hypocalcaemia, parathyroid cells secrete preferentially newly synthesized PTH and CgA without prior equilibration with the mature granule pool.

CgA is a highly acidic, glycosylated protein, widely distributed in the endocrine and neuroendocrine system. CgA is a precursor of bioactive peptides including parastatin (PARA, $pCgA_{347-419}$) that inhibits parathyroid cell secretion[2]. We recently reported that porcine parathyroid cells produce and secrete bioactive PARA-related peptides, suggesting that these peptides may act as autocrine inhibitors[3].

Several mammalian subtilisin-like serine proteases have been described that process proproteins to biologically active hormones. It has been reported that furin and PC7, but not PC1 and PC2, are candidates for processing of pro-PTH in the parathyroid. Porcine CgA contains one consensus sequence RRGWR at residues 364-368 that may be a potential furin/PC7 cleavage site. To determine if furin participates in CgA processing, purified pCgA was incubated *in vitro* with purified furin for 48 hours at 37C. Furin converted pCgA to small peptides within 24 to 48 hours (not shown). Without furin, pCgA remained unprocessed.

If PARA peptides act as physiological autocrine inhibitors of parathyroid secretion, blockage of CgA processing should release cells from autoinhibition, resulting in an enhanced PTH secretion, as we previously showed with CgA antiserum[4]. To test this, we used the cell-permeant furin inhibitor decanoyl-RVKR-chloromethylketone (dec-RVKR-CMK). Porcine parathyroid cells were stimulated at 0.5 mM Ca for 7 h in presence or absence of 100 μM dec-RVKR-CMK. Dec-RVKR-CMK potentiated secretion of both immunoactive PTH (iPTH, Fig. 1-A, left panel) and immunoactive CgA (iCgA, Fig. 1-A, right panel) 3-fold or more. Palm-FAKR-CMK that does not contain the furin recognition site was only one-tenth as potent as dec-RVKR-CMK (not shown).


A
CMK
Control
iPTH, ng/sample
Incubation time (h)
CMK
Control
iCgA, µg/sample
Incubation time (h)
B
Cpm/fraction (x 10-3)
Control
CMK
Fraction
Ctrl
CMK
72 kDa
69 kDa
CMK
Control
Fraction
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+ / + CMK
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ProPTH
PTH
C-Frgmt
Time (h): 0 ½ 1 2 3 4
Cell lysate
0 ½ 1 2 3 4
Secretion Medium

Figure 1. A) Parathyroid cells were incubated at 0.5 mM Ca in absence (Control) or presence of 100 μM dec-RVKR-CMK (CMK). Secreted iPTH (left panel) was measured using the C-mid molecule RIA kit from Nichols Institute, and iCgA (right panel) was measured by RIA using bCgA antiserum generated in our laboratory. * $p<0.001$ vs Control. **B)** Parathyroid cells were labeled at 0.5 mM Ca for 6 h in absence (Control) or presence of 100 μM dec-RVKR-CMK (CMK). Secretion media (left panel) and cell lysates (right panel) were immunoprecipitated using bCgA antiserum. Precipitates were separated by HPLC and fractions were analyzed for radioactivity. **C)** Parathyroid cells were either preincubated then labeled in absence (-/- CMK), or preincubated then labeled in presence (+/+ CMK), or preincubated in presence then labeled in absence (+/- CMK) of 100 μM dec-RVKR-CMK. Cell lysates (left panels) and secretion media (right panels) were immunoprecipitated using PTH antiserum. Precipitates were analyzed by SDS-PAGE and autoradiography. Positions of pro-PTH, PTH and PTH C-fragment are indicated.

We then studied the effect of dec-RVKR-CMK on the secretion of newly synthesized, radioactive CgA (Fig. 1-B) and PTH (Fig. 1-C). Parathyroid cells were stimulated in medium containing ^{3}[H]-Leu. Dec-RVKR-CMK decreased secretion of newly synthesized CgA at 0.5 mM Ca (Fig. 1-B, left panel). This is in contrast with the stimulation of immunoactive CgA observed in Fig 1-A. The decreased secretion of CgA was accompanied by an intracellular build-up of a 72 kDa CgA form (Fig. 1-B, right panel). Immunoprecipitation with PTH antiserum shows that dec-RVKR-CMK blocked the conversion of pro-PTH to PTH (Fig. 1-C, +/+ CMK). Newly synthesized pro-PTH accumulated intracellularly and no PTH or C-fragment was detected. No PTH was detected in the secretion medium. The effect of dec-RVKR-CMK was reversible (Fig. 1-C, +/- CMK). After removal of dec-RVKR-CMK at the beginning of the labeling period, pro-PTH was converted to PTH and then secreted, with a time lag of 2 h, the time required for synthesis and activation of new furin.

The data show that furin is involved in the conversion of pro-PTH to PTH and in the processing of CgA in parathyroid cells. The intracellular accumulation of newly synthesized pro-PTH and CgA in the presence of dec-RVKR-CMK suggests that inhibition of furin, a TGN resident protease, may affect the intracellular trafficking of secretory proteins in parathyroid cells. For proteins that have passed into the storage secretory granules, as measured for secretion of iPTH and iCgA, inhibition of furin affects the regulation of secretion. We

propose that inhibition of CgA processing, decreases the amount of PARA available to inhibit secretion. This results in increased secretion of proteins that are stored in secretory granules. These results agree with the hypothesis that CgA-derived peptides serve as autocrine inhibitors of parathyroid secretion. Autocrine regulation of parathyroid secretion could explain the physiological pulsatile mode of PTH secretion *in vivo*[5].

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Immunohistochemical studies with region-specific antibodies to chromogranins A, B and C in pheochromocytomas.

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Cell Biology of the Chromaffin Cell
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Chromogranins (Cgs) are a family of glycoproteins that occur in the secretory granules of neuroendocrine (NE) cells and NE tumours. Post-translational processing of Cgs has been reported in various human NE cell types and NE tumours. This processing appears to differ in various human NE cell types and NE tumours[1,2,3]. The plasma concentrations of CgA and CgB, and of a fragment of CgC (secretoneurin) have been reported to be increased in patients with pheochromocytomas[4,5].

The aim of the present investigation was to study the immunoreactivity to various fragments of the molecules of CgA, B and C in pheochromocytomas in order to analyse if the expression patterns differed between benign and malignant tumours.

MATERIALS AND METHODS

Antibodies raised in rabbits against specific regions of the molecules of CgA (CgA 116–130, chromostatin; 176–195, chromacins; 361–372, catestatin), CgB (CgB 244–255; 312–331; 542–552, PE-11; 568–577, chrombacin; 647–657, CCB) and CgC (CgC 154–165, N-terminal secretoneurin; 172–186, C-terminal secretoneurin) were used. The CgA and CgB region-specific antibodies have been described before[1,2]. Peptides corresponding to the N-terminal and C-terminal regions of secretoneurin were synthesised and rabbit polyclonal antibodies were raised as described earlier for the CgA and CgB region-specific antibodies. For comparison, a commercial antibody raised against the C-terminal half of CgA was also used (code no. A0430, DakoCytomation, Glostrup, Denmark). Tumour specimens from 25 patients operated on for clinico-pathologically benign pheochromocytomas and 4 for metastasizing ones were routinely fixed in 10% buffered neutral formalin and processed to paraffin. Adrenal medulla from 3 patients operated on for cortical adenomas were used as 'non-neoplastic' controls. Sections were immunostained with the streptavidin–biotin complex technique using diaminobenzidine as chromogen.

RESULTS AND DISCUSSION

All adrenal chromaffin cells from the 'non-neoplastic' controls were immunoreactive to all eleven region-specific antibodies. In the

pheochromocytomas, one or more fragments were lacking, but variations in the expression pattern occurred both in benign and malignant pheochromocytomas. The frequency of immunoreactive cells to the various region-specific antibodies did not differ significantly between benign and malignant Pheos. CgA 176–195 (chromacins), CgB 647–657 (C-terminal CgB) and CgC 175–186 (C-terminal secretoneurin) were expressed in all cells in both benign and malignant Pheos. As earlier reported[3], this investigation confirmed that CgA 176-195 is a good NE cell marker for both benign and malignant pheochromocytomas. In the malignant pheochromocytomas the antibodies raised against the C-terminal fragments of both CgB (CgB 647–657) and secretoneurin (CgC 175–186), visualized a distinct neoplastic chromaffin spindle cell population, characterized by elongated processes, a cell type which was demonstrated in all malignant pheochromocytomas, but only in one of the benign tumours. These spindle cells did not display S-100 immunoreactivity, *i.e.* were different from sustentacular cells which occur in some pheochromocytomas. This study shows that the use of antibodies to epitopes in the C-terminal regions of CgB and secretoneurin may facilitate the differential diagnosis of a malignant pheochromocytoma.

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Interaction of the N-terminal domain of chromogranin A (vasostatin derived peptides) with the rat posterior cerebral artery.

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Chromogranin A (CgA), an acidic granule protein of the regulated secretory pathway in the diffuse neuroendocrine system, is postulated to serve as a prohormone for vasoactive peptides derived from the hydrophobic N-terminal domain[1]. In rat, the first cleavage product is betagranin ($rCgA_{1-129}$), in which the first 76 amino acid residues are 86 % homologous to the bovine vasostatin I ($bCgA_{1-76}$), previously shown to be vasoinhibitory in human and bovine blood vessels[2]. The aim of the present study has been to characterize interactions of vasostatin I with rat posterior cerebral arteries (rPCA). Inverted fluorescence microscopy was applied to show the interaction of the rhodamine-labeled bCgA-derived peptides: $bCgA_{7-40}$ and $bCgA_{47-70}$ with the adventitial and luminal structures of rPCA. The results are consistent with a specific interaction of $bCgA_{47-70}$ with the smooth muscle cells but not with the endothelial cells.

MATERIALS AND METHOD

rPCA were removed under a dissecting microscope from the brain of male Wistar Kyoto rats (14 - 16 weeks of age). Segments of the isolated artery were loaded from the luminal or adventitial side with rhodamine (Rh)-labeled peptide in HEPES-PBS for 2 hours at room temperature. In a parallel series arteries were mechanically denuded using a fine metal wire prior to luminal peptide loading. After, completed loading the vessels were thoroughly rinsed with HEPES-PBS and placed on a cover glass in a drop of HEPES-PBS for analysis with an inverted fluorescence microscope.

RESULTS

Incubation of rPCA with peptide-free rhodamine (1 μM) resulted in an intensely fluorescent artery, whether added to the adventitial or the luminal side. Adventitial loading with Rh-CgA_{7-40} 1 μM did not give any fluorescence labeling of the artery. Loading with Rh-CgA_{47-70} revealed on the other hand a strong arterial fluorescence at both 1 nM and 1 μM peptide concentration. Luminal loading with Rh-labeled peptide failed to stain the artery, whether exposed to 1 μM Rh-CgA_{7-40} or 1 μM Rh-CgA_{47-70}. In denuded arteries luminal loading with 1 μM Rh-CgA_{47-70} resulted in an intense fluorescence, this was not observed with 1 μM Rh-CgA_{7-40}.

The endothelial lining of the rat cerebral artery did not interact with rhodamine-labeled N- and C-terminal peptides of vasostatin I. In contrast to the N-terminal peptide $CgA_{7-40,}$ the cationic C-terminal peptide Rh-CgA_{47-70} interacted with the rat vascular smooth muscle cells at a functionally relevant concentration (1nM). This suggests a role for the cationic domain of vasostatin I in regulation of smooth muscle function from the adventitial side. The positive charge of $bCgA_{47-70}$ (+2.5), as opposed to the negative charge of $bCgA_{7-40}$ (-2), might explain the selective interaction of CgA_{47-70} with the rat artery.

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Proteomic studies of the chromaffin granule demonstrates novel proteolytic processing mechanisms for chromogranin A and proenkephalin by secretory vesicle cathepsin L.

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Cell Biology of the Chromaffin Cell
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The chromaffin granule has been utilized by many investigators to understand molecular mechanisms by which neurosecretory vesicles synthesize, store, and secrete neurotransmitters and peptide hormones. Proteomics, in the post-genomic age, provides an ideal approach for elucidating the functional protein and peptide components of this regulated secretory vesicle system. Two proteomic approaches are presented in this study that have (1) defined novel chromogranin A (CgA) cleavage sites for production of the biologically active catestatin peptide[1] that regulates blood pressure, and (2) utilized an activity-based proteomic profiling approach for identification of the secretory vesicle cathepsin L as a proenkephalin and prohormone processing enzyme[2]. Moreover, the novel CgA cleavage sites are compatible with the prediction for CgA processing by secretory vesicle cathepsin L, as well as the prohormone convertases PC1 and PC2. Significantly, these studies demonstrate how selected proteomic studies combined with genetics can lead to novel findings of new regulatory components within neurosecretory vesicles.

Proteomics of the major proteins of chromaffin granules identifies distinct cleavage sites for chromogranin A (CgA) for biosynthesis of active catestatin. In proteomic studies of chromaffin granules, proteins were separated by 1-D and 2-D (one- and two-dimensional) SDS-PAGE gels and identified based on peptide sequence characterization via MALDI-TOF mass spectrometry combined with NH_2-terminal peptide sequencing by Edman degradation[1]. Multiple proteins of 70 kDa to 17 kDa were identified as catestatin-containing proteolytic fragments derived from chromogranin A (CgA). Catestatin itself, represented by $CgA_{344-364}$ was identified by gel filtration, NH_2-terminal sequencing by Edman degradation, and MALDI-TOF mass spectrometry. Results demonstrated that catestatin production requires cleavage of CgA at NH_2-terminal side of dibasic and monobasic residues. This cleavage specificity is consistent with the chromaffin granule 'prohormone thiol protease' (PTP) for proenkephalin processing[3-6] However, production of catestatin-containing intermediates appears to

utilize processing at the COOH-terminal sides of dibasic residues, resembling prohormone convertase 1 and 2 processing enzymes[1]. These proteomic studies of the major proteins of chromaffin granules demonstrate CgA-derived catestatin-containing intermediates and biologically active catestatin peptide as major components of chromaffin granules, which secrete catestatin in the regulation of blood pressure[7].

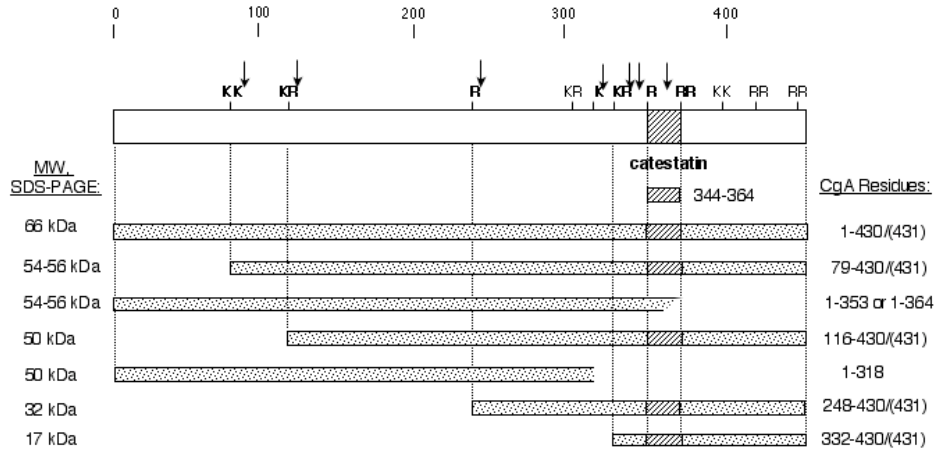

Figure 1. Proteomic approaches for understanding prohormone processing mechanisms in chromaffin granules. Catestatin-containing peptides derived from chromogranin A (CgA) were identified by NH_2-terminal sequencing and MALDI-TOF mass spectrometry. Chromaffin granules contain active catestatin peptide, $CgA_{344-364}$. Proteomics demonstrated catestatin-related peptides as full-length CgA, NH_2-terminally truncated forms of CgA (consisting of residues 79-430(431), 116-430(431), 248-430(431), and 332-430(431)), and COOH-terminal truncated forms of CgA (predicted as 1-353 and 1-318)[1].

Activity-based profiling proteomic approach identifies cathepsin L in chromaffin granules as the proenkephalin processing enzyme. In a focused proteomic study of cysteine protease activity for proenkephalin processing and production of the enkephalin opioid peptide in chromaffin granules, a high degree of enrichment and purification was

required. Moreover, selective activity-based affinity labeling of active cysteine proteases with DCG-04[8] was instrumental for identification of the enzyme responsible for the major proenkephalin cleaving activity in chromaffin granules. The enzyme was analyzed by tandem mass spectrometry of tryptic peptide digests, which indicated cathepsin L as the protease responsible for proenkephalin cleaving activity[2]. Secretory vesicle cathepsin L cleaved enkephalin-containing peptide substrates at prohormone processing sites occurring at the NH_2-terminal sides of dibasic or monobasic residues, as well as between the two residues of a dibasic site. These results show that the cleavage specificity of secretory vesicle cathepsin L results in enkephalin peptide intermediates that contain a basic residue extension at their NH_2-termini, thus, requiring further processing by an aminopeptidase B-like protease present in chromaffin granules[9]. The cathepsin L and aminopeptidase B proteolytic pathway represents an alternative pathway for prohormone processing, distinct from the prohormone convertase and carboxypeptidase E/H pathway for prohormone processing. The cleavage specificity of secretory vesicle cathepsin L is consistent with processing of CgA to catestatin.

The localization of cathepsin L to secretory vesicles of neuroendocrine chromaffin cells was visualized by immunogold electron microscopy for colocalization of cathepsin L with (Met)enkephalin in these vesicles[2]. Dual immunofluorescence microscopy demonstrated the high degree of colocalization of secretory vesicle cathepsin L with enkephalin-containing secretory vesicles.

Significantly, genetic studies with cathepsin L knockout mice illustrated the *in vivo* role of cathepsin L for enkephalin production. Brain levels of enkephalin were significantly reduced by 50% in cathepsin L knockout mice[2]. These proteomic and genetic findings demonstrate a novel biological function for secretory vesicle cathepsin L in prohormone processing in the regulated secretory pathway.

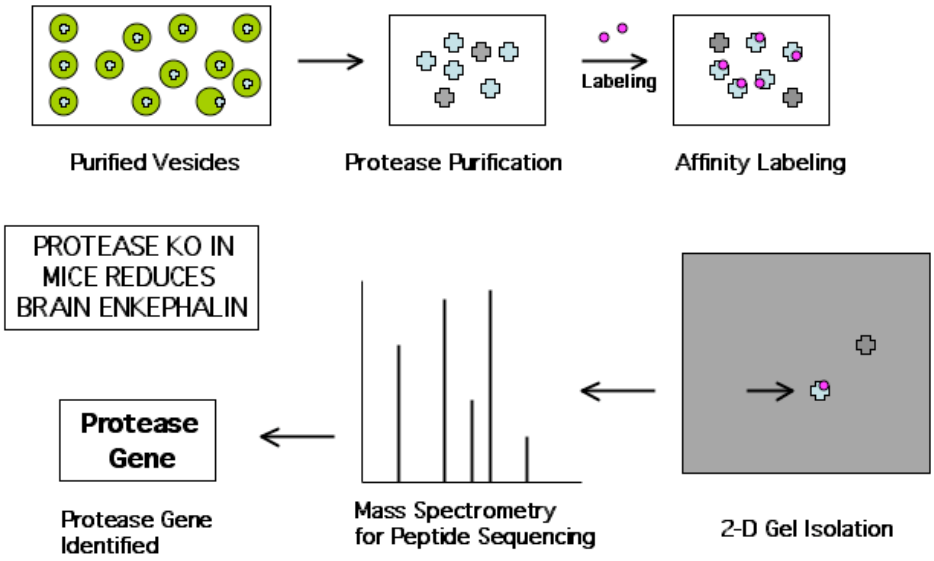

Figure 2. Activity-based profiling demonstrates cathepsin L in secretory vesicles for the production of the enkephalin opioid peptide. The cysteine protease activity in chromaffin granules responsible for proenkephalin processing was purified from chromaffin granules (secretory vesicles), identified with the activity-based probe DCG-04 for cysteine proteases, subjected to peptide sequencing by tandem mass spectrometry, and confirmed as the enkephalin-producing protease in genetic studies of cathepsin L knockout mice[2].

Future analyses of proteins and peptides in chromaffin granules. Continued proteomic studies of proteins and peptides involved in chromaffin granule function will require a step-wise approach to enrich the sample in proteins/peptides of moderate and low abundance. Resultant proteins can then be analyzed by separation via 1D or 2D gels, or LC (liquid chromatography) approaches, for peptide sequence characterization by mass spectrometry. Successive rounds of protein identification and enrichment of lower abundant proteins, by affinity-based removal of abundant proteins, will provide knowledge of proteins with differing levels of relative abundance. In any cellular system, strategies to examine

subsets of high or low abundant proteins, proteins of different gene families, or proteins localized to particular subcellular organelles are required to begin to obtain knowledge of the protein components of the functional chromaffin granule system that produces, stores, and releases biologically active peptide hormones and neurotransmitters.

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Neurotrophic factor GDNF and cAMP suppress glucocorticoid-inducible PNMT expression in a mouse pheochromocytoma model.

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Chromaffin cells and their precursors exhibit considerable plasticity of phenotype. In the developing adrenal medulla, specific arrays and timing of environmental cues contribute to the commitment of sympathoadrenal precursors to the chromaffin cell lineage. Those cues include neurotrophic factors, glucocorticoids, and intracellular messengers, including cAMP. The selective induction of developmental programs in pluripotent cells likewise implies that competing or opposing programs must necessarily be repressed or silenced at the genomic level. We hypothesize that neuronal inductive cues exert opposite, repressive influences on the glucocorticoid-stimulated profile of endocrine marker expression in those sympathoadrenal cells capable of becoming adrenergic chromaffin cells. We further report here that mouse pheochromocytoma cells (MPCs) exhibit cooperative silencing of phenylethanolamine N-methyltransferase (PNMT) expression in cell culture. This effect may serve as a model for developmentally regulated expression of PNMT in the manner of differentiating sympathoadrenal precursors *in vivo*.

Mouse pheochromocytoma (MPC) lines MPC 862L and 10/9 CRC1 (10/9s) were initially characterized by expression of PNMT and stimulus-induced secretion of epinephrine[1]. Additionally, neurotrophic factors and glucocorticoids (GCs) were found individually to elicit specific morphological responses. In MPC 862 cells, the neurite production evoked by treatment with glial cell-line-derived neurotrophic factor GDNF and cAMP distinctly contrasts with the characteristic polygonal or rounded endocrine phenotype of control (Figure 1, top).

Because these morphological changes are likely consequences of developmental commitment decisions, the objectives of this study were to resolve 1) whether expression of PNMT is regulated analogously in MPC cells and adrenal chromaffin cells and 2) whether initiation of a neuronal phenotype in MPC cells prohibits subsequent induction of an endocrine phenotype. GDNF and cAMP influences on the dexamethasone (DEX)-responsiveness of the PNMT gene were

evaluated by measuring steady state levels of PNMT mRNA and transcription from the PNMT promoter in MPC cell lines. Treatments were performed on consecutive days (Fig. 1, bottom) with cultures harvested 24 hr after beginning the third treatment interval.

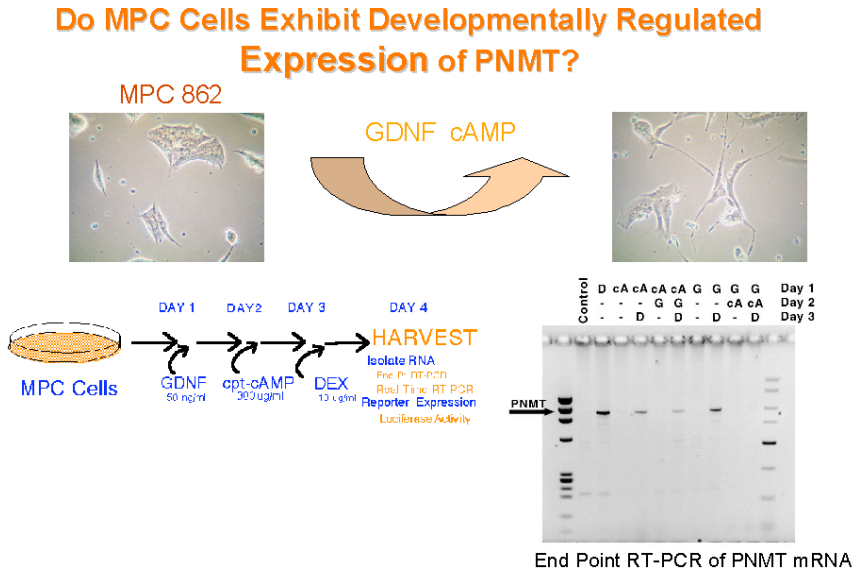

Figure 1. Neurotrophic factor GDNF (50 ng/ml) and cpt-cAMP (300 g/ml) elicit morphological changes including neurite extension in MPC 862 L cells (top). These neuronal inductive agents also significantly influence expression of PNMT mRNA in MPC cells. When MPC cells are treated sequentially with GDNF, cAMP, and DEX (bottom left), then harvested for isolation of total RNA, end point RT-PCR (bottom right) reveals that the induction of PNMT mRNA by DEX (D) is not observed following treatment with cAMP (cA) or GDNF (G). Notably, combined treatment with cAMP and GDNF prior to addition of DEX completely prevents induction of detectable levels of PNMT mRNA.

Semi-quantitative RT-PCR approaches quantified PNMT mRNA as the primary index of adrenergic response following

treatments with GDNF, cAMP, and DEX. End point RT-PCR revealed that DEX induced marked increases in PNMT mRNA levels in MPC 862s and in MPC 10/9s (Figure 1). No induction (<1-fold relative to control) occurred with GDNF or cpt-cAMP alone. However, when GDNF and cAMP together were evaluated for ability to alter responsiveness to DEX, treatment first with GDNF then with cAMP completely suppressed steady state levels of PNMT mRNA. (Individually, these neuronal agents reduced DEX-induction of PNMT mRNA by ~90% each). These neuronal cues, alone and in combination, therefore, effectively abrogated glucocorticoid-mediated induction of the endocrine phenotype in MPC cells.

To investigate the mechanism by which GDNF and cAMP alter DEX responsiveness of PNMT expression, stable transfectants of MPC 10/9 cells expressing ~ 1kb of the PNMT promoter were treated as before (Fig.1). While DEX alone elevated luciferase reporter gene activity approximately three-fold (indicating that GC signaling in MPCs analogous to previously studied chromaffin cells[2,3]), prior treatment with GDNF and cAMP significantly altered the magnitude of subsequent DEX induction. Sequential treatment of MPC 10/9s with GDNF, cAMP, then DEX produced ~50% reduction relative to DEX alone. Thus, neurotrophic factors and cAMP modulate PNMT transcription in MPC cells by decreasing the magnitude of GC induction.

Additional analyses of the 5' flanking region by transient transfection of nested deletion constructs revealed that the greatest effects of GDNF and cAMP on DEX inducibility of PNMT expression were achieved using the 'full length' ~1 kb promoter. Truncation of distal promoter regions successively reduced the magnitude of suppression up to the region of the GRE at -522 (For constructs in which DEX induction cannot occur, e.g. 3' to the GRE and with a mutated GRE, tests for DEX suppression did not differ from control expression.) These analyses demonstrate that the PNMT GRE plays a necessary, but not sufficient role in the transcriptional

aspects of this control mechanism. The greater responses observed with longer constructs, furthermore, implicate the participation of sequences in the distal 5' promoter of this gene.

In summary, MPC cells may provide important insight into regulatory mechanisms governing sympathoadrenal differentiation of neuronal and endocrine cells. In a manner that may parallel developmental responses for chromaffin cells *in vivo*, treatment of MPC cells with neurotrophic factor and cAMP effectively suppresses the induction of a specific gene, PNMT, that is critical to the acquisition of an endocrine phenotype. Moreover, the distinction between 100-fold induction of steady state mRNA levels vs. 3-fold stimulation of promoter activity argues for a regulatory mechanism in addition to transcriptional control. We have previously demonstrated that the cAMP-inducing peptide PACAP selectively enhances PNMT mRNA degradation[4] (while not influencing TH or DBH messenger half lives). That finding, coupled with our present observation that GDNF plus cAMP effectively prevent PNMT mRNA accumulation in MPC 862 and 10/9 cells, suggests that PNMT mRNA stability may represent an important locus for developmental regulation of the adrenergic phenotype.

The results of this study argue that the neuronal program of development predominates over the endocrine program of sympathoadrenal differentiation in MPC lines in a manner analogous to that observed in adrenal chromaffin cells. Furthermore, because these experiments have delineated both the individual and combined influences of specific neuronal and endocrine cues, it may additionally be noted that MPC cell lines 862 and 10/9 provide appropriate model systems for investigating the roles of intracellular signals in sympathoadrenal commitment.

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Mitochondrial mechanisms involved in nitric oxide (NO)-induced apoptosis in bovine chromaffin cells in primary culture.

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Nitric oxide (NO) is a signalling molecule that plays important roles in physiological processes including relaxation of smooth muscle, neurotransmission and host defence mechanisms against tumour cells and bacteria. Endogenous NO is synthesised from L-arginine by three isoforms of NO synthase (NOS), two of which are constitutively expressed, predominantly in neurones (nNOS) and endothelial tissue (eNOS), respectively. Generally, constitutive NOSs release small amounts of NO and are acutely regulated by calcium/calmodulin and phosphorylation. A third isoform (iNOS) is induced during inflammation and other oxidative stress events such hypoxia, producing large amounts of NO for up to long periods. NO exerts its physiological effects through the activation of guanylate cyclase and cGMP formation or through posttranslational modifications of proteins (*S*-nitrosylation and nitration). However, the induction of a high output system for NO in response to cytokines or a massive production of NO following accumulation of excitatory neurotransmitter glutamate can result in cell death. Neurones, pancreatic □-cells and macrophages seem to be particularly sensitive to NO toxicity. While in some systems, NO can react with some radicals and effectively cause cell death by necrosis, in others the progressive intra- or extra-cellular generation of NO causes apoptosis.

In bovine chromaffin cells, the presence of a constitutively expressed nNOS has been demonstrated by both biochemical and immunocytochemical methods[1-3]. In addition, the presence of NOS closely associated with ChAT-positive fibres innervating rat chromaffin cells has been reported[4,5]. In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and ACh-stimulated catecholamine (CA) secretion[1,6,8]. However, the exposure of these cells to high concentrations of NO donors, peroxynitrite or cytokines for a long time cause their death by a mixed necrotic and apoptotic mechanism, depending on NO concentration and time of exposure[7].

The cell death phenomenon, besides being an important feature in the development of the nervous system, seems to be a cause for many neurodegenerative diseases such as Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and

brain ischemia, where a gradual loss of specific sets of neurones results in disorders of movement and CNS function[9]. Regarding this subject, the study of the effects of NO on chromaffin cells, which share a common embryologic origin with neurones, is very interesting in order to examine whether these cells are good models for the study of the molecular mechanisms of catecholaminergic neuronal death underlying some neurodegenerative diseases.

Our results indicate that treatment of adrenal chromaffin cells with either NO donors or cytokines, which induce NO formation by both nNOS and iNOS activation, leads to a high output of NO and a dose-dependent apoptotic death. This apoptotic death was prevented by NO scavengers like CPTIO or haemoglobin in the case of NO donors, and by NOS inhibitors like L-NMMA and L-thiocitrulline in the case of cytokines, thus indicating that the effects are due to NO production (Vicente *et al.*, 1999). The NO-induced apoptosis in chromaffin cells takes place with an increase in hypodiploid cell number, activation of caspase-3 enzyme and DNA fragmentation, accompanied by arresting of cell cycle in the G_0G_1 phase and a decreased number of chromaffin cells in the G_2M and S phases[10]. Furthermore, the treatment of these cells with peroxynitrite mediates both necrosis and apoptosis depending on the dose and the time of stimulation. Therefore, we wondered whether the intensity of the initial insult could be related to the pathway leading to chromaffin cell death[10].

The molecular mechanisms of apoptosis involve several pathways and activation of caspases, a family of cysteine proteases, represents a common event for several pro-apoptotic stimuli. Regarding on the characterisation of the events upstream from caspase activation, mitochondrial damage has been reported to trigger this process. Consistent with this hypothesis, anti-apoptotic proteins such as Bcl-2 are located in the mitochondria, suggesting a role for this organelle in the induction of apoptotic death. Moreover, the release of mitochondrial proapoptotic factors, such as cytochrome c, is blocked by Bcl-2.

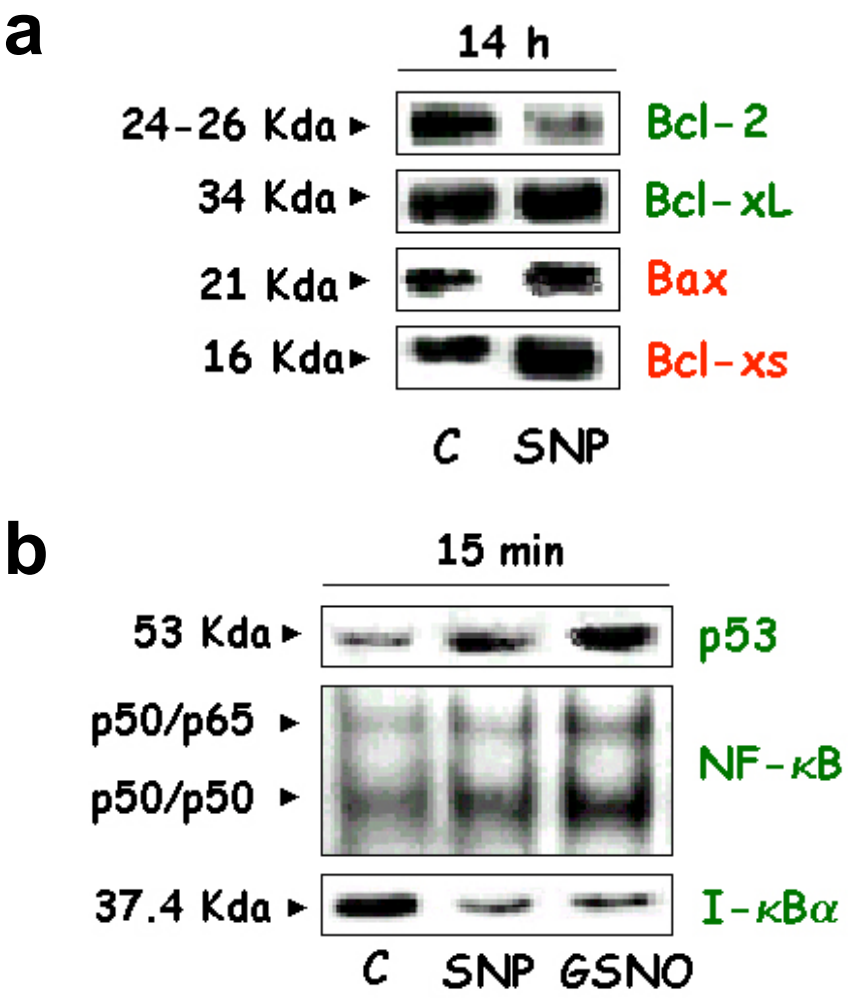

Figure 1: Effects of NO on expression of different protein involved in apoptosis. A) Western blots showing the effect of 1 mM SNP on expression of anti-apoptotic (Bcl-2 and Bcl-XL) and pro-apoptotic (Bax and Bcl-Xs) proteins of Bcl-2 family.at 14 h of incubation. B) Effect of 1 mM SNP on p53 expression and on NF-□B binding to iNOS promoter (EMSA) and I□B□ degradation at 15 min incubation..

The aim of this work was to assess the suspected involvement of mitochondrial mediators in the apoptotic death induced by NO in chromaffin cells, emphasizing the time course of these events. Us-

ing bovine chromaffin cells in primary culture and different NO donors (SNP, SNAP and GSNO) at apoptotic concentrations (100-1000 μM), we have demonstrated that NO induces a time-dependent decrease in trans-mitochondrial membrane potential ($\Delta\Psi_m$), measured by decrease in fluorescence of TMRM, being this effect detected after 4 hours of incubation with the NO donors. This effect preceded both NO donor-induced activation of caspase-3, which could be reversed by the inhibitor CPP32 at 50 nM, and appearance of hypodiploid cells, measured by flow cytometry. These events occurred after 8 hours of treatment and were maximal after 24 hours. The disruption of $\Delta\Psi_m$ is followed by cytochrome c release from the mitochondria to the cytosol, being this effect maximal after 14-16 h incubation with NO donors, and accompanied by a decrease in the mitochondrial content of the protein. Thus, both events occurred upstream from the caspase 3 activation and subsequent apoptosis in chromaffin cells.

The involvement of the Bcl-2 protein family in the NO-induced apoptosis was demonstrated by evaluating the effect of NO donors on the expression of different antiapoptotic (Bcl-2 and Bcl-x_L) and proapoptotic (Bax and Bcl-x_s) members of this family. Results (Figure 1a) show that NO donors mediate the inhibition of Bcl-2 expression, which was minimal after 14-16 hours incubation (25% of control value at 1 mM SNP). This effect was preceded by a time-dependent increase in the expression of this protein, thus indicating the activation of a survival pathway as an attempt to protect chromaffin cells against NO-induced apoptosis. The most significant effect was the dose-dependent induction of the proapoptotic protein Bcl-x_s expression, which was maximal between 14-16 hours of incubation with the NO donors (2,5 fold higher than the control value, at 1 mM SNP). On the other hand, the changes in the expression of the proapoptotic Bax and antiapoptotic Bcl-x_L were smaller (20-30% over the control). Main changes in the expression of Bax and Bcl-x_s preceded the decrease in Bcl-2, which indicates that both effects may be regulated by different cell signalling mechanisms.

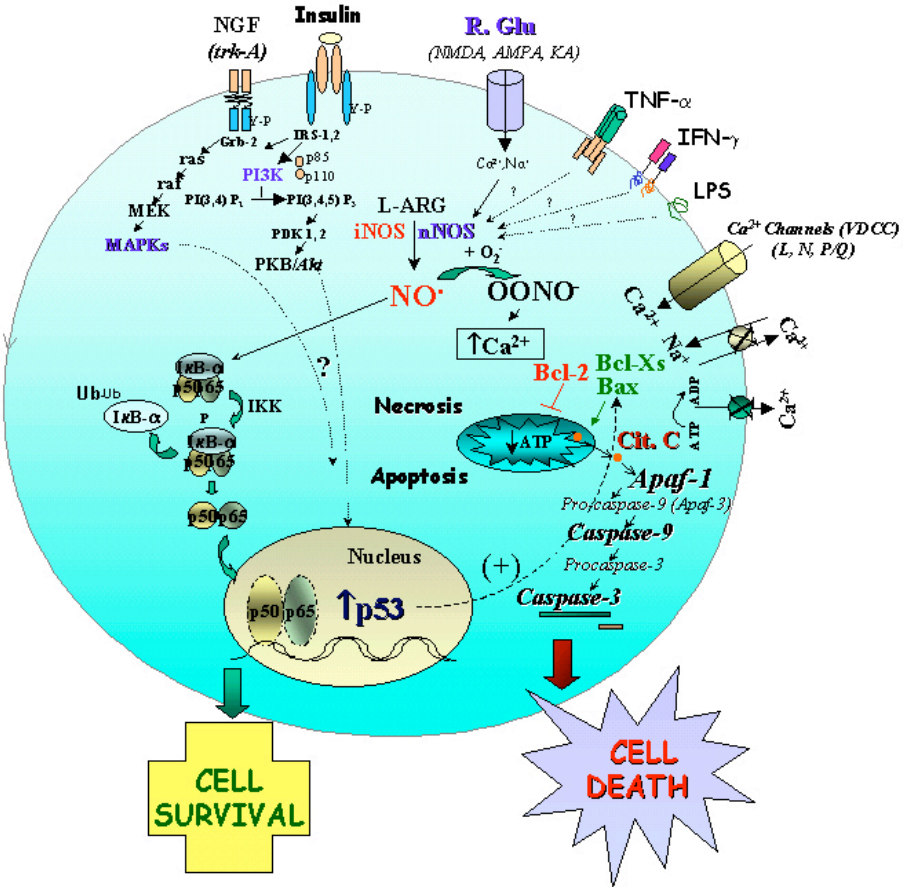

Figure 2: Summary of the proposed mechanism for the apoptotic events induced by NO in primary cultures of bovine chromaffin cells. NO, which could be generated in the cells by induction of nNOS and/or iNOS by cytokines or activation of glutamate receptors (Vicente 2000), mediates a decrease in $\Delta\Psi_m$, which is followed by a decrease in the Bcl-2 expression, an increase in cytochrome C (Apaf 2) release into the cytosol, the activation of caspase 9 and 3 and apoptotic cell death. It is likely that the induction of p53 expression is one of the earliest events after the initial insult, which could be responsible for the regulation of the expression of Bcl-2 family proteins. The expression of p53 could be, in turn, regulated by NF-κB. The growth factors NGF and IGF-1 are able to block these events, thus preventing both caspase activation and cell death.

One of the early events associated with NO-dependent apoptosis is a rapid rise and accumulation of the tumour suppressor protein p53, which reflects the cell stress elicited by NO. In chromaffin cells, an induction of the expression of p53 occurred between 15-30 minutes of incubation with different NO donors, and the effect was time-dependent and maximal between 1-24 hours, depending on the NO donor (Figure 1B). The NO-dependent increase of p53 has been consistently observed and it has been suggested that this accumulation leads to the expression of several proteins, which ultimately participates in apoptosis, like Bax. Following p53 upregulation, Bax levels could increase and heterodimerizate with other members of the Bcl-2 family, thus triggering the apoptosis. Indeed, as in chromaffin cells, in other cell types it has been observed that the overexpression of Bcl-2 inhibits p53-dependent apoptosis, which suggests a role for p53 as an initial step in the NO-dependent apoptotic process.

Finally, the activation of NF-κB to the nucleus, measured by the binding of NF-κB to nuclear proteins and I-Bα degradation, occurred after 15-30 minutes of incubation with NO donors and cytokines (Figure 1b), and seems to be another early mechanism involved in NO-induced apoptosis. However, NF-κB is a survival factor in these cells, since 10 μM SN50 (an inhibitor of NF-κB translocation) enhanced the NO-induced apoptosis in chromaffin cells. Therefore, in spite of its activation, NF-κB is not able to rescue cells from NO-induced apoptosis.

These results taken together strongly support the role of mitochondrial mediators in NO-induced apoptosis in chromaffin cells and point out these cells as good models for investigating the molecular mechanisms involved in neurodegenerative diseases with catecholaminergic neuronal death and the mechanisms of neuroprotection against apoptotic death underlying these important diseases.

Figure 2 summarises the molecular mechanism proposed for the involvement of mitochondrial mediators in NO-induced apoptosis in chromaffin cells.

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Characterization of enterochromaffin cells isolated from the rat ileum.

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Enterochromaffin (EC) cells are distributed throughout the digestive epithelium. This cell type releases serotonin besides a variety of peptides and hormones, thereby being a key messenger for intestinal motility as well as secretion. Studies using highly enriched, isolated EC cells from rat ileum now reveal a large spectrum of receptors specifically expressed on this cell type, for example β-adrenergic, muscarinic as well as GABA-ergic receptors. Interestingly, also EC cells seem to express several bitter taste and olfactory receptors, which might shed a light on a physiological function as a 'sentinel cell' in the gut. Current studies determine the response of these enriched cells towards receptor antagonists as well as antagonists, offering a large perspective to treat patients with motility disorders, diarrhoea or constipation. Gastric EC-like cells, in contrast, have been characterised since already ten years. This cell type releases histamine as a paracrine stimulant, thereby controlling the peripheral regulation of acid secretion. ECL cells respond to treatment with gastrin as well as PACAP with histamine release; somatostatin and galanin inhibit the release. So far, there is a lot of information regarding the physiological function; ECL cells, however, have not become a target for pharmacological therapy. Microarray techniques are currently being performed to determine key factors during the malignant transformation of these tumors.

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Enterochromaffin-like (ECL) cells: Lessons from the past. ECL cells are histamine-containing neuroendocrine cells located in the lower third of the acid-secreting gastric epithelium[1,2]. They constitute more then 30% of the neuroendocrine cell population of the gastric mucosa and are important for the peripheral regulation of gastric acid secretion. In response to gastrin and PACAP they release histamine in a calcium-dependent manner thereby stimulating acid secretion from parietal cells. Storage of histamine in secretory vesicles is dependent on transport via the vesicular monoamine transporter-2 (VMAT-2). Hypergastrinemia leads to proliferation and tumor development of ECL cells. These tumors infiltrate nearby structures but never metastatize. ECL cells can be isolated and highly enriched from the gastric mucosa. Previous studies have characterized the presence of certain exocytotic and endocytotic proteins specifically in ECL as well as other neuroendocrine cells, thereby serving as marker genes to identify the characteristic parameters during mircroarry techniques[3,4]. Thus, cell biology studies have served as a basis to determine changes during inflammatory or malignant transformations in the gut.

Enterochromaffin (EC) cells. EC cells are polarized neuroendocrine cells of the gastrointestinal epithelium, which synthesize, store and release serotonin (5-HT) as well as a variety of peptides such as guanylin[5,6]. They accumulate 5-HT in secretory vesicles via VMAT-1[7,8]. In vivo, 5-HT release is sensitive to mechanical stimulation or acidification of the gut lumen[9,10]. 5-HT regulates intestinal motility and transport in paracrine/endocrine and neuronal ways[11]. Activation of 5-HT receptors on intestinal crypt cells leads to chloride secretion[12]. The importance of 5-HT receptors on enteric nerves varies among intestinal regions and species[13,14]. Binding of 5-HT to the G-protein coupled 5-HT4 receptor on intrinsic neurons elevates intracellular cAMP levels and inhibits voltage gated K^+ channels, leading to depolarization and hence increased excitability. 5-HT4 receptors also appear to be important for the initiation of peristaltic reflexes. Furthermore, 5-HT is thought to play a role in generation of some diarrheal states caused by bacterial toxins [15,16] and several pathological conditions such as vomiting, diarrhea after radio- or chemotherapy[17,18] and irritable bowel syndrome[19,20].

A large number of stimulatory and inhibitory receptors have already been postulated as being present on EC cells [21]. However, the actual target and hence mechanism of action of most of the drugs used in intestinal therapy is not clear since only heterocellular tissue or organ preparations have been used[21,22]. The release of serotonin is thought to be calcium-dependent, suggesting the involvement of receptor-operated or voltage-dependent calcium channels, although a cellular attribution is difficult in the crypt preparations investigated[23]. An enriched preparation to investigate functions of and potential pharmacologic targets on EC cells of the small intestine has not been available to date.

Table 1: Enrichment of EC Cells from rat intestine as determined by Immunocytochemistry and serotonin content

	Mucosal cells	**EC Cells**
5-HT positive cells (%)	12 ± 4	84 ± 6
SNAP-25 positive cells (%)	42 ± 4	86 ± 8
5-HT content ($pg/10^5$ cells)	0.5 – 1	6 – 12

Isolation of enterochromaffin cells from the intestine. To obtain an enriched fraction of isolated EC cells, a combination of enzymatic digestion, counterflow elutriation, density gradient centrifugation and short-term culture was applied. The protocol used the small size and high cellular density of EC cells and was a modification of the isolation procedure for gastric ECL cells[24]. With this procedure, EC cells could be enriched ~7fold compared to the crude cell suspension (MUC) based on serotonin immunocytochemistry (table 1). Most of the contaminating cells in the enriched fraction were identified as goblet cells by PAS staining. The high percentage of SNAP-25 positive cells in the unfractionated cells indicates the presence of other neuroendocrine cells. While most of the isolated cells excluded Trypan blue, a loss of cell integrity occurred within 24 h in culture. This loss of survival seems characteristic of isolated intestinal epithelial cells[25]. Serotonin content was significantly enhanced in isolated EC cells when compared to

unfractionated mucosal cells from the ileum, yielding a 7-12 fold enrichment (see table 1).

Receptors present on EC cells. RT-PCR and imuno-cytochemistry were performed on EC cells after the enrichment procedure to determine possible receptors present on these cells. As shown in table 2, both methods gave positive results for alpha2A and beta1 adrenoreceptors as well as muscarinic M3 and the GABA-A receptors. Furthermore, RT-PCR revealed the presence of RNA for alpha2B and beta2 adrenoreceptors. No positive signal was obtained for the GABA-B receptor. The presence of adreno- and muscarinic receptors in EC cells has been deduced by Racke et al. based on data using isolated porcine or guinea pig tissue strips[26-28]. Thus, the isolated and enriched cells largely confirm these previous data. In contrast to previous studies[29], no 5-HT3 and 5-HT4 receptors were detected on isolated EC cells.

Table 2: Receptors expressed on EC cells

Receptor	Immunucytochemistry	RT-PCR	Affymetrix Gene chip
α_{2A}	+	+	+
α_{2B}	n.d.	+	+
β_1	+	+	+
β_2	n.d.	+	+
M_3	+	+	+
GABA-A	+	+	+
GABA-B	n.d.	-	?
5-HT_3	n.d.	-	-
5-HT_4	n.d.	-	-

Gene chip analysis. To further investigate gene expression in isolated EC cells, the Affymetrix GeneChip Rat Genome RG-U34A array, which allows detection of 5467 genes of known function, was used. Several genes were identified which showed both a relatively high level of expression (400 or greater) as well as a more than 4-fold

higher expression level in the EC cell fraction as compared to unfractionated mucosal cells (table 3). Expression of components necessary for storage of serotonin in EC cells was as expected. For example, the vesicular monoamine transporter 1 (VMAT-1), a specific marker of EC cells, was present, but not VMAT-2 which is present in gastric ECL cells[7]. In addition, a sodium dependent neurotransmitter transporter[30] appears to be expressed in EC cells. Animals lacking the serotonin reuptake transporter (SERT) still have the ability to accumulate serotonin in EC cells[31], suggesting a possible involvement of this plasmalemmal transmitter transporter found here.

Expected receptor expression on EC cells as determined by gene chip analysis. A variety of receptor genes were detected that were both highly enriched (> 7-fold) and expressed at high levels. It appears that the cells in the preparation selectively express the α_{1C} adrenoreceptor, nicotinic acetylcholine receptors, the histamine-2 receptor, GABA-A receptor, metabotropic and ionotropic glutamate (AMPA R1 and NMDA receptors R1 and others). Most of these receptor subtypes were also identified using IC and RT-PCR, similar to results in previous works using intestinal strips[29]. In contrast, GABA-B receptors were detected only in Gene chips, indicating there may be a cross-reacting with sequences to related receptor sequences such as glutamate receptors. GABA-B receptors are heterodimeric complexes with numerous splice variants. The heterogeneity of GABA-B receptors is quite complex and not fully investigated in detail yet[32]. Presence of "metabotropic" GABA-B receptors was determined by immunohistochemistry here in previous works[33]; however, several receptor subtypes exist which may bind various antibodies. N-methyl-D-aspartate receptors (NMDARs) are usually present at many excitatory glutamate synapses in the central nervous system, displaying unique properties that depend on their subunit composition[34]. Although not confirmed by specific immunostaining, the clustering of these genes suggests the selective expression

Table 3: Relative enrichment of EC cell mRNA expression in purified EC cells

	MUC (n=2)	EC (n=3)	x-fold
β_2 adrenergic receptor protein (RG20)	14.3	212.4	14.9
α_{2A} adrenergic receptor protein (promoter)	189.2	897.0	4.7
α_{1C} adrenergic receptor protein (promoter)	32.4	677.4	20.9
α_1 adrenergic receptor	34.9	155.3	4.5
β_2 adrenergic receptor gene	140.3	570.2	4.1
β_3 adrenergic receptor (spliced version)	118.9	696.5	5.9
M_1 muscarinic receptor	158.9	147.5	0.9
M_2 muscarinic receptor	131.0	440.1	3.4
M_3 muscarinic receptor	119.3	523.7	4.4
M_4 muscarinic receptor	229.1	460.6	2.0
Nicotinic acetylcholine receptor α_6 subunit	683	607.1	8.9
GABA-A receptor α_1 subunit	46.9	330.2	7.0
GABA-A receptor β_2 subunit	69.6	455.6	6.6
GABA-A receptor β_5 subunit	43.9	193.0	4.4
GABA-A receptor δ_6 subunit	22.8	277.7	12.2
GABA-A receptor γ subunit	172.8	1535.6	8.9
GABA-A receptor ρ_2 subunit	50.6	206.2	4.1
GABA-B receptor 1c	122.5	503.5	4.1
GABA-B receptor 1d	198.5	1178.8	5.9
metabotropic glutamate receptor mGluR7	15.55	291.5	18.7
glutamate receptor (GluR-B)	22.3	345.3	15.5
glutamate transporter	35.0	238.3	6.8
NMDA-R1	33.5	970.7	29.3
NMDA-R2C	198.2	991.2	5.0
NMDA-R2D1	148.6	645.6	4.3
NMDA-NR3	122.0	511.2	4.2
AMPA GluR1	35.6	818.7	23.2
olfactory OL1	84.1	535.4	6.4
olfactory QIL-LD1	161.7	1274.4	7.9
olfactory HGL-SL3	56.8	529.4	9.3
olfactory HGL-SL2	81.0	533.4	6.6
olfactory HVL-VN1	93.0	751.9	8.1
olfactory receptor-like protein	369.0	1622.1	4.4
olfactory EVA-TN1	191.5	876.3	4.6
olfactory SCR-D7	76.4	630.0	8.3
olfactory SCR-D8	164.6	734.0	4.5
olfactory receptor-like proteinSCR-D9	139.7	744.2	5.4
putative pheromone receptor	44.8	997.9	22.3
putative pheromone receptor	27.2	567.4	20.9
putative pheromone receptor	128.0	1139.5	8.9

of these receptors in EC cells. In addition, the metabotropic glutamate receptor subunit 4C appears to be related to EC cells. A possible effect of glutamate on EC cells may be related to the stimulation of serotonin secretion following neuronal stimulation.

A relative increase in mRNA was also detected for the muscarinic M3 and M5 acetylcholine receptor subtype and some subunits of the NMDA receptor. A high expression level and similar enrichment was also observed for the dopamine receptor D4. Most of the receptors demonstrated as present by gene chip analysis are not yet amenable to specific immunostaining, but the detection of many genes of several neuronal receptor families suggest that the cells in this EC cell enriched preparation have both neuronal and endocrine characteristics.

Un-expected receptor and protein expression on EC cells. Surprisingly, we also found clustering of several genes encoding olfactory and vomeronasal receptors. Such genes encoded olfactory SCR, HGL, HFL, QIL, and EVA receptor subtypes and pheromone receptors. The presence of these receptors was completely unexpected; however, the strong signal of several subtypes may suggest that some are selectively expressed in EC cells. It appears that EC cells may analyze chyme, similar to other intraepithelial sensors in the airway or blood system. The possibility exists that olfactory receptors may have a more general function in chemodetection similar to the situation observed in spermatids, taste buds and olfactory systems[35].

Besides the marker protein guanylin, secretin, VIP and galanin were detected as highly expressed and enriched in the EC cell fraction but other peptides were also present in the preparation such as vasopressin, secretogranin II and atrial natriuretic peptide (ANP). Vasopressin is a potent vasoconstrictor in the rat jejunum, enhances proliferation of mucosa cell lines and has been found previously in the gastrointestinal tract in nerve fibers and in enteroendocrine cells[36,37]. These findings may indicate that EC cells play an important role in blood flow and motility (via V_1 receptors in smooth muscle), resorption of electrolytes and fluid retention in the intestine. Secretogranin II, which is processed in the intestine to secretoneurin,

has previously been localized in D-cells of the human gut[38]. Secretoneurin has been described as a link to the immune system by inducing the migration of monocytes and leukocytes[38]. A possible function of ANP in the EC cell may be related to processes of electrolyte and water transport in the mucosa. Finally, factors involved in intracellular signaling were identified as highly expressed and enriched in the EC cell fraction. These include components participating in IP_3 metabolism and coupling via G-proteins (IP_3 binding proteins, inositol polyphosphate phosphatase, G protein coupled kinase 6). These components may participate in signal transduction after activation of olfactory or other receptors observed to be highly expressed in EC cells.

SUMMARY

The current data generated by gene chip analysis suggest the presence of certain specific genes in EC cells; many of which appear to be new to this cell type, some have already been assigned to EC cells by immuncytochemistry. Additional studies are needed to determine the accuracy of the gene chip readout and to assign specific proteins to EC cells rather than other small neuroendocrine cells. Our novel approach therefore defines several potential proteins of interest in regulation of EC cell function. Continuation of these studies at the proteomic level will likely yield a better understanding of the biology of this important neuroendocrine cell.

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Culturing Pheochromocytoma Cells.

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Cell Biology of the Chromaffin Cell
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Pheochromocytoma cells from humans, rats and mice rapidly cease proliferating in primary culture. In addition, variable proportions of the tumor cell populations undergo spontaneous neuronal differentiation [1-3]. Establishment of pheochromocytoma cell lines is therefore a challenging task. Immunocytochemical staining for tyrosine hydroxylase (TH) and bromodeoxyuridine (BrdU) after BrdU pulse-labeling [4] provides a means for rapidly assessing the success of attempts to establish cell lines and avoids the pitfall of propagating irrelevant types of cells while performing biochemical studies on persistent pheochromocytoma cells that are progressively diluted with successive cell passages. In most instances, proliferation of TH-positive cells ceases within two weeks, although the cells persist in cultures maintained for many months (Figure 1).

The NGF-responsive PC12 cell line, established from a rat pheochromocytoma [5], has for almost 30 years served as a tool for many aspects of neurobiology involving normal and neoplastic conditions. Recently developed mouse pheochromocytoma (MPC) lines from neurofibromatosis knockout mice supplement PC12 cells and have generated additional applications [6]. Advantages of the mouse lines include expression of substantial levels of phenylethanolamine N-methyl-transferase and expression of high levels of the receptor tyrosine kinase, Ret, which is characteristic of sporadic and familial human pheochromocytomas but not of PC12 cells. Disadvantages include an apparently less stable phenotype. Some MPC lines respond to the Ret-activating ligand, glial cell line–derived neurotrophic factor (GDNF), by ceasing to proliferate and undergoing neuronal differentiation similarly to NGF-treated PC12 cells[7].

The phenotype of PC12 cells has been remarkably stable for cells maintained as originally described in detail [5]. However, diminished NGF responsiveness, decreased numbers of large secretory granules or loss of other desired traits has occurred in some laboratories, emphasising the importance of freezing and storing early passages of any cell line. The characteristics of the cells have also been affected by culture conditions, most notably a switch made in some laboratories early in the history of

the cell line from RPMI 1640 medium to Dulbecco's modified Eagle's medium (DMEM), which increases cell flattening and cell-substratum adhesion because of its higher Ca^{++} concentration. PC12 cells maintained in DMEM often regain characteristics of canonical PC12 cells when re-introduced to RPMI. The properties of the cells are also transiently affected by different lots of serum, particularly horse serum, which may inhibit NGF responses, and by high plating density, which inhibits NGF responses while increasing catecholamine content.

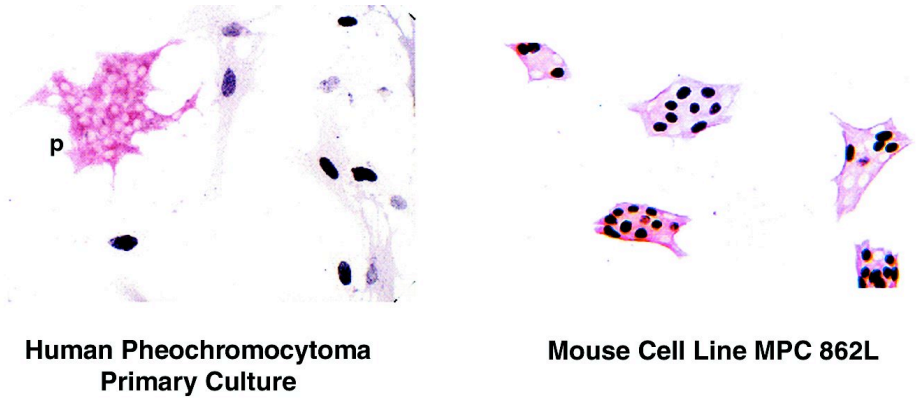

Figure 1. Double labeling for TH (pink cytoplasm) and BrdU (black nuclei) in primary culture of human pheochromocytoma compared to mouse pheochromocytoma cell line. A cluster of approximately 40 pheochromocytoma cells (p) in the human culture shows no BrdU incorporation, in contrast to adjacent, robustly proliferating but irrelevant cell types in the same culture and in contrast to the mouse cell line.

It should be borne in mind that PC12 cells and five of six lines of MPC cells arose from animals that had been irradiated postnatally, probably with resultant genetic damage that permitted the lines to be established. Pheochromocytoma cells from aged rats, non-irradiated *Nf1* knockout mice, MEN2B transgenic and *Rb* knockout mice as well as benign or malignant human pheochromocytomas persist in primary cultures but do not proliferate. This experience indicates that caution is warranted in drawing general conclusions from any single cell line, but

also suggests that understanding of factors that permit pheochromocytoma cells to proliferate might itself provide important insights for tumor biology.

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Genomics and proteomics of the chromaffin cell: characterization of cell differentiation and chromogranin peptide formation.

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Cell Biology of the Chromaffin Cell
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Chromaffin cells of the adrenal medulla have been widely used to decipher the mechanisms of neurotransmitter action as well as hormone biosynthesis and exocytosis. Moreover, the chromaffin cell-derived pheochromocytoma PC12 cells represent a useful model for studying neuronal and neuroendocrine cell regulation and differentiation in the sympathoadrenal lineage. We took advantage of the technological advances in the fields of genomics and proteomics, which allow the integration of a large amount of gene expression information in a unique and global view, to gain insight into the genetic program that governs sympathoadrenal cell differentiation. For this purpose, we compared the transcriptomes of undifferentiated rat pheochromocytoma PC12 cells and terminally differentiated rat adrenomedullary cells [1]. This analysis allowed the identification of more than one thousand differentially expressed genes (Fig. 1, A and B), including a large number of factors involved in cell proliferation that are mainly overexpressed in PC12 cells, *i.e.* components of mini-chromosome maintenance deficient complex or pituitary tumor transforming 1. On the contrary, the majority of the genes involved in cell adhesion and ion transport were more highly expressed in chromaffin cells, *i.e.* biglycan or ferredoxin reductase. It is worth noting that an important group of the identified genes that are differentially expressed between chromaffin cells and PC12 cells is associated with apoptosis and protein processing, *i.e.* proteasome subunits or glutathione peroxidases. In order to better understand the transcriptional events that accompany the differentiation of sympathoadrenal cells, we studied the modifications of the gene expression profile of PC12 cells following treatment with the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), a major regulator of adrenal activity [2] which is able to functionally differentiate these cells [3,4]. Microarray and subtractive hybridization analyses identified 145 genes regulated by PACAP in PC12 cells (Fig. 1, C and D), a large proportion of which are involved in cell proliferation and signaling, *i.e.* B-cell translocation gene 2 or bone morphogenetic protein 6. PACAP also regulated the expression of several genes associated with cell survival, *i.e.* Bcl2-associated athanogene 3, and motility/adhesion, *i.e.* kinesin-like 5 and embigin. Comparison of the transcriptome alterations in PC12 cells versus

adrenomedullary cells and undifferentiated PC12 cells versus PACAP-treated PC12 cells revealed that the majority of the common genes identified in both analyses are in fact more actively expressed in tumoral chromaffin cells and down-regulated by PACAP. These are mainly factors involved in cell proliferation, which are likely to be involved in the differentiation of sympathoadrenal cells during development.

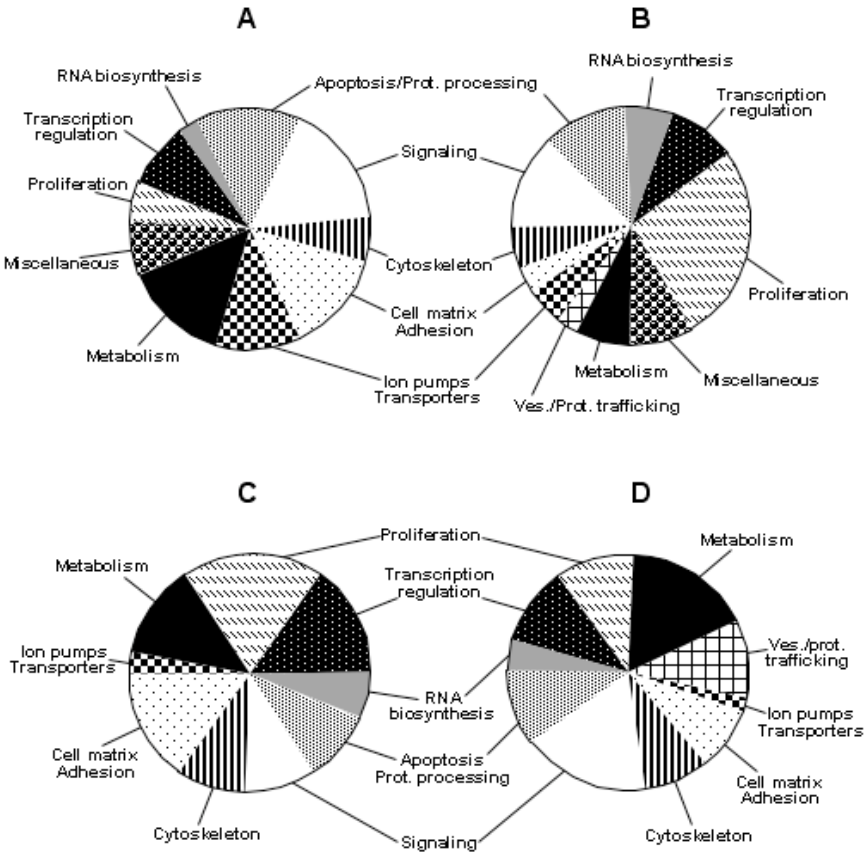

Figure 1. Functional clustering of the genes differentially expressed between rat adrenomedullary cells, PC12 cells and PACAP-treated (100 nM, 48 hrs) PC12 cells. A) genes over-expressed in adrenomedullary cells compared to PC12 cells. **B)** genes over-expressed in PC12 cells compared to adrenomedullary cells. **C)** genes down-regulated by PACAP in PC12 cells. **D)** genes up-regulated by PACAP in PC12 cells.

Among the genes differentially expressed in the three cell models studied, we identified various members of the chromogranin family of proteins which play a role in the acquisition and the function of the secretory phenotype [5].

The high homology of discrete sequences of chromogranins, that are delimited by basic residues whereas the rest of the proteins is poorly conserved in phylogenetically distant species, strongly suggests that these proteins are precursors of potentially active peptides [6,7]. In order to investigate the biological relevance of these conserved regions of chromogranins in sympathoadrenal cells, we first demonstrated by immunohistochemistry and HPLC analysis the occurrence of the peptides EL35 and WE14, which derive from chromogranin A, and EM66, which originates from secretogranin II processing, in both fetal and adult human adrenal gland. The possible implication of these peptides in pathological processes is supported by the presence of EM66 in various pheochromocytoma samples. Interestingly, a significant difference in EM66 concentration was observed between benign and malignant tumors, suggesting that measurement of EM66 levels may be of clinical value for the prognostic of pheochromocytoma progression [8-10].

In conclusion, genomics and proteomics approaches have been applied to identify genes and peptides associated with normal and tumoral chromaffin cells, in order to provide new insight in the molecular mechanisms involved in sympathoadrenal cell differentiation in normal and pathological condition. We are currently focusing on the functional characterization of the identified genes and peptides in this cell lineage.

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The *Virtual Chromaffin Cell*: analyzing Ca^{2+} transients in active secretory zones.

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Cell Biology of the Chromaffin Cell
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Although the importance of cytosolic Ca^{2+} signals in triggering hormone and neurotransmitter release was recognized some four decades ago, there remain critical unresolved issues regarding the dynamics and spatial distribution of the Ca^{2+} signal in sub-membrane secretory zones. Because Ca^{2+} enters the cell via voltage gated Ca^{2+} channels and moves down a steep gradient of four orders of magnitude (10^{-3} M extracellular to 10^{-7} M cytosolic), it's magnitude near exocytotic sites near the mouth of the Ca^{2+} channel is difficult to experimentally determine and remains poorly defined. This is due to limitations of spatial and kinetic resolution of experimental cytosolic Ca^{2+} measurements in sub-membrane microdomains. Yet it is this active zone Ca^{2+} that plays a critical role in evoking vesicle fusion with the plasma membrane and exocytotic secretion. An understanding of the spatio-temporal properties of Ca^{2+} signals in sub-membrane secretory zones could provide valuable information about the regulation of these signals, which in turn control the interaction of Ca^{2+} with its sensor in the exocytotic machinery.

In the present work we have developed the ***Virtual Chromaffin Cell***, (**VCC**) an advanced, multi-compartment, computational model of Ca^{2+} signaling in adrenal chromaffin cells[1]. The model represents a specific application to chromaffin cells of the more general cell modeling software known as the Virtual Cell[2]. The **VCC** facilitates a quantitative simulation of cytosolic Ca^{2+} transients with high spatial (10 nm) and kinetic ($\leq$ ms) resolution. It thus allows an evaluation of competing cellular processes that control the shape of sub-membrane Ca^{2+} transients. Unlike other simulation programs which assume uniform compartment concentrations, the **VCC** allows for concentration gradients within a compartment, thereby permitting an analysis of cytosolic Ca^{2+} gradients in sub-membrane microdomains near sites of exocytosis. These model simulations complement experimental measurements and provide new insights into the Ca^{2+} signal regulating secretion.

RESULTS AND DISCUSSION

In this work we have focused mainly on the fast (sub-second) Ca^{2+} transients in active secretory zones near the plasma membrane, which are

relevant to interactions with the Ca^{2+} sensor in the exocytosis mechanism. The specific Ca^{2+} control systems we have considered include: **a)** Ca^{2+} influx during repetitive activation of Ca^{2+} channels in various spatial arrays, **b**) diffusion, including sub-cellular diffusional barriers, **c)** binding to mobile and fixed Ca^{2+} buffers, including Ca^{2+}-binding proteins and charged membrane phospholipids (e.g., PS), **d**) Ca^{2+} sequestration by subcellular organelles, and **e**) efflux out of the cell via plasma membrane Ca-ATPase and Na-Ca exchange. Parameters quantifying transport and binding processes were chosen from the experimental literature to the extent possible. We found that the amplitude and kinetics of fast (ms-second), sub-membrane Ca^{2+} transients are dominated by the competition between diffusion and endogenous buffering as well as the spatial distribution and rate of repetitive activation of Ca^{2+} channels. Organelle Ca^{2+} sequestration (e.g., mitochondrial) and plasma membrane efflux mechanisms were slower (seconds – 10's of seconds) and found to contribute to a later phase of decay.

Figure 1 illustrates the effects of the rate of repetitive Ca^{2+} channel activation, on the sub-membrane Ca^{2+} signal adjacent to the Ca^{2+} channel, both in the presence and absence of endogenous Ca^{2+} buffers. Two repetitive activation rates were chosen: 3 Hz and 30 Hz. The slower rate is typical of maximal stimulation of chromaffin cells by the physiological transmitter, acetylcholine. The faster activation rate can result from stimulii that impose prolonged depolarization. A uniform array of Ca^{2+} channels was chosen with a membrane density of 16 channels/μ^2, a channel density close to the upper limit known for chromaffin cells. A fixed endogenous buffer was chosen with a Ca^{2+} binding affinity, Kd = 10 μM, unbinding rate constant, koff = 1000 s^{-1} and buffer concentration of 1 mM.

The top panel in Fig.1 shows that, in the absence of buffer, peak Ca^{2+} concentration near the mouth of the Ca^{2+} channel can rise to 120 μM in a 3 ms opening of the channel during an action potential, and then rapidly decays back to about 10 μM within 50 ms. Repetitive opening of Ca^{2+} channels at a rate of 3/s results in 9 μM increases in the Ca^{2+} signal above the previous values with each activation.

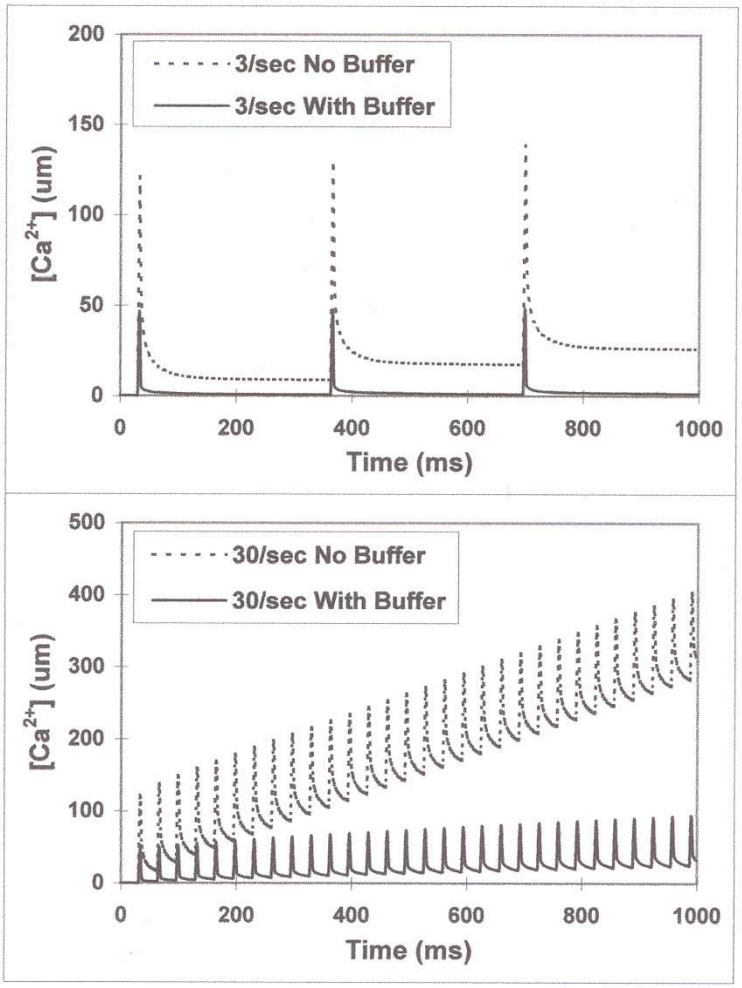

Figure 1. The effects of repetitive opening of calcium channels and Ca^{2+} buffering on the Ca^{2+} signal near mouth of channel. Top panel: Rate of repetitive channel activation = 3/s. Upper Ca^{2+} signal curve in the absence of buffer. Lower Ca^{2+} signal curve in the presence of buffer. Fixed buffer concentration = 1 mM with Kd = 10 μM and k_{off} = 1000 s^{-1}. **Bottom Panel**: Repetitive channel activation rate of 30/s. Upper curve in absence of buffer; lower curves in presence of same buffer as in top panel. VCC biomodel is radially oriented rectangular rod with 0.25 x 0.25 μm surface membrane and 8 μm deep into the cell, with reflecting boundaries, which create the equivalent of a large 2-D array of 16 channels/μ^2. Single channel current and open time = 0.3 pA and 3 ms, respectively. Computational unit volume element is 25nm x 25nm in plane of membrane x 80 nm depth.

Addition of buffer has a profound effect on the repetitive Ca^{2+} signal. It's peak value is lowered to less than 50 μM near the channel mouth and the signal returns close to resting levels (less than 1 μM) between openings of the channel, such that there is very little increase in signal at the 3 Hz repetitive activation rate.

Increasing the repetitive channel activation rate to 30 Hz dramatically raises the Ca^{2+} signal in the absence of buffers (bottom panel in Fig.1). Again the initial peak value is 120 μM and the increase with each activation is about 10 μM. Maintaining the 30 Hz activation rate for 1 second, in the absence of buffers, results in the peak Ca^{2+} signal rising to about 400 μM, with trough values between Ca^{2+} spikes reaching about 300 μM. Again, the presence of 1 mM of a Kd =10 μM fixed buffer substantially limits the rise of the 30 Hz repetitive Ca^{2+} signals, such that peak values remain under 100 μM and trough values reach only 25 μM at the end of 1 second of repetitive channel activation. These results provide a clear illustration of the critical importance of both the rate of repetitive channel activation and endogenous Ca^{2+} buffers on the Ca^{2+} signal in sub-membrane regions near Ca^{2+} channels and active secretory zones.

We have also explored the effects on the sub-membrane Ca^{2+} signal of a variety of other buffer binding parameters, buffer mobility, diffusion rates, diffusional barriers in the cytosol (e.g., organelles), organelle Ca^{2+} sequestration and plasma membrane efflux mechanisms (Na/Ca exchange, Ca-ATPase). The charged phospholipid, phosphatidylserine, was found to have a significant effect in reducing the Ca^{2+} signal. Diffusional barriers within 0.5 μm of the channel caused a large increase in the sub-membrane Ca^{2+} signal. Altering buffer binding affinity, binding rate constant, concentration or diffusional mobility had a profound effect on the sub-membrane Ca^{2+} signal and the distance it penetrated into the cytosol. Higher affinity or concentration of buffers confined the Ca^{2+} signal to less than 1 μm from the plasma membrane. The combined results illustrate the utility of the ***Virtual Chromaffin Cell*** in providing insight into the processes that regulate the sub-membrane Ca^{2+} signal for neurohormone secretion. The Virtual Cell is also applicable to analysis of Ca^{2+} signaling in other cell types, such as developing neural stem and progenitor cells,

which we have found exhibit anomalous Ca^{2+} signals that can influence nervous system development[3].

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A chromaffin cell model to simulate calcium dynamics and secretory responses in various conditions.

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We constructed a chromaffin cell model for computer simulation analyses of Ca^{2+} dynamics and secretory responses in various conditions. The model includes mechanisms involved in nicotinic excitatory synapse, voltage-dependent Na^+, K^+ and Ca^{2+} channels, Ca^{2+}-activated K^+ channel (SK type), buffered Ca^{2+} diffusion, Ca^{2+} uptake into and release from intracellular Ca^{2+} stores, extrusion of Ca^{2+} at the plasma membrane, fluorescent Ca^{2+} indicator and Ca^{2+}-triggered exocytosis. Membrane current properties and various modes of action potentials reported in the literature were simulated with the model. The model was also applied to simulate our experimental results obtained from chromaffin cells in the perfused rat adrenal medulla. Observed Ca^{2+} responses and secretory responses induced by electric stimuli to the splanchnic nerve or by 40 mM K^+ in the perfusate were adequately simulated. Moreover, in isolated rat chromaffin cells bathed in a Ca^{2+}-deficient medium, profiles of muscarine-induced Ca^{2+} transient were found to be altered from oscillatory to monophasic modes with increasing the muscarine concentrations, and the half-decay time of monophasic response became shorter in thapsigargin-treated cells. These characteristics were reproduced by simulation of Ca^{2+} dynamics involving Ca^{2+} stores with $InsP_3$ receptor/channel and Ca^{2+} pump. Thus, the presented model may provide a useful tool for analyzing and predicting quantitative relations in various events occurring in stimulation-secretion coupling in chromaffin cells.

MATERIALS AND METHODS

Monitoring of Ca^{2+} responses and secretory responses in the perfused rat adrenal medulla. The adrenal gland isolated from Wistar rats (330-390 g) was recurrently perfused for 60 min through the adrenal vein with 2 ml Krebs solution containing 10 μM fura-2 AM. After about half of the adrenal cortex was removed, the gland was mounted on a chamber. Chromaffin cells in the adrenal medulla were stimulated either by transmural electric stimulation of the presynaptic nerve elements in the tissue or by an elevation of KCl concentration in the perfusate. Changes in $[Ca^{2+}]_i$ of chromaffin cells were observed from an exposed portion of the adrenal medulla using an inverted fluorescence microscope. Catecholamines secreted in the perfusate were measured with a carbon fiber electrode.

Measuring $[Ca^{2+}]_i$ responses in isolated rat chromaffin cells. Changes in $[Ca^{2+}]_i$ in isolated rat chromaffin cells loaded with fluo-3 were measured with a laser confocal scanning unit (Zeiss LSM410).

Modeling of chromaffin cell functions.

***Synaptic mechanism*:** The time course of synaptic current in nicotinic transmission was parameterized with the rising and decaying time constants reported by Kajiwara *et al*.[1].

Voltage-dependent channels: Na^+ and K^+ channels of Hodgkin-Huxley's type were modeled so as to reproduce experimental results reported by Kajiwara *et al*.[1]. Cell averaged Ca^{2+} current properties in chromaffin cells were also modeled.

***Ca^{2+}-dependent K^+ channel*:** SK-type channel was modeled based on experimental results reported by Park [2], in which the channel gate was assumed to open depending on binding of Ca^{2+} with a Hill-type kinetics.

Buffered Ca^{2+} diffusion: Intracellular buffered Ca^{2+} diffusion was calculated by a simplified method, in which the model cell was divided into N (6 in this study) compartments by N-1 concentric spheres. The movement of Ca^{2+} with a rate associated with the Ca^{2+} diffusion constant occurred due to the Ca^{2+} concentration difference in adjacent compartments. The Ca^{2+} buffering action with a fixed Ca^{2+} buffer took place in each compartment.

***Ca^{2+} extrusion*:** The extrusion of Ca^{2+} by Ca^{2+} pump and Na^+/Ca^{2+} exchanger was assumed to occur in combination by following Michaelis-Menten's kinetics.

***Intracellular Ca^{2+} store*:** Functions of an $InsP_3$-sensitive Ca^{2+} store along with muscarine- induced $InsP_3$ generation were modeled [3]. The Ca^{2+} uptake into the store was expressed by a Hill-type equation (n=2), and the Ca^{2+} leak from the store was assumed to be proportional to the difference of Ca^{2+} concentrations between the store and the cytosol. The $InsP_3$ receptor/channel was designed to activate cooperatively with $InsP_3$ and Ca^{2+}, with a subsequent inactivation due to increased cytosolic Ca^{2+} concentrations. The Ca^{2+}-dependent inactivation was assumed to be removed in the presence of high concentrations of $InsP_3$.

***Secretory mechanism*:** A two-step secretory scheme proposed by Heinemann *et. al*.[4] was adopted. In the scheme, secretory granules in a reserve pool migrate into a release-ready pool in a Ca^{2+}-dependent manner and then secreted by exocytosis with a rate proportional to the third power of the submembrane Ca^{2+} concentration. The values of kinetic parameters provided by the original study were used without modification.

Detailed mathematical formulas and parameter values involved in the above-mentioned mechanisms are shown elsewhere[5]. Calculations of the modeled mechanisms were carried out using the NEURON simulation environment (http://www.neuron.yale.edu/neuron/).

RESULTS AND DISCUSSION

Simulations of membrane current properties and various modes of action potentials. Simulations using the present model reproduced basic electrophysiological properties of rat chromaffin cells reported in the literature, which included EPSP-induced action potential[1], aborted action potentials during a prolonged current injection[1], repetitive action potentials at elevated resting membrane potentials and SK channel current tails observed following 1-s depolarizing pulses to different voltages[2]. This indicates that the mechanisms for ionic channels and Ca^{2+} dynamics were adequately formulated in the model.

Simulations of Ca^{2+} and secretory responses in the perfused rat adrenal medulla. Chromaffin cells in the perfused rat adrenal medulla were stimulated transsynaptically and changes in fura-2 fluorescence intensity were observed. The record in Fig. 1A*a* was obtained with transmural application of 10 pulses at 400-ms intervals and that in Fig. 1B*a* with 20 pulses at 100-ms intervals. To simulate these results, the corresponding numbers of EPSPs were generated in the model cell. The EPSP-induced action potentials (Figs. 1A*d* and 1B*d*) and increases in the Ca^{2+} concentration in the submembrane compartment (solid line in Figs. 1A*c* and 1B*c*) and the inner compartment (dotted line). The cell-averaged Ca^{2+} responses calculated for fura-2 fluorescence signals are shown in Figs.1A*b* and 1B*b*, which mimic the observed Ca^{2+} responses in Fig. 1A*a* and 1B*a*.

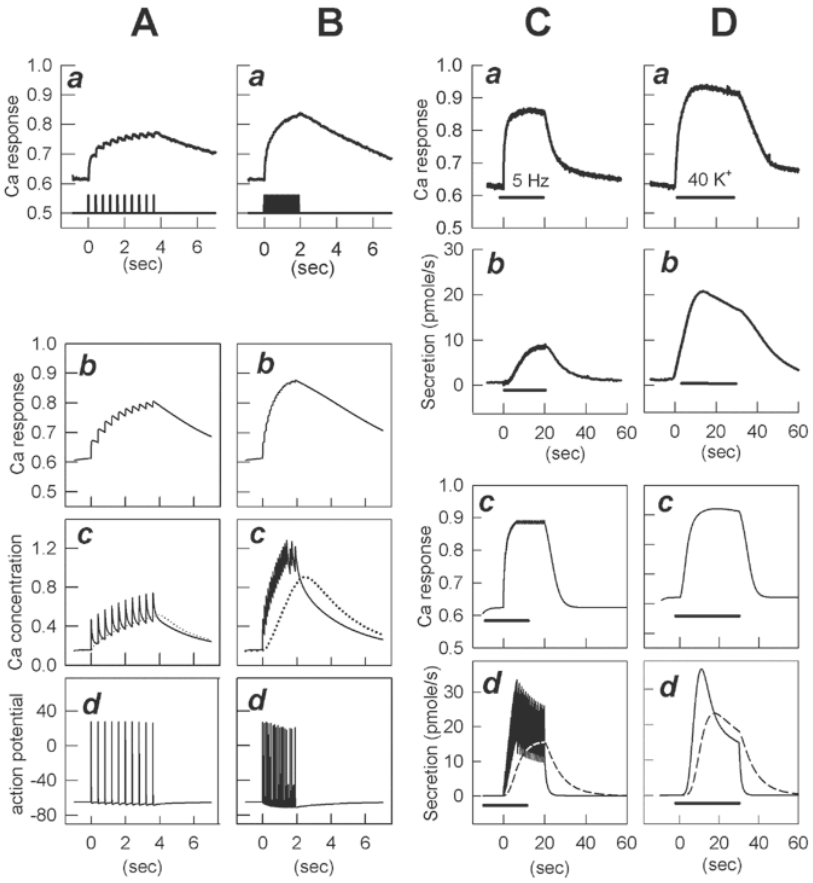

Figure. 1. Simulations of Ca^{2+} and secretory responses in chromaffin cells trassynaptically stimulated in the perfused rat adrenal medulla. (Column A) ***a*)** A normalized change in fura-2 fluorescence (342 nm excitation) intensity associated with 10 transmural stimuli at 400-ms intervals. ***b*)** Calculated cell-averaged Ca^{2+} response. ***c*)** Changes in Ca^{2+} concentrations (in μM) in the submembrane compartment (solid line) and in the 3rd compartment from the cell surface (dotted line). ***d*)** Calculated action potentials (in mV). **(Column B)** ***a*)** Observed Ca^{2+} response to 20 stimuli at 100-ms intervals. ***b, c* & *d*)** Simulation results are displayed in the same fashion as in A. **(Column C)** ***a*)** Observed Ca^{2+} response to transmural stimuli at 5-Hz for 20-s. ***b*)** The simultaneously observed secretory response. ***c*)** Calculated Ca^{2+} response. ***d*)** Calculated secretory response (solid line) was processed with numerical low-pass filter of a time constant of 7.5 s (dashed line). **(Column D)** Observed responses to 30-s stimulation with 40 mM K^+ and simulation results are displayed as in C. The ordinate scale in Figs. 1C***b***, 1C***d*,** 1D***b*** and 1D***d*** is pmole s^{-1} per gland.

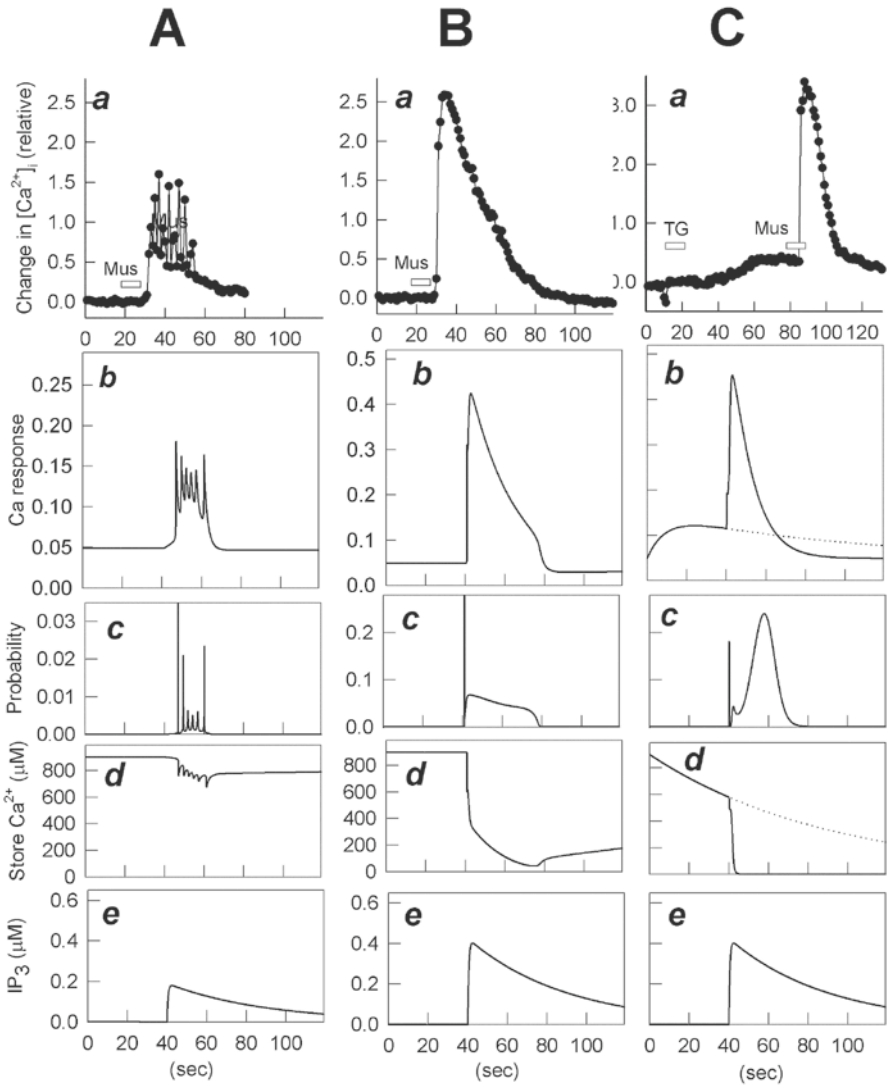

Figure. 2. Simulations of muscarine-induced Ca^{2+} responses in isolated rat chromaffin cells in Ca^{2+}-deficient medium. (Column A) ***a*****)** An oscillatory change in fluo-3 fluorescence intensity induced by 6 μM muscarine. ***b*****)** Calculated oscillatory Ca^{2+} response. ***c*****)** A change in the open channel probability of the Ca^{2+} release channel. ***d*****)** The associated change in the Ca^{2+} concentration of the store. ***e*****)** An assumed change in $InsP_3$ concentration following the muscarine stimulation. **(Column B)** Same as in A except that a cell was stimulated by 30 μM muscarine. **(Column C)** Same as in B except that a cell was pretreated with thapsigargin.

Figures 1C*a* and 1C*b* show the Ca^{2+} response and secretory response elicited by transmural application of 5-Hz pulses for 20 s. The calculated Ca^{2+} response in Fig. 1C*c* roughly approximates the time course of the observed record in Fig. 1C*a*, whereas the calculated secretory response in Fig. 1C*d* does not mimic the observed record in Fig. 1C*b*, exhibiting spiky responses associated with the respective stimuli. Since the experimental detection of catecholamines in the perfusate required a time constant of 7.5 s, the calculated response was processed by numerical low-pass filter with this time constant. The resultant shown by the dashed line in Fig 1C*d* approximates the time course of the observed response in Fig. 1C*b*.

Figures 1D*a* and 1D*b* show the Ca^{2+} response and secretory response observed in chromaffin cells stimulated by 40 mM K^+ in the perfusate. The calculated Ca^{2+} and secretory responses to depolarizing voltage to -30 mV (corresponding to E_K for 40 mM K^+) are displayed in Figs. 1D*c* and 1D*d*.

Simulation of muscarine-induced $[Ca^{2+}]_i$ changes in isolated rat chromaffin cells. Muscarine at 6 μM induced oscillatory changes in fluo-3 fluorescence intensity in isolated rat chromaffin cells in Ca^{2+}-deficient medium (Fig. 2A*a*), whereas 30 μM muscarine elicited monophasic responses (Fig. 2Ba). In thapsigargin-treated cells, the duration of the muscarine-induced monophasic Ca^{2+} responses was significantly reduced (Fig. 2C*a*). Ca^{2+} dynamics following muscarine stimulation were simulated with the model that included Ca^{2+} store function. Due to the assumed gating properties of $InsP_3$ receptor/channel, pulsatory increases in the open channel probability (Fig. 2A*c*) occurred with a low production of $InsP_3$ (Fig. 2A*e*), which caused an oscillatory Ca^{2+} release from the store as indicated by changes in the Ca^{2+} concentration in the store (Fig. 2A*d*) and in the cytosol (Fig. 2A*b*). With a higher production of $InsP_3$ (Fig. 2B*e*), no oscillation of the open channel probability (Fig. 2B*c*) occurred due to $InsP_3$-mediated removal of the Ca^{2+}-induced inactivation of $InsP_3$ receptor/channel. Consequently, a massive Ca^{2+} release form the store took place (Fig. 2B*d*) to produce a monophasic Ca^{2+} response (Fig. 2B*b*). It is noted that a robust reuptake of Ca^{2+} by the store delayed the depletion of the store and prolonged the duration of the Ca^{2+} response.

The reduction of duration of monophasic Ca^{2+} response by thapsigargin was reproduced in simulation with the model in which the Ca^{2+} uptake mechanism was removed ((Fig. 2C*b*).

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Automatic processing of amperometric data.

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Amperometry is a widely used technique for monitoring the secretion of catecholamines by exocytosis. Amperometric signals are generated by the oxidation of quantally released catecholamines close to tip of a carbon fibre electrode. Each event resulting from an exocytosis is called ‘secretory spike’. Several kinetic parameters can be extracted from spikes to get important information about the catecholamine storage and the time course of exocytosis. The large amount of data obtained from these experiments requires the use of computer programs. Here, we describe a software, written for Igor Pro (Wavemetrics, Lake Oswego, OR, USA) that allows the off-line analysis of amperometric signals, which includes: i) the automatic analysis of a large collection of experiments user independent, ii) the visual check and correction of the located spikes, iii) data pooling from several experiments to create galleries with hundreds of thousands of spikes[1].

Due to the high amplification of amperometric signals, they are usually contaminated with electromagnetic noise and its removal implies the use of digital filtering in order to improve the signal/noise ratio. Previous authors have used general filtering methods (FIR, smooth, band reject filter, etc.)[2] applied through the whole record affecting differently every spike that contains uneven frequency components. Here, we propose new filtering algorithms that apply low-pass digital filters with variable cut-off frequencies depending on the spectra of discrete segments of the record.

RESULTS AND DISCUSSION

We present the new version of our software for the automatic analysis of amperometrical records[1], which results much more flexible and user friendly. It includes a quick new way to choose folders and files. Now, the spike review becomes easier and faster due to its new method for applying corrections. In addition, the parameters can be plotted onto every event (figure 1A). The current version allows performing the statistical analysis either by spike- or cell-based way. The program and its user manual (Mac and PC compatible) are periodically updated and available as freeware at the web address:

http://webpages.ull.es/users/rborges/

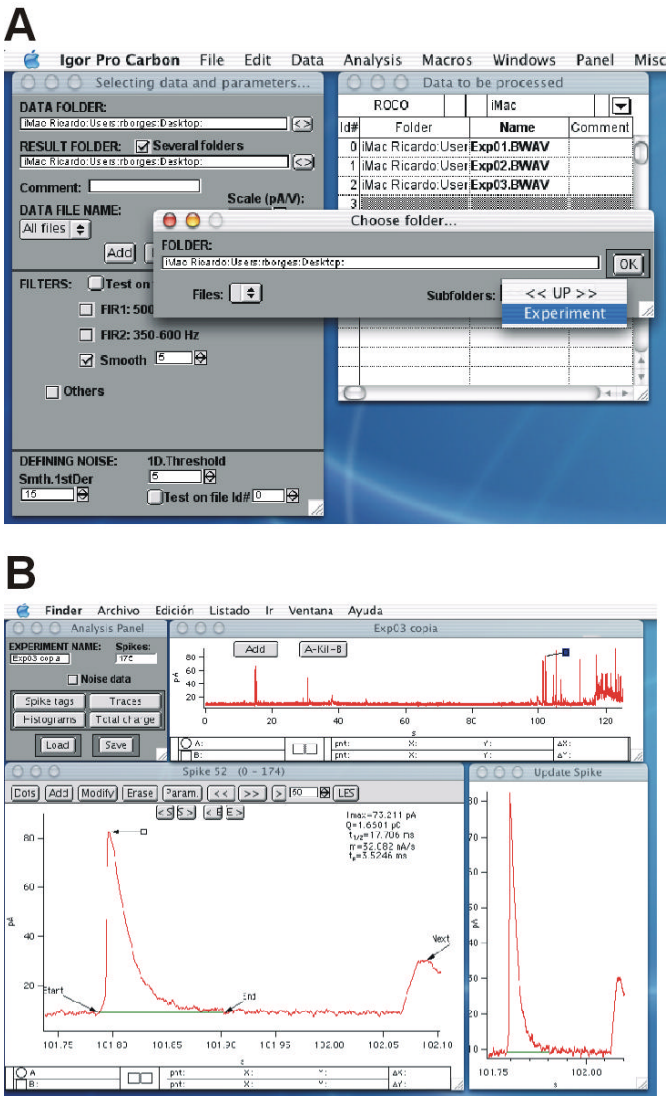

Figure 1. Analysis programs. The screens for the 'Spike Analysis' **A)** and for the 'Spike View' **B)** of the automatic analysis are presented. The 'Spike Analysis' panel is divided into three main parts: selection of data files, choosing of filters and specification of spike identification criteria. Once all previously mentioned fields have been filled or selected, user can start the analysis of the whole list of data files by pressing the 'Run' button. The researcher can check the spikes found by the previous automatic 'Spike analysis' and manual corrections can be introduced in the 'Spike View' screen.

The program is divided into three main parts (figure 1B) that appear in the 'Macros' menu of Igor: i) digital filtering, automatic localization and characterization of spikes from a number of experiments; ii) visual review of results for making manual corrections; iii) creation of galleries of spike parameters pooling results from a large number of experiments. User only needs to introduce the list of files to be processed, the type and characteristics of digital filter, and the spike identification criteria. The program iterates the analysis for every experimental file without any further user activity.

The program incorporates two of the most popular digital filters: binomial smooth and FIR. However, some noise frequencies are also present as components of the secretory events and these filters severely affect those spikes with rapid ascending/descending slopes. To avoid that, we have implemented two adaptive filters: YAIZA[3], which finds the presence of overfiltration by detecting the Gibbs phenomenon, and DAUTE which combines multi-low-pass filters for selective frequency rejection from discrete recording segments.

IMPROVED DIGITAL FILTERING METHODS

The design of the low-pass filter depends on spike features, because each spike has a different power spectrum, and drugs or experimental conditions could alter its form. The filter must be designed to get a better signal-to-noise ratio but not for adding perturbations or Gibbs phenomena. The Gibbs phenomenon is a distortion in the signal due to an overfiltration that rejects important high frequency components[4]. Consequently, Gibbs phenomenon appears in data regions where sudden signal variation occurs (i.e. fast ascending slope of a spike). We present here new algorithms to enhance the S/N ratio from individual amperometric spikes using different FIR (Finite Impulse Response) filters[5] as well as the spike features.

YAIZA algorithm.

We distinguish among three different regions in the amperometric records of bovine adrenal chromaffin cells: type I, segments where can be applied filters with frequencies under 50 Hz (where the basal trace and very slow spikes are); type II, regions with frequencies between 50 and 150 Hz (spikes with medium fast

ascending slopes); and type III**,** regions with frequencies over 150 Hz (spikes with fastest slopes).

YAIZA employs three fixed low-pass digital FIR filters**:** L1, whose cut-off frequency (*fc*) is 500 Hz, L2 (*fc* = 150 Hz) and L3 (*fc* =50 Hz). The correct filter will be chosen to avoid Gibbs phenomena. For instance, if Gibbs phenomenon is detected in a given spike region after use the L3 filter, YAIZA will apply the L2 just in this region. Whether Gibbs phenomenon persists L1 will be used.

DAUTE algorithm.

The DAUTE is a new signal-processing algorithm for the digital filtering of amperometrical records. This system uses a bank of low-pass FIR filters that act sequentially on discrete segments of data to suppress the noise, taking into account not modifying the original time course of secretory events.

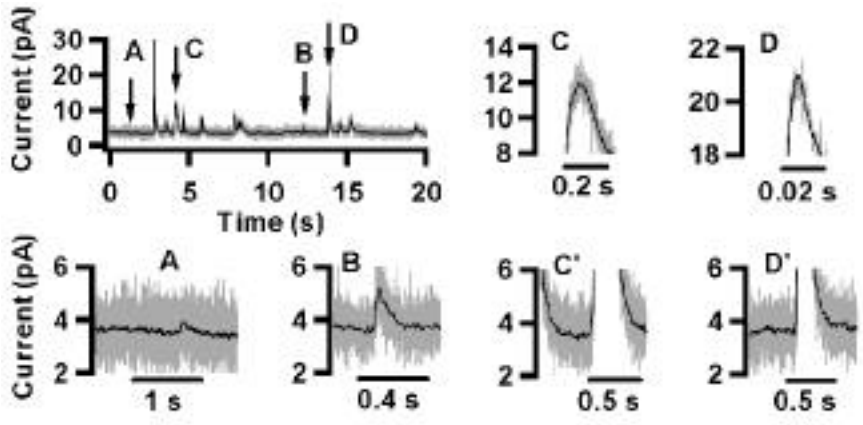

Figure 2. The filtered data with DAUTE algorithm. The gray and black traces are original and filtered data respectively. We show different zoomed segments of the record. The **A** plot shows a noise segment with no spikes, in this case the DAUTE has reduced considerably the noise allowing the detection of a small and slow spike, smaller than 2 pA, in **B**. The **C** and **C**' plots show the top and basal traces of the same spike respectively, In the same way but with other spike is plotted in **D** and **D**'. Therefore, DAUTE has filtered very fast and slow spikes in the same record without introducing Gibbs phenomena. On the other hand, we can now calculate parameters from very small spike (<2 pA) with reliability.

These discrete segments of high frequency are taken from a

bank of high-pass FIR filters. The high efficiency of DAUTE facilitates the automatic analysis of spikes and allows the extraction of reliable kinetics data even from very small secretory events (<2 pA; figure 2).

The selection of high-pass (*HPi*) and low-pass (*LPi*) filters is based on sampling frequency being *i*=1,...,*n* where n is the number of levels of the filter bank. We usually set n to 6 levels. Following the Nyquist principle, if *fs* is the sample frequency, *fs*/2 is the maximal detectable frequency thus cut-off frequency will be $(fs/2)/2^i$ for the level *i*.

When the high-pass filter bank is applied to data, we obtain *n* filtered signals, *DATAHPi*. DAUTE will use them for detecting the high frequency regions at each level. The next step consists in the application of the low-pass filter bank to data getting *DATALPi*. At the end, the best-filtered data is assessed by combining the *DATALPi*'s with all information picked up on the *DATAHPi's* (Figure 2).

In conclusion, analysis of amperometric records requires the use of software in order to process large amount of data. Therefore, we have described a program for the automatic analysis of individual secretory events. In addition, we have presented new approaches for digital filtering specifically designed for amperometric signals, the fixed level filter (YAIZA) and the self-corrected multi-low-pass digital filter (DAUTE).

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Gene analysis of rat chromaffin cells ELF MF differentiated using microarrays.

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Chromaffin cells have been extensively used as a model for the study of differentiation. Several reports have shown that such cells can change from an endocrine phenotype to a sympathetic neuron-like type when stimulated with either Nerve Growth Factor (NGF) or Extremely Low Frequency Magnetic Fields (ELF MF, 60 Hz, 0.7 mT, 4 hours/day, 7 days)[1-3].

We have recently demonstrated that ELF MF-neuron-like differentiated cells predominantly form dopaminergic cells[4] and develop neurites with a large number of tyrosine hidroxylase positive vesicles as well as augmented neurofilaments[5]. It has also been shown that this differentiation process largely depends on L-type Ca^{2+} channels[6].

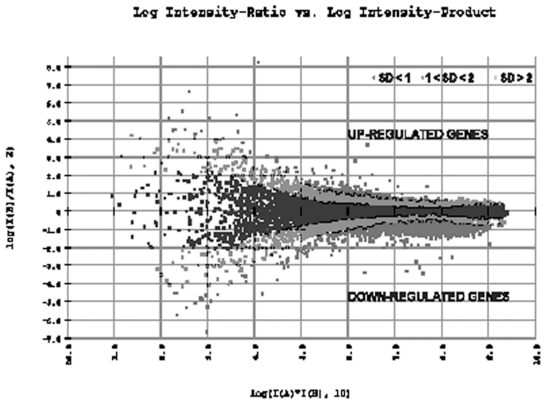
Thus, it can be expected that Electromagnetic Fields (EMF) stimulation must result in the induction of a variety of biochemical processes and changes in gene expression leading to short and long-term consequences on cellular behavior[7-9]. The mechanisms through which magnetic fields induce functional modifications have not been understood. The aim of the present work is to analyze the gene expression pattern obtained by ELF MF induction during chromaffin cells differentiation using microarray hybridization.

Microarrays are typically used to measure mRNA abundance, and this kind of experiments often provides a lot of information that cannot be completely analyzed in a single study [10].

RESULTS AND DISCUSSION

ELF MF treatment elicits a complex chromaffin cells trans-differentiation process that should imply important modifications in gene expression. As Figure 1-A shows, Wistar neonate rats' chromaffin cells cultures ELF MF-differentiated (experimental group) present differences on gene expression pattern compared with untreated chromaffin cells (control group). After filtering and analyzing four microarray slides using the TIGR-MIDAS software[11], with two standard deviation score we found that 170 genes modify their expression level between control and experimental group.

A



B

GENES DIFFERENTIALY EXPRESED ON CHROMAFFIN CELLS ELF MF TREATED	
Bone morphogenetic protein 4 (UR)	Pleiomorphic adenoma gene-like 1 (UR)
Cyclin G1 (UR)	Ribosomal protein L24 (UR)
Discoidin domain receptor family, member 1 (UR)	Ribosomal protein L5 (UR)
GTP cyclohydrolase 1 (UR)	Solute carrier family 12, member 2 (UR)
Nogo-A (UR)	Thioredoxin reductase 2 (UR)
Protein phosphatase 1, catalytic subunit, beta isoform (UR)	Transgelin 3 (UR)
Amyloid beta (A4) precursor protein-binding, family A, member 3 (X11-like 2) (UR)	Tumor protein, translationally-controlled 1 (UR)
Biglycan (UR)	v-akt murine thymoma viral oncogene homolog 1 (UR)
Cysteine rich protein 1 (UR)	Vesicle associated protein (UR)
Global ischemia induced protein GIIG15B (UR)	Vesicle-associated membrane protein 3 (UR)
Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (UR)	Growth associated protein 43 (DR)
Insulin induced gene 2 (UR)	Inositol polyphosphate multikinase (DR)

Figure 1. Differences between chromaffin cells untreated and chromaffin cells ELF MF differentiated using microarray analysis. A) Graphic of local variation as a function of intensity is used to identify differentially expressed genes by calculating an intensity-dependent Standard Deviation-score using the TIGR-MIDAS software. In this R-I plot, array elements are grey scale-coded depending on whether they are less than one standard deviation (SD) from the mean (black), between one and two SD (grey), or more than two SD from the mean (grey light). Zones of both up and down regulated genes are indicated. **B)** Table of known identified differentially expressed genes on chromaffin cells after ELF MF treatment. Both up (UR) and down (DR) regulation are indicated.

Of these genes, 24 are of known function and 146 correspond to ESTs sequences. We observed a mayor number of genes down regulated (22 genes) during ELF MF chromaffin differentiation (Fig. 1-A); in contrast with only 2 genes up regulated. Similar observations were made by Angelastro[12] on PC12 cells NGF-differentiated; they report only a 2% of genes up regulated during this process using SAGE method.

We observed that the genes with known function participating on ELF MF cellular differentiation are mainly involved on banding, cellular adhesion, catalytic activity and signal transduction (Figure 1-B). On PC12 cells have been reported genes that regulate the expression of adhesion molecules, membrane receptors and protein phosphorylation[13-15]. We found two up regulated genes: cyclin G1, involved on cellular processes as mitosis; and, bone morphogenetic protein 4, which participate on cellular development. Bone morphogenetic proteins (BMPs) are shown to promote NGF-induced neuronal differentiation on PC12 cells. Also, the addition of BMP4 or BMP6 robustly increased the neuritogenic effect on NGF within 2 days on PC12 cultures[16].

Our results indicate that ELF MF treatment elicits a peculiar transcriptional response where genes are manly down regulated; meanwhile few are up regulated, like BMP4. This observation supports the proposition that the large repulsive forces generated by ELF MF could determine a physical modification of the DNA structure of the region[17], which could either favor or hinder transcription. This suggests that the differentiation process elicited by ELF MF implicates changes in cellular programs that must turn off expression of complete sets of genes. Some other genes are over-expressed, thus the combination of the two may be responsible for the process.

The identification of the ESTs genes whose regulation is specifically increased by ELF MF treatment will surely shed light on the nature of the signal transduction pathway needed for ELF MF-dependent differentiation.

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β-subunit sequestration differently blocks Ca^{2+} channel current and exocytosis in chromaffin cells.

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Chromaffin cells represent a good neuron-like model for studying calcium channels and secretion. The identity of the calcium channels involved in chromaffin cell secretion has been investigated thoroughly[1-3]. Pharmacological studies suggest that all calcium channel subtypes are involved in the secretory process, but depending on the experimental conditions used, these channels behave at different ways. So far, it remains unclear why chromaffin cells require so many channel types to fulfil a single and fundamental process. The question is partly answered by the observation that, following inactivation by membrane depolarization, secretion can be induced by channel types less sensitive to inactivation[4].

High voltage activated calcium channels (HVA) are heteromeric complexes formed by at least three different subunits: α_1, β and $\alpha_2\delta$. The α_1 subunit forms the pore of the channel and confers the pharmacological properties, while the others ones are auxiliary subunits. The auxiliary β subunit is essential for functional expression of high-voltage activated Ca^{2+} channels. The β subunit binds to the α_1 subunit through the I-II loop of the α_1 subunit. All the I-II loops from the different α_1 subunits can bind all the β subtypes, with high affinity but with different selectivity between different α_1 subtypes. In addition the I-II loop contains a reticular retention signal that restricts its incorporation to the plasma membrane, and this sequence is antagonized by the β subunit, releasing the α_1 subunit from the endoplasmic reticulum and facilitating the incorporation of this subunit to the plasma membrane. This property suggests that an overexpression of a construct containing the loop I-II from an α_1 subunit would act as a lure sequestering the endogenous β subunit and altering its intracellular function. This is the case in heterologous expression systems like *Xenopus* oocytes, where the β subunit regulates the correct assembling and the localization of calcium channels in the membrane[5-8]. We describe here a lure sequence designed to sequester the β subunits in transfected bovine adrenal medulla chromaffin cells, and the functional consequences for the whole-cell inward Ca^{2+} channel current (I_{Ca}) and exocytosis (ΔCm) using the perforated configuration of the patch-clamp technique.

RESULTS AND DISCUSSION

We used the I-II loop of the α_1 subunit that forms the pore of the P/Q-type Ca^{2+} channels to design the β-lure sequence. The resulting chimeric protein contains extracellular and transmembrane region of the α chain of the human CD8, the I-II loop of α_{1A} subunit and the enhanced green fluorescent protein (EGFP). This construct has several advantages which are to be coupled to the membrane fraction of the cell and its detection is greatly facilitated by EGFP fluorescence. The presence of CD8 allows immunocytochemical detection using an anti-CD8 antibody.

When we evaluated the transfection efficiency of this construct in chromaffin cells maintained in culture, we saw that only around 2-5% of the cells expressed EGFP. The distribution of CD8-I-II-EGFP appeared often patchy and mostly intracellular.

We next wanted to evaluate the functional effects of the expression of the β-lure sequence onto native calcium channel currents in bovine chromaffin cells. Since expression of CD8-I-II-EGFP appeared reliable for only a few days, we evaluated its effect on current density 2 days after transfection. Figure 1A shows the protocol used; 200 ms duration depolarizing pulses were applied at 2 min intervals under the perforated-patch configuration of the patch-clamp technique, and the capacitance increase evoked by this pulse was recorded. The extracellular Ca^{2+} concentration to evoke the Ca^{2+} current and the capacitance increase was 2 mM. The peak of the current traces obtained in control untransfected cells was 441.5±47.9 pA, while in CD8-I-II-EGFP transfected cells averaged 271±69.1 pA, which correspond to a significant 62% reduction in calcium influx through calcium channels ($p<0.05$). When the current was normalized using the cell size, estimated by the capacitance of the resting cell (current density measured in pA/pF) before the pulse application, we observed a 54% reduction ($p<0.05$). These results show a direct effect of the CD8-I-II-EGFP protein to promote a reduction in the β subunits availability that leads to a reduction in the Ca^{2+} entry to the cell.

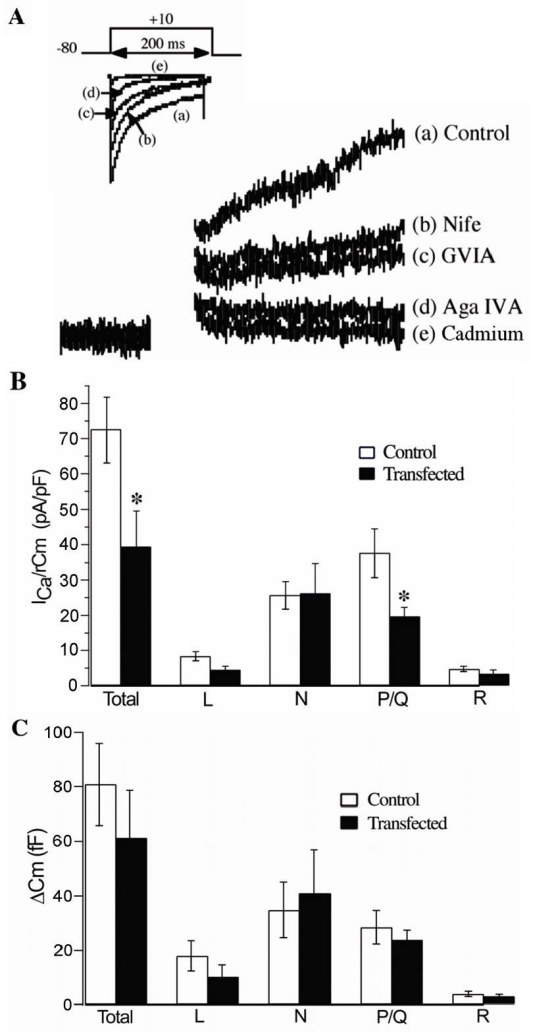

Figure 1. Effects of the CD8-I-II-EGFP construct on the Ca^{2+} current density (I_{Ca}/rCm) and the capacitance increase (ΔCm) in bovine chromaffin cells. **A)** The Protocol used for the estimation of the Ca^{2+} current density and the capacitance increase consisted in 200 ms duration depolarizing pulses that evoked an initial capacitance increase reduced by the accumulative application of the Ca^{2+} channel blockers (nifedipine, ω-conotoxin GVIA, ω-agatoxin IVA and Cd^{2+}). **B)** Average data of total calcium entry and contribution for the different Ca^{2+} channel subtypes to this entry in control and transfected cells. **C)** Total secretory response and contribution of the different Ca^{2+} channel subtypes to the exocytosis in control and transfected cells. *p<0.05.

Since the sequence of the I-II loop used in these studies derived from the α_{1A} subunit, that forms the pore of the P/Q Ca^{2+} channels, we wanted to verify if this sequence interfere with the formation of all calcium channel subtypes or if this inhibition affects preferentially to a specific calcium channel subtype (i.e. P/Q calcium channels). Pharmacological dissection of the whole-cell I_{Ca}, and its associated ΔCm response in the same cell, was achieved through the use of 3 μM nifedipine (L-type channel blocker), 1μM ω-conotoxin GVIA (N-type channel blocker), 1μM ω-agatoxin IVA (P/Q-type Ca^{2+} channel blocker) and 200 μM Cd^{2+} (blockade of residual current). These calcium channel blockers were applied sequentially and accumulatively to each cell, to evaluate the residual calcium current and the associate secretory response (R-type calcium channels).

Figure 1B shows the different blockade of the current density (I_{Ca}/rCm expressed in pA/pF) obtained for the different Ca^{2+} channel blockers. When comparing the pairs of bars we only observed significant difference in the case of the P/Q-type calcium channels that are reduced in CD8-I-II-EGFP transfected cells. The current density through P/Q-type calcium channels decrease from 37.7±8.9 pA/pF in control cells to 19.7±2.6 pA/pF in transfected cells ($p<0.05$). This result suggests a specific association between the I-II loop of α_{1A} subunit and a determinate isoform of β subunit, perhaps the β_4-type subunit. This inhibition is specific of the P/Q-type calcium channels and no compensation of any other calcium channel subtypes occurred (L, N, R). The sequestration of the β subunit preferentially associated to the α_{1A} subunit favoured the endoplasmic reticulum retention of this protein[9]. The L-type Ca^{2+} channels contributed to the total calcium entry with 8.4±1.3 pA/pF, the N-type Ca^{2+} channels 25.8±3.9 pA/pF and the R-type channels contribution were 4.8±0.7 pA/pF, in control cells. In transfected cells the contribution to the total current density for each channel was: L-type 4.5±1 pA/pF, N-type 26.3±8.5 pA/pF and R-type 3.5±1 pA/pF.

Then we wanted to evaluate the secretory response in control and CD8-I-II-EGFP transfected cells. Figure 1C shows the total capacitance increase (ΔCm) and the different blockade obtained using the indicated calcium channel blockers in control and transfected cells.

The total secretory response amounted to 80.8±15.1 fF and 61.24±17.27 fF, in control and transfected cells, respectively. We didn't find any different in the total secretory response when comparing control and transfected cells. The L-type Ca^{2+} channels were responsible for 17.8±5.6 fF, N-type for 34.8±10.2 fF, P/Q type for 28.5±6.2 fF and R-type 3.9±0.9 fF, in control cells. In transfected cells the L-type Ca^{2+} channels contribution were 10.2±4.4 fF, N-type Ca^{2+} channels were 41.1±15.8.4 fF, P/Q-type 23.8±3.6 fF and R-type Ca^{2+} channels contribution to the total secretory response were 3.1±0.7 fF. We didn't find any significant different when comparing control and transfected cells.

CONCLUSION

Our data suggest that CD8-I-II-EGFP acts as a specific represor of the expression of P/Q-type calcium channels and this effect is probably due to the ability of the I-II loop from the α_{1A} subunit to sequester a certain β subtype which is preferentially associated with the α_{1A} subunit, favouring its release from the endoplasmic reticulum and its incorporation to the plasma membrane.

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Multiple intracellular regulatory mechanisms of cell surface expression of sodium channels: therapeutic implications.

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Regulated expression of cell surface voltage-dependent Na^+ channels ensures development and differentiation of excitable cells; dysregulated expression of normal Na^+ channel isoforms or otherwise silent Na^+ channel isoforms causes the Na^+ channelopathies. In bovine adrenal chromaffin cells, (1) constitutive phosphorylation of extracellular signal-regulated kinase destabilized Na^+ channel □-subunit mRNA, thus negatively regulating steady-state level of Na^+ channels. (2) Protein kinase C (PKC)-□ down-regulated Na^+ channels via destabilization of □-subunit mRNA. (3) Cytoplasmic Ca^{2+} activated PKC-□ and calpain, and down-regulated Na^+ channels by promoting internalization of Na^+ channels. (4) Protein kinase A or insulin receptor tyrosine kinase up-regulated Na^+ channels via translational events. (5) Neuroprotective, antiepiletic, antipsychotic, and local anesthetic drugs up-regulated Na^+ channels via translational events, without or with elevating □- subunit mRNA levels. Thus, constitutive and inducible cellular signals, as well as therapeutic drugs up- and down-regulate cell surface Na^+ channel expression largely via regulating mRNA stability and intracellular trafficking of Na^+ channels.

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Regulated up- and down-regulations of voltage-dependent Na^+ channels play pivotal roles in the short- and long-term regulation of various cellular events (e.g. neuronal development, synaptogenesis and neuronal plasticity)[1]. Dysregulated up- and down-regulations of Na^+ channels are responsible for hypoxia/ischemia-induced neuronal injury[2], seizure, intolerable pain, and neurodegenerative diseases[3]. Although the molecular structures and biophysical properties of Na^+ channels have been well characterized, much remains unknown about the extracellular signals and intracellular mechanisms that regulate cell surface expression of Na^+ channels in these physiological and pathological states. In addition, Na^+ channels are molecular target for a growing number of therapeutic drugs (e.g. neuroprotective, antiepileptic, antipsychotic, and local anesthetic drugs). However, little is known whether therapeutic drugs could affect regulated expression of cell surface Na^+ channels. In this paper, we summarize our previous studies that multiple intracellular signals and therapeutic drugs cause up- and down-regulations of functional Na^+ channels via regulating mRNA stability and intracellular trafficking of Na^+ channels[4-17].

RESULTS AND DISCUSSION

Regulation of steady-state level of Na^+ channels: constitutive destabilization of Na^+ channel □-subunit mRNA by ERK. Serum deprivation caused time-dependent ($t_{1/2}$=12 h) increase (~52%) in cell surface [^{3}H]saxitoxin (STX) binding with no change in the Kd value; the increase was reversed after the addition of serum. Immunoblot analysis of mitogen-activated protein kinase (MAPK) family revealed that extracellular signal-regulated kinase (ERK), p38 MAPK (p38), c-Jun N-terminal protein kinase (JNK) were constitutively phosphorylated in quiescent chromaffin cells; however, serum deprivation rapidly (<15 min) lowered (~50%) the phosphorylation of ERK1 and ERK2 (but not p38, JNK1 and JNK2) without changing protein levels of MAPK molecules[4]. Treatment with PD98059 or U0126, a selective inhibitor of ERK kinase, lowered the phosphorylation of ERK1 and ERK2 (but not p38, JNK1 and JNK2), and increased cell surface [^{3}H]STX binding; the reduced extent of the former was inversely related to the increased extent of the latter. Cotreatment of serum deprivation with PD98059 or U0126 did not

further increase [^{3}H]STX binding, compared with either treatment alone. Serum deprivation, PD98059 or U0126 increased (~50%) Na^+ channel α- (but not α_1-) subunit mRNA level between 3 and 24 h; the half-life of α-subunit mRNA was prolonged from 17.5 to ~26 h, with no change in the α-subunit gene transcription[4]. Cycloheximide, an inhibitor of protein synthesis, increased α-subunit mRNA level, and nullified additional increasing effect of either treatment on α-subunit mRNA level.

Thus, constitutively phosphorylated/activated ERK destabilizes Na^+ channel α-subunit mRNA via translational event(s), which negatively regulates steady-state level of α-subunit mRNA and cell surface expression of functional Na^+ channels. Down-regulation of Na^+ channels by PKC: PKC-α-induced destabilization of α-subunit mRNA, and PKC-ε-induced internalization of Na^+ channels

Down-regulation of Na^+ channels by PKC: PKC$_\alpha$-induced destabilization of α-subunit mRNA, and PKC$_\varepsilon$-induced internalization of Na^+ channels. In adrenal chromaffin cells expressing only conventional protein kinase C (PKC)-α, novel PKC-ε, and atypical PKC-ζ [5], treatment with 1-1000 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) caused a rapid (<15 min) and sustained (>15 h) translocation of PKC-α and -ε (but not -ζ) from cytoplasm to membranes; in contrast, 100 nM thymeleatoxin (TMX) caused the similar, but selective membrane translocation of only PKC-ε [5].

TPA reduced [^{3}H]STX binding to a greater extent than did TMX [5,6]; TMX (100 nM)-induced reduction of [^{3}H]STX binding reached the maximum fall of 38% at 12 h, whereas TPA (100 nM)-induced reduction of [^{3}H]STX binding further developed into the almost maximum fall of 53% at 18 h. Gö6976, an inhibitor of PKC-α (but not -ζ), completely reversed the TMX-induced event; in contrast, TPA-induced event was partially prevented by Gö6976, but abrogated by H7, an inhibitor of PKC family.

Northern blot analysis revealed that 100 nM TPA (but not 100 nM TMX) lowered α-subunit mRNA level by 52% at 12 h in a monophasic manner, while increased α_1-subunit mRNA level in a time-dependent

manner. H7 (but not Gö6976) completely prevented TPA-induced reduction of □-subunit mRNA level [5,7]. TPA shortened the half-life of □-subunit mRNA from 18.8 to 3.7 h, without changing □-subunit gene transcription.

Table 1. Multiple mechanisms of Na^+ channel up- and down-regulations

Intracellular signals & Therapeutic drugs	[^{3}H]STX Binding	Subunit mRNA □	Subunit mRNA □$_i$	Mechanism	Ref.
Down-regulation					
ERK	↓	↓	→	□-Subunit mRNA stability ↓	4
PKC-□	↓	↓	↑	□-Subunit mRNA stability ↓	5,6,7
PKC-□	↓	→	→	Internalization ↑	5,7
$[Ca^{2+}]_i$ ↑	→				8
$[Ca^{2+}]_i$ ↑ ↑	↓	→	→	Internalization ↑	8
$[Ca^{2+}]_i$ ↑ ↑ ↑	↓ ↓	↓	↓	Internalization ↑	
				& □-, □$_i$-Subunit mRNA ↓	8
Calcineurin	↓	→	→	Internalization ↑	
				& Externalization ↓	9
Up-regulation					
PKA	↑	→	→	Intracellular trafficking	10
Insulin	↑	→	→	Intracellular trafficking	11
Therapeutic drugs					
Valploic acid	↑	↑	↑	□-, □$_i$□Subunit mRNA ↑	12
Riluzole	→				13
NS-7	↑	→	→	Intracellular trafficking	14
Carvedilol	↑	→	→	Intracellular trafficking	15
Bupivacaine	↑	→	→	Intracellular trafficking	16
Ropivacaine	↑				16
Lidocaine	→				16

Thus, TPA-induced, PKC-□-mediated, destabilization of □-subunit mRNA contributes to the elongation of Na^+ channel down-regulation, compared with TMX-induced, PKC-□-mediated, Na^+ channel down-regulation. Treatment (24 h) with cycloheximide progressively in-

creased α-subunit mRNA level, while continuously decreasing β_1-subunit mRNA level; in addition, cycloheximide completely reversed TPA-induced decrease of α-subunit mRNA level and increase of β_1-subunit mRNA level.

Because the reduction of [^{3}H]STX binding caused by TMX or TPA was rapid (<3 h) in onset, we measured internalization rate of cell surface Na^+ channels by using brefeldin A (BFA), an inhibitor of guanine nucleotide exchange protein of ADP-ribosylation factor 1, a monomeric GTPase. Concurrent treatment of BFA with TPA or TMX decreased [^{3}H]STX binding to a greater extent, compared with either treatment alone. Thus, PKC-α promotes endocytic internalization rate of cell surface Na^+ channels, causing down-regulation of Na^+ channels[5].

Cooperative down-regulation of Na^+ channels by Ca^{2+}-activated signals. Treatment with 1 μM A23187, a Ca^{2+}-ionophore, produced a rapid monophasic rise of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$), followed by the salient plateau increase lasting for 96 h[8]. Thapsigargin (TG) and DBHQ are two inhibitors of sarco(endo)plasmic Ca^{2+}-ATPase, but not of the plasma membrane Ca^{2+}-ATPase. TG (100 nM) produced a slowly-developing monophasic rise of $[Ca^{2+}]_i$, followed by the persistent (>48 h) plateau increase; in contrast, DBHQ (100 nM) produced only a rapid monophasic rise of $[Ca^{2+}]_i$. A23187 or TG (but not DBHQ) decreased [^{3}H]STX binding, with no change in the Kd value[8]. A23187 produced long-lasting (>96 h) time-dependent gradual decrease of [^{3}H]STX binding by 66% at 96 h, whereas TG-induced reduction of [^{3}H]STX binding leveled off by 35% at 48 h. A23187- or TG-induced event required long-lasting continuous increase of $[Ca^{2+}]_i$, because addition of BAPTA-AM, a cell membrane-permeable Ca^{2+} chelator, at 24 h abolished the subsequent decreasing effect of A23187 or TG at 48 h. The decreasing effect of A23187 or TG was partially prevented by calpastatin, an inhibitor of calpain, or Gö6976, and due to the increased internalization of Na^+ channels, as evidenced by using BFA, suggesting that PKC-α and calpain are involved in the Ca^{2+}-induced acceleration of Na^+ channel internalization. In addition, A23187 (but not TG) lowered Na^+ channel α- and β_1-subunit mRNA levels by ~50% between 3 and 48 h[8].

Thus, heterogeneous increases of $[Ca^{2+}]_i$ caused down-regulation of Na^+ channels via multiple mechanisms, depending on the amplitude and duration of $[Ca^{2+}]_i$ rise. Because down-regulation of Na^+ channels has been assumed to prevent hypoxia-induced neuronal injury [2], $[Ca^{2+}]_i$-induced down-regulation of Na^+ channels may be a defensive event against Ca^{2+} overload.

Up-regulation of Na^+ channels by activation of protein kinase A and insulin receptor tyrosine kinase. Chronic treatment with 1 mM dbcAMP (>12 h) or 100 nM insulin (>24 h) increased [^{3}H]STX binding by ~50%, without altering the Kd value [10,11]. The effect of dbcAMP or insulin was blocked by actinomycin D or cycloheximide, but was not associated with increased levels of Na^+ channel α- and β_1-subunit mRNAs. Thus, activation of PKA or insulin receptor tyrosine kinase up-regulates cell surface Na^+ channels via the de novo synthesis of as yet unidentified protein(s), which may be involved in the regulation of intracellular trafficking of Na^+ channels [5].

Up-regulation of Na^+ channels by antiepileptic, antipsychotic, neuroprotective, and local anesthetic drugs. Chronic treatment (>2 d) with therapeutic concentration of valproic acid, an antiepileptic drug, increased Na^+ channel α- and β_1-subunit mRNA levels by 74 and 83%, respectively, and caused up-regulation (~40%) of [^{3}H]STX binding, with no change in the Kd value [12]; it resulted in the enhancement of veratridine-induced $^{22}Na^+$ influx via Na^+ channels, thus augmenting veratridine-induced $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels and catecholamine secretion.

Acute treatment with neuroprotective drug, riluzole [13], NS-7 [14], or carvedilol [15] inhibited veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion. In contrast, chronic treatment (>12 h) with NS-7 or carvedilol (but not riluzole) increased [^{3}H]STX binding by ~86%, with no change in the Kd value. Bupivacaine enantiomers and ropivacaine, a propyl homolog of bupivacaine, are amide-type local anesthetics structurally similar to lidocaine, except that their amine-containing group is a piperidine, instead of a tertiary amine in lidocaine. Acute treatment with either local anesthetic inhibited veratridine-induced $^{22}Na^+$ influx with comparable potency; however, chronic treatment (>3 h) with bupivacaine or ropivacaine (but not

lidocaine) increased (~48%) [^{3}H]STX binding. Up-regulation of cell surface Na^+ channels caused by these Na^+ channel blockers was presumably due to the promotion of cell surface externalization of newly-synthesized protein(s) from the trans-Golgi network, because the increasing effect of either drug was prevented by cycloheximide or BFA, and was not associated with the increased levels of □- and □$_1$-subunit mRNAs.

CONCLUSION

Two major regulatory mechanisms of cell surface expression of Na^+ channels can be drawn from our study: the modulation of (1) □-subunit mRNA stability, and (2) intracellular trafficking, especially modulation of endocytic internalization rate of cell surface Na^+ channels (Table 1). Steady-state level of cell surface Na^+ channels is negatively regulated by constitutive activity of ERK; down-regulation of Na^+ channels by cooperative activation of PKC and Ca^{2+}-activated signals is significant in extent and rapid in onset, and thus may contribute to the neuroprotection[17]. Insulin-induced up-regulation of Na^+ channels may be informative for the down-regulation of Na^+ channels at the node of Ranvier in myelinated axons of insulin-deficient diabetic neuropathy[17]. Up- and down-regulation of cell surface Na^+ channels in our present study may provide a new avenue to understand the crucial roles of Na^+ channel modulation in the physiological and pathological states.

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Potassium channel activity from bovine chromaffin granules.

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Cell Biology of the Chromaffin Cell
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We characterized the large conductance potassium channel present in chromaffin granule membranes and the effect of pH on its kinetic. Granule membrane "ghosts" were incorporated into black lipid membrane (BLM) made of asolectin. In 450 / 150 mM KCl gradient the current / voltage relationship appeared to be linear for membrane potentials between -70 mV and +70 mV with conductance of ~360 pS. The effect of pH on the potassium channel was examined. After lowering pH value to pH = 6.4 from the intravesicular face potassium channel activity was blocked. Results show that K^+ channel with large conductance can play an important role in acidification of chromaffin granules interior by V-ATPase.

RESULTS AND DISCUSSION

The chromaffin granule membranes were added to the *trans*-side chamber. Upon reconstitution the potassium current flowing through membrane channels was observed. The conductance of the channel was determined on 360 ± 7 pS for 450 mM / 150 mM KCl and 432 ± 9 pS in symmetric 450 mM KCl, both in pH 7.0. Close and open- time distributions were voltage dependent. The channel activity was blocked by TEA^+ applied to the *cis* side (data not shown).

Figure 1A shows channel recordings in asymmetric 450 mM / 150 mM KCl at holding potential of 30 mV in control and after lowering pH to 6.4 from the *cis* side. KCl solution pH value in the *trans*-side chamber was always equal pH = 7.0. Traces 1 to 5 in the Figure 1B were measured at various pH of experimental solution of both sides of the planar lipid membrane as marked on the Figure 1. On the right side of each current trace the distribution of single channel current amplitude is presented. Lowering the pH from the *cis* side results in diminishing of both the open probability and the current amplitude. Only small changes of single channel current amplitude were observed when pH = 6.4 was applied to the *trans*-side chamber.

Our results indicate that measured channel activity was strongly inhibited by low pH applied to the *cis* side. Inhibition of K^+ channel by pH lowering was observed from the same side as TEA^+ inhibition. It was earlier shown that chromaffin granule K^+ channel is blocked by TEA^+ from intragranular side[1]. Thus, it seems that lowering pH in the intragranular side blocks the channel activity. Findings of the present

study may be important for understanding physiological role of potassium conductance in chromaffin granules.

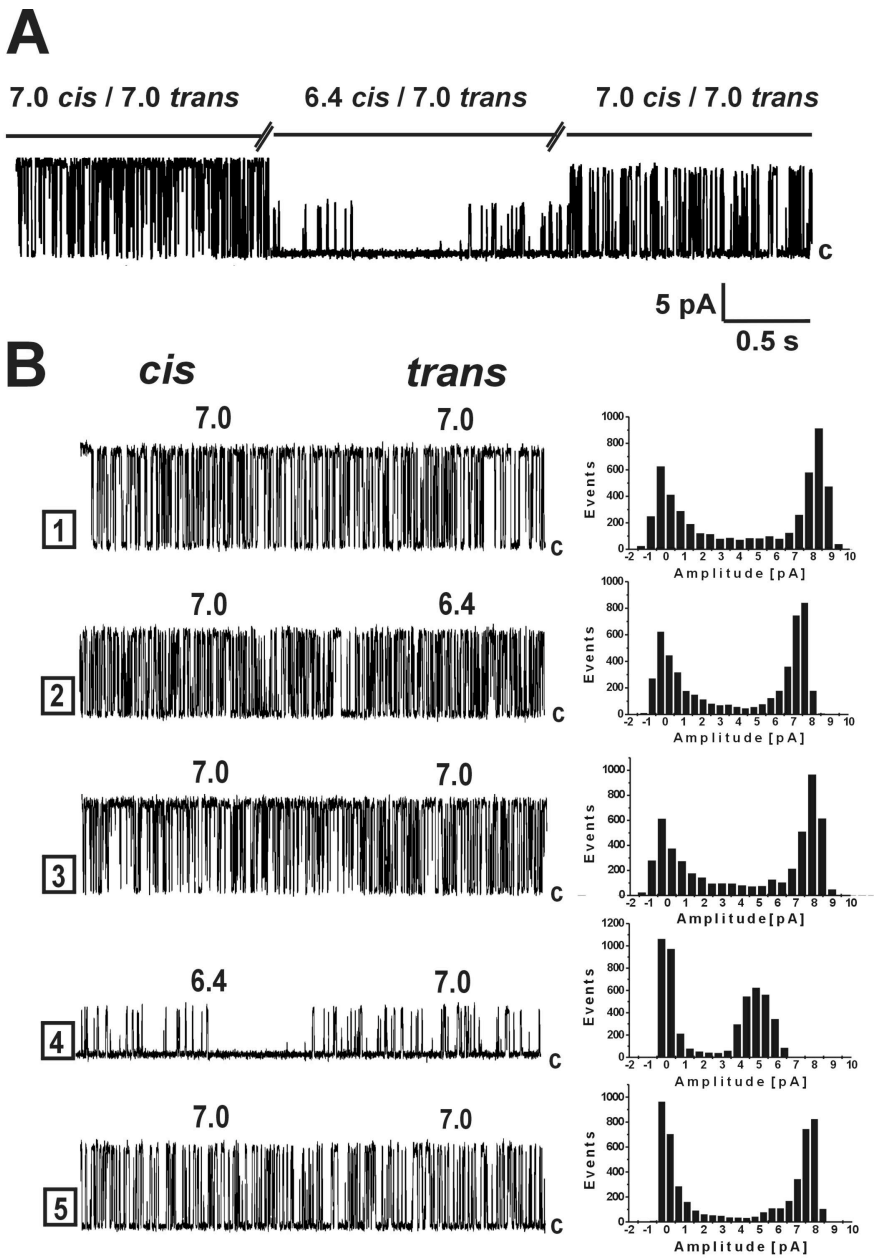

Figure 1. Regulation of large conductance chromaffin granules potassium channel by pH. Single channel recordings at a holding potential of 30 mV from "ghost" membranes incorporated into BLM in asymmetric 450 / 150 mM KCl. The closed levels, corresponding to currents though the lipid bilayer are indicated with **c**. **A** Changes in channel kinetic after changing pH from the *cis* side only. **B** Traces 1 to 5 measured at various pH of experimental solution of both sides of lipid bilayer as marked on the figure. All recordings filtered at 200 Hz. On the right side of each trace the distribution of single channel current amplitude is shown.

The chromaffin granule membranes contain a vacuolar-type (V-type) V-ATPase, which generates an electrochemical proton gradient, acidifying the granule interior. Potassium channel can play physiological role by compensating electric charge produced by the V-ATPase[1]. This enables the formation of a membrane potential ( ) and pH, sufficient to drive catecholamine uptake into the chromaffin granules. This hypothesis is also supported by the experiments on the effects of intra-granular cation composition on ATP-dependent acidification of chromaffin granules[1]. In fact, a much higher pH was observed with K^+ inside than with TEA^+ [1]. Our present observation on the pH-dependence of K^+ transport points to the fact that low pH should block this "charge compensation" mechanism. Blockage of K^+ channels by low pH would block further acidification of chromaffin granules. This channel would play a role of protective fuse to diminish over-acidification of granular lumen.

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The secretory responses to choline and acetylcholine show different patterns and calcium dependence in chromaffin cells.

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Cell Biology of the Chromaffin Cell
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Acetylcholine (ACh) is the physiological neurotransmitter at central and peripheral cholinergic synapses[1], and choline its catabolic metabolite; However, the role of choline in these synapses was changing in the time, and this change is due to the discovery of various activities of choline i.e. muscarinic effects on brain neurons[2], secretion of catecholamines from bovine adrenal medullary chromaffin cells[3], inward currents in neurons[4], or the increase of the cytosolic concentration of calcium ($[Ca^{2+}]_c$) in sympathetic neurons[5]. In oocytes expressing different subtypes of neuronal nicotinic receptors for ACh (nAChR)[6], choline does not activate $\alpha4\beta2$ nor $\alpha3\beta2$ receptors; it is, however, a partial agonist of several heteromeric receptors, including the $\alpha_3\beta_4$ subtype, and behaves as a full agonist at homomeric $\alpha7$ receptors[7].

In the study presented here, we used choline effects in chromaffin cells, a cholinergic neuronal model; using choline as a selective agonist for $\alpha7$ receptors and ACh as a non-selective agonist for $\alpha7$ and $\alpha3\beta4$ receptors, as well as high $[Ca^{2+}]_e$ solutions in order to further characterize the functional role of nicotinic receptors in bovine chromaffin cell. We compared the secretory responses, the confocal $[Ca^{2+}]_c$ signals and the changes in membrane potential elicited by choline and ACh in bovine chromaffin cells.

RESULTS

Ca^{2+}-depencence catecholamine release induced by choline. The catecholamine secretory response was studied in cell populations of bovine chromaffin cells. Choline, ACh and K^+ (as a functional control) were applied during 5-s, at 1 min intervals. Choline secretory responses exhibited a strong dependence on the extracellular concentration of Ca^{2+} ($[Ca^{2+}]_e$), those of ACh showed less dependence, and those of K^+ exhibited little Ca^{2+} dependence (fig.1A: 1 mM Ca^{2+}; fig 1B: 20 mM Ca^{2+}). The summary of pooled data is shown in the figure 1C (range 1-20 mM Ca^{2+}).

Intracellular Ca^{2+} increments elicited by choline and ACh, measured by confocal imaging in chromaffin cells. Cytosolic ($[Ca^{2+}]_c$) and nuclear ($[Ca^{2+}]_N$) confocal images of increments in the Ca^{2+} levels associated to patch clamp inward currents produced by

choline and ACh pulses given in high $[Ca^{2+}]_e$ solutions were measured. Under these conditions, changes in intracellular calcium levels are due to calcium entry through nicotinic receptors, especially α7 subtype that shows a high permeability to this cation.

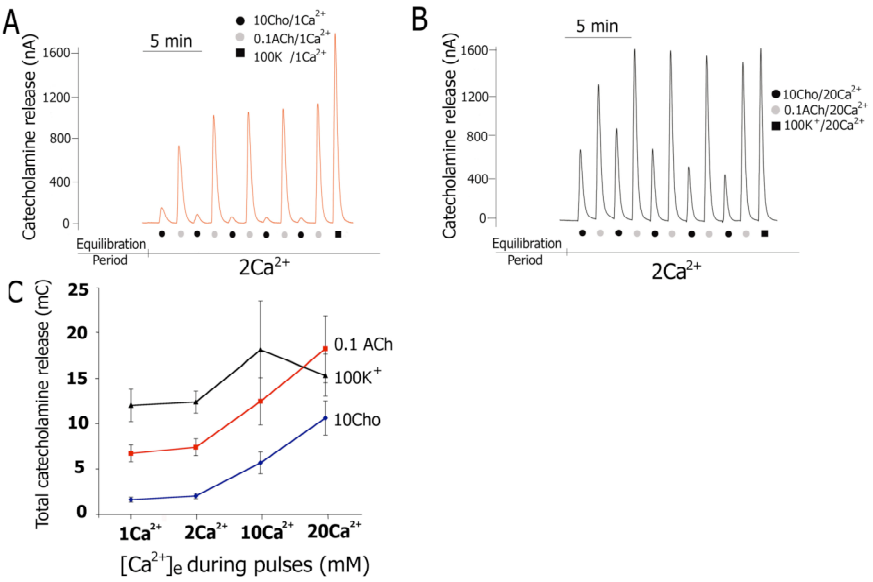

Figure 1: Cells were challenged at 1-min intervals with alternate 5-s pulses of a Krebs-Hepes solution containing 1 mM Ca^{2+} (panel A, 10Cho/1Ca^{2+}) or 20 mM Ca^{2+} (panel **B**, 10Cho/20Ca^{2+}). After applying 5 pulses of choline alternating with 5 pulses of ACh, a final pulse was given with a Krebs-Hepes solution containing 100 mM K^+ (with isoosmotic reduction of Na^+). Panel **C** shows pooled results on total secretion elicited by the three secretagogues, in the presence of four $[Ca^{2+}]_e$ tested. The secretion areas were calculated in each individual experiment for each stimulus and expressed in nA.s (μC). Data are means±SE, n=30 performed with at least 6 different batches of cells.

The holding potential was fixed at -80 mV. Figure 2A shows original inward current traces elicited by the application of 1-s pulses of ACh (0.1 mM) and Cho (10 mM). Panel B shows the changes in the fluorescence taken from an equatorial plane of the cell, before and after the applications of the agonist pulses. Panel C shows the differences on increments in $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ generated by choline and ACh pulses in different areas of the cytosol and the nucleus. Panel

D shows the pooled data of inward current generated by choline referred to the inward current elicited by 0.1 mM ACh (as %). Panel E shows the pooled data on cytosolic and nuclear calcium levels. Choline increased the $[Ca^{2+}]_c$ as well as the $[Ca^{2+}]_N$; the $[Ca^{2+}]_c$ induced by choline was about 20% of that induced by ACh and the $[Ca^{2+}]_N$ induced by choline about 70% of that of ACh.

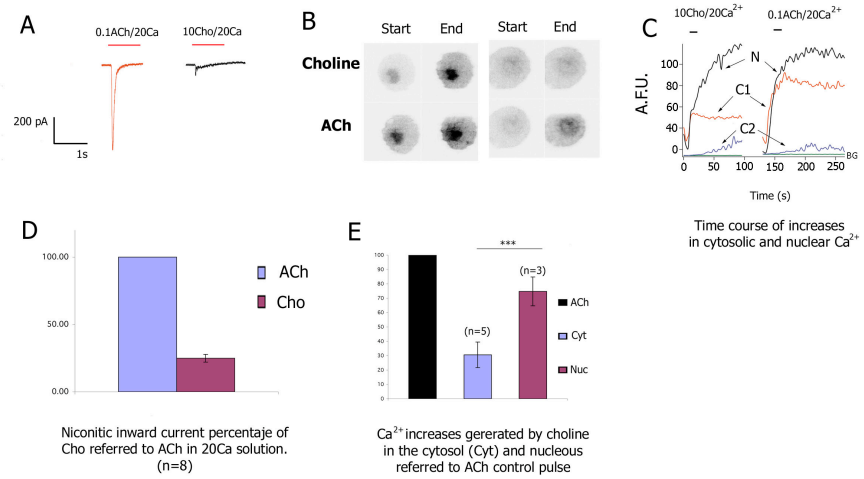

Figure 2: Choline increased the $[Ca^{2+}]_c$ as well as the $[Ca^{2+}]_N$. Cytosolic and nuclear confocal images of increments of the $[Ca^{2+}]_c$ associated to patch clamp inward currents produced by choline and ACh pulses given in high $[Ca^{2+}]_e$ solutions. Were recorded by using Fluo3 (5 µM), and acquired every 350 ms. Panel **A** shows original inward current traces elicited by application of 1-s pulses of 0.1ACh/20Ca^{2+} and 10Cho/20Ca^{2+} solutions. Panel **B** shows the changes in the fluorescence before and after the application of the agonist pulses. Panel **C** shows the differences on increments in $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ generated by choline and ACh pulses in different areas of the cytosol and the nucleus. Panel **D** show the pooled data of inward current generated by choline referred to ACh (as %). Panel **E** shown the pooled data of cytosolic and nuclear calcium increases of all cell (n=5 for cytosol and n=3 for nucleus).

Effects of choline and ACh on the membrane potential of chromaffin cells. Under the current clamp configuration of the whole cell patch clamp technique, we observed that in contrast to ACh, 10 mM choline (in low Ca^{2+} solutions) produced little or no depolarization, while ACh induce a large depolarization of membrane potential (fig. 3C-D). Those results change drastically when we

increase the extracellular Ca^{2+}; in these conditions, choline induce the same first phase of little or no depolarization followed by a second phase of strong hyperpolarization, while ACh only induced moderated hyperpolarizing phase (fig3. A-B).

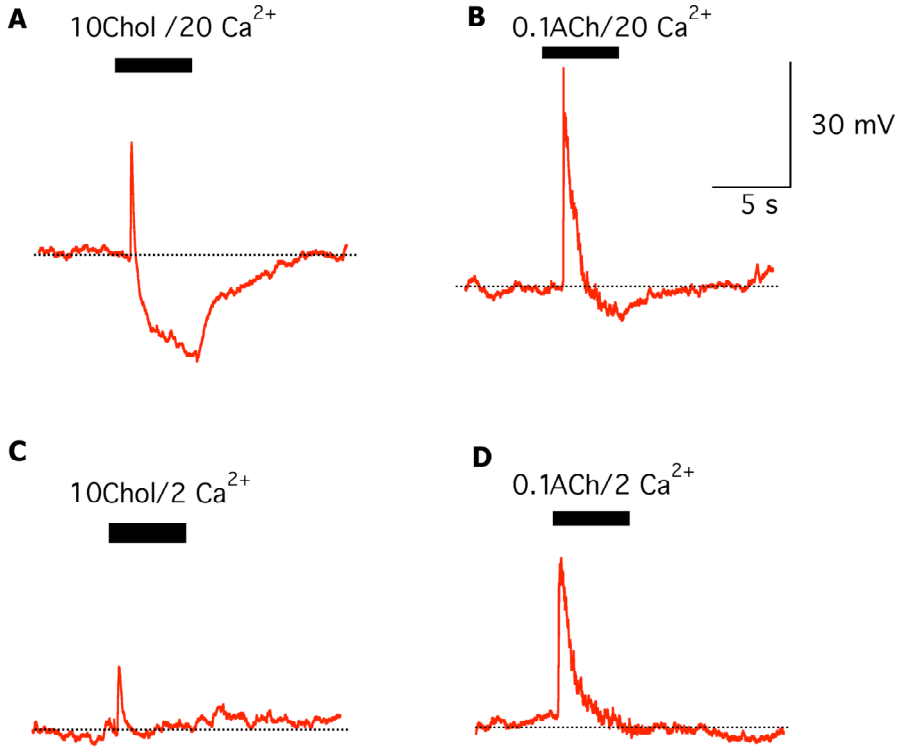

Figure 3: Original recordings of changes in membrane potential induced by nicotinic agonist applied in high-Ca^{2+}-solution (panels A-B) or low-Ca^{2+}-solution (panels C-D), choline elicited an hyperpolarizing response while ACh evoked a depolarizing followed a hyperpolarizing response. The high permeability of α_7 nicotinic receptors to Ca^{2+} ions could explain the pronounced hyperpolarization observed when 10Chol/20 Ca^{2+} was applied by means of the activation of co-localized Ca^{2+}-dependent K^+ channels.

In conclusion, choline acting as full agonist of $\alpha 7$ nAChR, shows striking differences respect to ACh, concerning their ability to generate depolarizing or hyperporalizing changes of the membrane

potential, inward currents, Ca^{2+} signals and exocytosis in bovine chromaffin cells. While the responses of choline were strongly dependent upon the $[Ca^{2+}]_e$, those of ACh were much less dependent on the changes of this cation. These differences are interpreted on the basis of a preferential selective activation of the highly Ca^{2+}-permeable α7 nicotinic receptor subtype, elicited by choline; this leads to activation of Ca^{2+}-dependent K^+ channels of the SK subtype, and to the choline hyperpolarizing response. These findings illustrates a novel mechanism of action of choline, that may be highly relevant to substantiate the proposal that it may have a physiological role in synaptic controlling neurotransmission

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Does an homomeric α7 nicotinic receptor exist in bovine chromaffin cells?

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The bovine adrenal chromaffin cell offers a unique model to explore the question of whether various subtypes of native and heterologously expressed neuronal nicotinic receptor subtypes share the same properties, as far as their ability to form a functional ion channel pore is concerned. This is so because, although from the molecular point of view various of the known subunits for the nAChR (α3, α5, α7, β4) seem to be present in bovine chromaffin cell[1-3], the subunit composition of the native receptor is still unknown, but it is assumed that it is formed by α3β4 and α7 independent receptors.

RESULTS AND DISCUSSION

In a previous work of our group[4], using short agonist applications (1s) and selective toxins, we see that the native nicotinic currents was generated by the simultaneous activation of a mixed pool of homomeric α7 and heteromeric α3β4 nicotinic acetylcholine receptors (nAChR); So the nicotinic native currents of the bovine chromaffin cells (BCC) should be similar to the nicotinic currents obtained when *Xenopus* oocytes are injected with the nAChRs present in the bovine chromaffin cells.

In this study, we intended to contribute to the knowledge of the neuronal nicotinic receptor composition in the BCC by comparing the effects of known nonselective nAChR agonist with those of selective α7 nAChR agonist. When we use different nonselective agonists (ACh, DMPP, Nicotine, and Epibatidine) we found that, ionic currents through nAChRs, measured with the whole-cell configuration of the patch-clamp technique in BCC, showed similar activation and inactivation kinetics with all nonselective agonist employed, and this kinetics are dependent on the agonist concentration employed. When we did the dose response curves with the different agonists and normalized the data with respect to the currents obtained in the same cell with 100 μM ACh, we observed a similar efficacy for Epibatidine, DMPP, and ACh, while nicotine showed 50% efficacy; This value was unexpected, and could not be explained because of receptor desensitization, because epibatidine is much more desensitizing than nicotine and appears to be as efficacious as ACh and DMPP. On the other hand the order of potencies for the different agonists was Epibatidine>DMPP>Nicotine>ACh. Surprisingly when we applied the

α7 selective agonists choline and 4-OH-GTS21, the response was less than 5% that induced by ACh 100μM; and when the current was evoked by cytisine, an α3β4 selective agonist, the current was only about 10% of the I_{ACh}.

On the other hand when the bovine nicotinic receptors α7 and α3β4 where expressed heterolousgy in *Xenopus* oocytes, neither of them showed a similar activation and inactivation kinetics that the present in CCB. Besides the selective agonists choline and 4-OH-GTS21 were check on bovine α3β4 and α7 nicotinic receptors heterolousgy expressed in *Xenopus* oocytes, and in this case the selective agonists activated only the α7 nicotinic receptors, producing an inward current comparable to that induced by ACh 100 μM, and not activated the α3β4 receptors. These experiments suggest that α7 subunits are not forming functional homomeric α7 receptor in bovine chromaffin cells and that they could be part of an heteromeric α3β4α7* nAChR.

Due to this surprised data and the absence of other pharmacological tools, we decided to further investigate the nature of the native nAChR to null the function the one of the other subtype of nAChR by incubing chronically the cells with selective antibodies against α3 subunits (mAb35) and against α7 subunits (H-302) (24-48h) of the nAChR. The results show a similar decline of the nicotinic response in both cases. In Addition, the catecholamine secretory response in cell population treated with specific antibodies against α3 or α7 subunits showed a similar decreased of ACh-evoked secretory responses, unrelated to the specific antibody used.

These results further support the presence of a heteromeric complex receptor formed by the combination of α3, α7, α4, and/or α5 subunits; this conclusion disagrees with the current hypothesis that assumes the existence of separated α 7 homomeric and α3β4 heteromeric receptors, based only in functional studies carried out in bovine Chromaffin cells.

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Choline as a tool to evaluate nicotinic receptor function in chromaffin cells.

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Choline is present in the synaptic cleft as a result of the rapid degradation of acetylcholine (ACh) by acetylcholinesterase (AChE). The hypotesis that choline will exert some modulatory function of synaptic neurotransmission is supported by few studies; for instance, choline has muscarinic effects on central neurons[1], evokes catecholamine release by acting as a partial agonist at neuronal nicotinic receptors for ACh (nAChRs), and blocks partially the release of catecholamines evoked by ACh, in cultures of bovine adrenal medullary chromaffin cells[2]. Choline has been also shown as a selective agonist of α7 nAChRs in brain neurons[3]. As far as we know, a detailed study of the effects of choline on the kinetic properties of inward currentes through α7 and α3β4 nAChRs is not available. Hence, we decided to express homomeric α7 and heteromeric α3β4 bovine nicotinic receptors in *Xenopus* oocytes and study the effects of choline on these receptors. We report here the results of such study that might be relevant in the frame of the growing number of physiological and physiopathological roles that are being adscribed to brain nAChRs, i.e. in neuroprotection, analgesia, addiction to nicotine, dementia or behaviour[4,5].

RESULTS AND DISCUSION

Application of brief (1-5 s) pulses of **c**holine (0.3-10 mM) elicited inward current in oocytes expressing α7 nAChRs, acting as a full agonist of these receptors (Figure 1) with an EC_{50} of about 0.6 mM. In contrast, in oocytes expressing α3β4 nAChRs, choline did not elicit any inward current by itself. However, choline blocked the inward current through these receptors, induced by 5-s pulses of 0.1 mM ACh (I_{ACh}) (figure 2), with an IC_{50} of about 0.38 mM. This blockade was quickly reversible after choline washout. The nature of I_{ACh} blockade by choline was further tested by challenging the oocytes with increasing concentrations of ACh (from 10 μM to 3 mM). In these experiment, α3β4 oocytes were sequentially stimulated with 5-s pulses of increasing concentrations of ACh, given first in the absence and subsequently in the presence of 1 mM choline. The blockade induced by choline was around 65-70% in all cases, suggesting a non-competitive nature of the blockade, as the case is for hexamethonium[6].

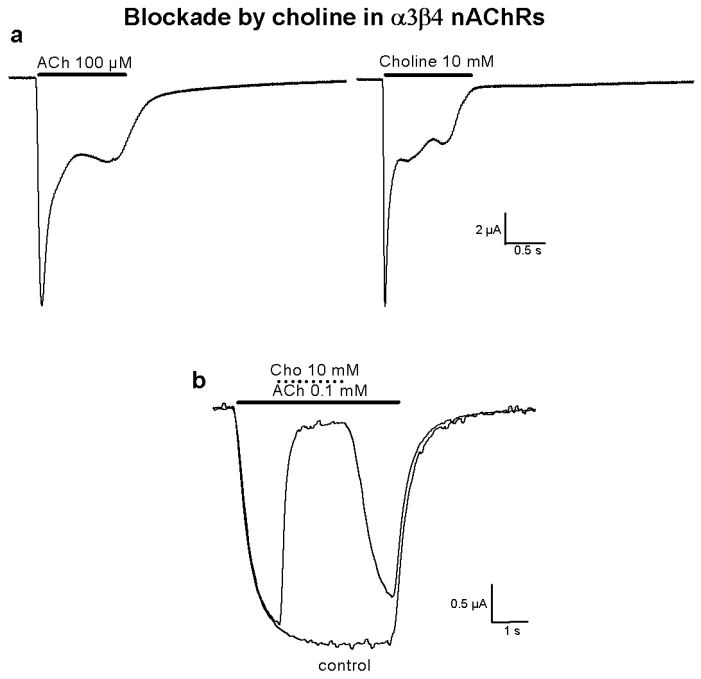

Figure 1. Effects of choline α7 and α3β4 nAChRs expressed in xenopus oocytes. a) Application of choline (10 mM) elicited an inward current in α7 nAChRs, acting as a full agonist of this receptor. In the same oocyte, ACh 0.1 mM elicited similar inward current. **b)** In an oocyte expressing α3β4 nAChRs, ACh 0.1 mM is capable to elicit a response (control) that was fully blocked by choline 10 mM. The blockade of the current was partially recovered when choline was removed from the perfusion solution.

We also wanted to study if the blockade induced by choline of I_{ACh} exhibited voltage-dependence, as has been decribe for different blockers of nAChRs[7-9]. For it, a voltage ramp protocol (-100 mV to +60 mV) was applied to α3β4 oocytes voltage-clamped at –80 mV and pulses of ACh (0.1 mM, 5-s) were applied either in the absence or in the presence of increasing concentrations of choline. The I-V curves for the traces are plotted, and calculations were made to estimate the degree of current blockade achieved by each concentration of choline at different voltages. That revealed that I_{ACh} blockade by choline was more pronounced at more hyperpolarised

potentials, as compared to depolarised potentials, in all of the three concentrations of choline tested.

Ours results reported here shown that, with a similar concentration range, choline exerted dual opposite effects in the two main receptor subtypes of nAChRs expressed by bovine chromaffin cells, α7 and α3β4[10-12].

The blockade of I_{ACh} through α3β4 receptors was voltage-dependent and was of a non-competitive nature, suggesting that choline had a binding site different to that for the agonist ACh, i.e. choline may bind to the ACh locus on the α7 receptor to cause its activation and to a site different to that of ACh on the α3β4 receptor to cause its blockade. The similar concentration at which choline exerts its dual action on α7 receptor activation and α3β4 receptor blockade, reinforces the hypothesis that choline has a role beyond that of being the simple precursor of ACh.

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Advances in cell culture for chromaffin cells and related cell types.

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Cell Biology of the Chromaffin Cell
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Studies making use of chromaffin cells (CCs), PC12 cells and enterochromaffin cell cultures continue to contribute significantly to our knowledge about basic mechanisms of neurotransmitter release and action[1-6]. These cells are derived embryologically from the neural crest[1,5] and serve as a convenient source of neural cells (paraneurons) for neurobiologists interested in exploring molecular and cellular mechanisms of neuronal processes including neuronal development and signal transduction. The combined use of techniques from biophysics and molecular biology has provided insight into synaptic transmission, in particular the molecular mechanisms of Ca^{2+}-triggered exocytosis[7].

Technical aspects of the isolation and culture of these cells are often lost in the fine print of Methods sections of published papers. Nevertheless this information is essential to anyone practicing the art of cell culture of these and related neuronal cells, including PC12 cells and enterochromaffin cells[6,7]. CCs have been succesfully maintained as primary monolayer cultures[2] and as suspension cultures[8]. In the latter, flow cytometry appears to be a useful technique to characterize noradrenaline and adrenaline cell subtypes[9] and to follow their phenotypic changes in response to growth factors[5,8].

Protocols have been refined in different labs but not officially published. CCs have been isolated primarily from bovine adrenal medullae but also from sheep, pig, cat, rat, mice, and even deer! Primary monolayer cultures of CCs and PC12 cells and suspension cultures of these and related cell types demand different protocols: specialized enzymes and plasticware, growth factors, substratum[10] and optimized culture media[2,5]. Catecholamine secretion and firing frequency can be analyzed at the single-cell level by means of perforated patch-clamp and carbon fiber electrochemical detection[11,12]. Tools such as electophysiology[7] and use of terrestrial and marine neurotoxins have been used to excite[13] and to inhibit[14,15] catecholamine secretion from CCs and to provide information about the subunit composition of the functional neuronal-type nicotinic receptors on these cells[14,16].

Aims. The aims of this Workshop were fourfold:

1. To review and update protocols for the isolation and maintenance of chromaffin and related cells.
2. To share our experience about technical aspects of chromaffin cell culture.
3. To provide working protocols for isolation and culture of chromaffin and related cells, and
4. To encourage on-going update of these protocols placed on the web.

Participants. The Discussion Leaders for this Workshop were Manuela G. López (Madrid), José-María Trifaró (Ottawa), Jakob Sørensen (Gottingen), Juan Antonio Gilabert (Madrid), Arthur Tischler (Boston) and Christian Prinz (Munich).

A brief history. To set the scene, Bruce Livett presented an outline of the history of development of techniques for isolation of chromaffin, PC12 and related cell types (see Table 1).

It is now timely to review and update the information gained by hundreds researchers who have used these cultures and related cell culture systems. The six Discusión Leaders addressed a number of important technical issues summarized below.

Experience in cell culture of chromaffin cells from bovine, mice and rat. *Manuela G. López* drew attention to enzymatic digestion as a limiting factor affecting yield, function and viability of isolated CCs. Gradient fractionation of NA and Ad cells on Urograffin[9] and Percoll provides a means of obtaining relatively pure populations of Ad cells but not as yet pure NA cells. Another important factor in isolation of CCs is the media. Ideally, the media should be at pH 7.2 – 7.4. Whether the serum is heat-inactivated or not and whether supplemented by serum, and for how long (24–48 h) affect the outcome.

Table 1. History of Cell Culture Techniques for Chromaffin and related cells.

Year	Advances in Cell Culture Techniques	Investigators
1967	Gerbil adrenal CCs, first isolated using 'Viokase'	*Kanno, Sampson, Douglas*
1970s	Bovine adrenal CCs first isolated by retrograde perfusion of bovine adrenal glands with collagenase	*Fenwick, Livett / Brookes / Zinder, Pollard / Viveros / Trifaro / Aunis / Garcia*
	PC12 cells	*Greene / Tischler*
	Rat chromaffin cells isolated	*Unsicker / Tischler*
1980s	Monolayer cultures bovine chromaffin cells	*Livett / Trifaro / Viveros /Aunis / Garcia*
	Pharmacology / patch-clamp electrophysiology of secretion	as above / Neher
	Suspension and 'flow-through' cultures of bovine chromaffin cells	*Westhead / Perlman*
	Electroporation *"Leaky Cells"*	*Baker, Knight / Kirshner /Schneider/ Holz*
	Fluorescence Calcium imaging	*Burgoyne / Morgan / Cheek*
1990s	NA and Ad cells 'separated'	*Moro / Krause*
	Cytometry on suspension cultures	Garcia / *Gilabert*
	Amperometry of released catecholamines	*Westerlink /Borges*
	Enterochromaffin cells	*Prinz*
2000s	Molecular mechanisms of Ca^{++} depdt. exocytosis	*Rettig, Neher*

Labelling catecholamine stores in chromaffin cells in culture. *José-María Trifaró* discussed optimal conditions for [^{3}H]NA labelling of amine stores in CCs as this affects release results[10]. Incubation with 10^{-7}-$5x10^{-7}$ M [^{3}H]NA for 5-10 min followed by 6 washes of 10 min. duration were found to label the stores are evenly. There was no activation of uptake 2, very little cytosolic or extra [^{3}H]NA, and importantly parallel release of endogenous amines and [^{3}H]NA. Moreover, there was a good correlation between the % total [^{3}H]NA released and the number of vesicles released.

A strategy for structure-function studies of exocytotic proteins using chromaffin cells from knockout mice and viral rescue techniques. *Jakob Sørensen* discussed ways of overcoming problems of working with transgenic mice. Problems include the peri-natal lethal phenotype, problems of not enough cells and lack of techniques for isolating and culturing mouse CCs for up to 3 days, suitable for single-cell measurements of exocytosis. Of particular interest, he provided a description of protocol for isolating CCs from K/O mice.

Conditions for maintaining rat adrenal chromaffin cells in primary culture. *Juan Antonio Gilabert* discussed conditions that affect yield and long-term survival of rat CCs in culture[8]. Dr. Gilabert is conducting and on-going survey of choice of rat strains, age, yield and tests of viability and encourages others to contribute. He has also devised an automated method for isolation of pig CCs.

Culturing pheochromocytoma cells. *Arthur Tischler* commented that "establishment of pheochromocytoma cell (PC) lines is challenging" because PCs from humans, rats and mice cease proliferating in primary culture. Only a proportion undergo spontaneous neuronal differentiation[5]. As a rapid index of success, he makes use of I.H. for TH and BrdU after BrdU pulse-labeling. He discussed the advantages and disadvantages of MPC lines from neurofibromatosis knockout mice, including precautions and cautions (including use of horse serum, prior irradiation, plating density) and cautioned that growth factors may give opposite responses in CCs and their corresponding tumours.

The art of culturing enterochromaffin cells. *Christian Prinz* asked "why do it?". Well, histamine release controls GI functions (acid secretion, motility, intestinal secretion), and isolated cells could prove useful both for basic research on mechanisms and as replacement therapy[6]. A protocol for cell isolation was described in which excised rat

stomach is enzymatically dispersed and fractionated by counterflow elutriation and density gradients.

Working protocols for isolation and culture of chromaffin and related cells. An outcome from this Workshop will be the posting of working protocols on the web, enabling sharing of techniques and on-going updates of these protocols.

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Necessary conditions to maintain rat adrenal chromaffin cells in primary culture.

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Cell Biology of the Chromaffin Cell
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The first successful attempts to isolate chromaffin cells from small rodents were carried out by Douglas and collaborators in mid-sixties using enzymatic digestion of gerbil adrenal medullae with Viokase (a mixture of pancreatic enzymes)[1]. Some years later, Livett and co-workers were able to obtain high yields of viable and pharmacologically responsive cells using retrograde perfusion of a collagenase solution through central lobular vein in bovine adrenal glands[2] (for a detailed review see ref. 3). Since then, the most popular technique for isolating chromaffin cells has been the use of collagenase, alone or in combination with another enzymes. However, due to the small size of rodents glands comparing with the bovine ones alternative methods to perfusion as the digestion of halved or sliced whole adrenal glands[4] or medullas, which have been previously separated from the capsule and cortex[5] have been used.

In spite of culturing rat chromaffin cells is not technically demanding, among the main disadvantages of rat chromaffin cultures are the low yield and short survival of cells in culture. However, rat model offers some advantages comparing to the bovine one. Due to some restrictions in the availability of bovine tissues after bovine spongiform encephalopathy crisis or the need to work with another animal models with an easier breeding or particular scientific interest (as new rat experimental disease models) doing of this preparation a good alternative [6].

In the next paragraphs, a detailed protocol will be presented pointing out those technical aspects that could be more critical to achieve long-term survival cultures. However, the quality of this culture is highly variable even in the same lab. The explanation could be the human factor and intrinsic variations in the manual procedures but sometimes it is not so clear, doing that a good culture is something almost “magic”.

About the animals and the isolation procedure. Any strain of rat can be used to isolate chromaffin cells from adrenal gland but young female animals usually offer better results with the advantage that they are more easily handled. The euthanasia procedure must be fast to avoid excessive stress to the animals, for what a well-trained person is required.

The location of adrenal glands is easy, both can be easily recognised just above the kidneys surrounded by fatty tissue. Once extracted the gland is important keep it always wet with cold saline solution (this preserve the gland and facilitate posterior removal of adipose tissue) and handle the gland with care to avoid damage it. Under a dissection stereomicroscope the adrenal glands are cleaned removing the surrounding adipose tissue using a scalpel blade or a small scissors. Then, the capsule is discarded doing a superficial incision along the gland and removing the capsule rolling the gland with the help of the scalpel blade. Finally, the cortex is also removed with a scalpel blade until the medulla is fully clean (the medulla is easily recognised because is usually clearer than the cortex) and placed in cold saline solution. This method give cultures with a reasonably good yield and enriched in chromaffin cells compared to those using whole adrenal glands.

About the enzymatic digestion. The most popular enzymatic digestion procedure involves the use of collagenase (IUB 3.4.24.3) from *Clostridium hystoliticum* alone or in combination with another enzymatic activities (trypsin, hyaluronidase, DNase or proteases). There is not doubt that this is the critical point to obtain good cultures.

One of the main problems with collagenase is that commercial crude collagenases are a blend of different enzymatic activities such as clostripain, neutral proteases and tryptic activities. According to the levels of collagenolytic and proteolytic activities commercial suppliers classify the collagenases in different types. Recently, purified collagenases with a reduced proteolytic activity are also available.

For these reasons, is very important test our enzymatic protocol to find the most suitable enzymatic blend and the best conditions in order to obtain a good yield with the minimum cell damage and keeping as intact as possible the properties of the cells. For it is important to let the enzymes work with minimal mechanical action.

Our digestion solution contains collagenase type I (Worthington Biochemical Co., NJ, USA) which contains caseinase too, bovine serum albumin, DNase type I (which reduces the amount of DNA fibres which favour aggregation and clumping of cells lowering the

yield) and hyaluronidase (often used in conjunction with collagenase to dissociate the extracellular matrix between cells of animal tissue).

The control of the digestion time is very important and the reaction should be stopped by dilution adding cold saline solution and preferably spin at 4ºC.

About the culture media. The most common medium used to keep chromaffin cells in culture is Dulbecco´s Modified Eagle´s Medium (DMEM). However, another media have been used alone (Eagle´s medium, RPMI 1640, M-199, etc) or combined in different proportions (DMEM/RPMI, DMEM/Ham´s F12). Depending of requirements of your experiments could be important to know the main differences between the formulations of several media available. Thus, DME is a modification of Basal Medium Eagle (BME) that contains four-fold higher concentration of amino acids and vitamins as well as another components.

Another important point for some experiments is to control just as possible the amount of serum or supplements that we are adding to the media. For this reason, culture media with an additional amount of known trophic factors can be prepared to eliminate the serum, which is always a more unspecific solution. Recently, new media formulations from main suppliers are appearing in the market that requires less amount of serum (usually, 2-5 times less). With our protocol (fig. 1) we are able to keep the cells in good conditions during several weeks, usually 1-4 weeks.

New approaches. One proof that the procedure is not technically demanding has been its recent automation. Jacqueline Sagen´s group from University of Miami have developed an adaptation of an automated method, previously described for isolation of islets of Langerhans[7], to isolate chromaffin cells from neonatal porcine adrenal glands[8]. The set-up consists in a digestion chamber where the tissue is placed, connected to a circuit in which a solution of purified collagenase at 37ºC is recirculating. When the cells are in small clusters or already isolated the flow permits their way through a metallic mesh being collected in a bottle containing collagenase-free saline solution.

Primary culture of rat chromaffin cells

1. Sacrifice the animals by cervical dislocation after stun them by concussion.
2. The adrenal glands must be immediately extracted after the sacrifice of the animal and placed on ice cold HBBS (Ca^{2+} and Mg^{2+} free) supplemented with antibiotics (Pen+Strep).
3. Under a dissection stereomicroscope, eliminate the surrounding adipose tissue with a stainless steel scalpel blade and decapsulate the adrenal glands, then remove the cortex to isolate the medullar tissue.
4. Place the adrenal medullas in a Falcon-type tube with cold HBBS.
5. Put 3 ml of dissociation solution (2,6 mg/ml collagenase type I (Worthington), 3mg/ml BSA, 0,15mg/ml DNase I, 0,15mg/ml hyaluronidase I-S in HBSS) in the lid of a small culture dish.
6. Put the medullas into the dissociation solution and cut them in four pieces each one with the help of a scalpel blade or two needles. Start to count the time of enzymatic digestion. Recover the medulla chunks and the solution and transfer them to a Falcon tube.
7. Place the tube in a thermostatic bath at 37ºC during 30 min approx.
8. Every 10 min the solution must be gently resuspended with a Pasteur pipette with a fire polished broken tip. During the last 5 min the solution is continuously resuspended until the medullary tissue becomes not visible.
9. Fill the tube with cold HBBS until a final volume of 10 ml to stop the enzymatic reaction.
10. Spin at 800 rpm during 10 min in a pre-cooled refrigerated centrifuge at 4ºC.
11. Discard the supernatant by decanting and homogenate the pellet with 800 □l pre-warmed DMEM+10% heat-inactivated FCS supplemented with antibiotics.
12. Put 1 or 2 drops in each poly-D-lysine treated coverslip and leave the cells settle down during 1 h at 37ºC.
13. Refill the culture dish with 1.5 ml of DMEM+10% FCS and incubate at 37ºC and 5%CO_2.

Figure 1. Our current lab protocol to prepare a primary culture of rat adrenal chromaffin cells.

This system is particularly useful to produce a high number of cells reducing the variability due to manual techniques for enzymatic digestion. This group have made another important contribution to use rat chromaffin cells. They obtained conditionally immortalized chromaffin cells from rat and bovine adrenals[9]. The rat RAD5.2 cell

line was obtained from E17 Sprague-Dawley rat using the temperature sensitive mutant of SV40 large T antigen (tsTag). This cell line is conditionally immortalized and can be later disimmortalized under specific conditions (i.e. temperature). This would particularly useful in cell lines used for neural transplants (in which chromaffin cells are having a renewed interest) because the RAD5.2 cells stops their growth at body temperature avoiding undesired effects.

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Extra-adrenal chromaffin cells of the Zuckerkandl´s paraganglion: morphological and electrophysiological study.

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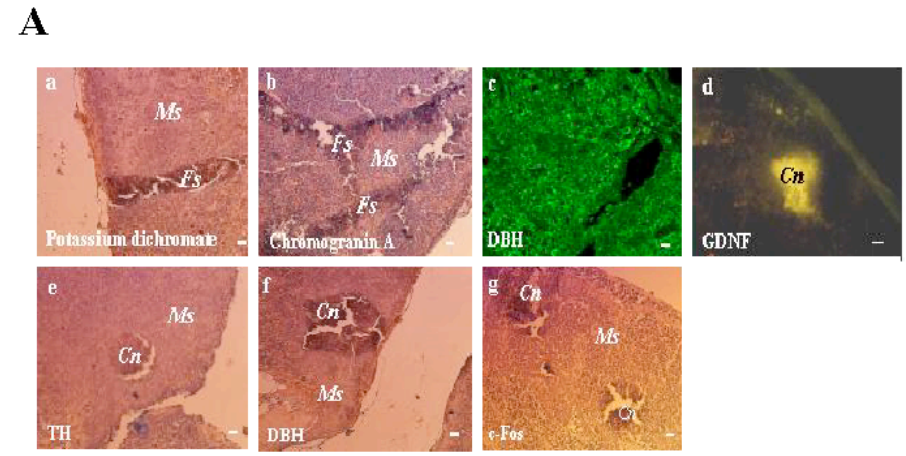
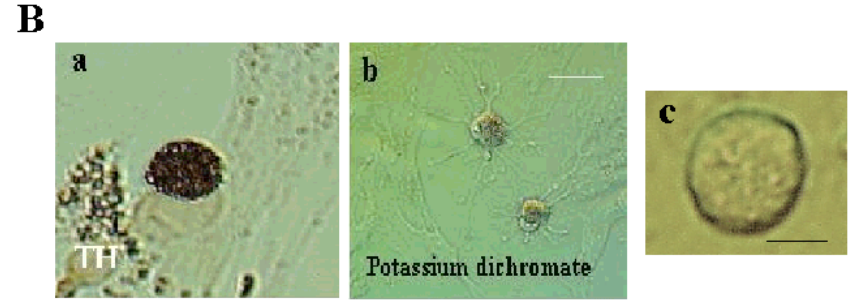
Parkinson´s disease is one of the most important neurodegenerative disorders that affects to one out of a hundred of the world population elder than 65. It has been observed in our laboratory, for the first time, that intrabrain transplantation of chromaffin cell aggregates from the Zuckerkandl´s organ, an extraadrenal paraganglion located adjacent to the lower abdominal aorta, induced gradual improvement of functional deficits in animal models of Parkinson´s disease[1]. This functional regeneration was likely caused by long-survival of grafted cells and chronic trophic action of dopaminotrophic factors, glial cell line-derived factor (GDNF)[2,3] and transforming growth factor beta1 (TGF-β_1)[4,5], which are expressed and delivered by long-surviving grafted chromaffin cells. The objective of this study is to discern the morphological and cytological characteristics of extra-adrenal cells of the Zuckerkandl's organ. On the other hand, long survival of extra-adrenal chromaffin cells could be related to resistance to hypoxia, since it is certainly know that hypoxia is a primary factor involved in cell death after intrabrain grafting. Thus, resistance to hypoxia seems to be a critical factor involved in survival fate after grafting because, among cells of the chromaffin lineage, adrenal medulla cells (non responsive to hypoxia) die shortly after transplantation, but carotid body cells (hypoxia responsive) present a sustained and long-term survival after grafting[6,7]. For this reason, we are currently studying the electrophysiological characteristics as well as sensitivity to hypoxia of these extra-adrenal chromaffin cells.

RESULTS

Paraganglia tissue was observed to be composed of chromaffin cells (size around 15 μm) and mesenchyma. Chromaffin tissue (around 22% of the organ) was found to be distributed making up longitudinal fascicles with the appearance of rounded "cell nests" on coronal sections. As typical chromaffin cells, they react with potassium dichromate (classical Orth's reaction), and was also detected the presence of chromogranin A, a chromaffin cell lineage protein that participates on exocytosis.


A
a
Ms
Fs
Potassium dichromate
b
Fs
Ms
Fs
Chromogranin A
c
DBH
d
Cn
GDNF
e
Ms
Cn
TH
f
Cn
Ms
DBH
g
Cn
Ms
Cn
c-Fos


B
a
TH
b
Potassium dichromate
c

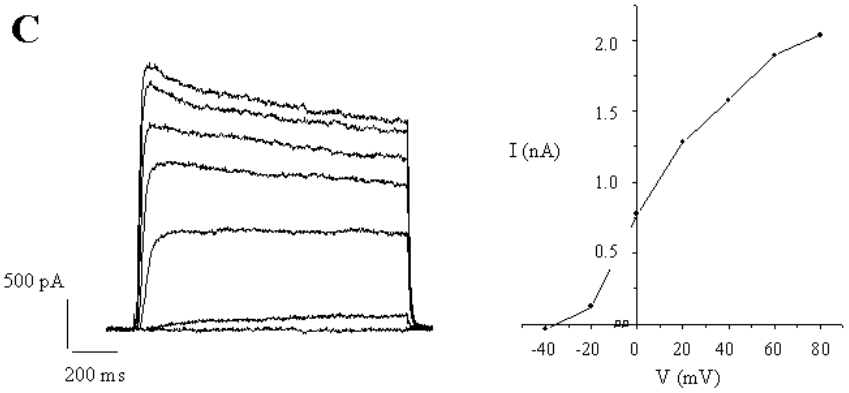

C
500 pA
200 ms
I (nA)
2.0
1.5
1.0
0.5
-40
-20
0
20
40
60
80
V (mV)

Figure 1. A) Morphological features of the Zuckerkandl's organ**.** Orth´s reaction showing that chromaffin cells clustered making up a fascicle (Fc), reacted with potassium dichromate (**a)**. Immunohystochemistry and immunofluorescence staining revealed that chromaffin cells, forming fascicles (Fc) or cell nests (Cn), are positive to chromogranin A **(b)**, dopamine-beta-hydroxylase (**c) and (f)**; glial cell line-derived neurotrophic factor (GDNF) **(d)**; tyrosine-hydroxylase (TH) **(e)**, and that they are transcriptionally active in vivo because they express c-fos **(g)**. Phenylethanolamine-N-methyl-transferase (PNMT, adrenaline synthesizing enzyme) is not expressed. Scale bars: 100 µm, except in c, 10µm. **B) (a, b)** Positive reaction to TH and potassium dichromate. **(c)** Isolated chromaffin cell of the Zuckerkandl´s organ. Bar, 10µm. **C**). **(a)** Ionic currents in extra-adrenal chromaffin cells. The currents traces are recorded every 8 s for 125 ms voltage steps in 20 mV increments from –40 to +80 mV from a holding potential of –70 mV. In all cells explored outward currents exhibiting sigmoid activation kinetics were observed with amplitude ranging from 0.5 to 2.5 nA. The outwards currents show activation threshold at approximately -40 to –35 mV. **(b)** Current-voltage relationship obtained by measuring the peak amplitude of traces shown in figure **C (a)**. These results suggest that these cells could possess different potassium channels.

Immunostaining (following standard procedures) also indicated that extra-adrenal chromaffin cells were noradrenergic (TH and DBH positive), and expressed the neurotrophic factor GDNF, that is known to be dopaminotrophic. Thus GDNF protects dopaminergic neurons from degeneration in vitro and in animal models of PD, when delivered by intraventricular injections or via transplanted cells or viruses[2,8].

Regarding isolated cell experiments (cytochemical and electrophysiological studies) chromaffin cells of the Zuckerkandl´s organ were cultured following a similar protocol to that used for isolating adrenal chromaffin cells, with few modifications[9]. Cultured extra-adrenal chromaffin cells were found to express TH and to react with potassium dichromate. An isolated typical chromaffin cell two days after cultured can be observed in Figure 1B (c).

Ionic currents from isolated cells were recorded using the whole cell variant of the patch-clamp technique[11] with an EPC-7 patch clamp amplifier. Data adquisition was performed by an ITC-16 computer interface and (Pulse + Pulsefit) software. Linear leak currents, through membrane capacitance, were cancelled on-line using P/4 procedure[12]. The

preliminary electrophysiological results are summarized in legend of figure 1C.

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CANSTAT-4: A four channels potentiostat for the on-line monitoring of catecholamine secretion.

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Instituto Teófilo Hernando, Spain, 2004

Amperometric techniques have been used for the analysis of the secretory responses from perfused adrenomedullary tissues like whole adrenal (cat [1], rat[2]) or cultured bovine cells. However, all of the commercially available equipments are electrochemical detectors used for HPLC (BAS, Metrohm). However, these apparatus are single-channel and the use of simultaneous recording will require the use of additional detectors with an increase in the price and bench space. Another disadvantage is the lack of proper software for the stimulation of the tissues and for multi-channel acquisition of secretory signal(s).

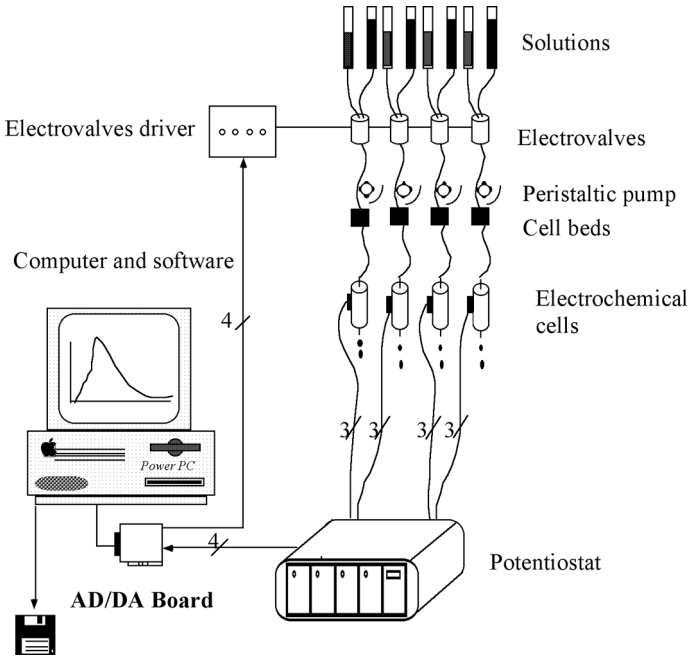

Figure 1. CANSTAT-4 setting-up. Basal incubating solutions or chemical stimuli are selected by the electro-valves. These valves can be manually operated or by a TTL signal generated by a computer digital output. A four-channel peristaltic pump is continuously perfusing the cell chambers or adrenal glands. The emanating fluid is passed through the electrochemical cells for catecholamine detection. The potentiostats send the analogic signal (± 12 V) to a computer through a 12-bit AD/DA board (PCI-1200, National Instruments)

We introduce here the CANSTAT-4, which has been designed and constructed in our lab to satisfy all of these

requirements. This is an integrated system for the simultaneous recording of the secretion of catecholamines from four perfused adrenal glands or cell beds.

CANSTAT-4 system includes: i) four potentiostat amplifiers, caged in individual shielded plug-in units, ii) new designed compact three-electrodes electrochemical cells, iii) computer-controlled drug administration by an electro-valve driver, iv) software for acquisition, v) variable applied voltage driven by the computer for voltammetry and vi) control of chemical stimulation protocol. Our system uses a graphic computer language (LabVIEW™, National Instruments, Austin, TX, USA) that can operate either under Macintosh or Windows environments.

The system requires some additional equipment: a peristaltic pump, a computer and a AD/DA board. Optionally, the program can trigger an electrical stimulator for the study of splanchnic nerve-chromaffin cell synapse.

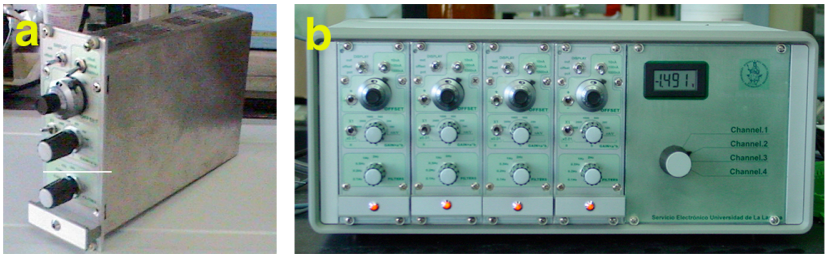

Figure 2. General view of the potentiostat amplifier. Panel **a** shows an individual plug-in module On panel **b** is shown the front view of the equipment with the four modules inserted and the common display. External dimensions 42 x 25 x 18 cm.

One important feature of the system is its modular structure. Hence it can operate with 1 to 4 channels independently and individual modules can be removed from the main box without alter the functioning of the other channels. Each module is a plug-in shielded box to avoid electromagnetic interferences (figure 2a). The oxidation/reduction voltage can be set from either an internal variable resistor or externally applied by the computer AD/DA

board. The front panel contains the gain, offset and filter controls whereas in the back of the main box are placed the plugs for the electrochemical cells and for the input/output terminals. A common display allows monitoring the electrode potential, the offset applied to the output signal and the output value for every channel (Figure 2b).

The electro chemical cells have been designed compact, light and small in order to be accommodated near the tissues thus reducing the liquid dead space. Each electrode can be removed for cleaning or replacement (figure 3a). The valve controller is a compact cage intended to be placed near the peristaltic pump. It can be used either in a manual or in a computer-operated mode.

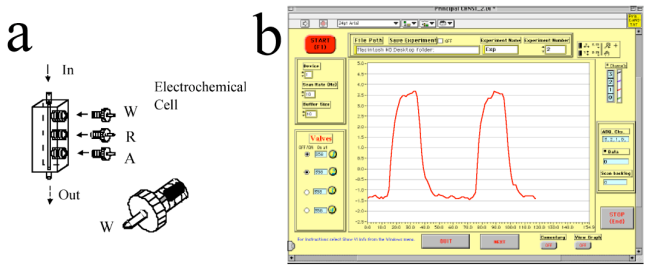

Figure 3. Some novelties of the CANSTAT-4. a) the system includes compact electrochemical cells for three electrodes: working (W, glassy carbon); reference (R, silver chloride) and auxiliary (A, stainless steel). Each electrode is built in a plastic screw that can be removed independently. **b)** the computer program allows the continuous acquisition and display of data. In the picture, a single channel record of catecholamine secretion shows two secretory peaks obtained after the stimulation of a cell bed (2 millions) with 30 µM DMPP for 15 s.

Although data obtained from CANSTAT-4 can be displayed by any acquisition program or chart recorder, we have created a specific system that combine data acquisition with stimulation protocols. The software designed for the CANSTAT-4 has been written using LabVIEW™. Data from all channels (oxidation/reduction currents) are online displayed on the computer screen and quantified in nA. The program returns and saves binary files ready for their analysis using Igor Pro (Wavemetrics, Lake Oswego, OR, USA).

The CANSTAT-4 constitutes a new system for the multiple online analysis of catecholamine secretion from adrenomedullary tissues. It opens the use of these tissues for simultaneous measurements useful for a wide range of purposes including drug testing.

ACKNOWLEDGMENTS

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