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.