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.

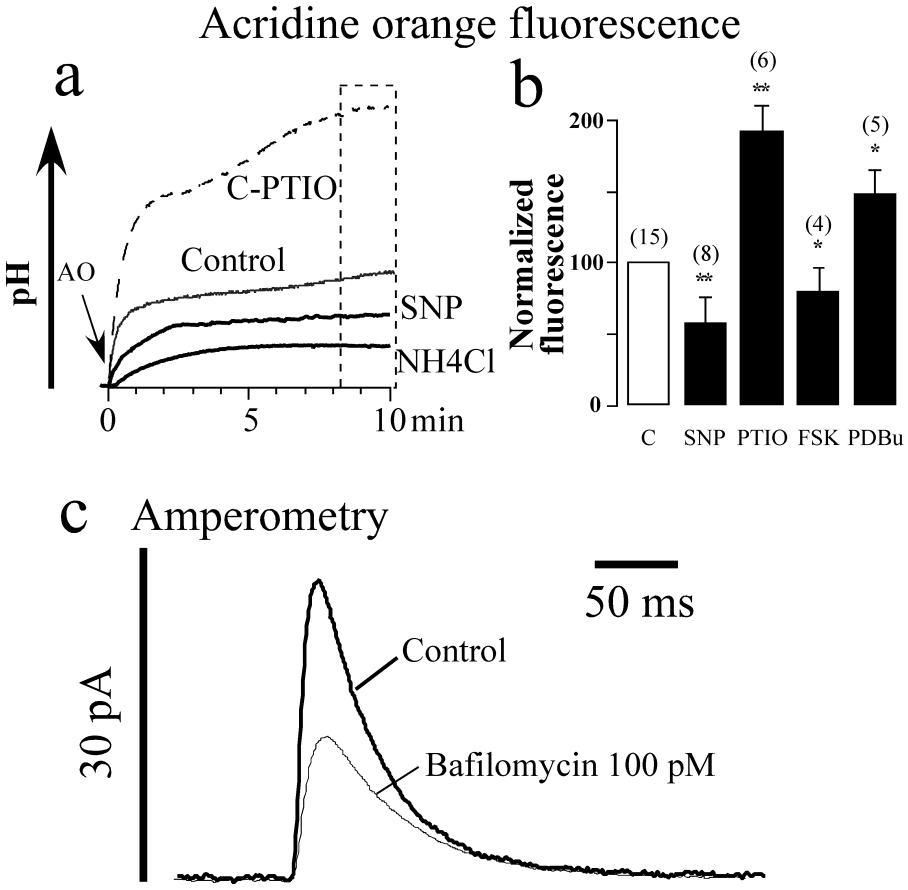

Figure 1. The effects of several drugs in the accumulation of acridine orange and in the kinetics of exocytosis in single chromaffin cell. a) Typical traces showing the cellular accumulation of acridine orange (AO, arrow; 10 nM, 30 s) measured using an inverted fluorescent microscope. Chromaffin cells 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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