Journal of Neurochemistry, 2006, **96**, 324–334 doi:10.1111/j.1471-4159.2005.03526.x

Intragranular pH rapidly modulates exocytosis in adrenal chromaffin cells

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Abstract

Several drugs produce rapid changes in the kinetics of exocytosis of catecholamines, as measured at the single event level with amperometry. This study is intended to unveil whether the mechanism(s) responsible for these effects involve changes in the intravesicular pH. Cell incubation with bafilomycin A1, a blocker of the vesicular proton pump, caused both a deceleration in the kinetics of exocytosis and a reduction in the catecholamine content of vesicle. These effects were also observed upon reduction of proton gradient by nigericin or NH_4Cl. pH measurements using fluorescent probes (acridine orange, quinacrine or enhanced green fluorescent protein–synaptobrevin) showed a strong correlation between vesicular pH and the kinetics of exocytosis. Hence, all maneuvers tested that decelerated exocytosis also alkalinized secretory vesicles and vice versa. On the other hand, calcium entry caused a transient acidification of granules. We therefore propose that the regulation of vesicular pH is, at least partially, a necessary step in the modulation of the kinetics of exocytosis and quantal size operated by some cell signals.

Keywords: acridine orange, ATP-dependent vesicular proton pump, bafilomycin A1, enhanced green fluorescent protein, quinacrine, secretion.

J. Neurochem. (2006) **96**, 324–334.

Exocytosis constitutes the main cellular mechanism for the secretion of neurotransmitters. This finding gave support to the classical quantal theory of neurotransmission, which maintained that neurotransmitters were released in discrete packages from the nerve terminals onto the postsynaptic cell (Del Castillo and Katz 1954). This implies that the modulation of a postsynaptic response can only take place through a change in the number of vesicles that undergo exocytosis at the nerve terminals. However, recent amperometric measurements at the single event level show that the concentration reaching the postsynaptic cell can be rapidly modulated without change in the number of released vesicles (Burgoyne and Barclay 2002). Two separate major mechanisms have been observed by which such a modulation is achieved; the first involves changes in the vesicular content (Sulzer and Pothos 2000), whereas the second acts through changes in the kinetics of exocytosis at the single event level (Machado *et al*. 2000). It is conceivable that either both mechanisms underlay the action of certain drugs or that they are the endpoint of cellular signaling routes.

Previous studies by others and our own laboratory have shown that certain drugs alter the vesicular content and others the kinetics of exocytosis (Borges *et al*. 2002; Burgoyne and Barclay 2002). However, the mechanisms underlying changes such as those caused by activation of PKA (cAMP-dependent protein kinase) or PKG (cGMP-dependent protein kinase) were not tested in this respect.

The vesicular pH could play a key role in the control of aggregation of vesicular solutes. The resting luminal pH of chromaffin granules is around 5.5 (Winkler and Westhead 1980), which roughly coincides with the pK_a of the major vesicular protein chromogranin A. This protein plays a key role in overcoming the osmotic gradient resulting from the

Received January 22, 2005; revised manuscript received August 1, 2005; accepted September 9, 2005.

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Abbreviations used: AO, acridine orange; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DMPP, dimethylphenylpiperazinium; EGFP, enhanced green fluorescent protein; NO, nitric oxide; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; QNC, quinacrine; SNP, sodium nitroprusside; V-ATPase, ATP-dependent vesicular proton pump.

massive accumulation of solutes within granules. The efficiency of this aggregating mechanism seems to be maximal at pH 5.5 (Helle *et al*. 1985; Yoo and Lewis 1996).

In this study we demonstrate a correlation between intravesicular pH and the kinetics of single exocytotic release events. We propose that several second messengers act by through this novel mechanism and thereby modify quantal release characteristics.

Materials and methods

Culture of chromaffin cells

Bovine adrenal chromaffin cells enriched in adrenaline were prepared and cultured as described elsewhere (Machado *et al*. 2000), and plated on 12-mm diameter glass coverslips at an approximate density of 5×10^4 cells/well and used at room temperature between 1 and 4 days of culture.

Generation of constructs of enhanced green fluorescent protein coupled to synaptobrevin

To produce an in-frame fusion of synaptobrevin to the N terminus of enhanced green fluorescent protein (EGFP), the coding region corresponding to synaptobrevin II (Archer *et al*. 1990) was amplified by PCR with the following primers: 5′-GCCGAATTCCCGCCATG-TCGGCTACC-3′ (sense) and 5′-GCCGGATCCGAGCTGAAGT-AAACGATGATG-3′ (antisense). The PCR product was digested with *Eco*RI and *Bam*HI and cloned into the same sites of the expression vector pEGFP-N1 (Clontech, Palo Alto, CA, USA). Primary cultures of chromaffin cells were infected with a herpes simplex virus (HSV-1) amplicon containing the construct mentioned above, as described (Gil *et al*. 2002).

Cell infection and expression of the recombinant protein was monitored by epifluorescence using a FITC filter set. EGFP fluorescence was detected in 80–95% of cells after 2 days of incubation. Experiments were performed within 48–72 h post infection.

Fluorescence microscopy

Cells were washed twice in Krebs-HEPES buffer solution containing (in mM): NaCl (140), KCl (5), $MgCl_2$ (1.2), $CaCl_2$ (2), glucose (11), and HEPES (10), adjusted to pH 7.35 with NaOH and placed in a perfusion chamber on the stage of an inverted epifluorescence microscope. Fluorescence due to the accumulation of the weak bases acridine orange (AO, 10 nM) or quinacrine (QNC, 10 μM) was measured with a standard FITC filter set using a 63 × oil immersion objective. Fluorescence was excited with 485 nm light from a monochromator (TILL Photonics, Munich, Germany) that was controlled by in house software written in LabVIEW for Mac (National Instruments, Austin, TX, USA).

When cell permeabilization was required, experiments were performed in a solution containing (in mM): K^+-glutamate (140), $MgCl_2$ (2), MgATP (5), glucose (11), EGTA (0.2) and PIPES (10), pH is adjusted at 6.6 or 7.2 with KOH. Coverslips were then incubated for 5 min with 10 μM digitonin dissolved in the same buffer.

To test the fluorescence properties of synaptobrevin-EGFP, the cells were fixed with 4% glutaraldehyde for 3 min followed by permeabilization with 0.01% Triton X-100. Cells were then washed thrice and calibrated with buffer solutions adjusted to various different pH. Intracellular distribution of synaptobrevin-EGFP was studied by excitation with a 488-nm laser line in a confocal microscopy (MRC 1025, Bio-Rad Laboratories, Madrid, Spain). The fluorescence was recorded at 1 Hz and expressed as arbitrary units of intensity or normalized to their own control cells. Statistical significance was evaluated with Student's *t*-test.

Amperometric detection of exocytosis

Glass-encapsulated carbon fiber microelectrodes were prepared as described (Kawagoe *et al*. 1993). A fixed potential of +650 mV was maintained between the carbon fiber and an Ag/AgCl pellet reference electrode. Calibration of the electrodes was essential to assure the reproducibility of results. Electrodes were tested and accepted for cell studies when the application of 50 μM noradrenaline resulted in an oxidation current of 300–400 pA. This oxidation current should be reduced by 80–100 pA under stopping flow conditions. Electrochemical recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), as described (Machado *et al*. 2000). Glass coverslips with adhering adrenal cells were rinsed in Krebs solution and placed in a perfusion chamber positioned on the stage of an inverted microscope.

Amperometric measurements were performed with the microelectrode gently touching the cell membrane. Unless otherwise stated, cell secretion was stimulated by 5-s pressure ejection of 5 mM Ba^{2+} from a micropipette situated 40 μm away from the cell.

Data analysis

Amperometric signals were low pass filtered at 1 KHz and collected at 4 KHz using a locally written program (LabVIEW). Data analysis was carried out using locally written software (Segura *et al*. 2000). These programs can be free downloaded from the web address: http://webpages.ull.es/users/rborges/.

A low-pass digital filter FIR with cut frequency from 500 to 850 Hz, implemented in the program, was applied to data prior to analysis. The analysis of individual exocytotic events was based on the measurements of the following parameters: I_{max}, maximum oxidation current; $t_{1/2}$, spike width at half height; Q, spike net charge and m, ascending slope of spike. An explanation of the biological significance of these parameters has been provided (Schroeder *et al*. 1996; Segura *et al*. 2000). Day-to-day changes in electrode sensitivity and cell responsiveness accounted for very large variations even among control cells. For this reason, effects of drugs on secretory spikes were always compared with control experiments carried out the same day and using the same electrode. Because of the high cell-to-cell variability in spike characteristics, we used the spikes recorded from a single cell to estimate one statistic per cell (kinetic parameters were given as mean from at least 20 spikes/cell) and compared this statistic between control and treated cells (Colliver *et al*. 2001). Kinetics parameters were expressed as percentage of their own controls. Statistical analysis was performed by the non-parametric Mann–Whitney rank sum.

Several precautions have to be kept in mind when performing and interpreting amperometry. The active surface of the carbon fiber is easily reduced by cell debris causing a reduction in the responsiveness; therefore, it is crucial to calibrate the electrode also after the experiment. The loss of sensibility of the electrode should not be

larger than 15%. However, to minimize the effect of this decrease, control and treated cells must be alternated along the experiment. The results obtained with two electrodes on two sets of cells from the same day can be statistically significant. Differences increase dramatically when un-calibrated electrodes are used. Data from different days should not be compared. Another important point is the inclusion criteria for spikes. Using a low noise amplifier (Axopatch 200B) and FIR digital filters (Segura *et al*. 2000) our current detection limit for spikes to obtain reliable parameter measurements is 3 pA of I_{max}. This means that our average values are usually smaller than in other studies (Graham and Burgoyne 2000), in which secretory spikes smaller than 40 pA were rejected. A small spike should not be considered, in principle, of less physiological importance than a big one. Whilst $t_{1/2}$ remains short these spikes are probably originated from granules that undergo exocytosis under beneath the glass-encapsulated electrode that is gently pressed against cell membrane.

In the analysis of secretory spikes it is important to separate changes in the kinetics of exocytosis that do not affect the granule content from those where the net charge is also affected (i.e. reduction). A secretory spike has roughly the shape of a triangle where I_{max} is the height and Q the area. One can assume that the base of that triangle is proportional to $t_{1/2}$. Then, when Q remains constant, an increase in I_{max} must be accompanied with a decrease in $t_{1/2}$. However, when the deceleration of secretion is accompanied by a reduction in Q, it is possible that the values of $t_{1/2}$ remain unaffected, as observed with bafilomycin. This should not be interpreted as unchanged kinetics. The parameter 'm' (the ascending slope between the 25–75% of the spike height) is associated with the initial part of the exocytosis. It usually behaves like $t_{1/2}$, although it is less affected by changes in Q. For instance, valinomycin caused a rapid increase in Q (10 min) without affecting the m. To preserve the 'triangle' dimensions, there are two possibilities: increase the height (I_{max}) or increase the $t_{1/2}$.

Results

Second messengers and drugs rapidly change the kinetics of exocytosis

In a previous study (Machado *et al*. 2000), we showed that nitric oxide (NO) promoted rapid deceleration of single granule release events acting through the classical cGMP route. The effects of NO were mimicked by the cGMP analog 8-Br-cAMP. Conversely, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO), which removed the NO present inside and outside the cell, accelerated exocytosis. I_{max} and m increased as $t_{1/2}$ decreased when cells were treated with C-PTIO, and the opposite was found when cells were incubated with NO or cGMP (Table 1).

In another study, we showed that stimulation of PKA also produced the deceleration of exocytosis (Machado *et al*. 2001). The activation of PKA produced complex effects on exocytosis. Mild stimulation affected only the kinetics, but strong stimuli, such as forskolin, also increased the net charge (Q) (Table 1), probably due to 'compound fusion' derived from an increase in Ca^{2+} entry (Borges *et al*. 2002).

The main question that emanated from these studies was how a given second messenger could perform effects on exocytosis within tens of seconds.

Different cell signals rapidly change the pH of secretory vesicles

We used three different fluorescence dyes to monitor changes in the vesicular pH. Weak bases, such as acridine orange (AO) or quinacrine (QNC), accumulate into acidic cellular compartments, so acidification of a highly abundant organelle such as chromaffin granules resulted in an increase in cellular fluorescence (Knight and Baker 1985; Sulzer and Rayport 1990). AO had the advantage of its high fluorescence, which made it ideal for rapid kinetic studies, even using very low concentrations of dye (10 nM); however, AO results are not useful for long recordings because, after a few minutes, it also stained nuclei (Neco *et al*. 2002). Although high concentration of AO modified the kinetic of exocytosis (reduced Q; Table 2), this effect was not significant at the dye concentrations used here for fluorescence measurements. Conversely, measurements with QNC required the incubation with higher concentrations of the dye (10 μM) in order to produce similar fluorescence intensities to 10 nM AO. However, QNC did not result in substantial nuclear labeling. In a third approach, we used EGFP coupled to the luminal

Table 1 Effects of several cell treatments on secretory spike parameters

	I_{max}	Q	$t_{1/2}$	m	*n* spikes	*n* cells
C-PTIO 10 nM[a]	288 ± 39**	100 ± 12	51 ± 4**	438 ± 17**	908/785	6/9
SNP 10 μM[a]	40 ± 4**	71 ± 6*	171 ± 11**	36 ± 1**	1121/881	16/15
8-Br cGMP 10 μM[a]	39 ± 5*	84 ± 14	153 ± 9**	31 ± 4**	453/319	8/10
Forskolin 100 nM[b]	96 ± 12	143 ± 21*	145 ± 7**	58 ± 7*	778/308	9/10

Drugs were applied for 10 min before and during data acquisition. For explanation of parameters used see Fig. 5(a). Data were normalized to their own control and are cell-based expressed (see Methods). *$p < 0.05$; **$p < 0.01$.

[a]Data recalculated from Machado et al. (2000).

[b]Data recalculated from Machado et al. (2001).

C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SNP, sodium nitroprusside.

Table 2 Effects of drugs that alter vesicular pH on secretory spike parameters

	I_{max}	Q	$t_{1/2}$	m	n cells
Acridine orange 10 nM	77 ± 12	98 ± 18	124 ± 5	77 ± 14	11/6
Acridine orange 1 μM	23 ± 4**	83 ± 16	269**	6 ± 2**	10/5
Acridine orange 10 μM	21 ± 5**	64 ± 16*	203 ± 29**	2 ± 1**	10/3
Quinacrine 10 μM	72 ± 8	86 ± 12	105 ± 12	67 ± 8*	19/20
NH_4Cl 10 mM	60 ± 7*	88 ± 9	135 ± 17*	55 ± 9**	26/29
DIDS 10 μM	74 ± 9	92 ± 15	103 ± 12	55 ± 9*	23/21
DIDS 100 μM	78 ± 14	91 ± 15	114 ± 15	52 ± 11*	9/7
Nigericin 500 nM	71 ± 9*	68 ± 8*	83 ± 6*	90 ± 8	8/7
Valinomycin 50 nM	97 ± 10	136 ± 15*	123 ± 11	*95 ± 14	10/9
NPPB 100 μM	73 ± 14*	105 ± 28	141 ± 18*	81 ± 25	6/6

Drugs were applied for 10 min at the given concentrations. Data are normalized to their own control and are cell-based expressed (see Methods). *$p < 0.05$; **$p < 0.01$ with respect its own control cells.

DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid.

side of synaptobrevin, which allowed the direct monitoring of pH in secretory vesicles with little background signal. Contrary to AO and QNC, this probe gives a stable baseline but increases its fluorescence when vesicular pH rises. The calibrated useful pH range for synaptobrevin-EGFP falls within the expected changes from 5.5 (secretory vesicles) to 7.4 (external solutions). Calibration properties are shown in Fig. S1(a) of the supplementary material. However, the amount of fluorescence recorded depends of the degree of expression of synaptobrevin-EGFP in the cell. The simultaneous measurement of luminal pH and catecholamine release from the same chromaffin granule is currently difficult to perform. For that reason, we have to carry out amperometry and pH measurements in parallel experiments.

The use of overexpressed synaptobrevin-EGFP as a pH probe (Kneen *et al*. 1998; Miesenbock *et al*. 1998) offers the advantage of a high selectivity for secretory vesicles (Gil *et al*. 2002). It also possesses a Boltzmann function that allows the correlation of changes in the emitting fluorescence with pH (Fig. S1, supplementary material). However, precise quantitative calibrations with a non-ratiometric dye are not easy because fluorescence will depend on the amount of expressed EGFP in a given cell. In addition, artifacts resulting from overexpression of the synaptobrevin-EGFP cannot be excluded. However, the kinetics of exocytosis was not altered (data not shown). In spite of its inverse fluorescence behavior (fluorescence increases as pH rises) changes in pH were qualitatively identical to those obtained with AO or QNC.

These three dyes seem to accumulate within the same cell structures, exhibiting the typical 'granular' pattern when examined with confocal microscopy. In addition our results employing these three dyes were consistent regardless of the method employed.

When AO was applied to cells, a progressive increase in the fluorescence was immediately observed. This uptake of AO is pH-dependent and can be inhibited by incubation with NH_4Cl (Fig. 1a). In solution, this compound dissociates to ammonia, which easily diffuses through membranes, accumulating within vesicles and promoting a transient disruption of the pH gradient and causing their alkalinization. Figure 1(b) shows the effect of a 10-s exposure of the cell to NH_4Cl (10 mM) on AO uptake. Immediately after application of NH_4Cl, a fall in the fluorescence signal was observed.

To address whether NO affects vesicular pH, we have used the same concentration of the NO donor sodium nitroprusside (SNP, 10 μM) that decelerates exocytosis; this amount of SNP yielded approximately 10 nM of free NO (Machado *et al*. 2000). Figures 1(c) and (d) show the effects of SNP and C-PTIO on the time course of AO accumulation. The data indicate that NO rapidly reduced the H^+ gradient across the vesicular membrane thus promoting its alkalinization. The same effect was observed using QNC (Fig. 2b). Changes in the vesicular pH (Fig. 1c) occurred with approximately the same time course that was observed measuring changes in $t_{1/2}$ by amperometry (Fig. 4 from Machado *et al*. 2000). The effects of SNP and C-PTIO also implied the presence of a basal concentration of NO in the bathing media that was revealed after its removal with the scavengers. Evidently, this basal presence of NO affected both pH and exocytosis.

The primary cellular compartment where AO and QNC accumulate appears to be the secretory vesicle, since the blockade of the ATP-dependent vesicular proton pump (V-ATPase) by bafilomycin A1 caused the rapid attenuation of the fluorescent signal (Fig. 2a). The time course of accumulation of QNC was slightly different than that observed with AO, although the effects of drugs on both signals were consistent. The application of a brief pulse of 10 μM QNC produced a rapid increase in the fluorescence signal followed by a steady state that lasted up to 10 min. Importantly, incubation with the V-ATPase inhibitor bafilomycin A1 (0.1–10 nM) inhibited the uptake of QNC in a concentration-dependent manner. This effect was also

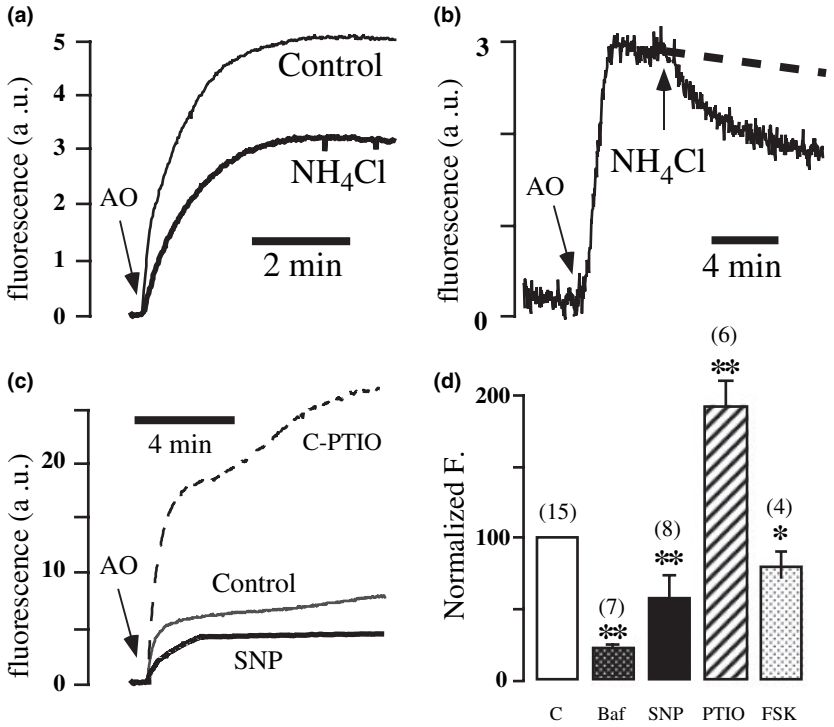


Fig. 1 Vesicular pH changes revealed by acridine orange (AO) accumulation in single chromaffin cells. (a) Chromaffin cells were incubated in Krebs solution in the absence and in the presence of NH_4Cl 10 mM during 10 min. Then cells received a 30 s pulse of 10 nM AO from a pipette situated near the cell (arrow, AO), and the accumulation of the dye in the cell was recorded. Traces represent the average of nine control cells and seven cells treated with NH_4Cl, expressed as arbitrary units of fluorescence (a.u.). (b) Typical trace showing the cellular accumulation of AO (10 nM, 30 s); NH_4Cl 10 mM was puffed for 10 s as indicated by the arrow. Dashed line depicts the predicted time course of fluorescence in the absence of NH_4Cl, taken from control cells. (c) Effects of incubation with the nitric oxide (NO) donor sodium nitroprusside (SNP, thick line) applied at 10 μM for 10 min or with 10 nM of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO, dashed line), traces are averaged from six to 15 experiments. (d) Pooled data from experiments similar as in (c). Measurements were done at the end of traces and are the average from the number of cells indicated in brackets; C, saline; PTIO, carboxy-PTIO 10 nM; FSK, forskolin 100 nM; SNP, sodium nitroprusside 10 μM. $^*p < 0.05$, $^{**}p < 0.01$, Student's *t*-test.

observed upon application of the ionophore nigericin (50 nM) and with NO donors (Fig. 2).

The basal pH of secretory vesicles remained stable during the time required to perform measurements as revealed by synaptobrevin-EGFP. Figure 3 shows that resting cells maintained a constant fluorescence intensity (Control). However, rapid changes were observed immediately after the addition of NH_4Cl. In spite of their different time course, both bafilomycin and SNP promoted the alkalinization of the vesicles (Figs 3a and b).

There were differences in the time course of pH changes caused by the different agents used. For instance, ammonia rapidly equilibrated the pH inside of the vesicles, whereas bafilomycin must enter the cell to block the V-ATPase, and SNP continuously donated NO, which causes a progressive alkalinization lasting minutes.

The fluorescence recorded from these experiments varied from cell to cell, depending on the amount of synaptobrevin-EGFP expressed. The experiments shown in Fig. 3 were performed with the same batch of cells and offer qualitative responses, but the values, expressed as arbitrary units, cannot be meaningfully compared from one cell to another.

Consistent with the changes in exocytosis observed by amperometry, adenylate cyclase stimulation with forskolin produced alkalinization of the vesicle lumen (Fig. 1d).

We next examined the effect of secretory stimuli on vesicular pH (Fig. 4). We chose dimethylphenylpiperazinium (DMPP) as nicotinic agonist because of its poor liposolubility, which allows application of brief pulses with little desensitization. In intact cells, stimulation with brief pulses of DMPP caused rapid and reversible acidification of the vesicles labeled with synaptobrevin-EGFP. These changes were not related to mechanical stimulation of the cell membrane or to movements of the cell produced by the pulse, because a brief puff with saline had no effect on the fluorescence (Fig. 4a).

To confirm that the mediator involved in these responses was Ca^{2+}, we applied pulses of 10 μM Ca^{2+} to cells

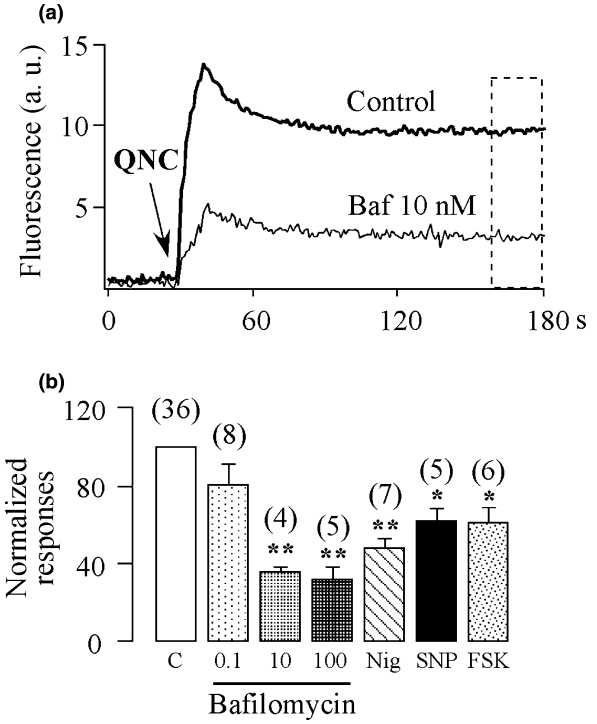


Fig. 2 Effects of several drugs on the time course of the accumulation of quinacrine (QNC) in chromaffin cells. A QNC pulse of 10 μM was applied for 10 s by pressure ejection in the vicinity of the cell, as indicated by the arrow. Panel (a) shows typical fluorescence intensity traces illustrating the effect of 10 nM bafilomycin A1 on quinacrine accumulation. Panel (b) shows the normalized to the control responses obtained during the last 20 s of records (dashed rectangle in panel a). Data (means ± SEM) are normalized to control cells from the same batch. Numbers in brackets indicate the number of cells employed. C, saline; bafilomycin (0.1–100 nM); Nig, nigericin (50 nM); SNP, sodium nitroprusside (10 μM). All drugs were present 10 min before and during the recording, except nigericine, which was washed out before QNC application. $^{*}p < 0.05$; $^{**}p < 0.01$, Student's *t*-test.

permeabilized with digitonin (Fig. 4b). Application of Ca^{2+} provoked a rapid acidification of chromaffin granules, whereas application of a control solution lacking Ca^{2+} had no effect on the vesicular pH. This indicated that the observed changes in fluorescence were not caused by cell displacement. Note that pH changes were quite modest, perhaps because the recorded area was the whole cell whereas the acidification occurred only on the granules close to the membrane, given the poor diffusion of Ca^{2+} through the cytosol. These experiments pointed to a rise in intracellular Ca^{2+} as the signal that caused the acidification observed upon nicotinic stimulation.

Bafilomycin A1 decelerates exocytosis and reduces the quantal size of single amperometric event levels

Acute incubation of cells with the vesicular H^+-pump inhibitor bafilomycin A1 resulted in a drastic deceleration of exocytosis and a reduction of vesicular content (Fig. 5). The effects of bafilomycin on the kinetics of exocytosis resembled those observed after a mild stimulation of PKG or PKA (Machado *et al*. 2000, 2001; summarized in Table 1), suggesting that the mechanism of action of these kinases might also be mediated by changes in vesicular pH. To test this hypothesis, we performed experiments monitoring the kinetics of exocytosis on cells treated with other drugs known to alter the intravesicular pH (Table 2).

The accumulation of QNC or AO is concentration-dependent and can be up to two orders of magnitude higher than their extracellular concentrations, thus affecting the mechanisms of vesicular packaging. Normalized data resulting from amperometric recordings of chromaffin cells treated with AO and QNC are shown on Table 2. The data indicate that the kinetics of exocytosis, as described by I_{max}, $t_{1/2}$ and *m*, were more sensitive to pH variations than the catecholamine content (*Q*). Note, however, that large concentrations of dyes or longer application times were also capable to produce changes in the quantal size of secretory spike.

The vesicular pH and the transmembrane potential can be manipulated using ionophores. Table 2 also shows the effects of nigericin, an ionophore that dissipates the pH gradient, on the kinetics of exocytosis and the quantal content of catecholamines. Nigericin (500 nM) was applied for 10 min prior and during the record; no changes were observed with 50 nM (not shown). The main effect observed was a decrease in the net spike charge, which manifests in smaller secretory spikes.

Conversely, incubation with valinomycin, a K^+ ionophore that impairs the potential gradient, led to acidification of the chromaffin granules and increases their catecholamine content. In order to examine the role of Cl^- as counter ion of H^+, two chloride channel blockers were used: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). The former is a potent blocker but permeates poorly through plasma membrane, producing little effects in exocytosis. However, NPPB caused a deceleration in exocytosis that was observed by an increase in $t_{1/2}$ and a decrease in I_{max} (Table 2). These data support the idea that interfering with Cl^- entry into the granules impairs their acidification Both Cl^- blockers tend to slow exocytosis, although the effects of DIDS are only evident on *m* whereas NPPB affects $t_{1/2}$ and I_{max}. These different effects of both Cl^- blockers could be explained perhaps because of the limited specificity of these drugs.

Alkalinization of secretory vesicles causes rapid displacement of catecholamines into the cytosol

In order to test whether the reduction observed in vesicular catecholamine content was due to its displacement to the cytosol, we carried out experiments in which the cells were incubated with bafilomycin and then permeabilized with digitonin. Cells permeabilized with digitonin released cytosolic soluble substances including catecholamines. This leak

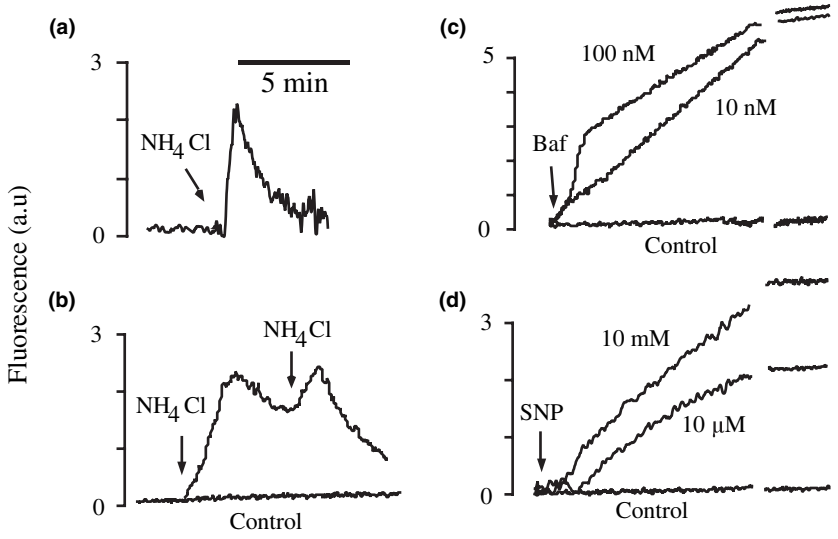


Fig. 3 Dynamic changes in vesicular pH detected by synaptobrevin-EGFP. Typical records obtained from cells expressing synaptobrevin-EGFP, 48 h after infection. Panel (a) shows the transient effect of a 5 s application of 10 mM NH_4Cl. Panel (b) shows that this effect was reproducible upon reapplication of NH_4Cl. Panel (c) shows the effects of 10 and 100 nM of the proton pump inhibitor bafilomycin A1 on the time course of intravesicular pH. Panel (d) shows the effects of two concentrations of sodium nitroprusside (SNP; 10 μM and 10 mM) applied for 5 s. Data are expressed in arbitrary fluorescence units (a.u.) and are typical observations from a minimum of five cells in each group. The gaps in traces from (c) and (d) correspond to 5 min. The time scale shown in (a) is the same for all panels.

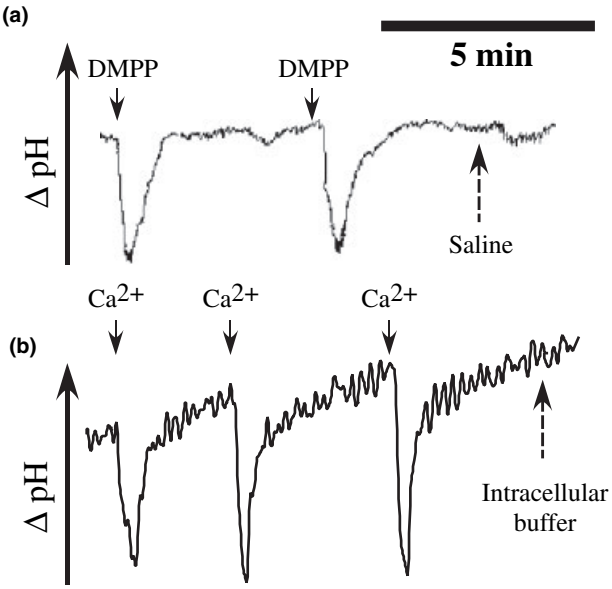


Fig. 4 Intracellular Ca^{2+} regulates the vesicular pH. Typical traces from four to seven different observations obtained from single chromaffin cells expressing synaptobrevin-EGFP. (a) A cell was stimulated with 100 μM of the nicotinic agonist dimethylphenylpiperazinium (DMPP) for 5 s or with normal Krebs solution (saline) as indicated by arrows. (b) Fluorescence traces recorded from a cell that was permeabilized with digitonin (10 μM, 5 min) and bathed in a 0 Ca^{2+} intracellular buffer, then pulses of 10 μM Ca^{2+} or intracellular buffer were applied as indicated by arrows.

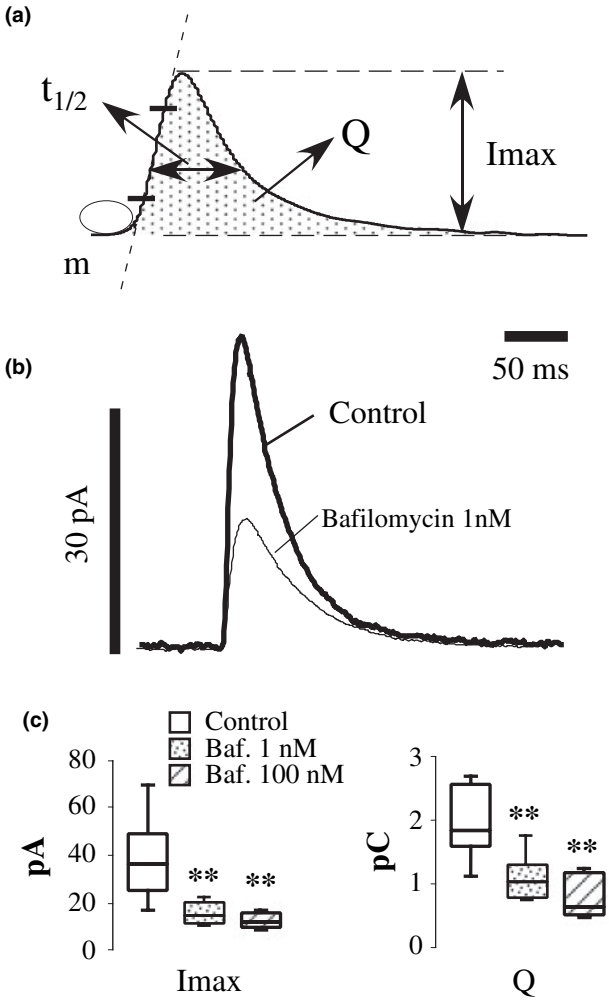
of free catecholamines was significantly augmented in cells treated with bafilomycin (Fig. 6a).

Blowing out the media surrounding the cell with saline evidenced the presence of free catecholamines leaked out from vesicles; this maneuver produced a decrease in the basal amperometric current (Fig. 6b). It is interesting that even in nominal zero Ca^{2+} (no added Ca^{2+} and 200 μM EGTA), some secretory spikes could still be observed even minutes after permeabilization, mostly from cells treated with bafilomycin.

The catecholamines detected originate from the cell studied and not from surrounding cells, as the amperometric signal decreased when the distance of the electrode from the cell membrane was progressively increased. These experiments indicate that the alkalinization caused by bafilomycin displaces catecholamines from the vesicles to the cytosol.

Discussion

Several attempts have previously been made to address a role of the pH gradient of vesicles on the secretory function (Pollard *et al*. 1979). However, the hypothesis that the acidic pH contributes to the fusion process was rejected by data obtained with permeabilized cells (Knight and Baker 1985). Nevertheless, these early studies were carried out by measuring the total secretion of catecholamines in the media surrounding millions of chromaffin cells (Holz *et al*. 1983) rather than on the exocytotic process itself. In the present work, we have re-examined the role of the intravesicular pH



	Imax	Q	$t_{1/2}$	m	n spikes	n cells
Bafilomycin 10 pM	120±20	103±15	95±23	142±27	930	11/3
Bafilomycin 100 pM	36±4**	68±9*	146±21	31±7*	1761	11/10
Bafilomycin I nM	40±4**	58±8*	109±7	28±3**	423	7/9
Bafilomycin 100 nM	32±2**	42±6**	95±10	23±2**	388	7/11

Fig. 5 Bafilomycin A1 slows exocytosis and reduces the quantal size. Cells were treated with bafilomycin at the concentrations indicated, for 10 min. Exocytosis was triggered by 5-s pressure ejection of 5 mM $BaCl_2$ in the vicinity of the cell. Panel (a) shows the kinetics parameters extracted from each secretory spike. Panel (b) illustrates the effects on secretory spikes; these traces were constructed using data from the inserted table. Panel (c) shows the 'box representation' of the effects of 1 and 100 nM of bafilomycin (Baf.) on the maximal amplitude and on the granule content. Boxes represent median ± 25–75% percentile distributions; vertical bars represent percentile 5–95% interval. Inserted table shows the values from the kinetics parameters normalized to their own control group (100%). *n* spikes accounts for the number of spikes; *n* cells indicates the number of cells used control/bafilomycin A1 as indicated in Methods, $*p < 0.05$, $**p < 0.01$, Student's *t*-test.

in the regulation of kinetics of single granule release event and found that it represents a necessary step in the action of several intracellular signals.

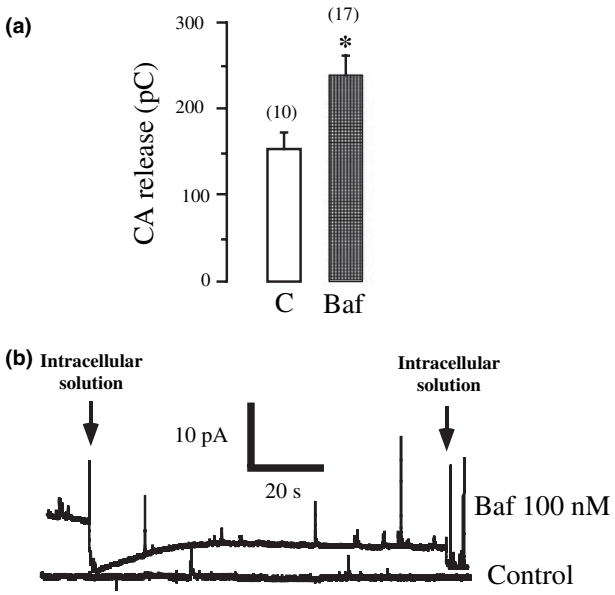


Fig. 6 Alkalinization displaces catecholamines from the vesicular compartment into the cytosol. Cells were bathed in a Ca^{2+}-free medium (5 mM EGTA) and permeabilized by a short (10 s) pulse of 20 μM digitonin in the presence and in the absence of 100 nM bafilomycin A1 (Baf). A carbon fiber electrode was placed onto the cell surface for the continuous recording of secretion. (a) The total catecholamine (CA) released was calculated by integrating amperometric traces, means ± SEM $*p < 0.05$, Student's *t*-test. (b) Five minutes after permeabilization, cells treated with bafilomycin were still leaking catecholamine, as evidenced by a brief puff of Ca^{2+}-free medium to remove accumulated catecholamines surrounding the electrode. Traces are representative from five independent records from both control and treated cells. In these experiments nominal zero Ca^{2+} was used (Ca^{2+} replaced with Mg^{2+} + 200 μM EGTA).

Acridine orange and QNC are fluorescent compounds that are widely used to monitor changes in the pH of secretory vesicles. We cannot explain the different time course of fluorescent signals observed with AO and QNC, although it might be caused by their different uptake and distribution throughout the cell. Even though both dyes accumulate also in lysosomes and early endosomes, most of the fluorescent signal is likely to originate from chromaffin granules, given their large number (17% of total cell volume).

We showed that mild activation of PKA or PKG rapidly promoted a slowing of exocytosis in chromaffin cells (Machado *et al*. 2000, 2001). We also described that strong PKA stimulation forced 'compound fusion' of several granules, thus promoting the increase in the observed *Q* (Machado *et al*. 2001). These data are summarized in Table 1. The cellular mechanisms involved in the regulation of the latest steps of exocytosis remain obscure. Two major targets have been proposed to explain how a second messenger can promote these effects: the fusion machinery (Graham *et al*. 2002; Barclay *et al*. 2003) and intravesicular

factors (Schroeder *et al*. 1994, 1996; Amatore *et al*. 2000). The first hypothesis is supported by studies over expressing proteins like of Munc-18 (Barclay *et al*. 2003) or mutations on SNAP-25 (Gil *et al*. 2002) affected the kinetic of exocytosis. However, other studies on mice lacking Munc18-1 (Voets *et al*. 2001) or SNAP-25 (Sorensen *et al*. 2003) did not report any difference in the kinetics of exocytosis.

At least for NO/PKG, there are experimental data indicating that fusion pore dynamics are not involved in the regulation of exocytotic kinetics. NO was able to modulate the kinetics of exocytosis even in prefused vesicles where secretion was 'frozen' under hypertonic solutions (> 750 mOsm; Borges *et al*. 1997). In addition, the duration of the pre-spike phenomenon (foot) did not change in response to NO (Machado *et al*. 2000). Moreover, NO modified the kinetics of exocytosis in rat mast cells without affecting the fusion pore kinetics recorded simultaneously by cell capacitance measurements (Fernández-Peruchena *et al*. 2003).

Assuming that intravesicular factors are, at least partially, regulating the kinetics of exocytosis it is necessary to identify the target(s) of these second messengers. The finding that the effects of bafilomycin on vesicular pH were accompanied by a deceleration of exocytosis pointed towards the vesicular pH as a probable mediator. Our experiments show that all treatments that reduce the vesicular gradient of pH (NH_4Cl, AO, QNC and low bafilomycin A1) resulted in deceleration of exocytosis (Table 2 and Fig. 5). We found that alkalinization also reduced the quantal size. Conversely, treatments that decelerated exocytosis also caused a rapid elevation in the intravesicular pH (Figs 1 and 2), the only exception was nigericin, see below. Alkalinization affected the whole population of vesicles released (Fig. S2, supplementary material), which was different from that found with the antihypertensive agent hydralazine that only affected the larger vesicles (Machado *et al*. 2002).

The vesicular acidification seems to accelerate exocytosis, thus reaching higher concentrations of the neurotransmitter at the postsynaptic element. Calcium is a candidate as a mediator in the acidification mediated by nicotinic stimulation, as shown in Fig. 4. These results are in agreement with those found by Sulzer's group (Pothos *et al*. 2002) who demonstrated a Ca^{2+}-dependent acidification of secretory vesicles prior to exocytosis. In the present study we confirm their data concerning the effects of NPPB in vesicular pH. However, other authors have reported that both Ca^{2+} and Ba^{2+} cause alkalinization (Han *et al*. 1999; Williams and Webb 2000).

We have manipulated the vesicular pH and the transmembrane potential with cation ionophores (Cidon and Sihra 1989). Although these tools are not very specific due to their actions on almost every membrane of cell and organelles, the results obtained also support the role of pH on the regulation of the kinetics and quantal size of exocytosis (Table 2). When the pH gradient was reduced with nigericin, we observed a slow down of exocytosis and a lost of quantal catecholamine content. However, the effects of nigericin were more complex, because it affected Q, I_{max} and $t_{1/2}$ but did not change m. We do not have an explanation but it can be caused by distortion of other transmembrane gradients such as K^+ caused by this ionophore.

Conversely, a reduction in the ψ gradient will result in an increase in the V-ATPase efficiency thus reducing the pH. Indeed, incubation with the K^+ ionophore valinomycin produced larger spikes. Note that the effect of both ionophores on $t_{1/2}$, a parameter that describes the speed of exocytotic events, was associated with an increase in net charge. Normally, net charge (Q) and spike height (I_{max}) are closely related parameters. The reason for this behavior is not easy to explain and probably was due to interactions of the cations carried by the ionophore with the intravesicular matrix.

We have no explanation that connects activation of a second messenger route with changes in the vesicular pH. However, the V-ATPase is a plausible candidate as the target for kinases to promote changes in vesicular pH. This proton pump is a complex protein that contains two subunits: the V_1 subunit where ATP hydrolysis takes place and the V_0 subunit where the H^+ carrier is localized (Futai *et al*. 2000). The former possesses several loci suitable for phosphorylation/dephosphorylation, whereas the latter is selectively blocked by bafilomycin A1 (Hicks and Parsons 1992). Nitric oxide is a potent inhibitor of V-ATPase in peritoneal macrophages (Swallow *et al*. 1991) and in the cortical collecting duct (Tojo *et al*. 1994). Conversely, a recent study describes the negative modulation of V-ATPase activity by PKA in rat osteoclasts (Kajiya *et al*. 2003). It would be plausible, therefore, that the second messenger routes involved in the modulation of the kinetics of exocytosis are acting on vesicular pH through the V_1 subunit. Other possible targets for these second messenger routes would be the vesicular Cl^- or K^+ channels, even it could affect the cytosolic trafficking of V_0 subunit.

The gradient of pH is the main driving force that maintains catecholamine accumulation into secretory vesicles. When the pH gradient is collapsed, a loss in quantal catecholamine content is observed (Fig. 5), probably because catecholamines leak out from the vesicles towards the cytosol, as suggest the experiments from Fig. 6. The presence of spikes in these experiments suggests that the Ca^{2+} released from vesicles or other intracellular organelles could induce exocytosis.

The deceleration of exocytosis seems to be a consequence of vesicle alkalinization (Fig. 5). This effect is also observed at the neuromuscular junction, where bafilomycin promotes the reduction of the quantal size and the inhibition of synaptic transmission (Hong 2001). Weak bases, such as tyramine (Mundorf *et al*. 1999), amphetamine (Sulzer and Rayport 1990) or hydralazine (Machado *et al*. 2002), also displace catecholamines. These drugs

also disturb the kinetics of exocytosis in adrenal chromaffin cells.

It is not easy to quantify the number of protons necessary to produce large pH changes. For a chromaffin granule (diameter, 200 nm; volume ≈ 4 aL) only 8 H^+ can account for 2 pH units (7.5–5.5) in an ideal non-buffered media, although it is likely that the presence of potent buffers will modify these calculations. Acidification is a process that occurs with a subsecond time course as observed immediately after endocytosis (Machado *et al*., unpublished results). Moreover, the high concentration of reticular solutes causes them to be almost in a solid state. In addition, the luminal matrix is expected to greatly reduce the available free space for H^+ dissolution. Therefore, small changes in the V-ATPase activity could account for notable changes in pH.

Changes in pH also promote conformational modifications in chromogranin A. As mentioned in the Introduction, chromogranins seem to play a crucial role in the complexation of catecholamines by a Donnan equilibrium that will be altered by alkalinization of the intravesicular media (Helle *et al*. 1985). Chromogranin A is in a monomer–tetramer equilibrium at pH 5.5 whereas it is in a monomer–dimer equilibrium at pH 7.5 (Yoo and Lewis 1992).

We have observed that PKA and PKG alkalinize, whereas a rise in intracellular Ca^{2+} acidified vesicles. These changes were accompanied by changes in the kinetics of single granule release event. Further investigations will be required in order to determine if the effect of granular pH could be a general and fine-tuned mechanism for the regulation of synaptic transmission. To our knowledge, this is the first report that links the regulation of intravesicular pH with the control of the kinetics of exocytosis by intracellular signals.

Acknowledgements

We thank the personnel from 'Matadero Insular de Tenerife' for providing us with bovine adrenals. Discussions with Drs De Potter (University of Antwerp), Barg (Vollum Institute) and Álvarez de la Rosa (University of La Laguna) are greatly appreciated. MC and MSM are recipients of a fellowship from the Spanish Ministerio de Ciencia y Tecnología. This work is supported in part by a grant from Spanish Ministerio deCiencia y Tecnología to RB (DGCYT BFI2001-3531 and Gobierno de Canarias) and Spanish Ministerio de Educación (PM98-0097/PM98-0104) and Generalitat Valenciana (Ctidib/2002/138 and GRUPOS03/038) to MC.

Supplementary Material

The following supplementary material is available for this article online.

Figure S1. Dependence of synaptobrevin-EGFP flourescent due to on pH.

Figure S2. Bafilomycin affects the Imax regardless of their quantal size.

This material is available as part of the online article from http://www.blackwell-synergy.com

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