

SYNAPTIC MECHANISMS

Calcium dynamics in bovine adrenal medulla chromaffin cell secretory granules

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Abstract

The secretory granules constitute one of the less well-known compartments in terms of Ca^{2+} dynamics. They contain large amounts of total Ca^{2+} , but the free intragranular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{\text{SG}}$), the mechanisms for Ca^{2+} uptake and release from the granules and their physiological significance regarding exocytosis are still matters of debate. We used in the present work an aequorin chimera targeted to the granules to investigate $[\text{Ca}^{2+}]_{\text{SG}}$ homeostasis in bovine adrenal chromaffin cells. We found that most of the intracellular aequorin chimera is present in a compartment with 50–100 μM Ca^{2+} . Ca^{2+} accumulation into this compartment takes place mainly through an ATP-dependent mechanism, namely, a thapsigargin-sensitive Ca^{2+} -ATPase. In addition, fast Ca^{2+} release was observed in permeabilized cells after addition of inositol 1,4,5-trisphosphate (InsP_3) or caffeine, suggesting the presence of InsP_3 and ryanodine receptors in the vesicular membrane. Stimulation of intact cells with the InsP_3 -producing agonist histamine or with caffeine also induced Ca^{2+} release from the vesicles, whereas acetylcholine or high- $[\text{K}^+]$ depolarization induced biphasic changes in vesicular $[\text{Ca}^{2+}]$, suggesting heterogeneous responses of different vesicle populations, some of them releasing and some taking up Ca^{2+} during stimulation. In conclusion, our data show that chromaffin cell secretory granules have the machinery required for rapid uptake and release of Ca^{2+} , and this strongly supports the hypothesis that granular Ca^{2+} may contribute to its own secretion.

Introduction

The increase in intracellular $[\text{Ca}^{2+}]$ triggers exocytosis of secretory vesicles in neuronal and neuroendocrine cells. The Ca^{2+} required for exocytosis usually enters from the extracellular medium through several kinds of plasma membrane Ca^{2+} channels (Garcia *et al.*, 2006). In addition, exocytosis may also be induced by the release of Ca^{2+} from intracellular Ca^{2+} stores, mainly the endoplasmic reticulum (ER), through either inositol trisphosphate (InsP_3) receptors or ryanodine receptors (RyRs). In fact, Ca^{2+} entry may activate Ca^{2+} release from the ER through RyRs, thus potentiating exocytosis. This phenomenon, known as Ca^{2+} -induced Ca^{2+} release, has been shown to be operative in chromaffin cells (Alonso *et al.*, 1999). However, it is hardly mentioned that the main Ca^{2+} store in some excitable cells resides in the secretory vesicles. In chromaffin cells, secretory granules contain about 60% of the total cellular Ca^{2+} (Haigh *et al.*, 1989), and the total Ca^{2+} concentration inside the granules has been estimated to be around 40 mM (Winkler & Westhead, 1980; Hutton, 1989). The reason for

this apparent oversight is that whereas the importance of Ca^{2+} dynamics in organelles such as the ER or mitochondria is now fully established (Rizzuto *et al.*, 2004), the physiological significance of the Ca^{2+} stored in secretory granules is still obscure.

An attractive possibility would be that intragranular Ca^{2+} could contribute to its own secretion by being released through Ca^{2+} channels situated in the granule membrane. In fact, it has been claimed that InsP_3 may release Ca^{2+} from chromaffin granules (Yoo & Albanesi, 1991), and the same authors recently described the presence of InsP_3 receptors in the secretory granule membrane (Huh *et al.*, 2005). Similarly, the presence of InsP_3 receptors has been reported in mast cell secretory granules (Quesada *et al.*, 2001, 2003) and in pancreatic acinar cell secretory granules (Gerasimenko *et al.*, 1996, 2006). However, results from other authors indicate that in chromaffin cells, InsP_3 receptors are present in the ER but not in the secretory granules (Endo *et al.*, 2006). Moreover, it was shown in PC12 cells that both the agonist-induced and the high- $[\text{K}^+]$ -induced $[\text{Ca}^{2+}]$ increases are identical in the cytosol and in the outer side of the granule membrane, suggesting that there is little Ca^{2+} release from the granules during stimulation (Pouli *et al.*, 1998).

We have investigated here the dynamics of intragranular $[\text{Ca}^{2+}]$ in primary cultures of bovine adrenal medulla chromaffin cells by using an adenoviral vector to express a vesicle-associated membrane protein

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(VAMP)-aequorin chimera targeted to the membrane of the secretory vesicles. This chimera was used for the first time to monitor intragranular $[Ca^{2+}]$ in MIN6 β -cells (Mitchell *et al.*, 2001, 2003), showing secretory granule $[Ca^{2+}]$ ($[Ca^{2+}]_{SG}$) values around 50 μM , and is the same that we have used before to study granule $[Ca^{2+}]$ dynamics in PC12 cells (Moreno *et al.*, 2005). However, we have now used a higher- Ca^{2+} -affinity form of that aequorin chimera, which we have found is much superior for monitoring the free $[Ca^{2+}]_{SG}$, and provides a considerable amount of new information on its dynamics.

Materials and methods

Cell culture and expression of aequorin and enhanced green fluorescent protein (EGFP) chimeras

Bovine adrenal glands were kindly provided by the veterinary of the slaughterhouse Justino Gutiérrez of Laguna de Duero (Valladolid, Spain). The study was approved by the ethical committee of animal experimentation of the Faculty of Medicine of the University of Valladolid. Bovine adrenal medulla chromaffin cells were isolated as described previously (Moro *et al.*, 1990) and plated on 12-mm glass polylysine-coated coverslips (0.25×10^6 cells per 1 mL of Dulbecco's modified Eagle's medium). Cells were cultured in high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 i.u. mL^{-1} penicillin and 50 i.u. mL^{-1} streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 .

The vesicle-associated membrane protein-mutated aequorin (VAMP-mutaeq) chimera has been described previously (Mitchell *et al.*, 2001; Moreno *et al.*, 2005). The VAMP-EGFP construct was made by inserting in frame the VAMP sequence in the *HindIII* site of the pEGFP-N1 plasmid. For construction of adenoviral vectors, full-length cDNA encoding these constructs was subcloned into the pShuttle vector and then used for construction of the corresponding adenoviral vector by using an AdenoX adenovirus construction kit (Clontech).

Chromaffin cells were infected with adenoviruses for expression of the constructs described above. Infection was carried out on the day after cell isolation, and $[Ca^{2+}]$ measurements were performed 48–72 h after infection. Efficiency of infection of chromaffin cells with the adenovirus carrying the VAMP-EGFP chimera was estimated to be about 60%.

$[Ca^{2+}]_{SG}$ measurements with aequorin

Cells were plated onto 12-mm polylysine-covered round coverslips and infected with adenoviruses to express VAMP-mutaeq. For aequorin reconstitution in intact cells (non- Ca^{2+} -depleted), cells expressing VAMP-mutaeq were incubated for 1–2 h at room temperature with either 1 μM native coelenterazine or 1 μM coelenterazine n, in standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). For experiments with Ca^{2+} -depleted cells, cells were treated for 10 min with the ionophores ionomycin (10 μM) and monensin (10 μM) and the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor *t*-butyl benzohydroquinone (BHQ, 10 μM) in Ca^{2+} -free standard medium (containing 0.5 mM EGTA instead of Ca^{2+}). Then, cells were incubated for 1–2 h in the same Ca^{2+} -free extracellular medium without the ionophores, but containing BHQ and 1 μM native coelenterazine. Cells were then placed in the perfusion chamber of a purpose-built thermostated luminometer. The temperature was 37°C for the experiments using coelenterazine n (Fig. 2) and 22°C for all the other experiments, performed using native coelenterazine. We have

shown before that this temperature change produces a small shift in the Ca^{2+} affinity of the aequorin, allowing monitoring of relatively high $[Ca^{2+}]$ values for a longer time (Barrero *et al.*, 1997). For experiments with permeabilized cells, cells expressing VAMP-mutaeq were reconstituted with coelenterazine for 1–2 h and then placed in the luminometer as indicated above in standard medium containing 0.5 mM EGTA instead of Ca^{2+} . Then, a 5-min pulse with 500 μM lanthanum was given, followed again by standard medium containing 0.5 mM EGTA for 3 min, prior to permeabilization for 1 min with 20 μM digitonin in intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM K_3PO_4 , 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 2 mM succinate, 20 mM HEPES, pH 7). Then, intracellular medium without digitonin and containing 100 nM Ca^{2+} (prepared with an EGTA/ Ca^{2+} mixture) was finally perfused to refill the vesicular Ca^{2+} store. Calibration of the luminescence data into $[Ca^{2+}]$ was performed using an algorithm as previously described (Alvarez & Montero, 2002). Calibration requires release of all the aequorin luminescence present in the cells at the end of every experiment. In intact cells, this is done by perfusing a water solution containing 20 μM digitonin and 10 mM Ca^{2+} . In previously permeabilized cells, a 10 mM Ca^{2+} solution is perfused at the end of the experiments.

Calibration of mutated aequorin at pH 7 and pH 5.5

Comparison of the luminescence obtained from mutated aequorin at either pH 7 or 5.5 was performed using HeLa cells expressing mutated aequorin targeted to the ER. HeLa cells were cultured and transfected with the ER-targeted mutated aequorin construct as described previously (Montero *et al.*, 1997a). The ER was irreversibly depleted of Ca^{2+} by incubation with 1 μM thapsigargin during reconstitution with 1 μM native coelenterazine. Cells were also incubated during reconstitution with 5 μM oligomycin, to avoid mitochondrial ATP production. Then, cells were placed in the luminometer, permeabilized as described above (except for the use of a higher digitonin concentration, 100 μM), and perfused with intracellular medium containing no ATP and no metabolic substrates, the ionophores ionomycin (1 μM) and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (2 μM), and the SERCA pump inhibitor BHQ. In this way, the ER becomes unable to accumulate Ca^{2+} and behaves simply as an aequorin container, freely permeable to Ca^{2+} and H^+ , and ready for the performance of a calibration in the desired conditions. Cells were then perfused under these conditions with different Ca^{2+} concentrations, from 5 to 200 μM , and using intracellular medium at either pH 7 (buffered with HEPES) or pH 5.5 (buffered with citrate).

Secretory granule pH measurements with VAMP-EGFP

Cells were plated onto 12-mm polylysine-covered round coverslips and infected with adenoviruses to express VAMP-EGFP. Forty-eight hours after infection, cells were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single-cell fluorescence was excited at 480 nm using a Cairn monochromator (200 ms excitation every 2 s, 10-nm bandwidth), and images of the emitted fluorescence obtained with a 40 \times Fluor objective were collected using a 495DCLP dichroic mirror and an E515LPV2 emission filter (both from Chroma Technology) and recorded with a Hamamatsu ORCA-ER camera. Single-cell fluorescence records were analyzed using the METAFLUOR program (Universal Imaging Corporation, Downingtown, PA, USA). Experiments were performed at 22°C.

Confocal measurements

Chromaffin cells expressing VAMP-EGFP were imaged on a Leica TCS SP2 confocal spectrophotometer using a 63× oil immersion objective. EGFP was excited with the 488-nm line of the argon laser, and the fluorescence emitted between 500 and 600 nm was collected. Images were then deconvoluted using HUYGENS software (Scientific Volume Imaging, Hilversum, The Netherlands).

Materials

Wild-type coelenterazine and coelenterazine n were obtained from Molecular Probes (OR, USA). Bafilomycin A was from Tocris (Bristol, UK). InsP₃, cADP-ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and FCCP were from Sigma (Madrid, Spain). Other reagents were from Sigma or Merck (Darmstadt, Germany). Statistical data are always given in mean ± SEM, and the number is indicated in each case. The ANOVA test was used to study the significance of the differences. To estimate the differences, the pre-stimulus [Ca²⁺]_{SG} was measured immediately before the addition of the stimulus.

Results

Subcellular distribution of VAMP-mutaeq in chromaffin cells

The subcellular distribution of VAMP-mutaeq in chromaffin cells was explored in two ways. First, we prepared an adenovirus able to express a construct containing EGFP instead of aequorin attached to VAMP. When we infected chromaffin cells with that virus and obtained confocal fluorescence images of cells expressing VAMP-EGFP, the

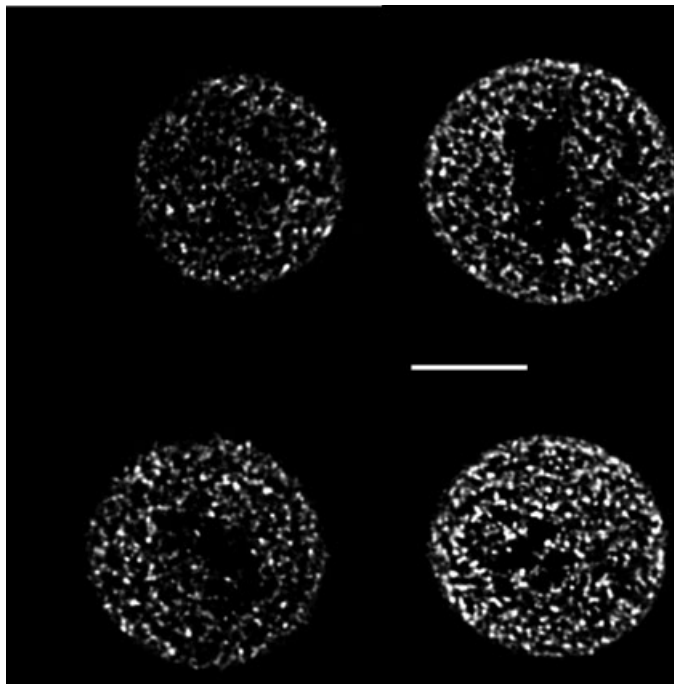


FIG. 1. Confocal fluorescence images of vesicle-associated membrane protein (VAMP)-enhanced green fluorescent protein (EGFP) in bovine chromaffin cells. Chromaffin cells were infected with adenovirus carrying the VAMP-EGFP construct, and confocal microscopy was performed 48 h later, as described in Materials and methods. The figure shows fluorescence images with a typical granular pattern obtained in four different confocal planes of a single labeled cell. Scale bar: 5 μm.

resulting images clearly showed a granular distribution (Fig. 1). This is consistent with previous results showing that this chimera colocalizes almost exclusively with secretory granules in chromaffin cells (Allersma *et al.*, 2004).

Both the images shown in Fig. 1 and previous results provide evidence that VAMP-EGFP, and thus probably also VAMP-mutaeq, is well targeted to the chromaffin granules. In addition, the good specificity of targeting of the VAMP-mutaeq chimera to secretory vesicles has been previously shown by immunofluorescence and immunoelectron microscopy in insulin-containing secretory vesicles of MIN6 β-cells (Mitchell *et al.*, 2001), where less than 3% of the probe was shown to be in the ER or Golgi compartments. On the other hand, functional evidence for the targeting of this aequorin chimera to the granules can also be provided by using the properties of aequorin luminescence, in particular the irreversible light emission from aequorin induced by Ca²⁺, the so-called aequorin consumption.

First, we found that reconstitution of intact chromaffin cells expressing VAMP-mutaeq with native coelenterazine produced hardly any functional photoprotein (data not shown). In contrast, when we used coelenterazine n to reconstitute mutaeq, we obtained exponentially decreasing records of aequorin luminescence like that shown in Fig. 2a. These results show that mutaeq is present in compartments with [Ca²⁺]_{SG} high enough both to preclude reconstitution with native coelenterazine and to produce a fast initial rate of consumption when reconstituted with coelenterazine n. The kind of record shown in Fig. 2a was very similar to those found before in PC12 cells (Moreno *et al.*, 2005). When this luminescence record was transformed into

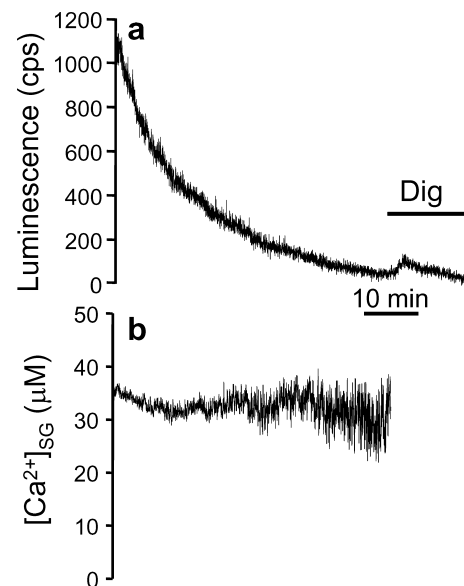


FIG. 2. Steady-state [Ca²⁺]_{SG} measured with low-Ca²⁺-affinity vesicle-associated membrane protein (VAMP)-mutated aequorin chimera (VAMP-mutaeq) in non-Ca²⁺-depleted cells. Bovine chromaffin cells expressing VAMP-mutaeq were reconstituted with coelenterazine n (see Materials and methods), and luminescence was then monitored under continuous perfusion with external medium containing 1 mM Ca²⁺ (cps, counts per second). (a) Crude luminescence record, which decreases exponentially due to aequorin consumption at the high intragranular [Ca²⁺]_{SG}. When indicated, standard medium containing 20 μM digitonin and 10 mM Ca²⁺ (Dig) was perfused, showing that little residual luminescence was left. (b) The calibrated [Ca²⁺]_{SG} values obtained from the same experiment, showing a stable steady-state [Ca²⁺]_{SG} around 35 μM. This experiment was performed at 37°C, and is representative of 45 similar ones.

[Ca²⁺] values (Fig. 2b), we obtained a steady-state [Ca²⁺] of $30 \pm 1 \mu\text{M}$ (mean \pm SEM, $n = 45$). Addition of digitonin and high [Ca²⁺] at the end of the experiment produced only a very small release of luminescence, showing that nearly all of the reconstituted aequorin molecules had already been consumed. This confirms the absence of significant amounts of reconstituted aequorin in lower-[Ca²⁺] compartments.

The problem of the resting [Ca²⁺] in the secretory granules

The conclusion from these experiments is that functional VAMP-mutaeq is present mainly in one compartment with a homogeneous [Ca²⁺] around $30 \mu\text{M}$. However, this [Ca²⁺] value is in the lower limit of the [Ca²⁺] range detected by this aequorin chimera (mutaeq reconstituted with coelenterazine n), and thus it should have very little sensitivity to [Ca²⁺] changes, particularly to Ca²⁺ release from the granules. In fact, we have used this probe before to monitor [Ca²⁺] in the ER, which is around $500 \mu\text{M}$ (Montero *et al.*, 1997a; Alonso *et al.*, 1999). Thus, a probe with higher Ca²⁺ sensitivity would be better for studying the dynamics of [Ca²⁺]_{SG}. However, as we have mentioned above, the resting [Ca²⁺]_{SG} did not allow reconstitution when an aequorin with higher Ca²⁺ affinity, such as mutaeq reconstituted with native coelenterazine, was used. Then, we decided to deplete the granules of Ca²⁺ prior to reconstitution, using the ionophores monensin and ionomycin (see Materials and methods). Cells were then reconstituted with native coelenterazine in the absence of extracellular Ca²⁺, and readdition of Ca²⁺ triggered a large luminescence peak (Fig. 3a). When calibrated into [Ca²⁺] values (Fig. 3b), the peak rose to above $100 \mu\text{M}$. Then, after the peak, [Ca²⁺] dropped rapidly and showed a prolonged shoulder starting at about $30 \mu\text{M}$ [Ca²⁺].

The presence of both a peak and a shoulder suggests that aequorin is situated in at least two different compartments, one of them reaching a much higher [Ca²⁺] than the other. Among the possible compartments where VAMP-mutaeq could be, it is quite obvious that some VAMP-mutaeq could have reached the plasma membrane following the secretory pathway. In that case, fusion of the vesicles with the plasma membrane would lead to exposure of the VAMP-linked aequorin to the extracellular medium, so that it would be rapidly burnt after the addition of extracellular Ca²⁺. In fact, the confocal images of VAMP-EGFP always show a certain amount of the fluorescence in the plasma membrane. In intact (non-Ca²⁺-depleted) cells, those VAMP-mutaeq molecules could never be reconstituted, because of the high extracellular [Ca²⁺]. Instead, in Ca²⁺-depleted cells, reconstitution is possible, as the extracellular medium is devoid of Ca²⁺. To distinguish between intracellular VAMP-mutaeq and that present in the plasma membrane, we gave a brief pulse containing lanthanum in order to trigger specifically the luminescence from aequorin present on the extracellular side of the plasma membrane (Shimomura & Johnson, 1973). Lanthanum is known to be unable to enter chromaffin cells (Marley *et al.*, 2000), and its effects on plasma membrane channels or transporters should be rapidly reversible after washout with EGTA-containing medium. The experiment shown in Fig. 3c demonstrates that perfusion of lanthanum generated, in fact, a peak of luminescence. Then, once lanthanum had consumed all the plasma membrane VAMP-mutaeq luminescence, it was washed out for 5 min and the subsequent addition of Ca²⁺ triggered a much smaller peak of luminescence, corresponding only to intracellular aequorin. After translation into [Ca²⁺] values (Fig. 3d), we obtained a peak of around $40 \mu\text{M}$ ($41 \pm 2 \mu\text{M}$, $n = 80$), followed by a prolonged and slowly decreasing shoulder with [Ca²⁺] values of $17.2 \pm 0.5 \mu\text{M}$ ($n = 80$) 5 min after the peak and $14.8 \pm 0.4 \mu\text{M}$ ($n = 80$) 10 min after the peak.

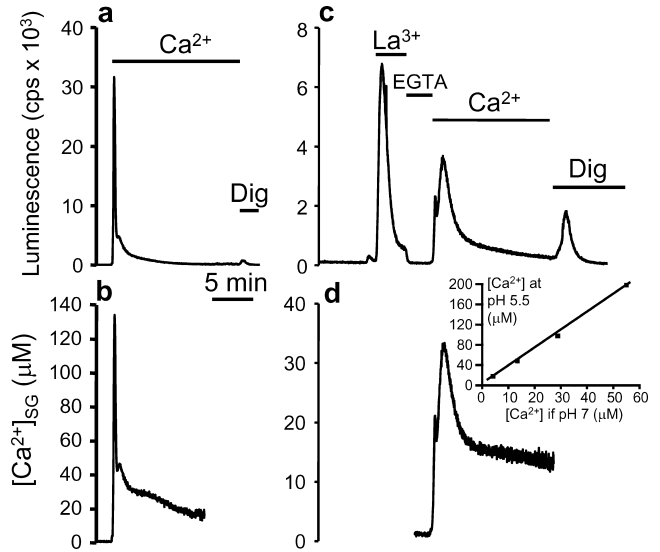


FIG. 3. Steady-state [Ca²⁺] in chromaffin secretory granules ([Ca²⁺]_{SG}) measured with high-Ca²⁺-affinity vesicle-associated membrane protein (VAMP)-mutated aequorin chimera (VAMP-mutaeq) in Ca²⁺-depleted cells. Bovine chromaffin cells expressing VAMP-mutaeq were depleted of Ca²⁺ before reconstitution with native coelenterazine, as described in Materials and methods. The upper panels show the luminescence records (cps, counts per second), and the lower panels the calibrated values. The luminescence was initially monitored under continuous perfusion of Ca²⁺-free (containing 0.5 mM EGTA) medium. Then, standard medium containing 1 mM Ca²⁺ was perfused as indicated (a), triggering a large peak of luminescence that consumed most of the available aequorin [later addition of digitonin and 10 mM Ca²⁺ (Dig) released hardly any additional luminescence]. Calibrated values of this experiment show a large peak followed by a shoulder (b). (c and d) A similar experiment, except for the 500 μM lanthanum pulse given prior to Ca²⁺ addition to release the luminescence of those VAMP-mutaeq molecules present on the outer side of the plasma membrane. After that, the peak induced by Ca²⁺ addition corresponded only to intracellular aequorin and was much smaller. These experiments were performed at 22°C, and are representative of five to nine similar ones of each kind (the statistical data shown in the text include experiments with different stimulation protocols in the steady-state). The inset shows a comparison of the calibrations of mutated aequorin at pH 7 and 5.5. The figure shows the correlations obtained between the calculated [Ca²⁺] values (with the pH 7 calibration, horizontal axis) and the actual values if aequorin was in a medium at pH 5.5 (vertical axis). There is a clearly linear correlation that represents a 3- to 4-fold increase in the [Ca²⁺] values required to produce the same luminescence levels when the pH shifts from 7 to 5.5. Each point was calculated using four to 10 experimental sets of data obtained at both pH levels as described in Materials and methods.

In accordance with the confocal microscope images and with data of other authors on the subcellular localization of VAMP chimeras (Mitchell *et al.*, 2001; Allersma *et al.*, 2004), most of the luminescence generating the [Ca²⁺] peak shown in Fig. 3d must come from the vesicular space. Moreover, other organelles in the secretory pathway, such as the ER or Golgi apparatus, reach much higher [Ca²⁺] values (Barrero *et al.*, 1997; Montero *et al.*, 1997a; Pinton *et al.*, 1998; Alonso *et al.*, 1999) and should produce much faster aequorin consumption. Therefore, these measures show that [Ca²⁺] in the vesicular space ranges between 15 and $40 \mu\text{M}$. The transient [Ca²⁺] peak that precedes the steady state in the experiment shown in Fig. 3d may reflect a gradient of [Ca²⁺] between different vesicles, but may also be just due to an overshoot of the granule Ca²⁺-accumulating system during refilling. Regarding the amount of VAMP-mutaeq present in the plasma membrane, the luminescence induced by lanthanum was $31 \pm 1\%$ ($n = 80$) of the total luminescence. However, we have observed that for the same cell preparation, this percentage

was always reduced to 10–20% when the duration of reconstitution with coelenterazine was longer (2–3 h), suggesting that plasma membrane VAMP-mutaeq is reconstituted faster than internal VAMP-mutaeq. Thus, 10–20% may be a more reasonable estimation for the amount of plasma membrane VAMP-mutaeq.

The above [Ca²⁺]_{SG} measured values may be an underestimation of the real ones, if we take into account the influence of vesicular pH in aequorin luminescence. Vesicular pH is thought to be around 5.5 in chromaffin cells (Winkler & Westhead, 1980; Wu *et al.*, 2001), and the luminescence of native aequorin is known to decrease when the pH is reduced (Moisescu & Ashley, 1977). We have compared here the calibrations of mutated aequorin reconstituted with native coelenterazine at both pH 7 and 5.5. The inset of Fig. 3 shows that decreasing the pH from 7 to 5.5 represents, in terms of calibration, a nearly lineal transformation of the [Ca²⁺] values multiplied by a factor between 3 and 4. Thus, if vesicular pH was 5.5, the real free [Ca²⁺]_{SG} would be 3- to 4-fold higher, about 50–100 μM. In addition, possible changes in vesicular pH induced by the stimuli or during the refilling period could interfere with [Ca²⁺]_{SG} measurements. To estimate the importance of this interference, we used the fluorescence of the VAMP–EGFP chimera as an intragranular pH probe, as has been described before (Camacho *et al.*, 2006). The single-cell traces of Fig. 4a show that no significant changes in VAMP–EGFP fluorescence were observed after addition of Ca²⁺ to Ca²⁺-depleted chromaffin cells or after the subsequent stimulation with high-[K⁺] depolarization. In contrast, direct alkalization of the granules with ammonium chloride (or FCCP, data not shown) produced a fast increase in fluorescence. Figure 4b shows single-cell VAMP–EGFP fluorescence data obtained in permeabilized Ca²⁺-depleted chromaffin cells. In that case, addition of an intracellular buffer containing 100 nM Ca²⁺, a procedure that rapidly increases intragranular [Ca²⁺] (see below), produced a transient increase in VAMP–EGFP fluorescence, suggesting vesicular alkalization, but that change was very small as compared with the large increase in fluorescence induced by ammonium chloride. Subsequent addition of InsP₃ produced no effects on intragranular pH. In conclusion, intragranular pH changes little during refilling with Ca²⁺ of the secretory granules or during cell stimulation. Thus, the observed changes in aequorin luminescence under these conditions can be confidently attributed to changes in intragranular [Ca²⁺]. Regarding the absolute values of [Ca²⁺]_{SG}, in this work we used the aequorin calibration at pH 7 throughout, so the [Ca²⁺]_{SG} values shown may be an underestimation of up to 3- to 4-fold, as described above, depending on the actual granule pH.

Dynamics of [Ca²⁺]_{SG} in intact chromaffin cells

The prolonged steady-state [Ca²⁺] values obtained under the conditions of the experiment shown in Fig. 3d are ideal for exploring the effects of different stimuli on the vesicular [Ca²⁺] dynamics. Moreover, if a small part of the aequorin probe was in earlier compartments of the secretory pathway, such as the Golgi apparatus, it should be fully consumed by that time because of the higher [Ca²⁺] of those compartments. Therefore, the rest of the experiments in this article were performed using this experimental approach. First, we investigated the mechanism of Ca²⁺ uptake and accumulation by the vesicles. Figure 5a–c shows experiments designed to test for the presence of thapsigargin-sensitive Ca²⁺ pumps in this compartment. In the experiments shown in Fig. 5a, we first consumed with lanthanum all the luminescence coming from plasma membrane VAMP-mutaeq,

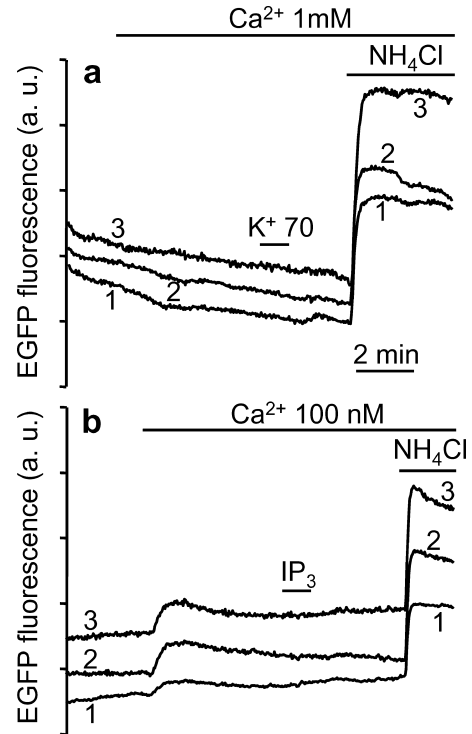


FIG. 4. Effect of Ca²⁺ refilling and several stimuli on intragranular pH measured with a vesicle-associated membrane protein (VAMP)-enhanced green fluorescent protein (EGFP) chimera. Bovine chromaffin cells expressing VAMP–EGFP were depleted of Ca²⁺, and then single-cell fluorescence was monitored as described in Materials and methods. (a) 1 mM Ca²⁺ was added when indicated to the extracellular medium to refill intracellular stores. Then, cells were stimulated with high-[K⁺] (70 mM) depolarization as indicated. None of these additions produced changes in the vesicular pH. Finally, 10 mM ammonium chloride was added to induce a maximum pH increase. (b) Ca²⁺-depleted cells were first permeabilized as described in Materials and methods (not shown). Then, addition of an intracellular buffer containing 100 nM Ca²⁺ produced a small increase in vesicular pH, but much smaller than that induced by 10 mM ammonium chloride. Addition of 2 μM inositol 1,4,5-trisphosphate (IP₃) had no effect. In each of the panels, three representative single-cell fluorescence traces corresponding to cells from the same microscope field (labeled 1, 2 and 3) are shown. These experiments are representative of nine similar ones of each kind.

and then added Ca²⁺ to refill the intracellular compartment, both in control cells and in cells treated for 5 min with 1 μM thapsigargin before the experiment. This treatment, which irreversibly blocks SERCA pumps, largely also blocked refilling of the vesicular compartment (initial peak of 9.6 ± 0.8 μM, *n* = 11, and [Ca²⁺]_{SG} value 10 min later of only 3.2 ± 0.4 μM, *n* = 11, *P* < 0.001 for the comparisons with the control values above, both at the peak and 10 min later), suggesting that VAMP-containing granules accumulate Ca²⁺ through this kind of Ca²⁺ pump. Further evidence for this idea was obtained from the effect of other two SERCA inhibitors, BHQ (Fig. 5b) and cyclopiazonic acid (CPA) (Fig. 5c). The figures show representative experiments in which addition of these inhibitors produced a rapid decrease in [Ca²⁺]_{SG} (BHQ, from 19 ± 3 μM to 6 ± 1 μM, *n* = 8, *P* < 0.001; CPA, from 22 ± 6 μM to 8 ± 1 μM, *n* = 5, *P* < 0.05), which was nearly complete in about 1 min. This effect was rapidly reversible in the case of BHQ and also reversible, although more slowly, in the case of CPA. Therefore, secretory granules require the continuous activity of SERCA pumps to maintain the intravesicular [Ca²⁺].

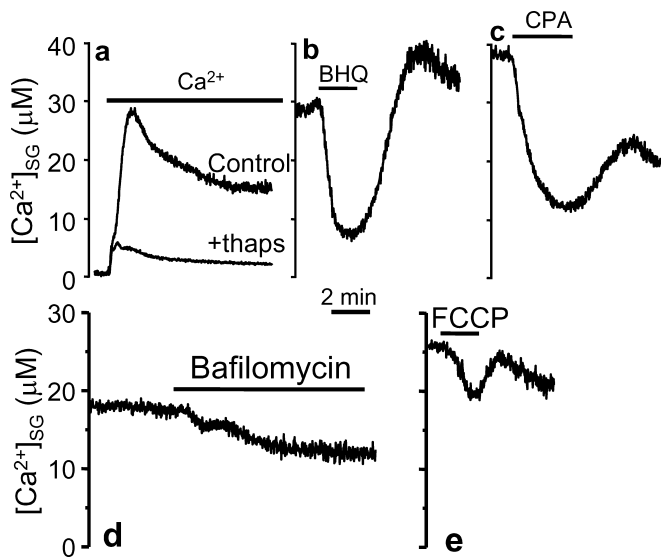


FIG. 5. Mechanisms of Ca^{2+} accumulation by the vesicles. Bovine chromaffin cells expressing vesicle-associated membrane protein (VAMP)-mutated aequorin chimera (VAMP-mutaeq) were depleted of Ca^{2+} before reconstitution with native coelenterazine, and the luminescence was initially monitored under continuous perfusion with Ca^{2+} -free (containing 0.5 mM EGTA) medium. A pulse of 500 μM La^{3+} was given to release the luminescence of the probe present in the plasma membrane (not shown). Then, after a 5-min wash with Ca^{2+} -free medium, standard medium containing 1 mM Ca^{2+} was perfused to refill the granules with Ca^{2+} , as indicated in (a). In the trace labeled '+thaps', it can be observed that refilling was largely blocked in cells treated with 1 μM thapsigargin for 5 min prior to the experiment. In (b), (c), (d) and (e), either 10 μM *t*-butyl benzohydroquinone (BHQ), 10 μM cyclopiazonic acid (CPA), 100 nM bafilomycin or 2 μM FCCP was added as indicated when the secretory granule $[\text{Ca}^{2+}]_{\text{SG}}$ ($[\text{Ca}^{2+}]_{\text{SG}}$) reached steady state. All of these treatments released Ca^{2+} from the vesicles. These experiments are representative of five to 13 similar ones of each kind.

We then explored the effect of bafilomycin, an inhibitor of the vesicular H^{+} -ATPase, on $[\text{Ca}^{2+}]_{\text{SG}}$. As shown in Fig. 5d, addition of bafilomycin induced a small and slow decrease in $[\text{Ca}^{2+}]_{\text{SG}}$. The rate of $[\text{Ca}^{2+}]_{\text{SG}}$ decrease changed from $0.37 \pm 0.06 \mu\text{M}/\text{min}$ to $1.1 \pm 0.2 \mu\text{M}/\text{min}$ ($n = 5$, $P < 0.01$) after the addition of 100 nM bafilomycin. However, we must take into account the fact that bafilomycin also produces a slow increase in the vesicular pH (Wu *et al.*, 2001; Camacho *et al.*, 2006) that should considerably increase aequorin luminescence (depending on the resting granular pH; see above). Thus, the small decrease in $[\text{Ca}^{2+}]_{\text{SG}}$ observed may in fact reflect a much larger drop, masked by the pH change. Finally, a direct collapse of the vesicular H^{+} gradient with the protonophore FCCP also produced a small drop in vesicular $[\text{Ca}^{2+}]$ (from 14 ± 2 to $11 \pm 2 \mu\text{M}$, $P > 0.05$, increasing again after FCCP washout to $16 \pm 3 \mu\text{M}$, $n = 13$; see Fig. 5e). The differences in this case were not statistically significant, because of the variability in the initial $[\text{Ca}^{2+}]$, but the pattern of the response was the same in all the experiments. Together with the effect of bafilomycin, this suggests that the vesicular H^{+} gradient may partially contribute to maintain the steady-state vesicular $[\text{Ca}^{2+}]$, perhaps through the activity of an $\text{H}^{+}/\text{Ca}^{2+}$ -exchanger. This mechanism could also be responsible for the small vesicular Ca^{2+} uptake occurring in thapsigargin-treated cells (Fig. 5a). The experiments of Fig. 5 also show the range of variability in the resting vesicular $[\text{Ca}^{2+}]$ among different single experiments, although mean values were quite similar (see above).

We then studied the effect of several chromaffin cell agonists on vesicular $[\text{Ca}^{2+}]$. In Fig. 6a, we show the effect of high- $[\text{K}^{+}]$

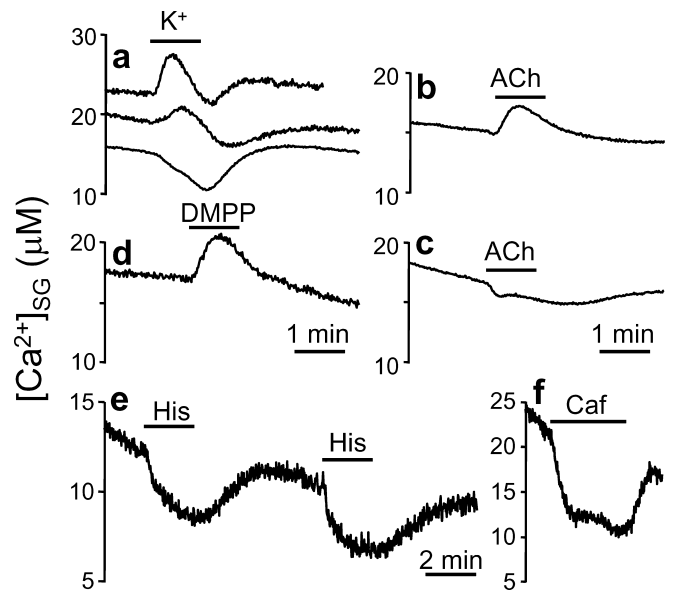


FIG. 6. Effect of high- $[\text{K}^{+}]$ depolarization, acetylcholine (ACh) and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) on secretory granule $[\text{Ca}^{2+}]_{\text{SG}}$ ($[\text{Ca}^{2+}]_{\text{SG}}$). Initial experimental details as in Fig. 5. When the $[\text{Ca}^{2+}]_{\text{SG}}$ reached steady state, medium containing either 70 mM K^{+} (a), 100 μM ACh (b and c), 10 μM DMPP (d), 100 μM histamine (His, e) or 20 mM caffeine (Caf, f) were added as indicated. Histamine and caffeine released Ca^{2+} from the vesicles, DMPP increased vesicular $[\text{Ca}^{2+}]$, and high- $[\text{K}^{+}]$ and ACh produced more variable responses (see text). These experiments are representative of five to 21 similar ones of each kind.

depolarization. The three different traces are representative of the different responses that we obtained. In some cell preparations (nine of 29 experiments, 31%), high- $[\text{K}^{+}]$ depolarization induced mainly a transient increase in $[\text{Ca}^{2+}]_{\text{SG}}$ (from 13.6 ± 1.4 to $21 \pm 2 \mu\text{M}$, $n = 9$, $P < 0.01$), in others (12 of 29 experiments, 41%), the response was mainly a transient decrease in $[\text{Ca}^{2+}]_{\text{SG}}$ (from 19.5 ± 1.2 to $15.0 \pm 0.6 \mu\text{M}$, $n = 12$, $P < 0.005$), and finally we also obtained (eight of 29 experiments, 28%) a mixed response, an initial increase followed by a later decrease in $[\text{Ca}^{2+}]_{\text{SG}}$ with final recovery to the initial levels (an initial increase from 13.7 ± 1.9 to $16.1 \pm 2.1 \mu\text{M}$, $P > 0.05$, a decrease to $11.8 \pm 2.0 \mu\text{M}$, and a final recovery to $13.9 \pm 2.2 \mu\text{M}$, $n = 8$). In the case of the mixed response, these changes were not statistically significant, but again the pattern of the response in all of these experiments was the same as that shown in Fig. 6a. A similar variable response was obtained when the natural agonist, acetylcholine (ACh), was used, although in this case the increase in $[\text{Ca}^{2+}]_{\text{SG}}$ was the predominant response (21 of 28 experiments, 75%, mean increase from 17.3 ± 0.8 to $24.3 \pm 1.6 \mu\text{M}$, $P < 0.001$). In five of 28 experiments (18%), the effect was the opposite; a small decrease in $[\text{Ca}^{2+}]_{\text{SG}}$ (mean decrease from 15.1 ± 2.2 to $13.8 \pm 2.2 \mu\text{M}$, $P > 0.05$), which was not statistically significant. Only in two experiments was a mixed response observed. Figure 6b and c shows representative traces demonstrating the two main effects of this agonist. The explanation for this heterogeneous behavior may perhaps be the presence of several populations of vesicles responding differently to the stimulus, some of them responding with an activation of SERCA pumps, and some of them responding with an activation of Ca^{2+} -induced Ca^{2+} release mechanisms. Stimulation of different cell preparations could preferentially activate one or the other mechanism. On the other hand, the nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) always induced an increase in $[\text{Ca}^{2+}]_{\text{SG}}$ (mean increase from $15.4 \pm 0.7 \mu\text{M}$ to $20.3 \pm 1.4 \mu\text{M}$, $n = 14$,

$P < 0.005$; see Fig. 6d), whereas the InsP₃-producing agonist histamine always induced a decrease in [Ca²⁺]_{SG} (mean decrease from 10.4 ± 0.6 to 7.4 ± 0.5 μM , $n = 11$, $P < 0.005$; see Fig. 6e). Similarly, addition of the RyR activator caffeine was also effective in releasing Ca²⁺ from the vesicles in intact cells (mean decrease from 13.7 ± 1.9 to 8.6 ± 1.6 μM , $n = 10$, $P < 0.05$; see Fig. 6f).

Dynamics of [Ca²⁺]_{SG} in permeabilized chromaffin cells

To investigate further the dynamics of [Ca²⁺]_{SG} and the mechanisms of Ca²⁺ uptake and release from the vesicles, we performed several experiments in permeabilized cells, in which the intracellular medium can be easily manipulated. To avoid interference from the VAMP-mutaeq chimera present in the plasma membrane, a pulse of lanthanum was always given prior to permeabilization. Then, cells were permeabilized with digitonin, and an intracellular medium containing a 100 nM Ca²⁺ buffer was perfused to mimic the physiological [Ca²⁺] in the cytosol. The control traces in Fig. 7a and b show that perfusion of the Ca²⁺ buffer induced a rapid increase in vesicular [Ca²⁺], which reached a peak around 40–50 μM (43 ± 4 μM , $n = 57$), followed by a slower decrease down to a steady state around 10 μM (9.2 ± 0.3 μM , $n = 55$, 10 min after the peak). The kinetics were very similar to those found in intact cells after perfusion of Ca²⁺-containing medium (Figs 3 and 5). In addition, the increase in vesicular [Ca²⁺] was also almost completely blocked by pretreatment of the cells with thapsigargin (Fig. 7a; peak of 2.2 ± 0.5 μM , $n = 10$, and [Ca²⁺]_{SG} value 10 min later 1.1 ± 0.2 μM , $n = 10$, $P < 0.001$ for the comparison with the corresponding control values above), confirming that SERCA pumps are mainly responsible for

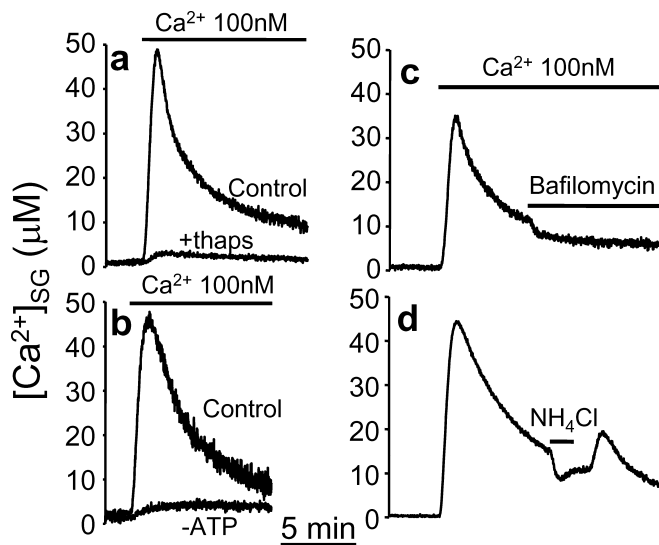


FIG. 7. Mechanisms of vesicular Ca²⁺ uptake in permeabilized chromaffin cells. Bovine chromaffin cells expressing vesicle-associated membrane protein (VAMP)-mutated aequorin chimera (VAMP-mutaeq) were depleted of Ca²⁺ and reconstituted with native coelenterazine, and the luminescence was initially monitored in Ca²⁺-free medium. A pulse of 500 μM La³⁺ was then given, followed by a 5-min wash with Ca²⁺-free medium and then by permeabilization as described in Materials and methods. Then, intracellular medium containing a 100 nM Ca²⁺ buffer was perfused to refill the granules with Ca²⁺, generating a peak of secretory granule [Ca²⁺] ([Ca²⁺]_{SG}). Pretreatment of the cells with 1 μM thapsigargin for 5 min (+thaps, a) or perfusion with medium lacking ATP and containing 5 μM oligomycin (-ATP, b) largely blocked refilling. (c) and (d) show that 100 nM bafilomycin or 10 mM ammonium chloride released Ca²⁺ from the vesicles when added after Ca²⁺ refilling. These experiments are representative of four to 10 similar ones of each kind.

Ca²⁺ uptake into the vesicular compartment. Consistently, the increase in [Ca²⁺]_{SG} was also blocked in the absence of ATP in the perfusion medium (Fig. 7b; peak of 3.3 ± 0.4 μM , $n = 6$, and [Ca²⁺]_{SG} value 10 min later 2.7 ± 0.3 μM , $n = 6$, $P < 0.001$ for the comparison with control values), confirming the requirement for an ATP-dependent mechanism for Ca²⁺ uptake into the vesicles.

As shown above for intact cells, addition of the H⁺-ATPase inhibitor bafilomycin to permeabilized cells induced a small decrease in vesicular [Ca²⁺] (from 10.7 ± 0.8 μM to 7.7 ± 0.3 μM , $n = 5$, $P < 0.01$; see Fig. 7c). This effect may be related to the pH changes induced by this inhibitor in the granules, as ammonium chloride produced similar effects (decrease from 11.8 ± 1.9 to 6.6 ± 1.0 μM , $P < 0.05$, followed by a rapid overshoot to 13.1 ± 2.6 μM after washout, $n = 4$; see Fig. 7d). As mentioned above, Ca²⁺ release induced by these agents may be underestimated, taking into account that the pH change that they induce should lead to an increase in aequorin luminescence. Finally, we tested the effect in permeabilized cells of InsP₃, caffeine, cADPR, NAADP, and micromolar Ca²⁺. The upper panels of Fig. 8 depict representative experiments showing that both InsP₃ and caffeine were able to induce fast and reversible Ca²⁺ release from the vesicles, suggesting the presence in the granules of both InsP₃ receptors and RyRs. Addition of 2 μM InsP₃ induced a rapid and reversible decrease of [Ca²⁺]_{SG} from 10.5 ± 0.8 to 5.5 ± 0.5 μM ($n = 14$, $P < 0.001$). Addition of 20 mM caffeine also induced a reversible decrease of [Ca²⁺]_{SG} from 12.7 ± 3.5 to 7.7 ± 2.0 μM ($n = 4$, $P > 0.05$). The difference in this case was not statistically significant, due to the variability in the initial [Ca²⁺], but the effect was consistently found in all the experiments (in terms of percentages, the mean [Ca²⁺]_{SG} decrease induced by caffeine was $39.8 \pm 2.5\%$, $n = 4$). The experiments included in the lower panels

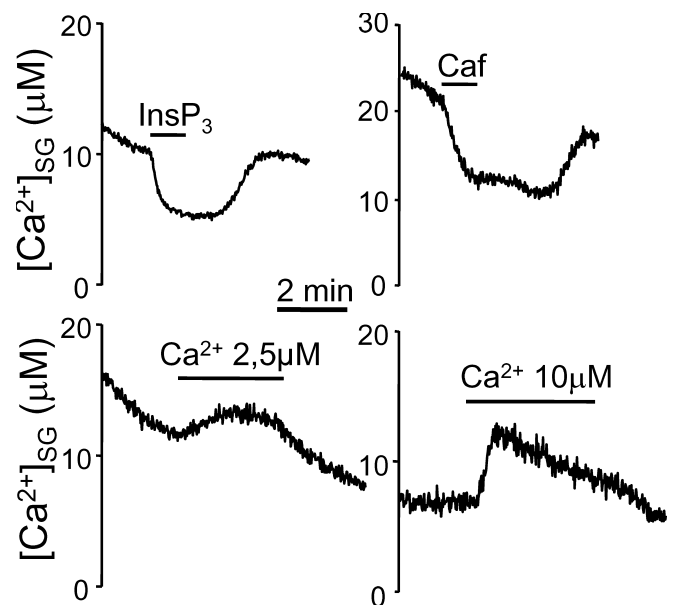


FIG. 8. Effects of inositol 1,4,5-trisphosphate (InsP₃), caffeine (Caf) and Ca²⁺ on secretory granule [Ca²⁺] ([Ca²⁺]_{SG}). Initial experimental details as in Fig. 7. The intracellular medium containing a 100 nM Ca²⁺ buffer was perfused until a quasi-steady state was reached (not shown). After this, addition of either 2 μM InsP₃ or 50 mM caffeine induced release of Ca²⁺ from the vesicles, whereas perfusion of Ca²⁺ buffers prepared to obtain 2.5 or 10 μM free Ca²⁺ induced an increase of [Ca²⁺]_{SG}. These experiments are representative of four to 14 similar ones of each kind.

of Fig. 8 show that perfusion of medium containing either 2.5 or 10 μM free Ca^{2+} produced a transient increase in $[\text{Ca}^{2+}]_{\text{SG}}$, which was larger at the higher $[\text{Ca}^{2+}]$. Perfusion of medium containing 2.5 μM Ca^{2+} induced a small increase in $[\text{Ca}^{2+}]_{\text{SG}}$ from 11.3 ± 1.0 to 13.5 ± 1.0 μM ($n = 6$) (19% increase), which was not statistically significant ($P > 0.05$). In the case of the 10 μM Ca^{2+} medium, $[\text{Ca}^{2+}]_{\text{SG}}$ increased from 9.6 ± 1.8 to 16.5 ± 2.9 μM ($n = 8$) (72% increase, $P < 0.05$), whereas neither 5 μM cADPR nor 100 nM NAADP produced any effect on $[\text{Ca}^{2+}]_{\text{SG}}$ (data not shown).

Discussion

In this study, we investigated the dynamics of $[\text{Ca}^{2+}]$ inside the secretory granules of primary cultures of chromaffin cells, using a targeted VAMP-mutated aequorin (VAMP-mutaeq) chimera. The same chimera has been used previously in MIN6 β -cells (Mitchell *et al.*, 2001, 2003), INS-1E cells (Iezzi *et al.*, 2005), and PC12 cells (Moreno *et al.*, 2005). In all of these studies, VAMP-mutaeq was reconstituted with the semisynthetic cofactor coelenterazine n, generating an aequorin chimera with very low Ca^{2+} sensitivity. This type of aequorin was originally designed and used to monitor the high $[\text{Ca}^{2+}]$ found in the ER, around 500 μM (Montero *et al.*, 1997a). However, the $[\text{Ca}^{2+}]$ found in the secretory vesicles is much lower, around 50–100 μM . This range of concentrations is in the lower limit of detection for that aequorin form, particularly if we take into account that the acidic granular pH reduces aequorin luminescence 3- to 4-fold, providing apparent $[\text{Ca}^{2+}]_{\text{SG}}$ values of only 20–30 μM . Thus, we have now found that it is much better to use an aequorin form with higher Ca^{2+} affinity to follow vesicular Ca^{2+} dynamics, and particularly to detect Ca^{2+} release from the vesicles.

We used here the same VAMP-mutaeq, but reconstituted with native coelenterazine. This combination increases by one order of magnitude the Ca^{2+} affinity of the probe and allows the monitoring of $[\text{Ca}^{2+}]_{\text{SG}}$ dynamics with much more sensitivity. Moreover, reducing the temperature to 22°C allowed fine tuning of the sensitivity in order to monitor adequately the dynamics of $[\text{Ca}^{2+}]_{\text{SG}}$ during stimulation. In fact, finding the aequorin form with the correct sensitivity is a critical point. In a recent study, a chromogranin–aequorin chimera with even higher Ca^{2+} affinity was used to monitor $[\text{Ca}^{2+}]$ in the secretory vesicles of PC12 cells (Mahapatra *et al.*, 2004). In that case, the native form of aequorin was used, and it was reconstituted with native coelenterazine, generating an aequorin form with very high Ca^{2+} affinity. That aequorin form, ideal for measuring cytosolic $[\text{Ca}^{2+}]$, becomes rapidly saturated and consumed when $[\text{Ca}^{2+}]$ rises to the micromolar range, as occurs in the secretory granules. This emphasizes again the importance of choosing the right aequorin form, adapted to the $[\text{Ca}^{2+}]$ in the compartment to which it is targeted. Otherwise, large discrepancies from the real $[\text{Ca}^{2+}]$ values can appear, as we have previously found using ER-targeted aequorins with different Ca^{2+} affinities (Montero *et al.*, 1997b).

Considerable evidence has previously been provided indicating that either VAMP–aequorin or VAMP–EGFP chimeras are correctly targeted to the secretory vesicles in different secretory cell types (Mitchell *et al.*, 2001, 2003; Allersma *et al.*, 2004). We have shown here by confocal microscopy that expression of VAMP–EGFP in chromaffin cells provides, in fact, a typical granular pattern, although a certain amount of fluorescence is always associated with the plasma membrane, as can be expected for a protein that follows the secretory pathway. The presence of VAMP-mutaeq in the plasma membrane was confirmed by the effect of lanthanum, an impermeable cation that immediately produced a peak of aequorin luminescence when added to the extracellular medium. Then, once the luminescence from the plasma membrane VAMP-mutaeq had been released with lanthanum, the

remaining intracellular luminescence was present in a compartment with an apparent $[\text{Ca}^{2+}]$ around 20–30 μM , much higher than in the cytosol but still one order of magnitude lower than in other compartments of the secretory pathway, such as the ER or Golgi apparatus.

The results of this study show several important and novel characteristics of $[\text{Ca}^{2+}]$ fluxes in secretory vesicles. The first question regards the free $[\text{Ca}^{2+}]$ in the vesicles. During Ca^{2+} refilling of the vesicular space, the $[\text{Ca}^{2+}]_{\text{SG}}$ traces showed a peak at 40 μM followed by a continuously decreasing trace in the 15–20 μM range (Fig. 3). This behavior suggests that the $[\text{Ca}^{2+}]$ in the vesicular population is not homogeneous, so that aequorin is first consumed in higher- $[\text{Ca}^{2+}]$ compartments and the final luminescence comes mainly from the lower- $[\text{Ca}^{2+}]$ compartments. Taking into account the influence on aequorin luminescence of the acidic intragranular pH of 5.5 (Winkler & Westhead, 1980; Wu *et al.*, 2001), the real values for the free $[\text{Ca}^{2+}]_{\text{SG}}$ should then range between 50 and 100 μM . Vesicular pH significantly modifies aequorin luminescence, but it appears to change little during Ca^{2+} refilling of the granules or during stimulation with high $[\text{K}^+]$ in intact cells or with InsP_3 in permeabilized cells. Thus, the aequorin luminescence changes observed under these conditions can be clearly attributed to changes in $[\text{Ca}^{2+}]_{\text{SG}}$. Similarly, the increases in $[\text{Ca}^{2+}]_{\text{SG}}$ induced by the nicotinic agonist DMPP and by Ca^{2+} in permeabilized cells are not due to pH changes, as these stimuli have been shown to produce only some acidification (Camacho *et al.*, 2006). On the other hand, the effects of vesicular pH changes on aequorin luminescence must be taken into account to interpret the effects of bafilomycin or FCCP, which are known to increase vesicular pH. In both cases, the observed release of Ca^{2+} from the vesicles may be underestimated because of the concurrent pH increase.

The measured values of $[\text{Ca}^{2+}]_{\text{SG}}$ represent only a tiny fraction of the total Ca^{2+} contained in the granules, which is around 20–40 mM (Fasolato *et al.*, 1991; Yoo & Albanesi, 1991), and is thus nearly 1000-fold the free $[\text{Ca}^{2+}]_{\text{SG}}$ measured here. This Ca^{2+} is bound in part to chromogranins, the most abundant proteins in the chromaffin vesicles (40% of the soluble intravesicular proteins) (Simon & Aunis, 1989), which bind 152 nmol Ca^{2+} /mg protein with a K_d of 54 μM (Bulenda & Gratzl, 1985; Simon & Aunis, 1989; Yoo & Albanesi, 1991). In addition, the vesicles also contain large amounts of ATP (130 mM) (Winkler & Westhead, 1980), which may also bind significant amounts of Ca^{2+} .

Our data provide evidence that Ca^{2+} accumulation in the vesicles is ATP-dependent and takes place mainly via SERCA-type pumps that are sensitive to the usual inhibitors: thapsigargin, BHQ, and CPA. Inhibition of the H^+ -ATPase with bafilomycin produced only slow Ca^{2+} release from the vesicles; this effect was mimicked by agents able to alkalinize the vesicles, such as the protonophore FCCP or ammonium chloride. These effects could be explained by the operation of an $\text{H}^+/\text{Ca}^{2+}$ exchanger, whose presence in the vesicular membrane has been suggested previously for synaptic vesicles (Goncalves *et al.*, 1998, 1999) and yeast vacuoles (Ungermann *et al.*, 1999). However, this system would make only a minor contribution to secretory granule Ca^{2+} uptake in these cells, perhaps because of its low Ca^{2+} affinity (Goncalves *et al.*, 1998, 1999). The presence of thapsigargin-sensitive Ca^{2+} pumps in the secretory vesicles was surprising, as this compartment is usually considered to have thapsigargin-insensitive secretory pathway Ca^{2+} -ATPases. In fact, in the original experiments performed with the same VAMP–aequorin chimera in the MIN-6 β -cell line, Ca^{2+} uptake into the vesicles was insensitive to thapsigargin or CPA. However, thapsigargin-sensitive Ca^{2+} uptake into the vesicles of PC12 cells has been also described in a previous study using a high- Ca^{2+} -affinity chromogranin–aequorin chimera (Mahapatra *et al.*, 2004). In addition, the presence of acidic Ca^{2+} stores (probably corresponding to

dense-core insulin storage granules) that are sensitive to thapsigargin, BHQ and CPA has also been recently described in primary mouse β -cells (Duman *et al.*, 2006).

From the physiological point of view, the main question regarding vesicular [Ca²⁺]_{SG} fluxes is whether the granular Ca²⁺ may or not be released under physiological conditions and contribute to secretion. We found here that both InsP₃ and caffeine are potent activators of Ca²⁺ release from the granules, suggesting the presence of functional InsP₃ receptors and RyRs in the vesicular membrane. The release of Ca²⁺ from the vesicles via InsP₃ receptors was also confirmed in intact cells, where the InsP₃-producing agonist histamine was able to induce a rapid and reversible [Ca²⁺]_{SG} decrease. In fact, the presence of InsP₃ receptors in the membrane of chromaffin cell secretory granules has been a matter of debate for a long time (Yoo & Albanesi, 1991; Pouli *et al.*, 1998; Huh *et al.*, 2005; Endo *et al.*, 2006), although they have been consistently described in mast cell granules (Quesada *et al.*, 2001, 2003) and pancreatic acinar granules (Gerasimenko *et al.*, 1996, 2006). However, we did not find effects with cADPR, which has been shown to activate RyRs in pancreatic acinar granules (Gerasimenko *et al.*, 1996, 2006) and MIN-6 cell granules (Mitchell *et al.*, 2001, 2003), or with NAADP, which has also been shown to release Ca²⁺ from pancreatic acinar granules (Gerasimenko *et al.*, 2006), MIN-6 cell granules (Mitchell *et al.*, 2003), and mouse pancreatic β -cells (Duman *et al.*, 2006). The discrepancy may perhaps be due to the cell type, as cADPR was also unable to release Ca²⁺ from the ER in chromaffin cells (Alonso *et al.*, 1999).

Our data also show that stimuli such as ACh or high-[K⁺] depolarization were able to induce a transient release of Ca²⁺ from the granules (Fig. 6). Actually, these stimuli induced a double response, an increase and/or a decrease of [Ca²⁺]_{SG}, a phenomenon that suggests a heterogeneous response of the vesicular population. We think that it is reasonable to speculate that vesicles close to the plasma membrane, in the releasable pool, would be more prone to open their InsP₃ receptors and RyRs after stimulation, because of the higher subplasmalemmal InsP₃ and Ca²⁺ concentrations obtained after the stimulus, whereas in more internal vesicles, activation of SERCA pumps by the increased cytosolic [Ca²⁺] may be predominant, leading to Ca²⁺ uptake by the vesicles, a mechanism that could be important for vesicle transport and maturation. Both effects, Ca²⁺ release in peripheral vesicles and increasing Ca²⁺ uptake in internal ones, should occur simultaneously in order to have physiological significance. The effects of histamine and caffeine show that the vesicles are able to rapidly release Ca²⁺ in intact cells after stimulation. The mixed response would then appear as the result of the two parallel effects in the particular cell conditions of each experiment.

From a theoretical point of view, it is difficult to speculate on how important Ca²⁺ release from the vesicles may be for secretion as compared to the large flux of Ca²⁺ entering the cell during an action potential. There are still many uncertainties regarding the percentage of vesicles able to release Ca²⁺, the amount of Ca²⁺ released, the kinetics of vesicular Ca²⁺ buffers, etc. However, it may be useful to make some simple estimations. For example, chromaffin cells have about 20 000 vesicles that each contain about 90 000 calcium ions (Winkler & Westhead, 1980). Assuming that 1% of the vesicles release 20% of their calcium ions (or 5% of the vesicles release 4% of it), this amounts to 3.6×10^6 Ca²⁺ released (6×10^{-18} mol). For chromaffin cells, with a diameter of about 15 μ m (volume 1.7×10^{-12} L), this corresponds to 3.5 μ mol/L cells. Regarding Ca²⁺ entry during an action potential, the average voltage-dependent Ca²⁺ current measured during chromaffin cell depolarization (250 pA) (Zhou & Neher, 1993) corresponds to about 700 μ mol/L cell/s for a

15- μ m-diameter cell. In these cells, a typical action potential lasts 5 ms, so the amount of Ca²⁺ entering the cells during an action potential would be about 3.5 μ mol/L cell, the same as the release from the vesicles under the assumptions made above. Of course, the real figures may be different, but this simple calculation shows that vesicular Ca²⁺ has the potential to influence secretion. In addition, we have to take into account that the Ca²⁺ released from the vesicles is necessarily closer to the exocytotic sites, and thus more effective, than Ca²⁺ entering from outside.

Therefore, in conclusion, secretory granules in chromaffin cells have mechanisms for fast Ca²⁺ release (InsP₃ receptors and RyRs) and are able to release Ca²⁺ after cell stimulation with ACh or high-[K⁺] depolarization. Evidence for the contribution of secretory granule Ca²⁺ to exocytosis has been reported before (Fossier *et al.*, 1998; Scheenen *et al.*, 1998; Mundorf *et al.*, 2000; Haynes *et al.*, 2006). Our data strongly support this hypothesis and suggest that granular Ca²⁺ may contribute to its own secretion.

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Abbreviations

ACh, acetylcholine; BHQ, *t*-butyl benzohydroquinone; [Ca²⁺]_{SG}, secretory granule [Ca²⁺]; CPA, cyclopiazonic acid; cADPR, cADP ribose; DMPP, 1,1-dimethyl-4-phenyl-piperazinium iodide; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; InsP₃, inositol 1,4,5-trisphosphate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; NAADP, nicotinic acid adenine dinucleotide phosphate; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca²⁺-ATPase; VAMP, vesicle-associated membrane protein; VAMP-mutaeq, vesicle-associated membrane protein-mutated aequorin chimera.

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