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ORIGINAL RESEARCH

Ca^{2+} Dynamics in the Secretory Vesicles of Neurosecretory PC12 and INS1 Cells

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Abstract We have investigated the dynamics of the free $[Ca^{2+}]$ inside the secretory granules of neurosecretory PC12 and INS1 cells using a low-Ca^{2+}-affinity aequorin chimera fused to synaptobrevin-2. The steady-state secretory granule $[Ca^{2+}]$ ($[Ca^{2+}]_{SG}$) was around 20–40 μM in both cell types, about half the values previously found in chromaffin cells. Inhibition of SERCA-type Ca^{2+} pumps with thapsigargin largely blocked Ca^{2+} uptake by the granules in Ca^{2+}-depleted permeabilized cells, and the same effect was obtained when the perfusion medium lacked ATP. Consistently, the SERCA-type Ca^{2+} pump inhibitor benzohydroquinone induced a rapid release of Ca^{2+} from the granules both in intact and permeabilized cells, suggesting that the continuous activity of SERCA-type Ca^{2+} pumps is essential to maintain the steady-state $[Ca^{2+}]_{SG}$. Both inositol 1,4,5-trisphosphate ($InsP_3$) and caffeine produced a rapid Ca^{2+} release from the granules, suggesting the presence of $InsP_3$ and ryanodine receptors in the granules. The response to high-K^+ depolarization was different in both cell types, a decrease in $[Ca^{2+}]_{SG}$ in PC12 cells and an increase in $[Ca^{2+}]_{SG}$ in INS1 cells. The difference may rely on the heterogeneous response of different vesicle populations in each cell type. Finally, increasing the glucose concentration triggered a decrease in $[Ca^{2+}]_{SG}$ in INS1 cells. In conclusion, our data show that the secretory granules of PC12 and INS1 cells take up Ca^{2+} through SERCA-type Ca^{2+} pumps and can release it through $InsP_3$ and ryanodine receptors, supporting the hypothesis that secretory granule Ca^{2+} may be released during cell stimulation and contribute to secretion.

Keywords Ca^{2+} · Secretory granules · PC12 cells · INS1 cells · Inositol 1,4,5-trisphosphate receptors · Ryanodine receptors · Aequorin

Abbreviations

BHQ	*tert*-Butyl benzohydroquinone
$[Ca^{2+}]_{SG}$	Secretory granule $[Ca^{2+}]$
DMPP	1,1-Dimethyl-4-phenyl-piperazinium iodide
$InsP_3$	Inositol 1,4,5-trisphosphate
SERCA	Sarco endoplasmic reticulum Ca^{2+} ATPase
VAMP	Vesicle-associated membrane protein
VAMP-mutaeq	VAMP-mutated aequorin chimera

Introduction

In neurosecretory cells, the largest Ca^{2+} store resides in the secretory granules. In chromaffin cells, for example, secretory granules contain approximately 60% of the total intracellular calcium (Haigh et al. 1989), and the total intragranular calcium concentration has been estimated

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to be around 40 mM (Winkler and Westhead 1980; Hutton 1989). In spite of this, very little evidence has been provided for the involvement of Ca^{2+}-release from the granules in stimulated secretion. In fact, intragranular calcium has always been considered to have a more structural than dynamic role. Instead, other organelles such as the endoplasmic reticulum or mitochondria are known to participate actively in the regulation of intracellular Ca^{2+} homeostasis in these cells and thus, in the regulation of secretion (Alonso et al. 1999; Montero et al. 2000).

The main reason for this apparent oversight of the Ca^{2+} contained in the secretory granules is the lack of knowledge on the dynamics of secretory granule $[Ca^{2+}]$ ($[Ca^{2+}]_{SG}$) and particularly on their mechanisms of Ca^{2+} uptake and release. Some authors have reported that chromaffin granules (Yoo and Albanesi 1990; Huh et al. 2005; Yoo 2010), mast cell secretory granules (Quesada et al. 2001, 2003) and pancreatic acinar cells secretory granules (Gerasimenko et al. 1996, 2006) possess functional inositol 1,4,5-trisphosphate ($InsP_3$) receptors. However, work from other authors suggests that $InsP_3$ receptors are present only in the endoplasmic reticulum but not in the secretory granules of chromaffin cells (Endo et al. 2006), or that cell stimulation does not produce any Ca^{2+} release from the granules in PC12 cells (Pouli et al. 1998).

We have shown recently (SantoDomingo et al. 2008) that chromaffin cell granules are able to take up Ca^{2+} actively from the cytosol through SERCA-type Ca^{2+} pumps, and can also release rapidly the accumulated Ca^{2+} via either $InsP_3$ receptors or ryanodine receptors. This clearly suggested that Ca^{2+} release from the granules could take place during the secretory process and probably contribute to its own secretion. We have extended here this study to two widely used cell lines: rat pheochromocytoma PC12 cells and rat insulinoma INS1 cells. Our results show that granules in these cells behave in a very similar way to chromaffin cell granules, although their steady-state free $[Ca^{2+}]_{SG}$ is somewhat smaller.

Methods

Cell Culture and Expression of Aequorin and EGFP Chimeras

The construction strategy of the 2-synaptobrevin-mutated aequorin (VAMP-mutaeq) chimera has been described previously (Mitchell et al. 2001; Moreno et al. 2005). PC12 rat pheochromocytoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, 7.5% horse serum, 2 mM glutamine, 100i.u. ml^{-1} penicillin, and 100i.u. ml^{-1} streptomycin. INS1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 25 mM Hepes, 1 mM sodium pyruvate, 2 mM glutamine, 100i.u. ml^{-1} penicillin, and 100i.u. ml^{-1} streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

The 2-synaptobrevin-mutated aequorin (VAMP-mutaeq) chimera has been described previously (Mitchell et al. 2001; Moreno et al. 2005). For construction of the adenoviral vector, full-length cDNA encoding this construct was subcloned into the pShuttle vector and then used for construction of the adenoviral vector by using an AdenoX adenovirus construction kit (Clontech, Palo Alto, CA).

Cells were infected with adenoviruses for expression of the constructs described above. Ca^{2+} measurements were performed 48–72 h after infection. Efficiency of infection with a similar adenovirus carrying the VAMP-EGFP chimera was estimated to be about 60%.

Secretory Granule $[Ca^{2+}]$ ($[Ca^{2+}]_{SG}$) Measurements with Aequorin

Cells were plated onto 13 mm polylysine-covered round coverslips and infected with adenoviruses to express VAMP-mutaeq. As reported before (SantoDomingo et al. 2008), reconstitution of this chimera requires previous Ca^{2+}-depletion of the cells by treating them for 10 min with the ionophores ionomycin (10 μM) and monensin (10 μM) and the sarco endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor *tert*-butyl benzohydroquinone (BHQ, 10 μM) in Ca^{2+}-free standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, and 10 mM HEPES, pH 7.4). 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 of native coelenterazine. Cells were then placed in the perfusion chamber of a purpose-built thermostatized luminometer (22°C) and perfused with Ca^{2+}-free standard medium. Then, a 5 min pulse with 500 μM lanthanum chloride was given to release the luminescence of the VAMP-mutaeq chimera present in the extracellular side of the plasma membrane (SantoDomingo et al. 2008), followed again by standard medium containing 0.5 mM EGTA during 3–5 min to wash out lanthanum.

The use of lanthanum to eliminate the luminescence of the aequorin chimera molecules present in the outer side of the plasma membrane, as a result of vesicle fusion, is an important technical point. When secretory granules fuse with the plasma membrane, the aequorin moiety linked to synaptobrevin becomes exposed to the extracellular medium. As aequorin is reconstituted with coelenterazine in Ca^{2+}-free medium, these aequorin molecules are fully reconstituted and then rapidly emit luminescence when Ca^{2+} is added back at the beginning of every experiment, introducing a significant artifact in the measurements. Lanthanum is able to trigger the luminescence from

aequorin but does not enter the cells. Therefore, a brief pulse with this cation in Ca^{2+}-free medium releases the luminescence from the probe in the plasma membrane, but does not affect the intracellular one. Then, when Ca^{2+} is added after that, only the luminescence from intracellular VAMP-aequorin is recorded. In previous works made with this chimera, the luminescence from those aequorin molecules (around 20% of the total, see Mitchell et al. 2001; SantoDomingo et al. 2008) could have made a significant contribution to the total signal after the readdition of Ca^{2+} to intact cells, thus obscuring the effect of Ca^{2+} pump inhibitors or other mediators.

At this point, cells were ready to start refilling with Ca^{2+} the vesicular store. For experiments with intact cells, standard medium containing 1 mM Ca^{2+} instead of EGTA was perfused to refill with Ca^{2+} the intracellular stores. For experiments with permeabilized cells, cells were treated 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 Ca^{2+} stores. Calibration of the luminescence data into [Ca^{2+}] was made using an algorithm as previously described (Alvarez and Montero 2002). To take into account the effect of the acidic intravesicular pH, around 5.5 (Winkler and Westhead 1980; Wu et al. 2001), on aequorin luminescence, the data obtained using the pH 7 calibration were then multiplied by a factor of 3.5. This factor was previously obtained by calibrating aequorin luminescence at both pH 7 and 5.5 (SantoDomingo et al. 2008). Calibration requires release of all the aequorin luminescence present in the cells at the end of every experiment. In intact cells, this was made by perfusing a water solution containing 20 μM digitonin and 10 mM Ca^{2+}. In previously permeabilized cells, a 10 mM Ca^{2+} solution was perfused at the end of the experiments.

Materials

Coelenterazine was obtained from Molecular Probes, OR, or Biotium, Hayward, CA, Other reagents were from Sigma-Aldrich, Madrid or Merck, Darmstadt.

Results

Mechanisms of Ca^{2+} Accumulation in the Secretory Granules

Measurement of $[Ca^{2+}]_{SG}$ required always previous depletion of Ca^{2+} of the cells in order to allow reconstitution of the chimeric aequorin with the cofactor coelenterazine. Then, after the release of the extracellular membrane-bound aequorin luminescence with lanthanum, refilling of the intracellular stores (including secretory granules) was accomplished either by perfusing extracellular medium containing 1 mM Ca^{2+} or by permeabilizing the cells and then perfusing an intracellular solution containing 100 nM [Ca^{2+}].

Figure 1 shows that readdition of Ca^{2+} by any of these methods to PC12 pheochromocytoma cells produced an smooth increase in $[Ca^{2+}]_{SG}$ to values of 30–60 μM, followed by a slow decrease. The mean $[Ca^{2+}]_{SG}$ values obtained 5 min after Ca^{2+} addition were 42 ± 4 μM ($n = 15$) in intact cells (protocol of a) and 25 ± 2 μM ($n = 11$) in permeabilized cells (protocol of b). At this point, addition of the SERCA pump inhibitor BHQ induced a fast and reversible decrease in $[Ca^{2+}]_{SG}$ (Fig. 1a, b mean $[Ca^{2+}]_{SG}$ decrease of 67 ± 7%, $n = 6$), suggesting that the activity of SERCA-type Ca^{2+} pumps is essential to maintain the steady-state [Ca^{2+}] within the secretory granules. Further evidence for this idea was obtained by showing that cell preincubation with thapsigargin, an irreversible inhibitor or SERCA-type Ca^{2+} pumps, reduced refilling with Ca^{2+} of the secretory vesicles to 18 ± 6% ($n = 5$) of that obtained in the controls (Fig. 1c). Consistently, the absence of ATP in the intracellular perfusion medium produced the same effects (11 ± 4% of the controls, $n = 4$, Fig. 1c). Therefore, continuous activity of the ATP-dependent SERCA pump is required both to refill the vesicles with Ca^{2+} and to maintain the steady-state $[Ca^{2+}]_{SG}$.

Figure 2 shows that the same results could be obtained in the rat insulinoma INS1 cells. In these cells, readdition of Ca^{2+} induced also a fast increase in $[Ca^{2+}]_{SG}$ to around 40–80 μM followed by a prolonged decrease that reached values of 29 ± 2 μM (intact cells, $n = 11$) or 33 ± 4 μM (permeabilized cells, $n = 9$) 5 min after Ca^{2+} addition. At this point, addition of BHQ induced also a fast release of Ca^{2+} from the vesicles (Fig. 2a, b mean $[Ca^{2+}]_{SG}$ decrease of 57 ± 3%, $n = 6$). Consistently, refilling of the secretory granules was significantly reduced in cells treated with thapsigargin (to 27 ± 2% of the control values, $n = 4$) or when ATP was absent from the perfusion medium (to 25 ± 2% of the control values, $n = 3$).

Dynamics of [Ca^{2+}] Inside the Secretory Granules

We have then studied if secretory granules in these cells have mechanisms for stimulated Ca^{2+} release. Figure 3 shows the effects of adding either inositol 1,4,5-trisphosphate ($InsP_3$) or caffeine to permeabilized PC12 or INS1 cells after refilling with Ca^{2+}. It can be observed that activation of $InsP_3$ receptors with $InsP_3$ or activation of ryanodine receptors with caffeine produced a fast decrease in the [Ca^{2+}] inside the secretory granules in both cell

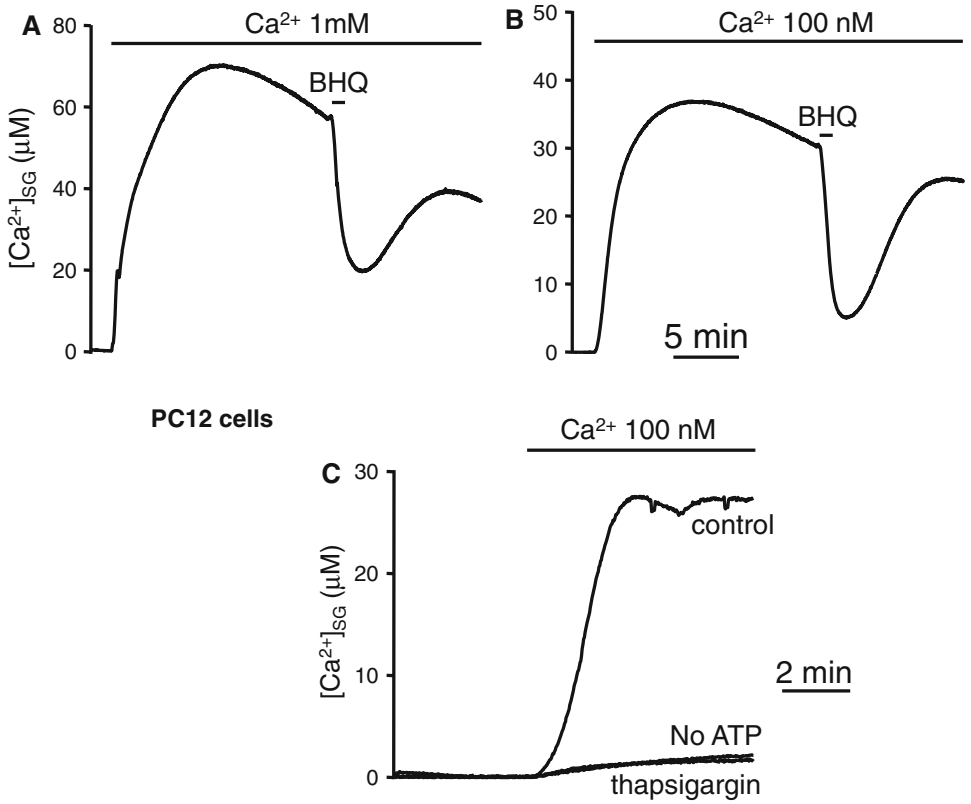

Fig. 1 Mechanisms of Ca^{2+} accumulation by the vesicles in PC12 cells. PC12 cells expressing VAMP-mutaeq were depleted of Ca^{2+} before reconstitution with native coelenterazine, and the luminescence was initially monitored under continuous perfusion of Ca^{2+}-free (containing 0.5 mM EGTA) medium. A pulse of 500 μM La^{3+} was given to release the luminescence of the probe present in the plasma membrane, followed by a 5 min wash with Ca^{2+}-free medium. Then **a** standard medium containing 1 mM Ca^{2+} was perfused to refill the granules with Ca^{2+} and 10 μM BHQ was added when indicated to inhibit the SERCA pump. In **b** cells were permeabilized as described in "Methods" section, perfused with intracellular medium containing 100 nM buffered Ca^{2+} and treated with 10 μM BHQ as indicated. In **c** cells were permeabilized and then perfused with intracellular medium containing 100 nM Ca^{2+} either in control cells or in cells previously treated for 5 min with 1 μM thapsigargin. In the trace labeled "No ATP", cells were perfused with intracellular medium lacking ATP and containing 5 μM oligomycin. Experiments are representative of 3–6 experiments of each kind. Statistics is provided in the text as mean ± s.e.m

types. Addition of $InsP_3$ induced a drop in $[Ca^{2+}]_{SG}$ of 26 ± 6%, $n = 4$, in PC12 cells, and of 29 ± 4%, $n = 3$, in INS1 cells. Addition of caffeine induced a decrease of $[Ca^{2+}]_{SG}$ of 22 ± 1%, $n = 4$, in PC12 cells, and of 27 ± 5%, $n = 4$, in INS1 cells. These effects were rapidly reversible once the activator was taken out, and $[Ca^{2+}]_{SG}$ returned then to the previous values. Therefore, as previously described in chromaffin cells, PC12 and INS1 cells may possess functional $InsP_3$ and ryanodine receptors in their secretory granules. As occurred also in chromaffin cells (SantoDomingo et al. 2008), the activator of ryanodine receptors cyclic ADP ribose (5 μM) had no effect in any of the cell types (data not shown).

We have then investigated the behavior of $[Ca^{2+}]_{SG}$ when intact PC12 or INS1 cells were stimulated. As we have previously shown to occur in chromaffin cells, the response was somewhat variable. Figure 4a shows that depolarization of PC12 cells with high-K^+ induced a transient decrease in $[Ca^{2+}]_{SG}$ (mean decrease of 23 ± 4%, $n = 3$), suggesting the activation of Ca^{2+}-induced Ca^{2+} release mechanisms. Instead, stimulation of these cells with the nicotinic agonist DMPP produced a small increase in $[Ca^{2+}]_{SG}$ (mean increase of 10 ± 3%, $n = 6$, Fig. 4b) and depolarization of INS1 cells with high-K^+ induced a significant and transient increase in $[Ca^{2+}]_{SG}$ (mean increase of 90 ± 20%, $n = 4$, Fig. 4c). Finally, increasing the glucose concentration in the INS1 insulinoma cells produced a biphasic response, with an initial small increase rapidly followed by a more prolonged decrease in $[Ca^{2+}]_{SG}$ (mean decrease of 27 ± 4%, $n = 6$, Fig. 4d), which was reversible after returning to a low glucose medium.

Discussion

In this paper we have extended our previous work in chromaffin cells (SantoDomingo et al. 2008) to investigate the dynamics of $[Ca^{2+}]$ inside the secretory granules of two

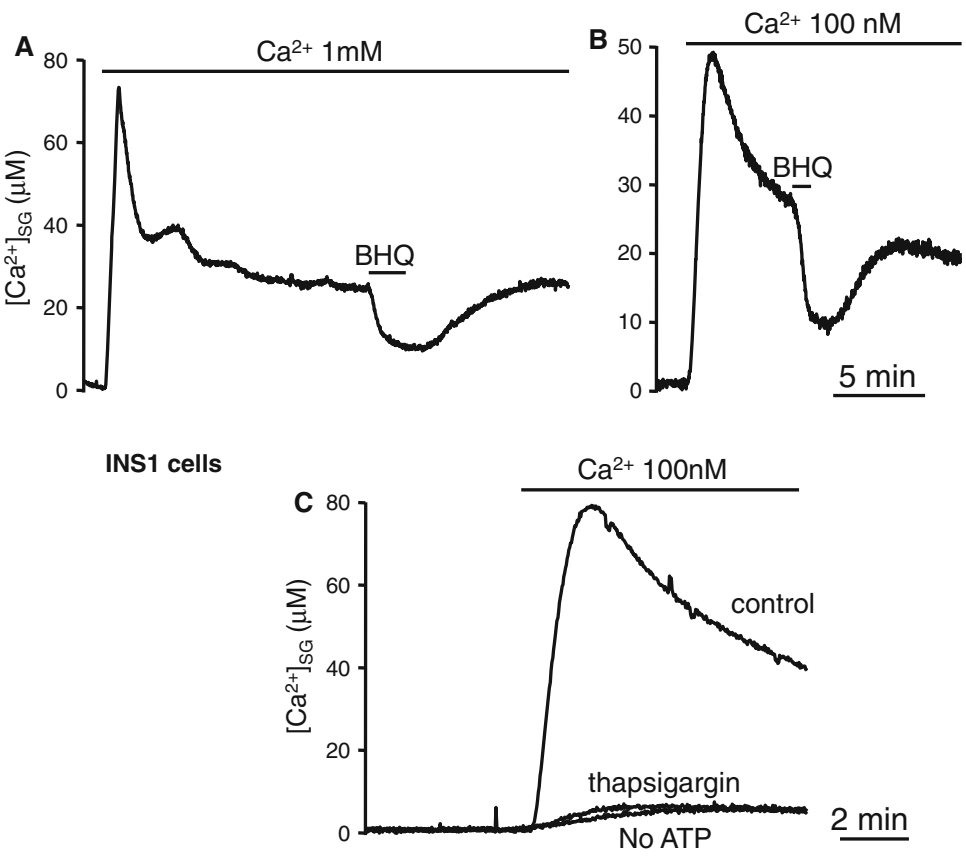

Fig. 2 Mechanisms of Ca^{2+} accumulation by the vesicles in INS1 cells. INS1 cells expressing VAMP-mutaeq were depleted of Ca^{2+} before reconstitution with native coelenterazine. Other experimental details as in Fig. 1. Experiments are representative of 3–6 experiments of each kind. Statistics is provided in the text as mean ± s.e.m

important neurosecretory cell lines, PC12 and INS1. For that, we have used a targeted VAMP-mutated aequorin (VAMP-mutaeq) chimera reconstituted with native coelenterazine in Ca^{2+}-depleted cells. The same chimera has been used previously to measure $[Ca^{2+}]_{SG}$ in MIN6 β-cells (Mitchell et al. 2001; Mitchell et al. 2003), INS-1E cells (Iezzi et al. 2005), and PC12 cells (Moreno et al. 2005). Its correct targeting to the secretory vesicles has been documented in MIN6 β-cells by immunofluorescence and immunoelectron microscopy (Mitchell et al. 2001). In chromaffin cells, Allersma et al. 2004, showed that the similar probe VAMP-EGFP largely colocalized with growth hormone and with dopamine-β-hydroxylase in large dense-core vesicles (see also López-Font et al. 2010), and we have also shown in PC12 and chromaffin cells that it generates a typical granular pattern when seen by confocal microscopy (Moreno et al. 2005; SantoDomingo et al. 2008). In addition, these studies also showed that the probe was also present in part in the plasma membrane, probably as a consequence of vesicle fusion with the plasma membrane.

In all the previous reports using the VAMP-mutaeq chimera, it was reconstituted with the semisynthetic cofactor coelenterazine *n*, which generates an aequorin chimera with a much lower Ca^{2+}-sensitivity. As we have shown previously (SantoDomingo et al. 2008), this apparently minor technical modification leads, however, to large changes in the results obtained because the resting $[Ca^{2+}]$ in the secretory granules is precisely in the lower limit of the dynamic range of measurement of the VAMP-mutaeq chimera reconstituted with coelenterazine *n* and therefore this aequorin form is hardly sensitive to the $[Ca^{2+}]_{SG}$ changes, particularly to Ca^{2+} release.

The importance of choosing the right aequorin form to measure $[Ca^{2+}]_{SG}$ is also emphasized by the fact that when a much higher-affinity aequorin chimera (native aequorin reconstituted with native coelenterazine) fused to chromogranin A was used to measure $[Ca^{2+}]_{SG}$ in PC12 cells (Mahapatra et al. 2004), saturation with Ca^{2+} of the probe and reduced reconstitution in high-Ca^{2+} compartments led to a large underestimation of the resting $[Ca^{2+}]_{SG}$. The aequorin form used here has intermediate Ca^{2+} affinity and covers precisely the $[Ca^{2+}]$ range present in the secretory granules.

Our results show that the $[Ca^{2+}]_{SG}$ under resting conditions in these cells is around 30–60 μM. Refilling with Ca^{2+} of the vesicular store is usually accompanied by a rapid overshoot in the $[Ca^{2+}]_{SG}$ followed by a prolonged decrease to lower levels. This kind of response may be due to heterogeneity in $[Ca^{2+}]$ among different vesicles. Given that the emission of luminescence by aequorin is Ca^{2+}-dependent and irreversible, if the probe is present in

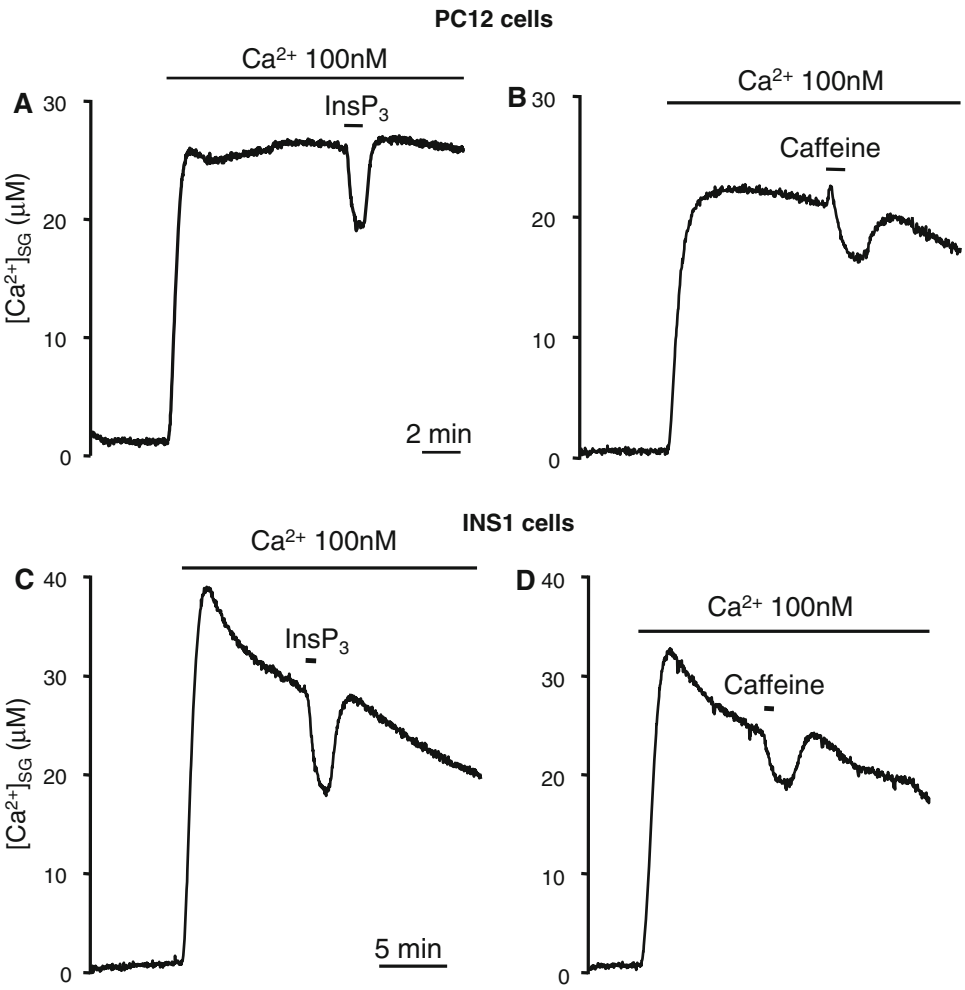

Fig. 3 Effects of $InsP_3$ and caffeine on $[Ca^{2+}]_{SG}$. Initial experimental details as in Fig. 1. Cells were permeabilized and perfused with intracellular medium containing a 100 nM $[Ca^{2+}]$ buffer. Then, either 2 μM $InsP_3$ or 50 mM caffeine were perfused as indicated. **a**, **b** PC12 cells. **c**, **d** INS1 cells. Experiments are representative of 3–4 similar ones of each kind. Statistics is provided in the text as mean ± s.e.m

compartments having different $[Ca^{2+}]$, it will be consumed first in the regions with higher $[Ca^{2+}]$ (providing higher initial values of $[Ca^{2+}]$) and after some time it will report mainly the $[Ca^{2+}]$ present in the compartments with lower $[Ca^{2+}]$. Therefore, the decreasing level of $[Ca^{2+}]_{SG}$ may probably reflect the transition in the measurements between vesicles with higher or lower $[Ca^{2+}]$. These different vesicle compartments could correspond to different states of granule maturation, to different types of vesicles (large dense-core vesicles or synaptic-like microvesicles), or both. The resting $[Ca^{2+}]_{SG}$ values obtained here are about half of those previously reported in chromaffin cells, between 50 and 100 μM (SantoDomingo et al. 2008).

Our results confirm our previous finding in chromaffin cells (SantoDomingo et al. 2008) that secretory vesicles take up Ca^{2+} through SERCA-type Ca^{2+} pumps, sensitive to both thapsigargin and BHQ. In fact, inhibition of SERCA pumps with thapsigargin produced the same effect as the lack of ATP, showing that SERCA pumps are the main responsible of Ca^{2+} uptake by the vesicles. In addition, inhibition of SERCA pumps under resting conditions, that is, when $[Ca^{2+}]_{SG}$ is high, produces an immediate release of Ca^{2+} from the vesicles. This indicates that SERCA pumps are also responsible of maintaining the resting $[Ca^{2+}]_{SG}$. Similar findings have also been reported using a chromogranin–aequorin probe in PC12 cells (Mahapatra et al. 2004). Consistently, the presence of acidic Ca^{2+} stores (probably corresponding to dense-core insulin storage granules) sensitive to thapsigargin and BHQ, has also been described in primary mouse *β*-cells (Duman et al. 2006). However, in rat pancreatic *β*-cells secretion was only partially sensitive to thapsigargin and a Ca^{2+}-storing compartment insensitive to thapsigargin was present (Xie et al. 2006). Our data also contrast with evidence obtained in single-granule studies using pancreatic acinar cells granules (Gerasimenko et al. 1996, 2006), mucin granules from goblet cells (Nguyen et al. 1998) and mast cell secretory granules (Quesada et al. 2001, 2003), where inhibition of SERCA pumps did not modify intragranular Ca^{2+} dynamics. The reasons for the discrepancy may rely in the different properties of the different kinds of secretory granules, and perhaps also in the different methodologies for measuring granular $[Ca^{2+}]$, either in a whole population of heterogeneous granules or in defined

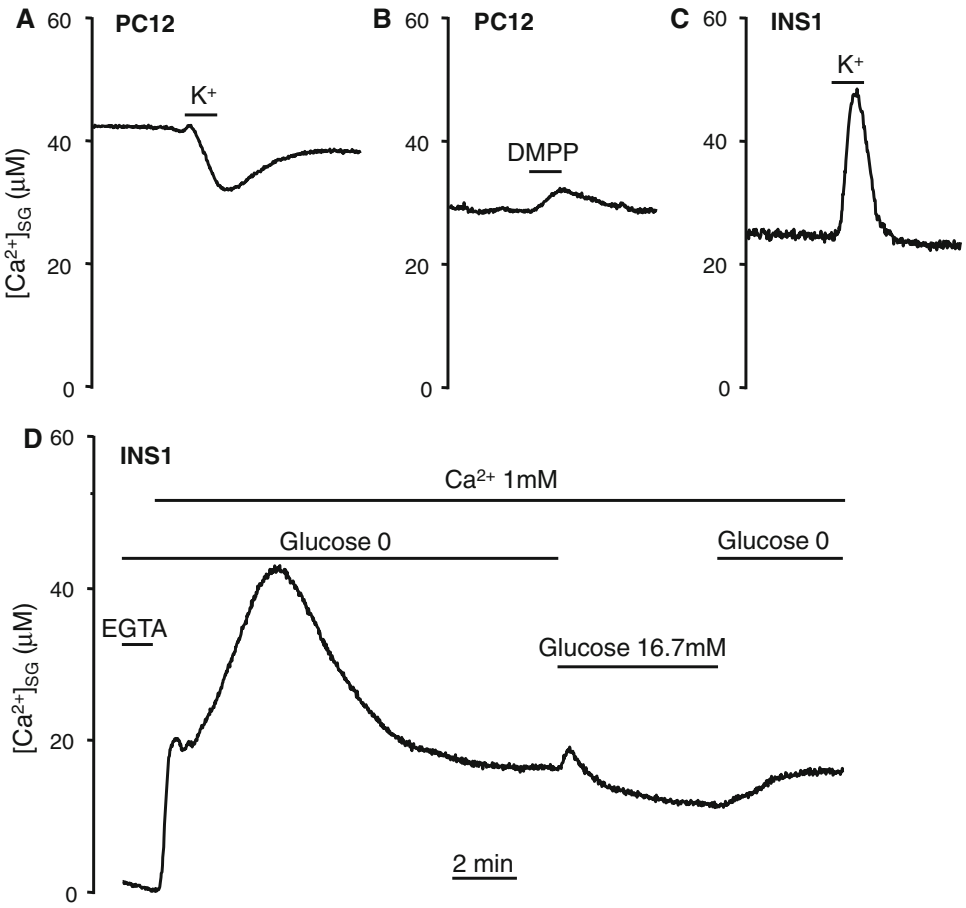

Fig. 4 Effect of cell stimulation on $[Ca^{2+}]_{SG}$. PC12 (**a**, **b**) or INS1 cells (**c**, **d**) expressing VAMP-mutaeq were depleted of Ca^{2+} before reconstitution with native coelenterazine. A pulse of 500 μM La^{3+} was then given, followed by a 5 min wash with Ca^{2+}-free medium and then by perfusion of standard medium containing 1 mM Ca^{2+} to refill the granules with Ca^{2+}. When $[Ca^{2+}]_{SG}$ was at steady-state, either medium containing 70 mM K^+ (**a**, **c**) or 10 μM DMPP (**b**) were added as indicated. In **d**, cells were perfused with medium containing no glucose during the treatment with lanthanum, and during the subsequent wash in Ca^{2+}-free medium and refilling with 1 mM Ca^{2+}. Then, medium containing a higher glucose concentration was perfused as shown in the figure. Experiments are representative of 3–6 similar ones of each kind. Statistics is provided in the text as mean ± s.e.m

single granules. Further work will be required to solve this conflicting point.

Regarding the mechanisms of Ca^{2+} release, we show here that the secretory granules in both PC12 and INS1 cells can release Ca^{2+} when stimulated with $InsP_3$, suggesting the presence of $InsP_3$ receptors in the vesicles. In fact, the presence of $InsP_3R$ in the membrane of chromaffin cell secretory granules has been a matter of debate for a long time (Yoo and Albanesi 1990; Pouli et al. 1998; Huh et al. 2005; Endo et al. 2006; Yoo 2010). Regarding ryanodine receptors, we show that caffeine releases Ca^{2+} from the vesicles, although cyclic ADP ribose did not. The same results were previously found in chromaffin cells, in which cyclic ADP ribose was also unable to release Ca^{2+} from the endoplasmic reticulum (Alonso et al. 1999) or the secretory granules (SantoDomingo et al. 2008). Thus, our results suggest that ryanodine receptors could be also present in the secretory granules, although further evidence is still required on this point. The presence of these Ca^{2+} channels in the membrane of the secretory granules would be very important in order to consider the possible participation of the Ca^{2+} released from the granules in the secretory process.

Stimulation of intact PC12 and INS1 cells by different mechanisms, such as high-K^+ depolarization, the nicotinic agonist DMPP (for PC12 cells) or changes in glucose concentration (for INS1 cells) produced variable responses in $[Ca^{2+}]_{SG}$, including in some cases a biphasic response, an initial increase followed by decrease. The reason for these variable responses, which were also observed in chromaffin cells (SantoDomingo et al. 2008), may rely in the heterogeneity of response among different vesicle populations. A possible explanation, though still speculative at this point, would be that vesicles closer to the plasma membrane would release Ca^{2+} during cell stimulation, but vesicles placed far from the membrane would only respond to the global increase in cytosolic $[Ca^{2+}]$ by activating SERCA pumps and increasing Ca^{2+} uptake. If that explanation applies, depending of the time course and the degree of activation of both mechanisms in each case, the global $[Ca^{2+}]_{SG}$ change measured by our probe would be variable.

In conclusion, or data show that the secretory vesicles of these neuroendocrine cell lines possess mechanisms for active Ca^{2+} uptake and stimulated Ca^{2+} release, and therefore can release Ca^{2+} in the active sites during stimulated secretion. Evidence for the contribution of secretory granules Ca^{2+} to exocytosis has been reported before (Fossier et al. 1998; Scheenen et al. 1998; Mundorf et al. 2000;

Haynes et al. 2006). It has also been shown recently that the Ca^{2+} released from the granules in the presence of bafilomycin dramatically increases granule motion and exocytosis (Camacho et al. 2008). Given that the secretory granules are one of the largest Ca^{2+} stores in neuroendocrine cells, the contribution of the Ca^{2+} released from the vesicles to the generation of local high-Ca^{2+} microdomains responsible of triggering vesicle fusion may be highly significant.

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