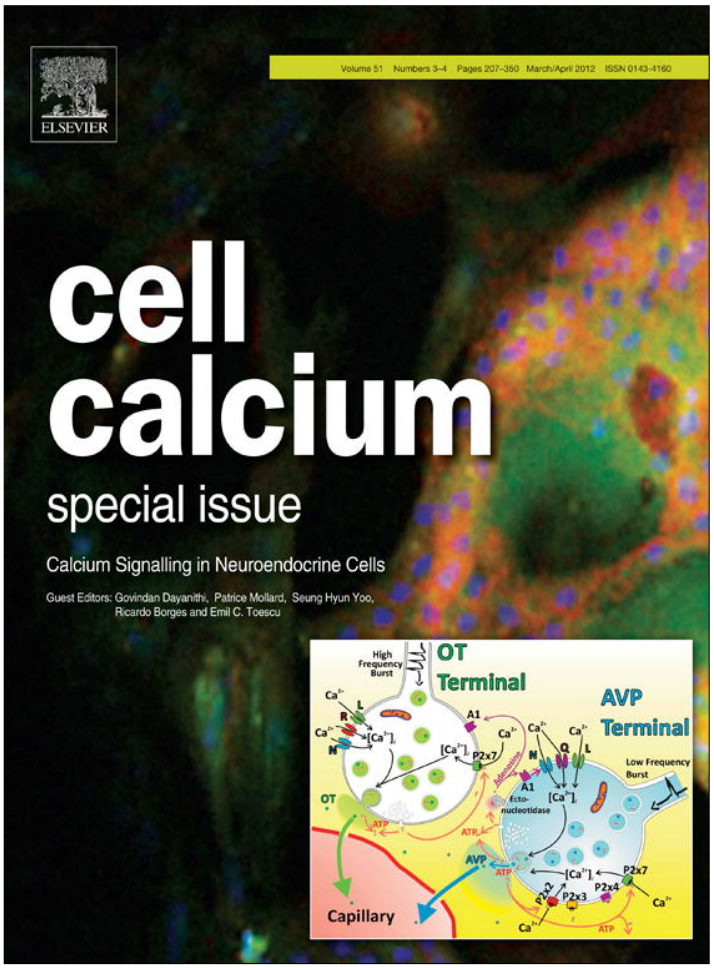

Cell Calcium 51 (2012) 331–337

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Calcium dynamics in the secretory granules of neuroendocrine cells

Javier Álvarez*

Instituto de Biología y Genética Molecular (IBGM), Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Ramón y Cajal, 7, E-47005 Valladolid, Spain

ARTICLE INFO

Article history:
Received 28 September 2011
Received in revised form
22 November 2011
Accepted 4 December 2011
Available online 30 December 2011

Keywords:
Ca^{2+}
Secretory granules
Neuroendocrine
Chromaffin
Inositol trisphosphate receptor
Ryanodine receptor
Ca^{2+}-ATPase

ABSTRACT

Cellular Ca^{2+}signaling results from a complex interplay among a variety of Ca^{2+} fluxes going across the plasma membrane and across the membranes of several organelles, together with the buffering effect of large numbers of Ca^{2+}-binding sites distributed along the cell architecture. Endoplasmic and sarcoplasmic reticulum, mitochondria and even nucleus have all been involved in cellular Ca^{2+} signaling, and the mechanisms for Ca^{2+} uptake and release from these organelles are well known. In neuroendocrine cells, the secretory granules also constitute a very important Ca^{2+}-storing organelle, and the possible role of the stored Ca^{2+} as a trigger for secretion has attracted considerable attention. However, this possibility is frequently overlooked, and the main reason for that is that there is still considerable uncertainty on the main questions related with granular Ca^{2+} dynamics, e.g., the free granular $[Ca^{2+}]$, the physical state of the stored Ca^{2+} or the mechanisms for Ca^{2+} accumulation and release from the granules. This review will give a critical overview of the present state of knowledge and the main conflicting points on secretory granule Ca^{2+} homeostasis in neuroendocrine cells.

1. Introduction

Ca^{2+} is an intracellular second messenger able to fulfill a variety of different functions in all cell types with no exception, both excitable and non-excitable cells. Let us just mention muscle contraction, neurotransmitter secretion, fertilization, proliferation, development, learning, memory or cell death [1]. This versatility can only be achieved thanks to a very precise control of its concentration at the subcellular level. In fact, activation of these processes usually takes place as a consequence of transient increases in the cytosolic $[Ca^{2+}]$ in specific subcellular locations, which are variable depending on the process. These hot spots or local $[Ca^{2+}]$ microdomains have a critical importance in Ca^{2+} signaling and their size and dynamics depends on the spatial disposition of the different Ca^{2+} transport systems (channels, exchangers, pumps, etc.) both in the plasma membrane and in intracellular organelles.

Because of the strong Ca^{2+} buffering of the cytosol (free Ca^{2+} constitutes only 1% of the total Ca^{2+} in the cytosol), diffusion of Ca^{2+} through the cytosol is severely limited, particularly during small, physiological, cell stimulations. Intracellular organelles are therefore essential to generate or control local $[Ca^{2+}]$ microdomains at different intracellular locations. The first organelle that was known to have an important role in Ca^{2+} homeostasis was the endoplasmic reticulum (ER) [2]. This organelle behaves as a dynamic Ca^{2+} store. It accumulates Ca^{2+} in its lumen through the SERCA pumps up to near millimolar levels [3], and is able to induce a rapid release of Ca^{2+} to the cytosol via several specific Ca^{2+} channels present in its membrane ($InsP_3$ and ryanodine receptors, mainly). In addition, both the ER and the sarcoplasmic reticulum of muscle cells contain Ca^{2+}-binding proteins with high-capacity and low-affinity that make the total calcium content of the ER to be about 10-fold larger than the free $[Ca^{2+}]_{ER}$. The more abundant, calreticulin in the ER and calsequestrin in the SR, have Ca^{2+}-affinities of 1–4 mM [2], close to the free $[Ca^{2+}]_{ER}$, thus facilitating fast binding and release of Ca^{2+} following the changes in free $[Ca^{2+}]_{ER}$.

Another organelle with an important role in cell Ca^{2+} homeostasis is mitochondria, the organelle responsible of aerobic energy production in the cell. The ability of mitochondria to take up large amounts of Ca^{2+} was known since the sixties. However, the low Ca^{2+}-affinity of the mitochondrial Ca^{2+}uniporter (K_M above 10 μM, 100-fold higher than the resting cytosolic $[Ca^{2+}]$, 100 nM, and 10-fold higher than the usual peak cytosolic $[Ca^{2+}]$ found during cell stimulation, about 1 μM) led to most researchers in the 1970s and 1980s to exclude a significant role in Ca^{2+} homeostasis for this organelle, at least under physiological conditions. Then, in the beginning of the 1990s, recombinant aequorin targeted to the mitochondria showed that mitochondrial $[Ca^{2+}]$ was able to undergo rapid changes during cell activation [4]. That was the origin of a series of works by several research groups demonstrating that mitochondria were very active players in the control of the global cell Ca^{2+} homeostasis. In particular, because of the low-Ca^{2+}-affinity of their Ca^{2+} uptake mechanism, mitochondria are very well

* Tel.: +34 983 423085; fax: +34 983 423588.
E-mail address: jalvarez@ibgm.uva.es

0143-4160/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2011.12.002

adapted to take up Ca^{2+} from local high-Ca^{2+}microdomains, such as those formed after the activation of plasma membrane or endoplasmic reticulum Ca^{2+} channels. Mitochondria can therefore modulate important cellular functions such as neurotransmitter secretion by acting as local Ca^{2+} sinks [5].

The subject of this review, the secretory granules, constitutes one of the less known compartments in terms of Ca^{2+} dynamics. It is widely known that the increase in intracellular [Ca^{2+}] levels triggers exocytosis of a pool of docked secretory vesicles in neuronal and neuroendocrine cells. These vesicles are attached to the plasma membrane and are also very close to portions of ER and mitochondria. Thus, it is generally assumed that the Ca^{2+} required for exocytosis may enter from the extracellular medium through several kinds of plasma membrane Ca^{2+} channels [6] or be released from the endoplasmic reticulum through either inositol trisphosphate ($InsP_3$) or ryanodine receptors [7]. Nearby mitochondria may also contribute to modulate the size of the local [Ca^{2+}] microdomain responsible for vesicle fusion and secretion. 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 calcium [8], and the total calcium concentration inside the granules has been estimated to be around 40 mM [9], much higher than that in the ER (about 5–10 mM, see [2]). Of course, an attractive possibility would be that intragranular calcium could contribute to its own secretion by being released through Ca^{2+} channels placed in the granule membrane. In that way, Ca^{2+} would be released precisely in the right place to trigger secretion. What is then the reason for this apparent oversight? As we will discuss in this review, the main reason for that is that there is still little and sometimes contradictory information on the dynamics of Ca^{2+}in the granules. Thus, the main aspects of Ca^{2+} homeostasis in the granules, that is, the free $[Ca^{2+}]_{SG}$ and the mechanisms for Ca^{2+} accumulation and release, are still controversial.

2. The problem of the free and total calcium concentration in the secretory granules

Studies in the 1980s showed that the total calcium concentration in the secretory granules is 40–80 nmol/mg of protein [10,11], which considering a protein/water relationship in the granules of about 0.5 mg/μl [12], implies that the total calcium concentration in the granules is 20–40 mM. Winkler and Westhead [9] calculated a total of 90,000 Ca^{2+} ions for a vesicle with 270 nm internal diameter, which means a concentration of 15 mM. Studies of total calcium made by electron energy loss imaging spectroscopy in PC12 cells show also clearly that dense granules have a high calcium content (>10 mM), with a signal comparable to that obtained in the Golgi complex and some regions of the ER [13]. Accordingly, in PC12 cells, the acidic calcium pool accounted for 170 μmol/l cell water, which corresponds to an internal total concentration of about 30 mM [14]. This high calcium content is not exclusive of chromaffin granules but instead it is also found at similar or even higher values in most types of secretory granules, e.g. zymogen granules of pancreatic acinar cells [15], small synaptic vesicles of the neuromuscular junction [13], insulin granules [16] and many others (see [17] for a review).

Therefore, secretory granules appear to be the organelles with the higher calcium content of the cell. Other organelles with high calcium content are the endoplasmic reticulum and Golgi apparatus. Electron microscopy techniques have shown that the total calcium content of the ER from different cell types is in the range of 5–50 mM, with the highest values in the terminal cisternae of the sarcoplasmic reticulum and mean values of 5–10 mM [2,18], that is, 5–10-fold lower than in the secretory granules. The free [Ca^{2+}] in the ER has been also measured with low-Ca^{2+}-affinity aequorin and is in the 500–800 μM range [7], so that the Ca^{2+}-bound/Ca^{2+}-free relationship in the ER is about 10. In addition, the total calcium distribution in the ER is heterogeneous, with strongly positive cisternae (including the nuclear envelope) lying in the proximity of or even in direct continuity with other, apparently negative cisternae [13]. This heterogeneity must surely reflect the distribution of the Ca^{2+}-binding proteins in the lumen of the ER, as most of the calcium content of the ER is bound with low affinity (K_d = 1–4 mM) to several Ca^{2+}-binding proteins, mainly calreticulin in the ER and calsequestrin in the SR [2,18]. The K_d for Ca^{2+} binding of these proteins is therefore close to the free [Ca^{2+}] in the ER, assuring an efficient binding.

Many studies have tried to estimate the free [Ca^{2+}] in the secretory granules. Bulenda and Gratzl [10] and Haigh et al. [8], using null-point titration techniques, found values of 24 μM and 5.6 μM, respectively, in isolated acidic chromaffin vesicles. In mast cell granules, studies with fluorescent dyes showed a resting intraluminal free [Ca^{2+}] of 25 μM [19]. In the β-cell line MIN6, data obtained with targeted aequorin indicated a free [Ca^{2+}] in the vesicles around 50 μM [20]. These values contrast with the 1.4 μM [Ca^{2+}] value obtained by Mahapatra et al. [21] in PC12 chromaffin granules, using also targeted aequorin. The discrepancy may probably be due to the higher Ca^{2+}-affinity of the aequorin type used in the last study. Because aequorin is consumed faster in high-Ca^{2+} environments, high-Ca^{2+}-affinity aequorin is rapidly consumed in those regions and finally tends to reflect only the behavior of minor compartments with low [Ca^{2+}] [22]. Using an aequorin with lower Ca^{2+}-affinity and correcting for the effect of acidic pH on aequorin luminescence, we have reported free [Ca^{2+}] values of 50–100 μM in chromaffin granules [23] and 20–40 μM in PC12 and INS1 cells [24]. We should note also that the small size of some of the vesicles poses some restrictions to the possible values of free granular [Ca^{2+}]. Let us just mention that for a small synaptic vesicle, with a diameter of 50 nm, 25 μM [Ca^{2+}] would be obtained with only 1 Ca^{2+} ion, and even for a large dense core vesicle of 200 nm diameter, just 2–3 Ca^{2+} ions per vesicle would represent 1 μM [Ca^{2+}] (that would give figures of 100–300 free Ca^{2+} ions per vesicle).

In conclusion, the free [Ca^{2+}] in several types of secretory granules appears to range between 20 and 100 μM. These values are significantly lower, nearly by one order of magnitude, than the free [Ca^{2+}] in the endoplasmic reticulum, which is about 500 μM [3,25]. Given that the total calcium content is larger in the vesicles than in the ER also by nearly one order of magnitude in terms of concentration, this means that the Ca^{2+} bound/Ca^{2+} free relationship is much higher (almost two orders of magnitude) in the vesicles than in the ER, from values of 10–20 in the ER to nearly 1000 in the vesicles. The question then is where all those Ca^{2+}-binding sites are. It is generally assumed that the large calcium storing capacity of the secretory granules is mainly due to the presence of high concentrations (about 2 mM) of the proteins chromogranin A and B and secretogranin [26]. In bovine chromaffin cells, chromogranin A constitutes about 90% of these Ca^{2+}-binding proteins (1.8 mM), and it is able to bind 55 mol of Ca^{2+} per mol of protein, with a K_d of 4 mM [26]. The Ca^{2+}-binding capacity of these proteins is therefore very large and could store up to 100 mM total calcium. However, the affinity is too low if we consider that the free $[Ca^{2+}]_{SG}$ remains below 100 μM. In fact, even considering a free granular [Ca^{2+}] of 100 μM, binding to a protein with a K_d of 4 mM would hardly fill 2.5% of the available binding sites. In our case, this binding would explain 2.5 mM of the stored calcium, that is, less than 10% of the calcium present in the granules.

How can then we explain the large Ca^{2+}-storing capacity of the granules? There are several alternatives: (i) the Ca^{2+} affinity of these Ca^{2+}-binding proteins could be different in the conditions of the secretory granules, that is, at very high protein concentrations (0.5 mg/μl) and in the presence of 500–1000 mM catecholamines

and 125–300 mM ATP; (ii) early studies described a significant number of higher affinity Ca^{2+}-binding sites in vesicle matrix proteins, that would be able to bind up to 180 nmol Ca^{2+}/mg protein with affinities in the 100–300 μM range depending on the pH and ionic conditions [27,28]; and (iii) part of the calcium could also be bound to other vesicle components, such as nucleotides or lipids. Regarding the nucleotides, they are present at high concentrations and can bind Ca^{2+} with a K_d in the 100–300 μM range depending on the pH and ionic conditions [27]. However, catecholamines and ATP have been shown to interact at the concentrations found in the granules forming highly non-ideal solutions that considerably reduce osmotic pressure [29]. Given that this interaction does not require calcium, and that most of vesicle ATP and catecholamines appear to be involved in these interactions, probably only a small part of the total ATP is available to bind Ca^{2+} in the vesicles.

A final point regards the availability of the stored calcium for exchange from the binding sites. In the ER and SR, the Ca^{2+} bound to calreticulin or calsequestrin is in fast equilibrium with the free Ca^{2+}, and it is therefore readily available for release from the binding sites when the luminal free [Ca^{2+}] is reduced. In that way, when the Ca^{2+}-channels in the ER or SR (e.g., $InsP_3$ or ryanodine receptors) are activated, all the stored Ca^{2+} can contribute to the release into the cytosol. In the case of the secretory granules, some reports suggest that at least part of the stored calcium is relatively immobile or exchanges very slowly with the free Ca^{2+} pool. Clemente and Meldolesi [15] found that zymogen granules have a very slow rate of ^{45}Ca accumulation and release compared with other organelles, suggesting that the stored Ca^{2+} could play mainly a structural role in the architecture of the organelle. Similarly, ^{45}Ca flux experiments performed in PC12 cells showed that the granule Ca^{2+} pool was extraordinarily stable in periods of hours in EGTA-containing medium, and very slow to label [14], concluding that the stored Ca^{2+} would only serve to keep granule components together and to be discharged to the extracellular space by exocytosis. The question then relies in the nature and the physical state of the contents of the granule. Evidence from several techniques indicates that no important fraction of the small molecules of the granule is in an immobilized or precipitated form (see [9] for a review). However, complexes between ATP and catecholamines and perhaps other components must necessarily exist to explain the osmotic properties of the granule [29]. In addition, chromogranins have been shown to aggregate or form tetramers in a Ca^{2+}-dependent way [28,30,31]. These complexes could perhaps trap in a stable form some of the stored calcium.

As a conclusion, Ca^{2+} ions in the secretory granules can be in three different states: (i) a stable, slowly exchanging pool which may be important for the structure of the granular components; (ii) a pool bound to chromogranins or other granular components with an affinity close to the free $[Ca^{2+}]_{SG}$, readily available for exchange and whose size may depend on $[Ca^{2+}]_c$ variations or other cellular conditions; and (iii) the free Ca^{2+} pool. The relative size of these pools may depend on the type of granule and the cellular conditions.

3. Mechanisms of Ca^{2+} accumulation in the secretory granules

If the free $[Ca^{2+}]_{SG}$ is in the 20–100 μM range, there should be in the membrane of the secretory granules active mechanisms to accumulate this ion in the matrix, given that its concentration in the cytosol is nearly 1000-fold lower. Several candidates have been proposed for this role (see the cartoon in Fig. 1).

Krieger-Brauer and Gratzl [32] found evidence for the presence of a $2Na^+/1Ca^{2+}$ exchange in the membrane of the secretory granules of bovine chromaffin cells. The system was pH-independent and had high affinity for Ca^{2+} (0.28 μM [33]), so that it was still partially active at 100 nM extragranular [Ca^{2+}], although the rate increased at higher concentrations. Haigh and Phillips [34] proposed that Na^+/Ca^{2+} exchange could be coupled to the H^+ pump via a Na^+/H^+ exchange, but the rates of uptake they found in chromaffin granule ghosts were very slow. The capacity of this system to accumulate Ca^{2+} in the vesicles depends on the magnitude of the Na^+ gradient. The intragranular [Na^+] was estimated by Kriegler-Brauer and Gratzl [32,33] about 50 mM. Haigh et al. [8] found also that the sum of the intragranular [Na^+] and [K^+] was 50–60 mM, the actual amount of each one depending of the isolation procedure. In any case, this means that the [Na^+] gradient between the granule matrix and the cytosol is no larger than 3-fold. Thus, with a stoichiometry of $2Na^+/1Ca^{2+}$, this system would not be able to accumulate Ca^{2+} more than 10-fold inside the granules, much less than the existing gradient. In fact, with a [Ca^{2+}] gradient of 1000-fold, this system would mainly work releasing Ca^{2+} from the vesicles in exchange for Na^+. It has been actually proposed that Na^+/Ca^{2+} exchange may mediate the increase in intravesicular [Na^+] that coincides with the onset of secretion in neurohypophysial nerve endings [35].

The presence of a Ca^{2+}/H^+antiport has been reported in synaptic vesicles of brain cortex [36]. Combined with the H^+-ATPase and assuming a stoichiometry of $1Ca^{2+}/2H^+$, this system would be able to accumulate Ca^{2+}nearly 1000-fold into the vesicles. However, that transport system showed very low Ca^{2+} affinity for Ca^{2+} entry, starting to be active at cytosolic [Ca^{2+}] around 100 μM. Thus, under these conditions it could not be involved in physiological Ca^{2+} uptake into the vesicles, although it may perhaps mediate Ca^{2+}-release from the vesicles following changes in the [H^+] gradient. In fact, the disruption of the vesicular pH gradient induces a significant leak of Ca^{2+} from the vesicles [37,38].

The last possibility is a direct ATP-dependent mechanism for Ca^{2+} accumulation, that is, a Ca^{2+}-ATPase. The sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor 2,5-diterbutyl benzohydroquinone (BHQ) has been shown to inhibit Ca^{2+} uptake into isolated synaptic vesicles [39]. In platelets, the acidic Ca^{2+} pool was controlled both by a Ca^{2+}/H^+ exchange and by a BHQ-sensitive SERCA 3 [40,41]. Similarly, loading with Ca^{2+} of dense-core insulin storage granules of mouse pancreatic β-cells was sensitive to both SERCA inhibitors BHQ and thapsigargin [42]. More recently, using aequorin to monitor intragranular [Ca^{2+}] we have also described both in chromaffin cells and in the cell lines PC12 and INS1 [23,24], that Ca^{2+} uptake by the secretory granules was ATP-dependent and fully sensitive to thapsigargin and BHQ (see Fig. 1). On the other hand, in the β-cell line MIN6, Ca^{2+} uptake was shown to be ATP-dependent but insensitive to thapsigargin [20], suggesting the involvement of a different type of Ca^{2+}-ATPase, the secretory pathway Ca^{2+}-ATPase or ATP2C1, which is also present in the Golgi apparatus together with the SERCA [43].

Recent data obtained from proteomic studies of the secretory granules in several cell types provide new important information on this point, though do not clarify it. A recent proteomic study in the secretory granules of chromaffin cells has identified 371 distinct soluble proteins and 384 distinct membrane proteins [44]. However, among them, the only membrane protein able to accumulate calcium in the vesicles was a sodium/potassium/calcium exchanger. Previous studies in pancreatic zymogen granules [45] (371 proteins identified) and corticotropes dense-core secretory granules [46] (150 proteins identified) had not identified any protein related with Ca^{2+} accumulation. Instead, a proteomic study that identified 270 proteins in secretory vesicles from insulinoma NIT-1 cells showed the presence of SERCA2 in the membrane of the vesicles [47], but another study in the insulin-secreting cell line INS-1E that identified 150 vesicular proteins failed to find any Ca^{2+} transport protein [48].

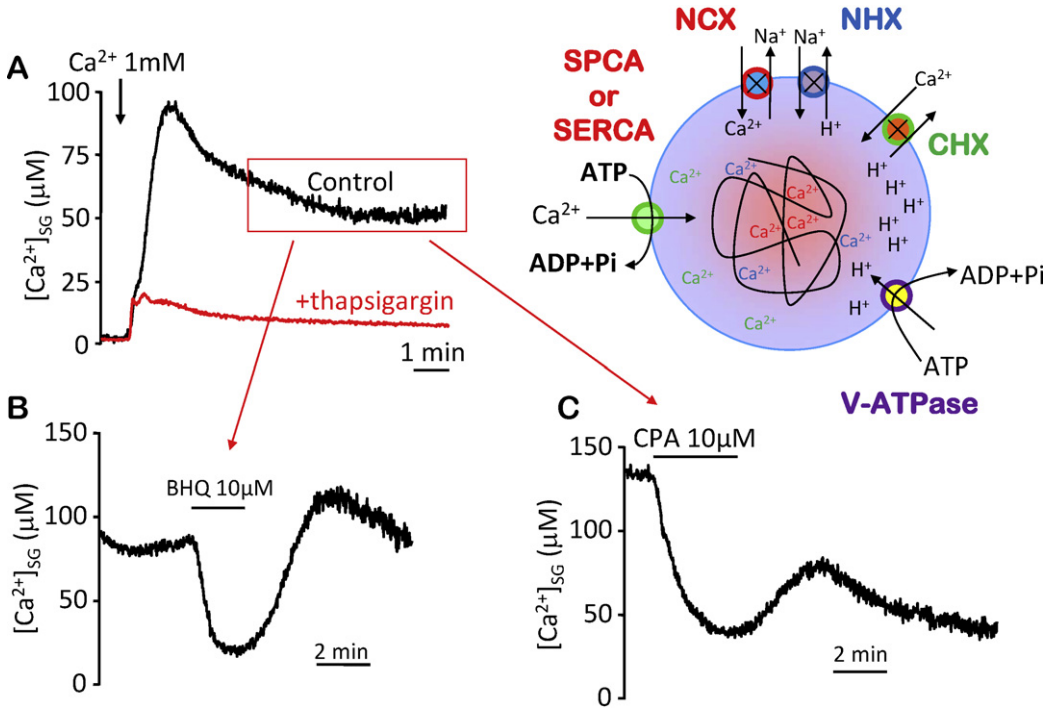

Fig. 1. Mechanisms of Ca^{2+} accumulation in the granules. Traces correspond to $[Ca^{2+}]_{SG}$ measurements made with chromaffin cells expressing aequorin targeted to the secretory granules (modified from Ref. [23]). Panel A shows the increase in $[Ca^{2+}]_{SG}$ that occurs upon readdition of Ca^{2+} 1 mM to the extracellular medium in Ca^{2+}-depleted cells, and its inhibition by thapsigargin. Panels B and C show that the SERCA inhibitors 2,5-diterbutyl benzohydroquinone (BHQ) and cyclopiazonic acid (CPA) both reversibly inhibit Ca^{2+} uptake by the granules when added after refilling of the granules with Ca^{2+} (at the point marked by the red rectangle in panel A). The cartoon resumes the possible mechanisms for Ca^{2+} accumulation in the granules: Ca^{2+} pumps, either SERCA or secretory pathway Ca^{2+}-ATPase (SPCA), Na^+/Ca^{2+} exchange coupled with Na^+/H^+ exchange, and Ca^{2+}/H^+ exchange. The exchangers would be energized by the H^+ gradient created by the H^+-ATPase. Ca^{2+} inside the granules is represented in three possible forms, strongly bound (red), bound and readily releasable (blue) and free (green).

4. Mechanisms of Ca^{2+} release from the secretory granules

If the Ca^{2+} stored in the granules has to be released during cell stimulation to cooperate with Ca^{2+} coming from other sources in triggering vesicle fusion and secretion, the granules need to have Ca^{2+} transport systems that are able to open or activate during cell stimulation (see the cartoon in Fig. 2). The first system to be proposed for that role in chromaffin cells was the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). The presence of $InsP_3R$ in secretory granules has been a highly conflicting point over the

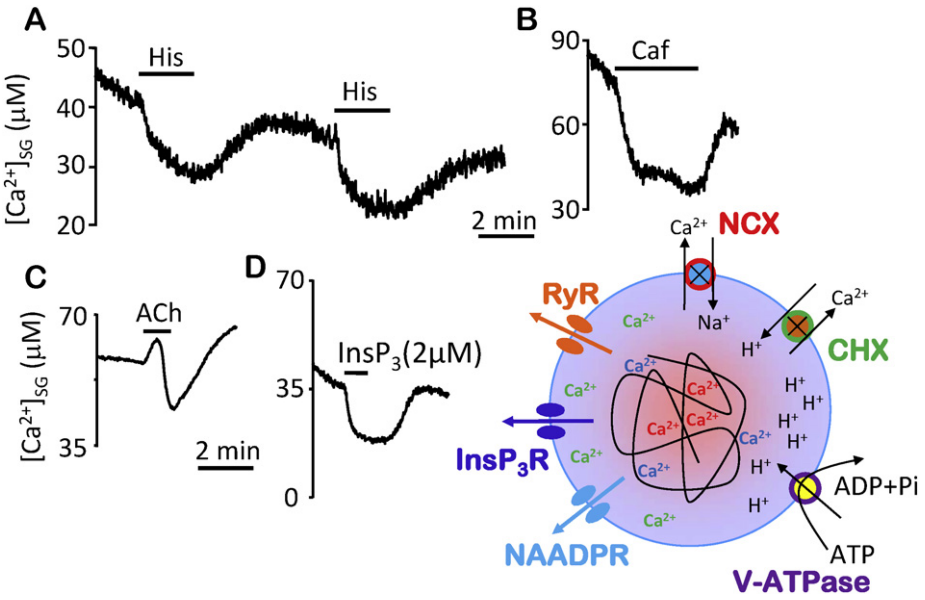

Fig. 2. Mechanisms of Ca^{2+} release from the granules. Traces correspond to $[Ca^{2+}]_{SG}$ measurements made with chromaffin cells expressing aequorin targeted to the secretory granules (modified from Ref. [23]). Panels A and B show the Ca^{2+} release from the granules induced by histamine (via $InsP_3$ production) and by caffeine (RyR activator). Panel C shows the effect of the natural agonist acethylcholine, and panel D shows the Ca^{2+} release induced directly by $InsP_3$ in permeabilized cells. The cartoon resumes the possible mechanisms for Ca^{2+} release from the granules: three Ca^{2+} channels ($InsP_3$, ryanodine and NAADP receptors), and 2 exchangers, Na^+/Ca^{2+} exchanger and H^+/Ca^{2+} exchanger, that under some circumstances, namely after an increase in granular pH, may act extruding Ca^{2+} from the vesicles.

last 20 years. In 1990, Yoo and Albanesi [49] reported that inositol 1,4,5-trisphosphate ($InsP_3$) released Ca^{2+} from chromaffin cells secretory vesicles. Immunocytochemical localization of all three types of $InsP_3R$ in serially sectioned bovine chromaffin cells showed that the secretory granules contained about 60% of the $InsP_3R$ in the cell [50], and additional evidence for the presence of $InsP_3R$ in the granules was the finding that both chromogranin A and B interact with $InsP_3R$ and modulate its activity [26,51–54]. More recently, using targeted aequorin to measure [Ca^{2+}] in the secretory granules of chromaffin cells and the neurosecretory cell lines PC12 and INS1 [23,24], we have shown that $InsP_3$ and $InsP_3$-releasing agonists can release Ca^{2+} from the granular compartment (see Fig. 2).

$InsP_3R$ have been also detected in other types of secretory granules. Isolated zymogen granules were found to release Ca^{2+} in the presence of $InsP_3$ [55], although the same authors have also reported that in intact cells most of the Ca^{2+} released by $InsP_3$ comes from the ER [56]. In mast cell granules [19] and mucin granules of goblet cells [57], $InsP_3$ induced periodic oscillations of luminal [Ca^{2+}] by coupling Ca^{2+} release with the activity of a Ca^{2+}-dependent K^+ channel in the granules. Finally, $InsP_3R$ have been also localized in the secretory granules of insulin-secreting β-cells and somatostatin-secreting cells [58–60], and also in dense-core vesicles of astrocytes [61].

However, there is also a large body of evidence against the presence of $InsP_3R$ in secretory granules. In chromaffin cells, Endo et al. [62] found that $InsP_3R$-2 was present in the ER but not in the secretory granules. In rat insulinoma cells, $InsP_3$ was found to release Ca^{2+} from the microsomal fraction but not from the secretory granules [63]. Ravazzola et al. [64] found that antibodies against $InsP_3R$ cross-reacted with insulin, and that could be a source for artifacts in immunostaining (see also [65]). Measuring the extragranular [Ca^{2+}] with a phogrin–aequorin chimera, Pouli et al. [66] found no difference among the Ca^{2+} transients in the granule membrane and in the bulk cytosol after insulin-secreting MIN6 or phaeochromocytoma PC12 cells stimulation, indicating that no significant local Ca^{2+} release takes place in these cells. Similarly, using a targeted aequorin to monitor granule [Ca^{2+}], no effect of $InsP_3$ was observed in MIN6 cells [20]. Regarding zymogen granules, $InsP_3R$ were not found using highly pure granule preparations [67] and the absence of zymogen granules did not modify the cytosolic Ca^{2+} transients in rat parotid acinar cells [68]. Finally, in platelets, $InsP_3R$ are present in the dense tubular system (the equivalent of the ER) but not in the acidic granules [41].

There is also evidence for the presence of ryanodine receptors in secretory granules. The ryanodine receptor activator cyclic ADP ribose (cADPR) has been reported to release Ca^{2+} from zymogen granules [55,69]. Both cADPR and caffeine released also Ca^{2+} from the secretory granules of insulin-secreting MIN6 cells [20], and caffeine, but not cADPR, released Ca^{2+} from secretory granules in chromaffin cells [23]. The lack of effect of cADPR may reflect an insensitivity to this agonist of the ryanodine receptors in these cells, as cADPR was also unable to release Ca^{2+} from the ER in chromaffin cells [7]. The novel agonist nicotinic acid adenine dinucleotidephosphate (NAADP) was also effective to release Ca^{2+} from zymogen granules [69], platelet acidic stores [41], insulin-secreting MIN6 cells [70] and the acidic Ca^{2+} stores of PC12 cells [71]. The identity of the NAADP receptor is still unknown. It was initially associated with the ryanodine receptor, although other alternatives have recently appeared (two pore channels, TPCs or TPCNs, see [72]).

What do proteomic studies tell us on this point? None of the proteomic studies made up to date has detected the presence of $InsP_3R$ in the secretory granules of chromaffin [44], insulin [47,48], zymogen [45] or corticotrope granules [46]. Instead, the largest study [44] found in chromaffin granules the presence of ryanodine receptors together with several types of Ca^{2+} channels (voltage-dependent and transient receptor potential). However, none of these proteins were detected in the other studies. Of course, if granular [Ca^{2+}] is nearly 1000-fold higher than in the cytosol, at least some mechanism to accumulate Ca^{2+} in the granules must exist. This means that the sensitivity of these studies may not be still enough to detect proteins at very low concentrations, and further improvement of this technique may provide in the future more useful information.

5. Conclusion

In neuroendocrine cells, the secretory granules constitute one of the largest Ca^{2+} stores and its total calcium concentration is even higher than that in the ER. Given that Ca^{2+} is the key signal that triggers secretion of granule contents, the possible functional role of the calcium stored in the granules has attracted considerable attention for more than thirty years. The available evidence suggests moreover that intragranular Ca^{2+} is important for secretion [37,73–75]. However, in spite of the efforts of many research groups, most of the key questions related with granule Ca^{2+} homeostasis, e.g., the free granular [Ca^{2+}], the physical state of the stored Ca^{2+} or the mechanisms for Ca^{2+} accumulation and release from the granules, remain obscure. Further work will be necessary to clarify all these uncertainties. Besides the biochemical and physiological approaches, the new proteomic techniques look promising, but the data obtained up to date on proteins related to Ca^{2+} dynamics is still scarce and too variable among secretory granules of different cell types.

Acknowledgments

This work was supported by grants from Ministerio de Educación y Ciencia (BFU2008-01871) and from Junta de Castilla y León (GR105).

References

[1] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling, Nat. Rev. 1 (2000) 11–21.
[2] T. Pozzan, R. Rizzuto, P. Volpe, J. Meldolesi, Molecular and cellular physiology of intracellular calcium stores, Physiol. Rev. 74 (1994) 595–636.
[3] M. Montero, M.J. Barrero, J. Alvarez, [Ca^{2+}] microdomains control agonist-induced Ca^{2+} release in intact HeLa cells, FASEB J. 11 (1997) 881–885.
[4] R. Rizzuto, A.W. Simpson, M. Brini, T. Pozzan, Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin, Nature 358 (1992) 325–327.
[5] M. Montero, M.T. Alonso, E. Carnicero, I. Cuchillo-Ibañez, A. Albillos, A.G. Garcia, J. Garcia-Sancho, J. Alvarez, Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion, Nat. Cell Biol. 2 (2000) 57–61.
[6] A.G. García, A.M. García de Diego, L. Gandía, R. Borges, J. García-Sancho, Calcium signaling and exocytosis in adrenal chromaffin cells, Physiol. Rev. 86 (2006) 1093–1131.
[7] M.T. Alonso, M.J. Barrero, P. Michelena, E. Carnicero, I. Cuchillo, A.G. García, J. García-Sancho, M. Montero, J. Alvarez, Ca^{2+}-induced Ca^{2+} release in chromaffin cells seen from inside the ER with targeted aequorin, J. Cell Biol. 144 (1999) 241–254.
[8] J.R. Haigh, R. Parris, J.H. Phillips, Free concentrations of sodium, potassium and calcium in chromaffin granules, Biochem. J. 259 (1989) 485–491.
[9] H. Winkler, E. Westhead, The molecular organization of adrenal chromaffin granules, Neuroscience 5 (1980) 1803–1823.
[10] D. Bulenda, M. Gratzl, Matrix free Ca^{2+} in isolated chromaffin vesicles, Biochemistry 24 (1985) 7760–7765.
[11] H. Krieger-Brauer, M. Gratzl, Influx of Ca^{2+} into isolated secretory vesicles from adrenal medulla. Influence of external K^+ and Na^+, FEBS Lett. 133 (1981) 244–246.
[12] R.G. Johnson, A. Scarpa, Ion permeability of isolated chromaffin granules, J. Gen. Physiol. 68 (1976) 601–631.
[13] R. Pezzati, M. Bossi, P. Podini, J. Meldolesi, F. Grohovaz, High-resolution calcium mapping of the endoplasmic reticulum-Golgi-exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells, Mol. Biol. Cell 8 (1997) 1501–1512.
[14] C. Fasolato, M. Zottini, E. Clementi, D. Zacchetti, J. Meldolesi, T. Pozzan, Intracellular Ca^{2+} pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release, J. Biol. Chem. 266 (1991) 20159–20167.

[15] F. Clemente, J. Meldolesi, Calcium and pancreatic secretion-dynamics of subcellular calcium pools in resting and stimulated acinar cells, Br. J. Pharmacol. 55 (1975) 369–379.
[16] J.C. Hutton, E.J. Penn, M. Peshavaria, Low-molecular-weight constituents of isolated insulin-secretory granules. Bivalent cations, adenine nucleotides and inorganic phosphate, Biochem. J. 210 (1983) 297–305.
[17] G. Nicaise, K. Maggio, S. Thirion, M. Horoyan, E. Keicher, The calcium loading of secretory granules. A possible key event in stimulus-secretion coupling, Biol. Cell 75 (1992) 89–99.
[18] J. Meldolesi, T. Pozzan, The endoplasmic reticulum Ca^{2+} store: a view from the lumen, Trends Biochem. Sci. 23 (1998) 10–14.
[19] I. Quesada, W.C. Chin, J. Steed, P. Campos-Bedolla, P. Verdugo, Mouse mast cell secretory granules can function as intracellular ionic oscillators, Biophys. J. 80 (2001) 2133–2139.
[20] K.J. Mitchell, P. Pinton, A. Varadi, C. Tacchetti, E.K. Ainscow, T. Pozzan, R. Rizzuto, G.A. Rutter, Dense core secretory vesicles revealed as a dynamic Ca^{2+} store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimaera, J. Cell Biol. 155 (2001) 41–51.
[21] N.R. Mahapatra, M. Mahata, P.P. Hazra, P.M. McDonough, D.T. O'Connor, S.K. Mahata, A dynamic pool of calcium in catecholamine storage vesicles: exploration in living cells by a novel vesicle-targeted chromogranin A/aequorin chimeric photoprotein, J. Biol. Chem. 279 (2004) 51107–51121.
[22] M. Montero, J. Alvarez, W.J. Scheenen, R. Rizzuto, J. Meldolesi, T. Pozzan, Ca^{2+} homeostasis in the endoplasmic reticulum: coexistence of high and low [Ca^{2+}] subcompartments in intact HeLa cells, J. Cell Biol. 139 (1997) 601–611.
[23] J. SantoDomingo, L. Vay, M. Camacho, E. Hernández-SanMiguel, R.I. Fonteriz, C.D. Lobatón, M. Montero, A. Moreno, J. Alvarez, Calciumdynamics in bovine adrenal medulla chromaffin cellsecretory granules, Eur. J. Neurosci. 28 (2008) 1265–1274.
[24] J. SantoDomingo, R.I. Fonteriz, C.D. Lobatón, M. Montero, A. Moreno, J. Alvarez, Ca^{2+} dynamics in the secretory vesicles of neurosecretory PC12 and INS1 cells, Cell. Mol. Neurobiol. 30 (2010) 1267–1274.
[25] J. Alvarez, M. Montero, Measuring [Ca^{2+}] in the endoplasmic reticulum with aequorin, Cell Calcium 32 (2002) 251–260.
[26] S.H. Yoo, Secretory granules in inositol 1,4,5-trisphosphate-dependent Ca^{2+}signalingin the cytoplasm of neuroendocrine cells, FASEB J. 24 (2010) 653–664.
[27] F.U. Reiffen, M. Gratzl, Ca^{2+} binding to chromaffin vesicle matrix proteins: effect of pH, Mg^{2+}, and ionic strength, Biochemistry 25 (1986) 4402–4406.
[28] J.S. Videen, M.S. Mezger, Y.M. Chang, D.T. O'Connor, Calcium and catecholamine interactions with adrenal chromogranins. Comparison of driving forces in binding and aggregation, J. Biol. Chem. 267 (1992) 3066–3073.
[29] W.N. Kopell, E.W. Westhead, Osmotic pressures of solutions of ATP and catecholamines relating to storage in chromaffin granules, J. Biol. Chem. 267 (1982) 5707–5710.
[30] S.H. Yoo, M.S. Lewis, Effects of pH and Ca^{2+} on monomer–dimer and monomer–tetramer equilibria of chromogranin A, J. Biol. Chem. 267 (1992) 11236–11241.
[31] S.H. Yoo, M.S. Lewis, Effects of pH and Ca^{2+} on heterodimer and heterotetramer formation by chromogranin A and chromogranin B, J. Biol. Chem. 271 (1996) 17041–17046.
[32] H. Krieger-Brauer, M. Gratzl, Uptake of Ca^{2+} by isolated secretory vesicles from adrenal medulla, Biochim. Biophys. Acta 691 (1982) 61–70.
[33] H. Krieger-Brauer, M. Gratzl, Effects of monovalent and divalent cations on Ca^{2+} fluxes across chromaffin secretory membrane vesicles, J. Neurochem. 41 (1983) 1269–1276.
[34] J.R. Haigh, J.H. Phillips, Indirect coupling of calcium transport in chromaffin granule ghosts to the proton pump, Neuroreport 4 (1993) 571–574.
[35] S. Thirion, J.D. Troadec, N.B. Pivovarova, S. Pagnotta, S.B. Andrews, R.D. Leapman, G. Nicaise, Stimulus-secretion coupling in neurohypophysial nerve endings: a role for intravesicular sodium? Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 3206–3210.
[36] P.P. Goncalves, S.M. Meireles, P. Neves, M.G. Vale, Distinction between Ca^{2+} pump and Ca^{2+}/H^{+}antiport activities in synaptic vesicles of sheep brain cortex, Neurochem. Int. 37 (2000) 387–396.
[37] C.L. Haynes, L.A. Buhler, R.M. Wightman, Vesicular Ca^{2+}-induced secretion promoted by intracellular pH-gradient disruption, Biophys. Chem. 123 (2006) 20–24.
[38] M. Camacho, J.D. Machado, J. Alvarez, R. Borges, Intravesicular calcium release mediates the motion and exocytosis of secretory organelles, J. Biol. Chem. 283 (2008) 22383–22389.
[39] P. Fossier, M.F. Diebler, J.P. Mothet, M. Israel, L. Tauc, G. Baux, Control of the calcium concentration involved in acetylcholine release and its facilitation: an additional role for synaptic vesicles? Neuroscience 85 (1998) 85–91.
[40] J.J. López, C. Camello-Almaraz, J.A. Pariente, G. Salido, J.A. Rosado, Ca^{2+} accumulation into acidic organelles mediated by Ca^{2+}- and vacuolar H^{+}-ATPases in human platelets, Biochem. J. 390 (2005) 243–252.
[41] J.A. Rosado, Acidic Ca^{2+} stores in platelets, Cell Calcium 50 (2011) 168–174.
[42] J.G. Duman, L. Chen, A.E. Palmer, B. Hille, Contributions of intracellular compartments to calcium dynamics: implicating an acidic store, Traffic 7 (2006) 859–872.
[43] P. Pinton, T. Pozzan, R. Rizzuto, The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store, with functional properties distinct from those of the endoplasmic reticulum, EMBO J. 17 (1998) 5298–5308.
[44] J.L. Wegrzyn, S.J. Bark, L. Funkelstein, C. Mosier, A. Yap, P. Kazemi-Esfarjani, A.R. La Spada, C. Sigurdson, D.T. O'Connor, V. Hook, Proteomics of dense core secretory vesicles reveal distinct protein categories for secretion of neuroeffectors for cell–cell communication, J. Proteome Res. 9 (2010) 5002–5024.
[45] M.J. Rindler, C.F. Xu, I. Gumper, N.N. Smith, T.A. Neubert, Proteomic analysis of pancreatic zymogen granules: identification of new granule proteins, J. Proteome Res. 6 (2007) 2978–2992.
[46] D.J. Gauthier, J.A. Sobota, F. Ferraro, R.E. Mains, C. Lazure, Flow cytometry-assisted purification and proteomic analysis of the corticotropes dense-core secretory granules, Proteomics 8 (2008) 3848–3861.
[47] H.S. Lee, J. Jeong, K.-J. Lee, Characterization of vesicles secreted from insulinoma NIT-1 cells, J. Proteome Res. 8 (2009) 2851–2862.
[48] Y. Brunner, Y. Coute, M. Iezzi, M. Foti, M. Fukuda, D.F. Hochstrasser, C.B. Wollheim, J.-C. Sanchez, Proteomics analysis of insulin secretory granules, Mol. Cell. Proteomics 6 (2007) 1007–1017.
[49] S.H. Yoo, J.P. Albanesi, Inositol 1,4,5-trisphosphate-triggered Ca^{2+} release from bovine adrenal medullary secretory vesicles, J. Biol. Chem. 265 (1990) 13446–13448.
[50] Y.H. Huh, J.A. Yoo, S.J. Bahk, S.H. Yoo, Distribution profile of inositol 1,4,5-trisphosphate receptor isoforms in adrenal chromaffin cells, FEBS Lett. 579 (2005) 2597–2603.
[51] S.H. Yoo, Y.S. Oh, M.K. Kang, Y.H. Huh, S.H. So, H.S. Park, H.Y. Park, Localization of Three types of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} channel in the secretory granules and coupling with the Ca^{2+} storage proteins chromogranins A and B, J. Biol. Chem. 276 (2001) 45806–45812.
[52] E.C. Thrower, H.Y. Park, S.H. So, S.H. Yoo, B.E. Ehrlich, Activation of the inositol 1,4,5-trisphosphate receptor by the calcium storage protein chromogranin A, J. Biol. Chem. 277 (2002) 15801–15806.
[53] S.H. Yoo, Coupling of the IP_3 receptor/Ca^{2+} channel with Ca^{2+} storage proteins chromogranins A and B in secretory granules, Trends Neurosci. 23 (2000) 424–428.
[54] S.H. Yoo, Y.H. Huh, Y.S. Hur, Inositol 1,4,5-trisphosphate receptor in chromaffin secretory granules and its relation to chromogranins, Cell. Mol. Neurobiol. 30 (2010) 1155–1161.
[55] O.V. Gerasimenko, J.V. Gerasimenko, P.V. Belan, O.H. Petersen, Inositol trisphosphate and cyclic ADP-ribose-mediated release of $Ca^{2+}$from single isolated pancreatic zymogen granules, Cell 84 (1996) 473–480.
[56] O.V. Gerasimenko, J.V. Gerasimenko, R. Rizzuto, M. Treiman, A.V. Tepikin, O.H. Petersen, The distribution of the endoplasmic reticulum in living pancreatic acinar cells, Cell Calcium 32 (2002) 261–268.
[57] T. Nguyen, W.C. Chin, P. Verdugo, Role of Ca^{2+}/K^{+} ion exchange in intracellular storage and release of Ca^{2+}, Nature 395 (1998) 908–912.
[58] O. Blondel, M.M. Moody, A.M. Depaoli, A.H. Sharp, C.A. Ross, H. Swift, G.I. Bell, Localization of inositol trisphosphate receptor subtype 3 to insulin and somatostatinsecretory granules and regulation of expression in islets and insulinoma cells, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 7777–7781.
[59] O. Blondel, G.I. Bell, S. Seino, Inositol 1,4,5-trisphosphate receptors, secretory granules and secretion in endocrine and neuroendocrine cells, Trends Neurosci. 18 (1995) 157–161.
[60] L. Xie, M. Zhang, W. Zhou, Z. Wu, J. Ding, L. Chen, T. Xu, Extracellular ATP stimulates exocytosis via localized Ca^{2+} release from acidic stores in rat pancreatic β-cells, Traffic 7 (2006) 429–439.
[61] Y.S. Hur, K.D. Kim, S.H. Paek, S.H. Yoo, Evidence for the existence of secretory granule (dense-core vesicle)-based inositol 1,4,5-trisphosphate-dependent Ca^{2+} signaling system in astrocytes, PLoS ONE 5 (2010) 1–13.
[62] Y. Endo, K. Harada, N. Fujishiro, H. Funahashi, S. Shioda, G.D. Prestwich, K. Mikoshiba, M. Inoue, Organelles containing inositol trisphosphate receptor type 2 in adrenal medullary cells, J. Physiol. Sci. 56 (2006) 415–423.
[63] M. Prentki, T.J. Biden, D. Janjic, R.F. Irvine, M.J. Berridge, C.B. Wollheim, Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate, Nature 309 (1984) 562–564.
[64] M. Ravazzola, P.A. Halban, L. Orci, Inositol 1,4,5-trisphosphate receptor subtype 3 in pancreatic islet cell secretory granules revisited, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 2745–2748.
[65] J. Meldolesi, T. Pozzan, IP_3 receptors and secretory granules, Trends Neurosci. 18 (1995) 340–341.
[66] A.E. Pouli, N. Karagenc, C. Wasmeier, J.C. Hutton, N. Bright, S. Arden, J.G. Schofield, G.A. Rutter, A phogrin–aequorin chimaera to image free Ca^{2+} in the vicinity ofsecretory granules, Biochem. J. 330 (1998) 1399–1404.
[67] D.I. Yule, S.A. Ernst, H. Ohnishi, R.J.H. Wojcikiewicz, Evidence that zymogen granules are not a physiologically relevant calcium pool. Defining the distribution of inositol 1,4,5-trisphosphate receptors in pancreatic acinar cells, J. Biol. Chem. 272 (1997) 9093–9098.
[68] A. Nezu, A. Tanimura, T. Morita, K. Irie, T. Yajima, Y. Tojyo, Evidence that zymogen granules do not function as an intracellular Ca^{2+} store for the generation of the Ca^{2+} signal in rat parotid acinar cells, Biochem. J. 363 (2002) 59–66.
[69] J.V. Gerasimenko, M. Sherwood, A.V. Tepikin, O.H. Petersen, O.V. Gerasimenko, NAADP, cADPR and IP_3all release Ca^{2+}fromtheendoplasmicreticulumandanacidicstore in the secretory granule area, J. Cell Sci. 119 (2006) 226–238.
[70] K.J. Mitchell, F.A. Lai, G.A. Rutter, Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca^{2+} release from insulin-containing vesicles in living pancreatic beta-cells (MIN6), J. Biol. Chem. 278 (2003) 11057–11064.
[71] E. Brailoiu, D. Churamani, V. Pandey, G.C. Brailoiu, F. Tuluc, S. Patel, N.J. Dun, Messenger-specific role for nicotinic acid adenine dinucleotide phosphate in neuronal differentiation, J. Biol. Chem. 281 (2006) 15923–15928.

[72] P.J. Calcraft, M. Ruas, Z. Pan, X. Cheng, A. Arredouani, X. Hao, J. Tang, K. Rietdorf, L. Teboul, K.T. Chuang, P. Lin, R. Xiao, C. Wang, Y. Zhu, Y. Lin, C.N. Wyatt, J. Parrington, J. Ma, A.M. Evans, A. Galione, M.X. Zhu, NAADP mobilizes calciumfrom acidic organelles through two-pore channels, Nature 459 (2009) 596–600.
[73] W.J. Scheenen, C.B. Wollheim, T. Pozzan, C. Fasolato, Ca^{2+} depletion from granules inhibits exocytosis. A study with insulin-secreting cells, J. Biol. Chem. 273 (1998) 19002–19008.
[74] M.L. Mundorf, K.P. Troyer, S.E. Hochstetler, J.A. Near, R.M. Wightman, Vesicular Ca^{2+} participates in the catalysis of exocytosis, J. Biol. Chem. 275 (2000) 9136–9142.
[75] J.D. Machado, M. Camacho, J. Alvarez, R. Borges, On the role of intravesicular calcium in the motion and exocytosis of secretory organelles, Communicative Integrative Biol. 2 (2009) 1–3.