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Cytosolic organelles shape calcium signals and exo–endocytotic responses of chromaffin cells

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ABSTRACT

The concept of stimulus–secretion coupling was born from experiments performed in chromaffin cells 50 years ago. Stimulation of these cells with acetylcholine enhances calcium (Ca^{2+}) entry and this generates a transient elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) that triggers the exocytotic release of catecholamines. The control of the $[Ca^{2+}]_c$ signal is complex and depends on various classes of plasmalemmal calcium channels, cytosolic calcium buffers, the uptake and release of Ca^{2+} from cytoplasmic organelles, such as the endoplasmic reticulum, mitochondria, chromaffin vesicles and the nucleus, and Ca^{2+} extrusion mechanisms, such as the plasma membrane Ca^{2+}-stimulated ATPase, and the Na^+/Ca^{2+} exchanger. Computation of the rates of Ca^{2+} fluxes between the different cell compartments support the proposal that the chromaffin cell has developed functional calcium tetrads formed by calcium channels, cytosolic calcium buffers, the endoplasmic reticulum, and mitochondria nearby the exocytotic plasmalemmal sites. These tetrads shape the Ca^{2+} transients occurring during cell activation to regulate early and late steps of exocytosis, and the ensuing endocytotic responses. The different patterns of catecholamine secretion in response to stress may thus depend on such local $[Ca^{2+}]_c$ transients occurring at different cell compartments, and generated by redistribution and release of Ca^{2+} by cytoplasmic organelles. In this manner, the calcium tetrads serve to couple the variable energy demands due to exo–endocytotic activities with energy production and protein synthesis.

1. Introduction

Stressful conflicts trigger a surge of the catecholamines adrenaline and noradrenaline that mobilize the body to survive by combating an enemy or to flee from danger, the so-called "fight or flight" response. This response is the end result of a secretory event that takes place in the adrenal medulla, the inner part of the two adrenal glands located just above the kidneys. The adrenal medulla is composed of chromaffin cells that secrete adrenaline and noradrenaline. These cells are of interest not only to explore the mechanisms underlying the "fight or flight" response, but also because they have been used for decades as excellent models to study the working of other secretory cells, in particular neurons.

Acetylcholine, the physiological neurotransmitter at the splanchnic nerve-chromaffin cell synapse [1], causes the release of catecholamines from the adrenal gland. This secretory response is suppressed in the absence of extracellular calcium (Ca^{2+}) [2]. Also, acetylcholine enhances Ca^{2+} entry into adrenal medullary chromaffin cells [3]. On the basis of these and other pioneering experiments William W. Douglas coined the expression "stimulus–secretion coupling" as the basic mechanism involved in neurotransmitter and hormone secretion; Ca^{2+} was the coupling ion between the stimulus and the exocytotic response [4]. Since then, adrenal chromaffin cells from various mammalian species but mostly from bovine, rats and mice have extensively been used to study the relationship between the changes of cytosolic concentrations of free Ca^{2+} ions in the cytosol ($[Ca^{2+}]_c$), its redistribution into organelles, its clearance from the cytosol and the exocytotic and endocytotic responses triggered by acetylcholine and other nicotinic and muscarinic receptor agonists, various agonists for G-protein coupled receptors and different depolarising stimuli including high concentrations of potassium (K^+), square depolarising pulses or action potentials.

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Chromaffin cells are excitable cells and fire action potentials that open various of the neuronal-type voltage-dependent calcium channels (VDCCs) and produce Ca^{2+} entry; the resulting $[Ca^{2+}]_c$ signal triggers exocytosis. Because cytoplasmic organelles can take up and release Ca^{2+} to the cytosol, understanding the $[Ca^{2+}]_c$ signal requires understanding of the Ca^{2+} redistribution between the cytosol and the different organelles. The coding of the photoprotein aequorin gene [5] made it possible to introduce targeting sequences, and measuring selective $[Ca^{2+}]$ changes in different organelles [6]. This methodology has been applied during the last decade to gain insight into the role of organelles in shaping $[Ca^{2+}]_c$ signalling and exocytosis in chromaffin cells. This review focuses on the pathways for Ca^{2+} entry into the chromaffin cell, on the intracellular organelles that contribute to the redistribution of the Ca^{2+} entering the cell, and on the mechanisms that terminate the $[Ca^{2+}]_c$ signals and extrude the cation outside the cell. We also analyse the influence of this Ca^{2+} trafficking between the different organelles on the exocytotic responses. Finally, we analyse the kinetics of Ca^{2+} handling at different cell compartments, trying to obtain a unified picture of Ca^{2+} handling and the exo–endocytotic responses of chromaffin cells. Several reviews of some of these questions have been published [7–11].

2. Calcium influx

The most relevant Ca^{2+} entry pathways in chromaffin cells are VDCCs, store-operated Ca^{2+} channels (SOCCs) and ligand-gated calcium channels. The characteristics and regulation of the various VDCC subtypes will extensively be described by E. Carbone in this special number of Cell Calcium. So, we will only make a brief mention to them.

2.1. Voltage-dependent calcium channels

As in neurons [12], multiple VDCCs are expressed in chromaffin cells [13]. Significant differences exist in the densities of each channel subtype in cells from different species. For instance, L channels (α_{1D}, Cav1.3) carry near 50% of the whole-cell current in cat, rat and mouse chromaffin cells. In contrast, P/Q channels (α_{1A}, Cav2.1) account for 50–60% of the current in bovine and human chromaffin cells. N-type channels (α_{1B}, Cav2.2) contribute 80% in pig, 45% in cat and 30% in bovine, rat, mouse and human chromaffin cells. Finally, R-type channels (α_{1E}, Cav2.3) are present only in mouse chromaffin cells [9].

2.2. Store-operated calcium channels

In many non-excitable cells, inositol 1,4,5-trisphosphate ($InsP_3$) generated by agonist stimulation causes a biphasic elevation of $[Ca^{2+}]_c$. The initial peak is due to ER Ca^{2+} release via the $InsP_3$ receptor channel while the subsequent maintained plateau phase is associated to Ca^{2+} entry through SOCCs [14–16]. The plateau phase is produced by a small-conductance, voltage-independent Ca^{2+} release activated Ca^{2+} current (I_{CRAC}), that serves to replenish the Ca^{2+} store [17–19]. Having multiple types of VDCCs, excitable cells could be refilling their depleted ER Ca^{2+} store by Ca^{2+} entering through those high-conductance channels. This has been shown to apply for some neurosecretory cells [20,21] including bovine chromaffin cells; in these cells loaded with ER-targeted aequorin, high K^+ accelerates the ER Ca^{2+} store refilling upon Ca^{2+} reintroduction [22].

Early experiments demonstrated Ca^{2+} influx through SOCCs upon ER Ca^{2+} depletion of bovine chromaffin cells [23]; this was corroborated by later experiments [22,24–28]. A direct proof for the presence of SOCCs was obtained from voltage-clamped bovine chromaffin cells where a small-amplitude, voltage-independent I_{CRAC} carried by Ca^{2+} and Na^+, was characterised under conditions of Ca^{2+} store depletion [29]. A Ca^{2+} entry pathway triggered by histamine and independent of the ER Ca^{2+} store is also present in these cells [25,30].

A few studies have explored the role of Ca^{2+} entry through SOCCs in triggering exocytosis in bovine chromaffin cells. Thus, histamine and angiotensin II stimulate exocytosis by a combination of ER Ca^{2+} release and additional Ca^{2+} entry through SOCCs [24]. More convincing evidence arises from experiments performed in voltage-clamped cells, where angiotensin II-induced exocytosis was associated with an uncharacterised leak current [27]. In addition, exocytosis could be elicited in the absence of depolarisation by photolysis of caged $InsP_3$ [31] or by bradykinin [32]. But the most convincing evidence comes from experiments done with stimulation of Ca^{2+} entry through SOCCs by store depletion that produces exocytosis at negative membrane potentials that maintain closed the VDCCs [29].

Why a bovine chromaffin cell expressing L, N and P/Q high-conductance VDCCs [9] should still require additional pathways for Ca^{2+} entry is puzzling. The fact such pathways can be physiologically activated by action potentials or sustained depolarisation triggered by acetylcholine is even more puzzling. Combining aequorins and confocal microscopy, Ca^{2+}-induced Ca^{2+} release (CICR) was shown to be activated by K^+ or 50-ms depolarising pulses in bovine chromaffin cells [22]. Upon repetitive stimulation with bursts of action potentials under stress, CICR may produce partial ER Ca^{2+} depletion and give rise to SOCC activation. A modulatory role of this capacitative Ca^{2+} entry on exocytosis in chromaffin cells has been suggested, but other pathways for Ca^{2+} entry were not under control in these experiments [26]. Later, direct experiments demonstrated that receptor-free activation of Ca^{2+} entry via SOCCs is sufficient to trigger and or facilitate exocytosis in these cells [29]. In this context, it is interesting that hyperpolarisation is associated with histamine receptor stimulation that is coupled to ER Ca^{2+} release and activation of small-conductance Ca^{2+}-activated K^+ channels [33]. This mechanism could amplify Ca^{2+} influx via SOCCs, thus facilitating the exocytosis triggered by bursts of action potentials, in a kind of long-lasting modulatory mechanism for stimulus–secretion coupling.

2.3. Ligand-gated calcium channels

Nicotinic receptors for acetylcholine (nAChRs), as well as receptors for glutamate and ATP, underlie excitatory transmission at central and peripheral synapses. These receptors are ion channels permeable to cations. The fraction of the inward cation current carried by Ca^{2+}, triggered by agonists in various cell types is about 5% for nAChRs and ATP receptors and around 10% for N-methyl-D-aspartate (NMDA) receptors [34,35]. In bovine chromaffin cells the fraction of acetylcholine-elicited inward current carried by Ca^{2+} accounts for about 5% [36]. Ca^{2+} entering through nAChRs may contribute to augment vesicle movement and the size of the ready-release vesicle pool [37,38]. Furthermore, glutamate receptors seem to mediate an increase of $[Ca^{2+}]_c$ and exocytosis in bovine chromaffin cells [39]. On the other hand, various purinoceptor subtypes that respond to ATP with a $[Ca^{2+}]_c$ increase have also been found in these cells [40]. Remarkable differences among species have been found. For instance, rat chromaffin cells lack P2X receptors while in the guinea-pig, ATP generates an inward current that seems to be associated to $P2X_2$ receptors [41]. Na^+ influx through P2X channels causes depolarisation of bovine chromaffin cells, enhances Ca^{2+} entry through VDCCs and catecholamine release [42]. On the other hand, P2X receptors seem to be preferentially expressed by noradrenergic cells, while adrenergic cells preferentially contain P2Y metabotropic receptors [43]. These latter

receptors exert an autocrine regulatory inhibition of inward Ca^{2+} currents through VDCCs of bovine cells [44,45].

$GABA_A$ receptor agonists also cause cell depolarisation, an elevation of $[Ca^{2+}]_c$, likely due to opening of VDCCs and the release of catecholamines [46–48]. Furthermore, GABA enhances the $[Ca^{2+}]_c$ elevation elicited by low-frequency electrical field stimulation of perfused rat adrenals [49]. It is still unclear how GABA can exert those modulatory effects; a paracrine role has been suggested for GABA co-stored and co-released with catecholamines during electrical stimulation of the splanchnic nerves at the adrenal medulla [49].

3. Calcium redistribution

The abrupt $[Ca^{2+}]_c$ transient generated by depolarising stimuli elicited by either action potentials or sustained depolarisations, are controlled not only by the different subtypes of VDCCs expressed by chromaffin cells, but also by cytosolic calcium buffers, Ca^{2+} sequestration or release by cytoplasmic organelles and extrusion by plasmalemmal calcium transporters. We will separately analyse these calcium regulatory elements (Fig. 1).

3.1. Cytosolic calcium buffers

Ca^{2+} buffering and diffusion in bovine chromaffin cells has been studied extensively by Neher and coworkers (see Section 5). However, the molecular nature of the cytosolic calcium buffers is unknown. Only a few reports have been devoted to the study of calcium-binding proteins in chromaffin cells. For instance, in bovine cells parvalbumin contains Ca^{2+}/Mg^{2+} mixed sites that show slow Ca^{2+}-binding kinetics under physiological conditions. Parvalbumin acts as a Ca^{2+} source during relaxation of $[Ca^{2+}]_c$ peaks and extends the $[Ca^{2+}]_c$ transient by conversion of a monoexponential decay in a biexponential one [50]. Another study reported that calbindin-D28k is homogeneously distributed in the cytosol of bovine cells while its distribution was preferentially concentrated at submembrane sites in mouse cells. The clearance of the K^+-evoked $[Ca^{2+}]_c$ transients was slower in bovine cells, but the initial quantal secretory response was faster in mouse chromaffin cells. Thus, the different distribution of calbindin-D28k does certainly affect Ca^{2+} signalling and exocytosis in both cell types [51].

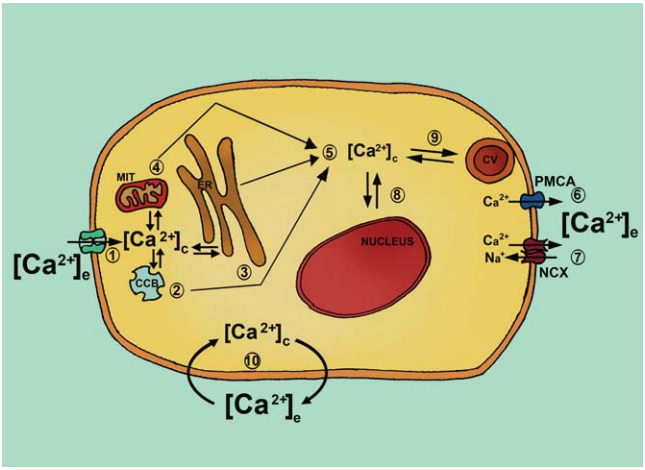

Fig. 1. Calcium (Ca^{2+}) cycling in the chromaffin cell. Upon cell depolarisation, extracellular Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels (1, VDCCs). This generates a local cytosolic Ca^{2+} transient ($[Ca^{2+}]_c$), with activation and clearance phases exhibiting spatial and temporal patterns that are tightly regulated by nearby poorly defined immobile cytosolic calcium buffers (2, CCB), the endoplasmic reticulum (3, ER) and the mitochondrion (4, MIT). Ca^{2+} taken up by organelles and cytosolic calcium buffers is released back into the cytosol allowing its redistribution towards the cell core (5). Finally, to re-establish the cell Ca^{2+} balance, the plasmalemmal Ca^{2+} pump (6, PMCA) and Na^{2+}/Ca^{2+} exchanger NCX (7) drive Ca^{2+} efflux back to the extracellular space. The nucleus (8) and chromaffin vesicles (9, CV) may also contribute to Ca^{2+} redistribution. Pathways for Ca^{2+} entry other than VDCCs, such as store-operated calcium channels (SOCCs), nicotinic acetylcholine receptors, purinergic receptors, GABA and glutamate receptors have also been reported to be present in chromaffin cells; they are not represented for the sake of simplicity. Being an excitable cell driven by the sympathetic nervous system, the Ca^{2+} cycling must be continuously going on in the intact adrenal medullary tissue. The velocity of such Ca^{2+} cycling (10), depends on the rate of action potential firing and the sympathetic cholinergic input at different stress situations. Such variations in the velocity of Ca^{2+} cycling serve to adapt the bioenergetic needs of the cell, in order to secure the rapid release of catecholamines into the circulation, to prepare the body for the fight or flight response.

3.2. Nucleus

Ca^{2+} has relevant functions in the regulation of gene expression in the nucleus. In addition, a few studies have approached the nuclear Ca^{2+} kinetics [52]. For instance, there is consensus that the nuclear envelope may somewhat delay the propagation of Ca^{2+} waves from the cytosol to the nucleus [53–55]. In PC12 and other cell types, half-equilibrium times for Ca^{2+} fluxes through the nuclear envelope are in the range of seconds [54]. Under these conditions, strong stimuli such as K^+ depolarisation or stimulation with UTP or bradykinin generate Ca^{2+} signals that are quickly transmitted to the nucleus. On the contrary, the progression of high-frequency $[Ca^{2+}]_c$ oscillations to the nucleus may be dampened by the nuclear envelope [54]. The nuclear matrix also differ from the cytosol in having a larger Ca^{2+}-buffering capacity [56], which would also result in an obvious slowing in the progression of the Ca^{2+} wave. It is interesting to note that selective nuclear signalling might be achieved by Ca^{2+} release from nuclear stores in certain cells [57].

3.3. Chromaffin vesicles

Chromaffin vesicles of bovine chromaffin cells contain as much as 40 mM calcium [58]. Most of this calcium (>99.9%) is bound to chromogranins and the free Ca^{2+} concentration is about 40 μM [59,60]. At pH 7.5, chromogranin A binds 32 mol of Ca^{2+}/mol protein, with a K_D of 4 mM; the binding capacity increases to 55 mol of Ca^{2+}/mol protein with a K_D of 2.7 mM at the intravesicular pH of 5.5 [61]. Thus, an increase of intravesicular pH increases the free Ca^{2+} concentration, thereby facilitating its release into the cytosol. This has been experimentally demonstrated with alkalinising agents and protonophores, which enhance vesicular Ca^{2+} release, vesicle motion and exocytosis [62–66]. As much as 20–30% of the basal chromaffin cells volume is occupied by about 20,000 chromaffin vesicles [67] that store around 60% of total cell Ca^{2+} [59,68]; however, scarce data are available to support the original hypothesis stating that intravesicular Ca^{2+} could be involved in the exocytotic process [69]. Experiments with alkalinising agents are certainly interesting; but it is difficult to envision the physiological context that they could mimic. The presence of $InsP_3$ receptors in the chromaffin vesicle membrane [70,71] and $InsP_3$-induced vesicular Ca^{2+} release [60,72,73] suggest that the $InsP_3$ pathway may be physiologically relevant. It seems likely that vesicular Ca^{2+} release could be involved in slow pre-exocytotic steps aimed at mobilizing vesicles from a reserve pool to a ready-releasable pool, as it is the case for Ca^{2+} release from the ER (see Section 3.4). However, it is unlikely that this slow Ca^{2+} release can compete with the rapid high-Ca^{2+} microdomains (HCMDs) formed at subplasmalemmal exocytotic sites nearby VDCCs and docked vesicles ready to undergo fast exocytosis. Experimental protocols and techniques capable of distinguishing the various Ca^{2+} sources contributing

to such HCMDs may enlighten the contribution of vesicular Ca^{2+} release in the various steps of exocytosis. For further analysis of this topic, see two recent reviews [74,75].

3.4. Endoplasmic reticulum

Earlier observations established that Ca^{2+} uptake occurred in the sarcoplasmic reticulum of skeletal muscle [76,77] through a Mg^{2+}- and ATP-dependent P-type transport Ca^{2+} ATPase, the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [78]. This led to the concept of intracellular calcium stores that was soon extended to most nonexcitable and excitable cells including neurons and neurosecretory cells [79]. Two channels are mainly responsible for the release of Ca^{2+} from the ER store namely, the $InsP_3$ receptor channel, which is activated by the $InsP_3$ generated as a result of G-protein coupled membrane receptor activation, and the ryanodine receptor channel (RyR) that is activated by enhanced $[Ca^{2+}]_c$, caffeine and ryanodine. Ca^{2+} binding to RyR opens the channel, thereby triggering the release of Ca^{2+} into the cytosol through the Ca^{2+}-induced Ca^{2+} release mechanism (CICR).

Considerable efforts have been devoted to clarify the kinetics of Ca^{2+} fluxes of the ER Ca^{2+} store, and its role in controlling pre-exocytotic and the last exocytotic steps in chromaffin cells. For instance, histamine, angiotensin II, bradykinin and carbachol have been shown to augment the production of $InsP_3$ in bovine chromaffin cells [80–82]. A parallel increase of $InsP_3$ and $[Ca^{2+}]_c$ occurs upon challenging these cells with histamine or angiotensin II [83]. The augmentation of $[Ca^{2+}]_c$ elicited by stimulation with histamine is mimicked by direct stimulation with $InsP_3$, suggesting that stimulation of histamine receptors is coupled to $InsP_3$ generation and the subsequent stimulation of $InsP_3$ receptors to cause ER Ca^{2+} release [84,85]. Pituitary adenylate cyclase-activating polypeptide (PACAP) has also been shown to enhance both $InsP_3$ production and enhanced $[Ca^{2+}]_c$ [86]. In rat chromaffin cells, stimulation of muscarinic and β_2 adrenergic receptors modulates the amplitude of $[Ca^{2+}]_c$ oscillations [87]; such Ca^{2+} oscillations are dependent on ER Ca^{2+} release from heparin-sensitive Ca^{2+} stores [88].

The functional correlate of histamine-elicited ER Ca^{2+} release has also been studied. For instance, this $[Ca^{2+}]_c$ signal activates small-conductance Ca^{2+}-activated K^+ channels leading to hyperpolarisation of bovine chromaffin cells [33]. In this line is the finding that muscarine produces a $[Ca^{2+}]_c$ elevation and an outward K^+ current, due to activation of Ca^{2+}-activated K^+ channels in guinea-pig chromaffin cells [89]. These channels are regulating the nicotinic and muscarinic secretory response of cat and bovine chromaffin cells [90–92]. While ER Ca^{2+} release by histamine causes a mild and transient catecholamine release response [93], a more sustained application causes a longer effect [93–95]. This greater effect could be explained by the fact histamine-elicited $[Ca^{2+}]_c$ elevations has two components: an initial transient phase due to ER Ca^{2+} release and a late more sustained phase due to Ca^{2+} entry [30,83,96,97]. The second component has been associated to inhibition of an M-current by sustained histamine application, leading to cell depolarisation, discharge of action potentials and opening of VDCCs in bovine chromaffin cells [98], although stimulation of SOCCs by ER emptying could also contribute to this effect (see Section 2.2). Finally, it is interesting that histamine has been used as a tool to elicit subthreshold $[Ca^{2+}]_c$ elevations in voltage-clamped bovine chromaffin cells. This $[Ca^{2+}]_c$ signal does not elicit exocytosis by itself, but potentiates the subsequent exocytotic response to a depolarising stimulus, likely due to an acceleration of the flow of new vesicles towards exocytotic subplasmalemmal sites [99]. Also, angiotensin II augments $[Ca^{2+}]_c$ and secretion in bovine chromaffin cells but to a lesser extent than histamine [24,100]. On the other hand, the nicotinic response seems to have a component linked to ER Ca^{2+} release [101].

Concerning RyR channels, it has been known for long that bovine chromaffin cells possess a powerful caffeine-sensitive calcium store [102]. The release of ER Ca^{2+} by caffeine was later shown to follow a quantal pattern, suggesting that the caffeine-sensitive Ca^{2+} pool is composed of functionally discrete stores with heterogeneous sensitivities to caffeine [103,104]. Additionally, the presence of separate or overlapping Ca^{2+} pools responsive to either caffeine, $InsP_3$ or cyclic ADP ribose, their differential sensitivity to SERCA inhibitors such as thapsigargin, and the physiological significance or the different Ca^{2+} release mechanisms, have been subject of debate for many years [85,105–108].

Direct monitoring of changes in the ER Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) in bovine chromaffin cells transfected with ER-targeted aequorin, permitted clarification of some of those issues [22,109]. Thus, Ca^{2+} entry elicited by depolarisation triggers a transient Ca^{2+} release from the ER that is highly dependent on $[Ca^{2+}]_{ER}$ and sensitised by low caffeine concentrations. On the other hand, caffeine-induced Ca^{2+} release was quantal in nature due to modulation by $[Ca^{2+}]_{ER}$. Whereas caffeine releases essentially all the Ca^{2+} from the ER, $InsP_3$-producing agonists release only 60–80%. However, in digitonin-permeabilised cells both $InsP_3$ and caffeine emptied completely the calcium store while cyclic ADP ribose has no effect. Finally, the wave of Ca^{2+} elicited by 100 ms depolarising pulses measured with confocal microscopy, is delayed and reduced in intensity in ryanodine-treated cells. These data suggest that the ER of bovine chromaffin cells behaves as a single thapsigargin-sensitive calcium pool that can release Ca^{2+} both via $InsP_3$ receptors or CICR. A later report showed that mouse chromaffin cells in the intact gland exhibited a smaller or nonexistent CICR [110]. However, in a recent study performed on cultured mouse chromaffin cells the expression of RyRs and a functional CICR mechanism was shown [111].

In isolated bovine chromaffin cells, caffeine causes a mild secretory response [102], and this effect is also observed in the absence of extracellular Ca^{2+} [112,113]. Activation of CICR during cell depolarisation may have functional consequences for the control of the exocytotic process. In this context, it is interesting that when the Ca^{2+} store has been depleted by sustained caffeine stimulation, a subsequent depolarisation by high K^+ elicits a smaller secretion. Consistently, after full ER Ca^{2+} depletion, the first two or three initial depolarisations contribute to refill the ER with Ca^{2+} and therefore, the ER behaves as a sink, reducing the amount of Ca^{2+} available for secretion [113].

In voltage-clamped bovine chromaffin cells, exocytosis is unaffected by previous ER Ca^{2+} depletion with thapsigargin [114,115]; however, a later study show depressed secretion [115]. In bovine chromaffin cells stimulated with acetylcholine, severe ER Ca^{2+} depletion with a mixture of caffeine, ryanodine and thapsigargin halves the catecholamine release responses. However, the K^+ responses are little affected. This may be due to the fact that acetylcholine elicits discrete and more localised $[Ca^{2+}]_c$ elevations, whereas K^+ pulses produce higher $[Ca^{2+}]_c$ transients that spread quickly throughout the cytosol [116]. This difference may be explained considering that acetylcholine evokes action potentials [117] while K^+ produces sustained cell depolarisation [118] in bovine chromaffin cells. Thus, it is plausible that the contribution of CICR to the exocytotic response is more visible under conditions of physiological stimulation of chromaffin cells with acetylcholine.

3.5. Mitochondria

Mitochondria are the main energy-producing centres of eukaryotic cells [119,120]. They are capable of accumulating vast amounts of Ca^{2+} in their matrix through their Ca^{2+} uniporter, that uses the driving force of the electrical potential across the mitochondrial membrane [121]. The matrix is more negative than the cytosol,

with a large transmembrane potential difference (near −180 mV) that is generated by the respiratory chain or by ATP hydrolysis. Ca^{2+} accumulated in mitochondria is then released back into the cytosol by electroneutral antiporters that export Ca^{2+} from the matrix by swapping one Ca^{2+} ion for two Na^+ through the mitochondrial Na^+/Ca^{2+} exchanger (mNCX). A Na^+/H^+ exchange mechanism does also exist, but it is less active than mNCX [122,123]. Additionally, mitochondrial Ca^{2+}-induced Ca^{2+} release mediated by the calcium uniporter has also been observed [124].

During cell activation, some mitochondria take up Ca^{2+} from cytosolic HCMDs that are created by the opening of nearby VDCCs [109,125]. In rat chromaffin cells, mitochondria act as rapid and reversible Ca^{2+} buffers during cell stimulation [126,127]; they also contribute to the clearance of large Ca^{2+} loads in bovine chromaffin cells [109,128]. However, early measurements of $[Ca^{2+}]$ changes in the mitochondrial matrix ($[Ca^{2+}]_M$) provides values only in the low micromolar range [126], most probably because underestimation by saturation of the measuring fluorescent Ca^{2+} probe. By using mitochondrially targeted aequorins with different Ca^{2+} affinities, which have a much wider dynamic range [52,129], it was later on shown that bovine chromaffin cell mitochondria exhibit surprisingly rapid millimolar Ca^{2+} transients upon cell stimulation with acetylcholine, caffeine or K^+ [130].

This avid Ca^{2+} uptake by mitochondria surely has functional consequences. For instance, dissipation of the proton gradient by protonophores decreases the Ca^{2+} buffering capacity of mitochondria [130,131] and drastically augments the exocytotic response in voltage-clamped bovine chromaffin cells stimulated with depolarising pulses [132]. In perifused populations of bovine chromaffin cells stimulated with acetylcholine, caffeine or K^+, mitochondrial protonophores enhance 3–5 fold the release of catecholamines [116,130,133]. Blockade of the mitochondrial calcium uniporter also enhances the K^+-evoked secretion in single bovine chromaffin cells [131]. K^+-elicited secretion is particularly augmented by protonophores when Ca^{2+} entry via L-type VDCCs is enhanced by FPL64176 [134]. In mouse chromaffin cells however, protonophores halved the K^+-evoked $[Ca^{2+}]_c$ and catecholamine release responses [135]; this could be explained by differences in the expression of VDCC subtypes in bovine versus mouse chromaffin cells, and/or different rates of inactivation of VDCC subtypes during blockade of mitochondrial Ca^{2+} uptake by protonophores [136].

4. Calcium efflux

The main transporters used by cells to extrude Ca^{2+} from the intracellular to the extracellular compartment are the plasmalemmal Ca^{2+} pump or Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger (NCX) (Fig. 1). Both transporters contribute to maintain the long-term Ca^{2+} homeostasis through a well balanced Ca^{2+} influx and Ca^{2+} efflux activities. The functional expression of these two transporters was first demonstrated using plasma membrane vesicles from bovine adrenal medulla [137]. The plasmalemmal Ca^{2+}-ATPase has a high Ca^{2+} affinity (K_D in the 10^{-7} M range) and operates as an electrogenic Ca^{2+}/H^+ exchanger with a 1:1 stoichiometry [138].

The NCX uses the energy provided by the Na^+ gradient to achieve an electrogenic exchange of 3 Na^+ ions for 1 Ca^{2+} ion. Under physiological conditions Na^+ is transported into the cell and Ca^{2+} is extruded from the cytosol [139]. However, when the electrochemical gradient for Na^+ is reversed, such as during membrane depolarisation or the opening of gated Na^+ channels, the exchanger moves Na^+ out of the cell and Ca^{2+} into the cell [140]. The Ca^{2+} exit mode is referred to as the forward mode, and the Ca^{2+} entry mode as the reverse mode of the NCX [141]. Bovine chromaffin cells express the major isoform of the NCX, namely NCX1 [142], which can mediate Na^+-dependent Ca^{2+} influx [143] or Ca^{2+} export [144], depending on the circumstances.

The cardiotonic steroid ouabain, the classical inhibitor of the plasmalemmal Na^+/K^+-ATPase (NKA) or Na^+ pump [145] has been widely used to infer the role of the NCX in various cell types. Although ouabain upsets primarily the Na^+ and K^+ gradients across the plasma membrane, the collapse of the Na^+ gradient can secondarily drive Ca^{2+} entry though NCX. This is the mechanism underlying the heart inotropic effect of cardiac glycosides. On the other hand, repeated action potential firing leading to Na^+ accumulation, can also force NCX to work in reverse mode, thereby increasing $[Ca^{2+}]_c$ and favouring the replenishment with Ca^{2+} of the sarcoplasmic reticulum. During subsequent action potentials, augmented CICR, which is potentiated by the increased $[Ca^{2+}]_{ER}$, leads to enhanced cardiac contraction [146].

Since long we know that ouabain enhances both the spontaneous [147–149] and the K^+-evoked catecholamine release from cat chromaffin cells [150] and bovine chromaffin cells [151,152]. These effects were initially interpreted as a secondary activation of the NCX by ouabain [143,153,154], through a mechanism similar to that occurring in the heart. However, an alternative explanation can be inferred from the recent observation that NKA co-localises with subplasmalemmal regions of the ER [155,156]. Recent data on bovine chromaffin cells show that ouabain causes the release of Ca^{2+} from the ER and augments the catecholamine secretory responses to sequential K^+ pulses. ER Ca^{2+} depletion prevents such potentiation and causes a gradual decrease of the responses to K^+. Furthermore, ouabain enhances the number of docked vesicles at subplasmalemmal regions, as revealed with TIRF microscopy [152]. All these data support earlier suggestions that the ER Ca^{2+} store contributes to maintain healthy secretory responses elicited by depolarising pulses applied to bovine chromaffin cells [115,116]. The fact that endogenous ouabain has been identified in human plasma [157,158] and that bovine adrenal cortex is particularly rich in endogenous ouabain [159], suggests a physiological role of this mediator in the control of Ca^{2+}-dependent vesicle flow from a reserve pool towards ready-release and immediate-release vesicle pools at subplasmalemmal sites [7].

Bovine chromaffin cells express the major isoform of the NCX, NCX1 [160]. In bovine chromaffin cells NCX1 can favour Na^+-dependent Ca^{2+} influx [143] or Ca^{2+} export [144] and has been proposed to participate in the regulation of $[Ca^{2+}]_c$ and exocytosis in cat [150,161,162] and bovine chromaffin cells [142,143,153,163–165]. In addition, chromaffin cells co-express NCX and the retinal rod-type K^+-dependent Na^+/Ca^{2+} exchanger [166]. Attempts to clarify the participation of the NCX in physiological and pathological processes have been hampered by the lack of potent and selective blockers. The antagonist KB-R7943 preferentially inhibits, at low micromolar concentrations, the reverse mode of the NCX [167]. Unfortunately, this compound also blocks other transporters, such as the mitochondrial uniporter [168], and the nicotinic receptors of bovine chromaffin cells [169]. Novel and more selective inhibitors such as SEA0400, SN-6 and YM-244769 [170] should help to further clarify the role of the NCX in Ca^{2+} signalling and exocytosis in chromaffin cells. In fact, SEA0400 inhibits Na^+-dependent Ca^{2+} uptake and catecholamine release in bovine chromaffin cells, with IC_{50} of 40 and 100 nM, respectively, compared with IC_{50} of 1.8 and 3.7 μM for KB-R7943, that was 40-fold less potent [171].

5. A functional tetrad shapes calcium gradients and calcium microdomains

In the intact organism, the fight or flight stress response is triggered by acetylcholine, the physiological neurotransmitter at the

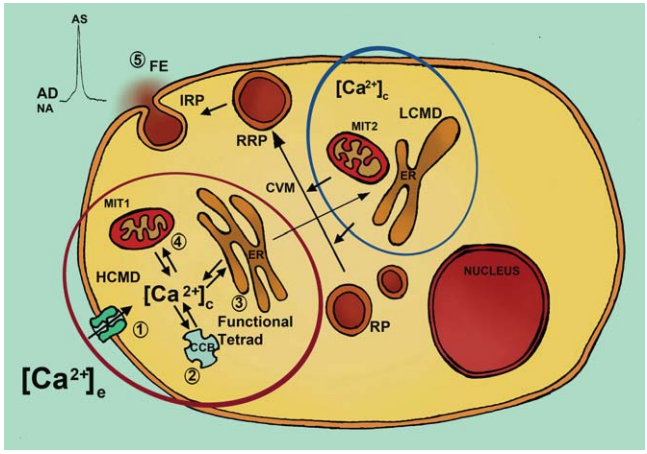

Fig. 2. Functional tetrads to shape the high-Ca^{2+} microdomains (HCMDs) an low-Ca^{2+} microdomains (LCMDs) that determine, respectively, the fast exocytosis (FE) release of adrenaline (AD) and noradrenaline (NA), from an immediately releasable vesicle pool (IRP), and chromaffin vesicles movement (CVM) from a reserve pool (RP). Tetrads are formed by voltage-dependent Ca^{2+} channels (1, VDCCs), cytosolic Ca^{2+} buffers (2, CCB), the endoplasmic reticulum (3, ER) and the mitochondrial pool 1 (MIT1) located nearby the plasmalemma (4). This tetrad (red line) is responsible for generating and shaping the HCMD transients (10–100 μM), nearby subplasmalemmal exocytotic sites, to trigger fast catecholamine release from the IRP, that can be monitored at the single-vesicle level as amperometric spikes (AS) with a carbon fibre microelectrode (5). The LCMD (<1 μM) is located at cytosolic sites away from the plasmalemma, and facilitate the Ca^{2+}-dependent CVM. Crosstalk between HCMD and LCMD is needed to secure the supply of new vesicles to the secretory machinery under different stimulation rates of chromaffin cells (see text for further details).

synapse of the splanchnic nerve and chromaffin cells [1]. Direct membrane depolarisation or action potentials fired by the interaction of acetylcholine with nicotinic receptors on the surface of chromaffin cells [117] is likely the primary stimulus that induces the $[Ca^{2+}]_c$ transient, thus triggering the discharge of adrenaline and noradrenaline into the circulation [38]. Ca^{2+} entry through the various subtypes of VDCCs is the primary determinant for the extent and shape of the initial $[Ca^{2+}]_c$ transient. However, cytosolic calcium buffers, Ca^{2+} sequestration or release from the cytoplasmic organelles, and plasmalemmal Ca^{2+} extrussion have a prominent role in the fine tuning of the Ca^{2+} signal. On the other hand, correct Ca^{2+} signalling is critical to warrant the adaptation of the entire organism to a stress response which determines its survival. We will emphasise here our present integrative view of the biophysics of Ca^{2+} redistribution, which is the ultimate regulator of the exocytotic response (Fig. 2).

Essential to the understanding of Ca^{2+} function in chromaffin cells is the concept that organelles and cytosolic calcium buffers shape $[Ca^{2+}]_c$ transients at different cell locations, the so-called HCMDs, that do not necessarily crosstalk. Several kinds of these HCMDs have been described in different cell systems and given evocative names, such as sparks, puffs, sparklets and syntillas [172,173]. Syntillas are brief focal $[Ca^{2+}]_c$ transients elicited by localised ER Ca^{2+} release via RyR channels, first reported in neurohypophysial terminals at magnocellular neurons [174]. These focal Ca^{2+} transients were later on found in mouse chromaffin cells [175] and, paradoxically, they seem to block spontaneous exocytosis in these cells [176]. Because CICR is present in bovine chromaffin cells, it could be of interest to investigate whether the Ca^{2+} wave that extends from subplasmalemmal sites to the inner cytosol following a 100 ms depolarising pulse and CICR activation [22], is composed of elementary syntillas. We have recently found that nanomolar concentrations of the wine grape polyphenol resveratrol causes ER Ca^{2+} release in bovine chromaffin cells and, at the same time, it blocks the quantal catecholamine release response [177]. It would be interesting to clarify whether these effects of resveratrol are linked to the production of Ca^{2+} syntillas. It seems however that the presence and functional role for Ca^{2+} syntillas are seriously questioned and controversial. In fact, caffeine or ryanodine do not augment $[Ca^{2+}]_c$ and neuropeptide release at neurohypophysial terminals [178,179].

The rate of Ca^{2+} fluxes between different chromaffin cell compartments have been estimated using more or less direct approaches, and under temperature conditions (i.e. room temperature) that might affect the activity of some Ca^{2+} transporters. Even with these limitations, putting together the estimates of the different fluxes allows for several interesting predictions [109]. For instance, for a 15-μm diameter bovine chromaffin cell, a rate of Ca^{2+} entry of 700 μmol L cells^{-1} s^{-1} can be computed from the measured Ca^{2+} inward current [180]. A similar value (400 μmol L cells^{-1} s^{-1}) was estimated by measuring $^{45}Ca^{2+}$ uptake into K^+ depolarised bovine chromaffin cells [181]. Ca^{2+} entry would be focused at the channels location and then diffuse through the surrounding cytosol.

Regarding progression of the Ca^{2+} wave generated by Ca^{2+} entry through plasma membrane Ca^{2+} channels, binding to cytosolic calcium buffers is a most important determinant. The cytosol of bovine chromaffin cells has a Ca^{2+} binding capacity of ~4 mmol/L cells. The cytosolic calcium buffers are scarcely mobile and have a low Ca^{2+} affinity (K_D ~ 100 μM) with an activity coefficient of ~1/40 [128,180]. The two-dimensional diffusion coefficient is ~40 μm^2/s and shows inhomogeneities at the nuclear envelope and at the plasma membrane [53]. Brief openings of VDCCs generate HCMDs near the channel mouth that can be detected in Ca^{2+} imaging measurements [182]. These HCMDs can reach concentrations as high as 10–100 μM [7,182] Because of rapid diffusion of Ca^{2+} towards the surrounding cytosol, the HCMDs are highly restricted in time and space [7,183]. The presence of mobile calcium buffers accelerates diffusion and opposes the development of HCMDs [180,184–186]; for example, at concentrations of 50 μM, fura-2 increases the apparent rate of Ca^{2+} diffusion four times [180].

Ca^{2+} entering the cell redistributes among the different cell compartments. The increase of $[Ca^{2+}]_c$ activates the SERCA and the ER avidly takes up Ca^{2+} from the cytosol. For example, during stimulation of bovine chromaffin cells [22,109,128] and rat chromaffin cells [187], the maximal Ca^{2+} uptake by the ER ranges between 40 and 80 μmol L cells^{-1} s^{-1}. At rest, the rate of Ca^{2+} exchange between ER and cytosol at steady state is 2–3 μmol L cells^{-1} s^{-1}. The net Ca^{2+} influx upon maximal stimulation with caffeine or $InsP_3$-producing agonists is 10–20 times faster [22].

Concerning mitochondria, it is notorious that the Ca^{2+} activity coefficient (free Ca^{2+}/bound calcium) in the matrix is very low, in the 1/1000 range [109,126]. Mitochondria are very effective in the clearing of $[Ca^{2+}]_c$ transients, although drastic differences have been reported between bovine and rat chromaffin cells. For instance, in experiments with photorelease of caged Ca^{2+} in bovine chromaffin cells, rates of $[Ca^{2+}]_M$ increase as high as 4800 μmol L cells^{-1} s^{-1}, at saturating $[Ca^{2+}]_c$ (200 μM), were found [128]. In contrast, in rat chromaffin cells, mitochondrial uptake rates are 150–300 fold slower but at $[Ca^{2+}]_c$ of only 0.2–2 μM, were found [187]. These differences are consistent with dependence of the rate of uptake through the uniporter on the second power of $[Ca^{2+}]_c$ [123,124,130,188]. Using mitochondria-targeted aequorin to specifically monitor $[Ca^{2+}]_M$, we found that mitochondria took up about 1100 μmol L cells^{-1} s^{-1} upon maximal stimulation of Ca^{2+} entry into bovine chromaffin cells depolarised with K^+ [109,124,130]; this value is comparable with the rate of Ca^{2+} entry through VDCCs. The maximal rate of Ca^{2+} release from mitochondria trough the mNCX at 37 °C in bovine chromaffin cells is about 800 μmol L cells^{-1} s^{-1}. Regarding the kinetics of this mitochondrial Ca^{2+} efflux, the dependence on $[Ca^{2+}]_M$ is exponential and K_{50} approaches 200 μM [109]. Transport through the uniporter is usually unidirectional (entry); however, when mitochondria are

completely depolarised, the uniporter may allow Ca^{2+} exit from the matrix in a sort of mitochondrial CICR mechanism [124].

Ca^{2+} extrusion from the cell to the extracellular medium is due to joint operation of both Ca^{2+}-ATPase and NCX. The joint action of both transport systems has been estimated to decrease $[Ca^{2+}]_c$ to a maximal rate of 20 μmol L cells^{-1} s^{-1}, in rat chromaffin cells at 27 °C [127,187]. At 37 °C the rate can be close to 100 μmol L cells^{-1} s^{-1} [109].

At each and every moment the $[Ca^{2+}]_c$ is defined by the rate of Ca^{2+} redistribution into chromaffin cell compartments which in turn depends on fluxes between the extracellular medium, the cytosol, cytosolic calcium buffers and organelles. At rest, a steady state with Ca^{2+} exchange rates below 10 μmol L cells^{-1} s^{-1} and $[Ca^{2+}]_c$ near 0.1 μM is established; $[Ca^{2+}]_M$ is similar to $[Ca^{2+}]_c$ while $[Ca^{2+}]_{ER}$ is much higher, reaching 500–1000 μM. Consequently, there are enormous electrochemical gradients favouring Ca^{2+} diffusion to the cytosol from both, the ER and the extracellular medium where the Ca^{2+} concentration is above 1 mM.

At low-frequency stimulation with action potentials, the rate of Ca^{2+} diffusion through the cytosol and binding by the endogenous Ca^{2+} buffers are the main determinants of the $[Ca^{2+}]_c$ signal [7,183]. Under these conditions, global $[Ca^{2+}]_c$ goes up to about 1 μM and Ca^{2+} clearance is primarily achieved through the high-affinity Ca^{2+}-ATPase and SERCA. Upon strong stimulation (high-frequency action potentials or prolonged depolarisation), global $[Ca^{2+}]_c$ may approach 10 μM, a concentration high enough to activate Ca^{2+} uptake through the mitochondrial uniporter. Under these conditions, most of the Ca^{2+} that enters chromaffin cells is taken up by mitochondria [109,127,130,187]. For example, mitochondria-targeted aequorin revealed that 90% of the Ca^{2+} that enters a bovine chromaffin cell stimulated with a 10-s K^+ pulse is taken up by mitochondria. Later, when the stimulation ceases, the Ca^{2+} accumulated in mitochondria is released back to the cytosol during a period of seconds or even minutes [109]. The Ca^{2+} accumulated in mitochondria stimulates respiration until Ca^{2+} extrusion from the mitochondrial matrix is complete [109]. It can be speculated that the extra energy provided in this way may be used for clearing the Ca^{2+} load and restoring Ca^{2+} homeostasis after the activity period.

In bovine chromaffin cells, the opening of VDCCs generates HCMDs of about 0.3 μm diameter and 10 μM $[Ca^{2+}]_c$ [99,182,189–191]. Building of HCMDs may be favoured by co-localisation of VDCC clusters and chromaffin vesicles [31,192,193]. Evanescent microscopy has shown fast ($t_{1/2} \sim 100$ ms) and localised (~350 nm) HCMDs beneath the plasma membrane of stimulated chromaffin cells [194]. These HCMDs selectively trigger the release of vesicles docked within 300 nm, indicating that some vesicles are docked but not primed. It is interesting that HCMDs reduce the distance between docked vesicles and Ca^{2+} entry sites, suggesting a role for stimulation-dependent facilitation of exocytosis in chromaffin cells [193,194].

Mitochondria located nearby VDCCs at subplasmalemmal sites can sense HCMDs during physiological stimulation [109,127,130,187,195]. Through measurements of aequorin consumption upon repeated stimulation of bovine chromaffin cells, the cumulative history of Ca^{2+} uptake may be traced. Using this approach, two pools of mitochondria with different subcellular distribution were evidenced. Pool M1, located nearby exocytotic sites, accumulates $[Ca^{2+}]_c$ at a rate of 2000 μmol L cells^{-1} s^{-1}, while pool M2 located at inner cytosolic areas takes up Ca^{2+} at a much lower rate, 12 μmol L cells^{-1} s^{-1} [109,130]. These rates are reached at concentrations of 20 and 2 μM $[Ca^{2+}]_c$ respectively, which are coincident with the concentrations reached at subplasmalemmal sites and the cell core during cell stimulation. The M1 pool would tune the mitochondrial function to match the local energy needs for exocytosis and Ca^{2+} redistribution whereas the M2 pool, located at the bulk cytosol, could serve to redistribute Ca^{2+} and canalize it towards inner cytosolic regions to serve other cell functions, i.e. transport of new secretory vesicles to plasmalemmal exocytotic sites.

ER Ca^{2+} fluxes could also contribute to the regulation of HCMDs formed during cell stimulation. For instance, under K^+ depolarisation of bovine chromaffin cells transfected with ER-targeted aequorin, reductions of 60–100 μM $[Ca^{2+}]_{ER}$ are observed (about 10–15% of the total ER Ca^{2+} content) [22], suggesting Ca^{2+}-induced Ca^{2+} release. Although the decrease of $[Ca^{2+}]_{ER}$ may seem quite small, it could correspond to large release at certain subcellular ER locations compensated by strong uptake in others. CICR sites seem to co-localise with plasmalemmal VDCCs and the M1 mitochondrial pool. Thus, complex functional tetrads including VDCCs, cytosolic calcium buffers, the mitochondrial uniporter and the RyR are essential for the efficacious regulation of adequate local $[Ca^{2+}]_c$ transients to control the rate and extent of exocytotic catecholamine release (Fig. 2).

6. Relationship between calcium and the exo–endocytotic responses

A few studies have addressed the question of the quantitative relationship between Ca^{2+} and the exo–endocytotic responses triggered by chromaffin cell stimulation. One approach consists in the dialysis of bovine chromaffin cells with solutions containing known $[Ca^{2+}]$ to elicit secretion, measured as an increase of membrane capacitance (ΔCm) [32]. Also caffeine is used to augment $[Ca^{2+}]_c$ and measure ΔCm [196]. Both approaches lead to a $[Ca^{2+}]_c$-exocytosis relationship that scaled to a power function with an exponent of 3. Still other studies use voltage steps (square depolarising pulses) to boost Ca^{2+} influx (Q_{Ca}) and exocytosis; they found a Q_{Ca}/ΔCm relationship that fitted a power function with an exponent of 1.5 [197,198]. There are additional studies in bovine chromaffin cells stimulated with single or trains of depolarising pulses [199] or action potential waveform trains as well as 100 ms depolarising pulses [132]. The longer depolarising pulses produced Q_{Ca}/ΔCm relationships that fitted to power functions of 1.2–2. In line with these conclusions is the observation in rat chromaffin cells stimulated with single depolarising pulses of increasing length (10–150 ms), showing a linear Q_{Ca}/ΔCm relationship [200]. Flash photolysis of caged Ca^{2+} has also been used to study the kinetic components of a fast exocytotic burst [201]. Depolarising pulses are known to be much less efficient than Ca^{2+} photorelease in triggering exocytosis [202,203].

Other studies have used action potential waveforms to correlate the stimulation frequency in bovine chromaffin cells with amperometric spike secretion in rat chromaffin cells [192] or with capacitance increase in bovine chromaffin cells [204]. In addition, depolarising pulses have been used in transgenic mice to study the role of exocytotic proteins on the kinetics of ΔCm [203]. On the other hand, a study comparing depolarising pulses of increasing length with acetylcholine-type action potentials in voltage-clamped bovine chromaffin cells, found less Ca^{2+} entry and slower activation of $[Ca^{2+}]_c$ transients with faster delayed decay. With action potentials a linear relationship is found between Q_{Ca} and stimulus duration, capacitance increase and stimulus duration and Q_{Ca} and capacitance increase. These relationships are nonlinear with depolarising pulses. Furthermore, capacitance increase responses elicited by action potential trains are followed by little slow endocytosis, while those induced by depolarising pulses are followed by a pronounced endocytosis, particularly at the longer pulses [205].

Controversy exists over the manner in which membrane retrieval during endocytosis is affected by Ca^{2+}. For instance, compensatory and excess endocytosis represent two independent Ca^{2+}-regulated mechanisms of rapid internalisation in bovine

chromaffin cells [204,206]. The existence of these two Ca^{2+} sensors is consistent with the fact that Ca^{2+} and Ba^{2+} support excessive membrane retrieval in bovine chromaffin cells [207]. In contrast, a previous study in the same cells showed that rapid endocytosis was supported by Ca^{2+} but not by Sr^{2+} or Ba^{2+} [208]. A recent study shows a linear correlation between endocytosis and Q_{Ca} in voltage-clamped bovine chromaffin cells [209].

It is interesting that Ca^{2+}-dependent endocytosis triggered by single long depolarising pulses in voltage-clamped bovine chromaffin cells seems to be coupled to L-type VDCCs, whereas N- or PQ-type of calcium channels seem to play little role [210,211]. Lack of co-localisation between VDCC subtypes and clathrin or dynamin suggests a functional, rather than physical coupling between L-type calcium channels and the endocytotic machinery. In bovine chromaffin cells, L-type calcium channels undergo a Ca^{2+}-dependent inactivation slower than N- or PQ-type of calcium channels [136,212]. It is therefore plausible that a slower but more sustained Ca^{2+} entry through slowly inactivating L-type calcium channels, rather than through higher but fast-inactivating N- and PQ-type of calcium channels, is a requirement to trigger endocytosis efficiently, at least in bovine chromaffin cells [211]. This Ca^{2+}-dependent endocytotic response is enhanced by sphingosine dialysis, that seems to play a permissive role for endocytosis by acting on an endocytotic pathway different to those of dynamin- and calmodulin-signalling pathways [209].

7. Conclusions and perspectives

A number of studies have clarified the role of several families of ion channels and transporters in shaping the $[Ca^{2+}]_c$ signals and the exo–endocytotic responses occurring during chromaffin cell stimulation. From the 1970s onwards most of the studies were performed in ready available bovine chromaffin cells. During the last two decades, however, chromaffin cells from rats have also been thoroughly used. It is surprising, however, that only few studies on Ca^{2+} handling in mouse chromaffin cells have been performed. Transgenic mice lacking or over-expressing a given protein have extensively been used to clarify molecular mechanisms of the secretory machinery. It would be very interesting to use chromaffin cells as models to identify alterations of Ca^{2+} homeostatic mechanisms and the release of catecholamines in mouse models of disease. For instance, in transgenic mouse models of Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and other neurodegenerative diseases, the expectation is that high and low Ca^{2+} microdomains may differently affect pre- and exocytotic steps, which could be a peripheral marker of a brain synaptic dysfunction. There is increasing concern on the involvement of Ca^{2+} dyshomeostasis in these diseases [213–216].

Whether the large Ca^{2+} concentrations in chromaffin vesicles play a function other than the mere packing of catecholamine such as for instance, contributing to regulation of the last steps of exocytosis, requires further clarification. We also know little on the role of Ca^{2+} fluxes in the chromaffin cell nucleus, although they are likely involved in the control of gene expression. It would be nice to know, for example, whether such nuclear Ca^{2+} signalling is involved in the expression of the enzymes of catecholamine synthesis and degradation.

Efforts should also be done to extrapolate the numerous data obtained in cultures of chromaffin cells to more physiological preparations such as adrenal slices or even the intact adrenal, using electrical stimulation of the sympathetic cholinergic nerve terminals that innervate chromaffin cells to regulate secretion. Attempts to establish organotypic cultures of adrenal slices should also be pursued, as this could facilitate chronic treatments to study novel aspects of catecholamine synthesis, storage and release and on the role of Ca^{2+} signalling under these more physiological conditions of preservation of tissue structure.

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