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SYMPOSIUM REVIEW

The coupling of plasma membrane calcium entry to calcium uptake by endoplasmic reticulum and mitochondria

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Key points

- Cross-talk between organelles and plasma membrane Ca^{2+} channels modulates cytosolic Ca^{2+} signals in different ways.
- In chromaffin cells Ca^{2+} entry through voltage-operated channels is amplified by Ca^{2+} release from the endoplasmic reticulum (ER) and generates subplasmalemmal high Ca^{2+} microdomains (HCMDs) as high as 20–50 μM, which trigger exocytosis. Subplasmalemmal mitochondria take up Ca^{2+} from HCMDs and avoid progression of the Ca^{2+} wave towards the cell core.
- In non-excitable HEK293 cells activation of store-operated Ca^{2+} entry triggered by ER Ca^{2+} emptying also generates subplasmalemmal HCMDs of about 2 μM. In this case most of the Ca^{2+} is taken up by the ER rather than by mitochondria. This outcome may be explained because sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) has much higher Ca^{2+} affinity than mitochondria. The relative positioning of organelles, channels and accessory proteins may also play a role.

Abstract Cross-talk between organelles and plasma membrane Ca^{2+} channels is essential for modulation of the cytosolic Ca^{2+} ($[Ca^{2+}]_C$) signals, but such modulation may differ among cells. In chromaffin cells Ca^{2+} entry through voltage-operated channels induces calcium release from the endoplasmic reticulum (ER) that amplifies the signal. $[Ca^{2+}]_C$ microdomains as high as 20–50 μM are sensed by subplasmalemmal mitochondria, which accumulate large amounts of Ca^{2+} through the mitochondrial Ca^{2+} uniporter (MCU). Mitochondria confine the high-Ca^{2+} microdomains (HCMDs) to beneath the plasma membrane, where exocytosis of secretory vesicles happens. Cell core $[Ca^{2+}]_C$ is much smaller (1–2 μM). By acting as a Ca^{2+} sink, mitochondria stabilise the HCMD in space and time. In non-excitable HEK293 cells, activation of store-operated Ca^{2+} entry, triggered by ER Ca^{2+} emptying, also generated subplasmalemmal HCMDs, but, in this case, most of the Ca^{2+} was taken up by the ER rather than by mitochondria. The smaller size of the $[Ca^{2+}]_C$ peak in this case (about 2 μM) may contribute to this outcome, as the sarco-endoplasmic reticulum Ca^{2+} ATPase has much higher Ca^{2+} affinity than MCU. It is also possible that the

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relative positioning of organelles, channels and effectors, as well as cytoskeleton and accessory proteins plays an important role.

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Abbreviations $[Ca^{2+}]_C$, $[Ca^{2+}]_M$, $[Ca^{2+}]_{ER}$, Ca^{2+} concentration in cytosol, mitochondria and ER, repscetively; AEQ, aequorin; CICR, Ca^{2+}-induced Ca^{2+} release; CREB, cAMP response element-binding protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; HCMDs, high Ca^{2+} microdomains; ICS, intracellular calcium store; MCU, mitochondrial Ca^{2+} uniporter; NCX, Na^+/Ca^{2+} exchanger; nFAT, nuclear factor of activated T-cells; VOCCs, voltage-operated Ca^{2+} channels; PMCA, plasma membrane Ca^{2+} ATPase; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; SOCs, store-operated channels; SOCE, store operated Ca^{2+} entry.

Introduction

Cell activation is often triggered by an increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). At rest, $[Ca^{2+}]_c$ is kept low ($\sim 10^{-7}$ M) by the operation of the Ca^{2+}-dependent ATPases, which pump Ca^{2+} away from the cytosol, to the extracellular medium and to the intracellular Ca^{2+} stores (ICSs) (Alvarez *et al.* 1999; Berridge *et al.* 2003; Clapham, 2007; Berridge, 2009). During cell activation Ca^{2+} channels located either at the plasma membrane or at endomembranes open, and Ca^{2+} flows into the cytosol. In excitable cells Ca^{2+} dynamics is directed by plasma membrane voltage-operated Ca^{2+} channels (VOCCs), but ICSs can cooperate to amplify the $[Ca^{2+}]_C$ signal. In non-excitable cells Ca^{2+} release from ICSs is often the trigger of activation, but the $[Ca^{2+}]_C$ signal is amplified by Ca^{2+} entry, which is activated by ICS emptying [store-operated calcium entry (SOCE)] (Clapham, 2007; Cahalan, 2009). Therefore, cross-talk between plasma membrane and organelles is essential for Ca^{2+} homeostasis (Rizzuto *et al.* 1998; Alvarez *et al.* 1999; Berridge *et al.* 2003; Singaravelu *et al.* 2011; Rizzuto *et al.* 2012). We shall refer here to the role of endoplasmic reticulum (ER) and mitochondria. ER acts as normally filled ICSs and can release Ca^{2+} to the cytosol to generate or reinforce $[Ca^{2+}]_c$ signals. Mitochondria acts as a normally empty ICS, but can accumulate large amounts of Ca^{2+} when $[Ca^{2+}]_c$ increases above 10^{-6} M. Mitochondrial Ca^{2+} accumulation is usually transient, following $[Ca^{2+}]_c$ peaks generated during cell activation (Alonso *et al.* 2006; Rizzuto *et al.* 2012). The increase of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_M$) stimulates respiration, thus contributing to adjust ATP synthesis to the increased energy demand, and Ca^{2+} overload produces mitochondrial damage and may trigger apoptotic mechanisms (Alonso *et al.* 2006; Duchen *et al.* 2008; Spat *et al.* 2008; Rizzuto *et al.* 2012).

Recent research on Ca^{2+} homeostasis has evolved from global (cellular) to local (subcellular) signalling, produced by opening of a few Ca^{2+} channels located near the effector of a physiological action (Alvarez *et al.* 1999; Garcia *et al.* 2006; Clapham, 2007; Berridge, 2009). In this way, Ca^{2+} control of different cellular processes is made possible by coincidental compartmentalization of the pertinent Ca^{2+} microdomain with its physiological target. Control of cell secretion illustrates this spatiotemporal specificity (Garcia *et al.* 2006, 2012; Parekh, 2011). The affinity of the secretory machinery for Ca^{2+} is low (10^{-5} to 10^{-4} M) and, because of physical constraints imposed by diffusion and intracellular Ca^{2+} buffering, these concentrations can only be reached in the vicinity of Ca^{2+} channels (Garcia *et al.* 2006). Generation of high $[Ca^{2+}]_c$ microdomains (HCMDs) near the exocytic machinery allows secretion to proceed without disturbance of other Ca^{2+}-triggered functions, which have a different subcellular location. Certain geometric or structural arrangements may favour the generation of HCMDs. This is the case for synaptic bulbs in neurones or the particular arrangement of T tubules and terminal cisternae in muscle. On the other hand, the signal generated by Ca^{2+} release from ICSs by SOCE (Clapham, 2007; Cahalan, 2009) requires reinforcement via SOCE at the immunological synapse for normal lymphocyte function (Oh-hora & Rao, 2008).

Measuring $[Ca^{2+}]$ inside organelles

Selective $[Ca^{2+}]$ measurements at subcellular locations often requires the use of targeted protein probes. As discussed in detail elsewhere (Alonso *et al.* 2006), aequorin (AEQ) probes are very well suited for measurement of Ca^{2+} inside cytoplasmic organelles and/or in specific subcellular microdomains. AEQ emits photoluminescence in response to Ca^{2+} according to the reaction:

$$\text{AEQ} + \text{coelenterazine} + O_2 \xrightarrow{Ca^{2+}} \text{apoAEQ} + \text{coelenteramide} + CO_2 + \text{light}.$$

AEQ has three active Ca^{2+} binding sites and hence gain of the light output/$[Ca^{2+}]$ relation is very steep, making it ideal for detection and measurement of Ca^{2+} microdomains (Alonso *et al.* 2006). Other advantages

include a dynamic range much larger than other Ca^{2+} probes, no need of illumination to excite light emission and the possibility of being engineered to modify Ca^{2+} affinity and/or to target different locations. Fusion with green fluorescent protein (GFP) or other fluorescent proteins improves stability and allows easy tracing of distribution by fluorescence microscopy (Alonso *et al.* 2006). Figure 1 shows some of the available variants, which allow extending the range of $[Ca^{2+}]$ measurements from under 10^{-7} to over 10^{-3} M. Constructs with viral vectors allow transfection *in vitro*, *ex vivo* or *in vivo* (Alonso *et al.* 1998). Because of the small light output, imaging is difficult, but possible (see sample movies with $[Ca^{2+}]_M$ oscillations in Supplemental material; Villalobos *et al.* 2001, 2002; Nunez *et al.* 2007).

Functional triads and their role in control of secretion in adrenal chromaffin cells

Depolarization of chromaffin cells triggers a series of effects that end to catecholamine secretion. The principal player is a triad made up of plasma membrane VOCCs, subplasmalemmal mitochondria and ER (Fig. 2; Montero *et al.* 2000; Garcia *et al.* 2006, 2012). The joint action of these three elements generates HCMDs besides the plasma membrane that are adequate for triggering exocytosis of secretory vesicles, but that do not propagate to the cell core, and therefore do not interfere with other Ca^{2+}-dependent processes. The sequence of events is as follows: Ca^{2+} entering through VOCCs is taken up by a pool of mitochondria close to the plasma membrane (M1 in Fig. 2) and this stops progression of the Ca^{2+} wave towards the cell core. This is revealed by mitochondrial AEQ decay, which detects two very different rates of consumption that must correspond to two different mitochondrial pools, M1 and M2. These two pools take up Ca^{2+} at very different velocities, 2000 and 12 μmol l^{-1} cells s^{-1}, respectively (Montero *et al.* 2000; Villalobos *et al.* 2002). These rates are the ones reached at about 30 and 2 μM $[Ca^{2+}]_C$, respectively, which should correspond to the Ca^{2+} concentrations attained at the subplasmalemmal region, where exocytosis takes place, and at the cell core (Fig. 2*A*). The increase of $[Ca^{2+}]_M$ would keep respiration increased, thus providing the basis for subcellular tuning of mitochondrial function to match the local energy needs. A similar compartmentalization of Ca^{2+} signalling by mitochondrial function has been suggested in pituitary cells (Villalobos *et al.* 2001), pancreatic acinar cells (Park *et al.* 2001; Walsh *et al.* 2009; Petersen, 2012), beta cells (Quesada *et al.* 2008) and sympathetic neurons (Nunez *et al.* 2007).

Calcium-induced calcium release (CICR) amplifies the Ca^{2+} signals generated by Ca^{2+} entry through VOCCs (Alonso *et al.* 1999). Measurements of $[Ca^{2+}]_{ER}$ during stimulation with high K^+ show net decreases of 60–100 μM (10–15% of the total content) with each stimulating pulse. This is an averaged value that may consist of strong liberation in certain places partly compensated for by strong uptake in others. CICR was sensitized by low concentrations of caffeine or by increasing the load of Ca^{2+} stored inside the ER, and it was blocked by ryanodine (Alonso *et al.* 1999). The mitochondrial AEQ pool sensitive to CICR overlaps with the pool reached by plasma membrane depolarization (Montero *et al.* 2000; Villalobos *et al.* 2002), suggesting physical co-localization of VOCCs and the ryanodine receptors (RyRs). Recent studies on the subcellular organization of the secretory machinery suggest that it accumulates at preferential sites that co-localize with the Ca^{2+} channel clusters. The cortical F-actin cytoskeleton may play a role in the association (Torregrosa-Hetland *et al.* 2011, 2013).

Therefore, the local $[Ca^{2+}]_C$ hotspots result from the joint action of the functional triad elements: the calcium channel acts as the trigger, the RyR, located at strategic places, is the signal amplifier, and the cortical mitochondria act as a contention barrier that avoids propagation of the high Ca^{2+} tide to the cell core, where such a huge signal is not required. Using the kinetic parameters for transport of Ca^{2+} in every organelle (Villalobos *et al.* 2002), we have modelled concentrations at different cell locations, near the cytosolic opening of the channel pore (0 μm) and 5 and 10 μm away, in cytosol, mitochondria and ER (Fig. 2*B–D*). $[Ca^{2+}]_C$ reaches

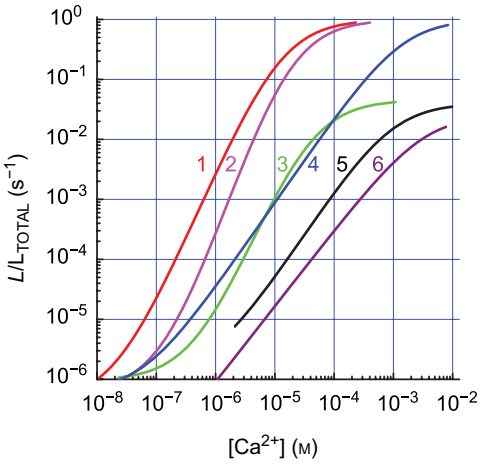

Figure 1 Different AEQ/coelenterazine pairs cover a broad Ca^{2+} concentration range with a high gain
1, native AEQ + native coelenterazine at 37°C (red); 2, same at 22°C (pink); 3, native AEQ + coelenterazine at 22°C (green); 4, low Ca^{2+} affinity mutated AEQ at 22°C + native colenterazine (blue) (Montero *et al.* 2000); 5, GFP fusion to mutated low affinity AEQ + colenterazine at 22°C (black) (Manjarres *et al.* 2008); 6, low Ca^{2+} affinity mutated AEQ + colenterazine at 22°C (purple). Light emissions (ordinate) are quantified as rates of photoluminescence emission/total c.p.s remaining at each time and divided by the integration period (L/L_{TOTAL} in s^{-1}).

a very high level at the mouth of the channel (Fig. 2*B*; note log scale) and a much smaller one at the core, 5 or 10 μm away. Mitochondria take up most of the Ca^{2+} load and $[Ca^{2+}]_M$ increases quickly to very high levels (Fig. 2*C*). Much of the Ca^{2+} that enters mitochondria at the subplasmalemmal locations may diffuse through the mitochondrial matrix to other cell locations and be eventually extruded from mitochondria closer to the cell core. $[Ca^{2+}]_{ER}$ first decreases by release during CICR, and then increases due to Ca^{2+} uptake through the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Fig. 2*D*). Note that once VOCCs close, $[Ca^{2+}]_C$ decreases quickly because of Ca^{2+} pumping from the cytosol both to the ER (through SERCA) and out of the cells (through plasma membrane Ca^{2+} ATPase, PMCA) (Fig. 2*A*). It takes several seconds to clear out the Ca^{2+} load that entered through VOCCs. This is due to the fact that most of the load is inside mitochondria by that time, and has to exit from the matrix to the cytosol (through the mitochondrial Na^+/Ca^{2+} exchanger) before being available to PMCA and SERCA (Villalobos *et al.* 2002). Because of the action of PMCA, the subplasmalemmal $[Ca^{2+}]_C$ reaches, during the post-stimulation period, lower levels than the core $[Ca^{2+}]_C$ (Fig. 2*B*). This faster decrease may be important for termination of the secretory process by the end of the stimulus.

Ca^{2+} released from the ER through inositol-trisphosphate receptors (IP_3Rs) is closely coupled to mitochondrial uptake through the mitochondrial calcium uniporter (MCU; Rizzuto *et al.* 1993). It has been discovered recently that dynamin-related mitofusins are responsible for the tethering mechanism between both organelles, thereby ensuring rapid and high-fidelity Ca^{2+} signalling between them (Parekh, 2009). This

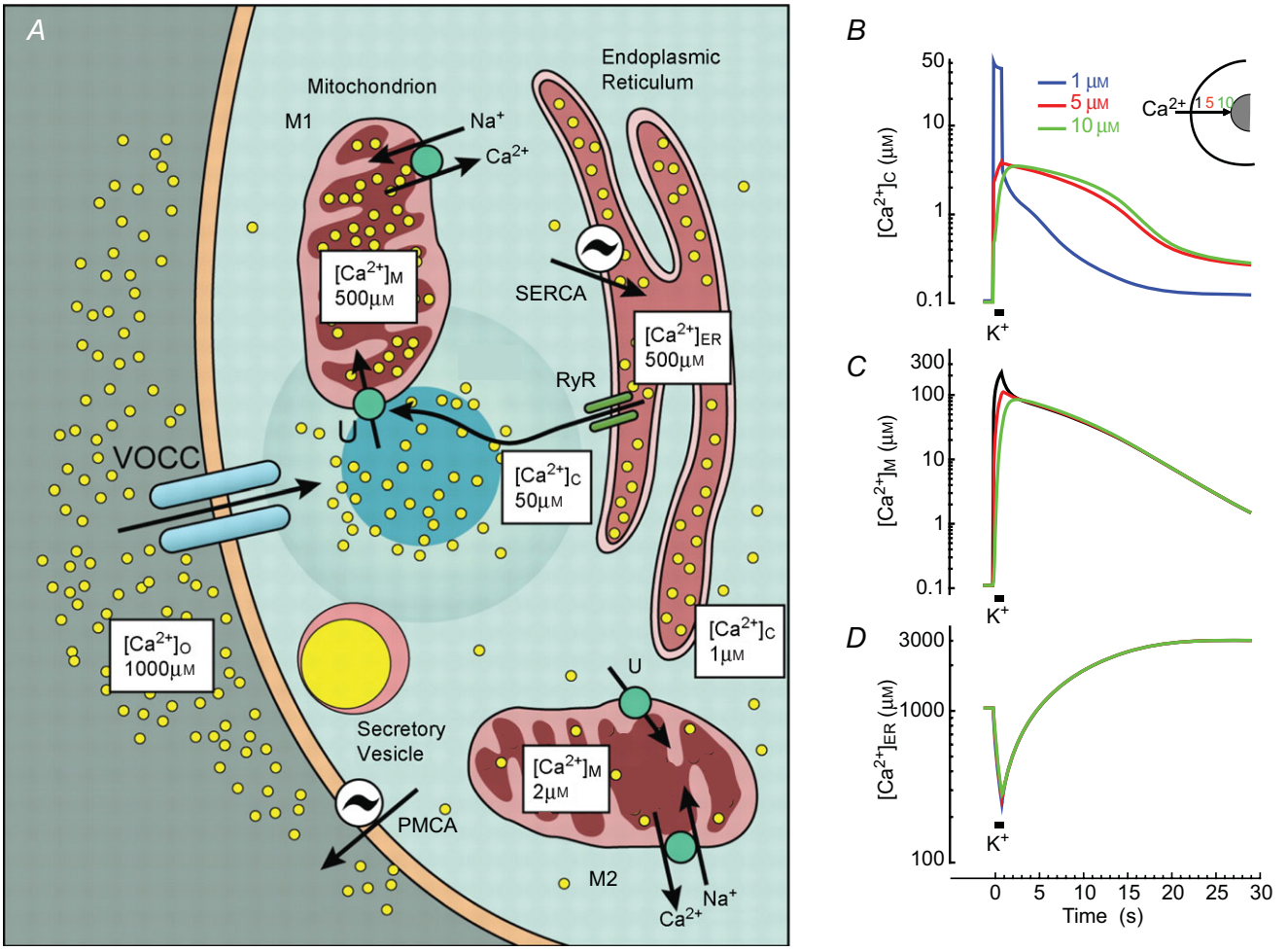

Figure 2. Functional triads shape Ca^{2+} stimuli and regulate secretion in adrenal chromaffin cells
A, schematic representation of the contribution of VOCCs, mitochondria and ER. *B–D*, simulations of $[Ca^{2+}]_C$ (*B*), $[Ca^{2+}]_M$ (*C*) and $[Ca^{2+}]_{ER}$ (*D*) changes during stimulation of a chromaffin cell by a depolarizing 1 s high K^+ pulse. For details see Villalobos *et al.* (2002). The $[Ca^{2+}]$ near the mouth of the channel (blue) and 5 (red) and 10 μm away (green) are shown. *A*, reproduced with permission from García *et al.* (2006), *Physiol Rev* 86, 1093–1131.

ER–mitochondria cross-talk has been studied extensively in several tissues (Csordas *et al.* 2006; Rizzuto & Pozzan, 2006; Csordas *et al.* 2010; Chen *et al.* 2012; Rizzuto *et al.* 2012). It is relevant here to ask whether this cross-talk implies, in chromaffin cells, the same mitochondrial subpopulation as the one involved in the functional triads described above. It seems that the response to histamine, which is mediated by IP_3Rs, includes a pool of mitochondria that amounts to about 30% and responds with $[Ca^{2+}]_M$ increases larger than 1 μM, while the rest respond with smaller changes. These mitochondria may overlap with the triads, as previous stimulation with caffeine occludes the response to histamine. However, only 2–3% of these mitochondria are able to generate $[Ca^{2+}]_M$ transients comparable in size to those observed after high K^+ or caffeine stimulation (Montero *et al.* 2002), suggesting that coupling of mitochondria to IP_3Rs is poorer than to RyRs. The situation is complicated further by the fact that the responses to histamine are not homogeneous, but much larger in adrenergic than in noradrenergic cells (Nunez *et al.* 1995).

Chromaffin cells have a plasmalemmal Na^+/Ca^{2+} exchanger (NCX) which may mediate either Ca^{2+} entry or Ca^{2+} extrusion, depending on the relative Na^+ and Ca^{2+} concentrations and membrane potential. NCX, activity of which is highly dependent on temperature, may contribute to shape the $[Ca^{2+}]_C$ transients and the exocytic responses elicited by Ca^{2+} entry through VOCCs. NCX blockade potentiates the secretion elicited by caffeine or histamine, whereas it either did not modify or antagonized the secretion elicited by high K^+ pulses (Padin *et al.* 2013).

Privileged coupling between SOCE and SERCA in non-excitable cells

Figure 3 illustrates measurements of coupling between plasma membrane Ca^{2+} entry (through SOC) and calcium uptake into the nucleus, the cytosol (same values as for nucleus; not shown for clarity), mitochondria or ER of HEK293 cells. The slope of the signal strength/flow relationship was much larger for ER (about 30 times larger, note double log scale) and approached the excitation–response coupling efficiency found in muscle or in synaptic transmission (Manjarres *et al.* 2011; Alonso *et al.* 2012). This is not surprising given the close relationships between Orai-1, the plasma membrane Ca^{2+} channel of SOC, STIM1, the ER Ca^{2+} sensor of SOCE and activator of Orai1, and SERCA, which is the third element of SOCE (Sampieri *et al.* 2009; Manjarres *et al.* 2010). As a consequence, Ca^{2+} entering through SOC goes almost directly to the ER and mitochondrial uptake is very small (Manjarres *et al.* 2010; Alonso *et al.* 2012). This contrasts with the behaviour of chromaffin cells, where most of the calcium is taken up by mitochondria rather than ER (see above). These differences are in part explained by the size and location of Ca^{2+} microdomains. Figure 4 compares the kinetics of Ca^{2+} accumulation by ER and mitochondria, which is very similar in both models, the excitable chromaffin cells and the non-excitable HEK293 cells. At $[Ca^{2+}]_C$ concentrations near 10^{-7} M, found at rest (box 1 in Fig. 4), ER takes up Ca^{2+} much faster than mitochondria. When $[Ca^{2+}]_C$ reaches 10^{-6} M, as in HCMDs associated with SOCs, the calcium uptake is still faster (about 10 times as much) in the ER than in mitochondria (box 2 in Fig. 4). When $[Ca^{2+}]_C$ exceeds 10^{-4} M, as in the HCMDs generated by VOCC activation plus CICR amplification in chromaffin cells, then transport into mitochondria through the low-affinity/high-capacity MCU dominates (3 in Fig. 4) and most of the Ca^{2+} is taken up by mitochondria.

Relative positioning of the effector could also be relevant. We have mentioned above arguments for the close proximity, both physical and functional, of SOCs and SERCA (Manjarres *et al.* 2010; Alonso *et al.* 2012). By contrast, it has been reported that mitochondria are seldom located close to the plasma membrane or Orai1 channels (Korzeniowski *et al.* 2009; Csordas *et al.* 2010; Singaravelu *et al.* 2011). It has been proposed that the

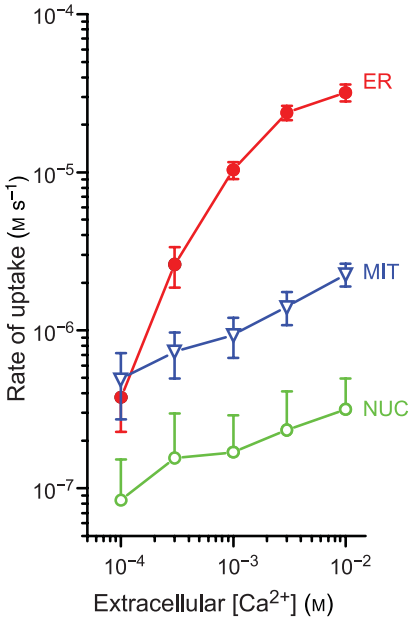

Figure 3. Coupling of Ca^{2+} entry through SOCs to calcium uptake by different organelles in HEK293 cells
Measurements were performed using aequorins targeted to ER, mitochondria (MIT) and nucleus (NUC). SOC was activated by Ca^{2+} emptying of the ER by treatment with 10 μM tert-butyl hydroquinone (TBH), and Ca^{2+} entry was started by washing TBH and adding different extracellular Ca^{2+} concentrations (0.1 to 10 mM), as shown. The rate of entry should increase linearly with $[Ca^{2+}]$ (abscissa). Reproduced with permission from Alonso *et al.* (2012), *Mol Cell Endocrinol* 353, 37–44.

cytoskeleton and microtubule end tracking protein EB1 could play a role in regulating association and dissociation of the SOCE components and other protein complexes (Sampieri *et al.* 2009; Vaca, 2010). On the other hand, nuclear factor of activated T-cells (nFAT) migration to nucleus is exquisitely sensitive to $[Ca^{2+}]_C$ microdomains generated near SOCs (Kar *et al.* 2011, 2012), and selective cross-talk with cAMP signalling (Willoughby, 2012) and with RyRs (Thakur *et al.* 2012) has been suggested. In the case of chromaffin cells, association among VOCCs, mitochondria and the exocytic machinery has been repeatedly reported (Montero *et al.* 2000; Villalobos *et al.* 2002; Garcia *et al.* 2006; Torregrosa-Hetland *et al.* 2011, 2013; Garcia *et al.* 2012), and communication between L-type VOCCs and cAMP response element-binding protein (CREB) regulation of excitation transcription coupling via a Ca^{2+}/calmodulin-dependent protein kinase II acting near the plasma membrane channel has been brilliantly demonstrated (Wheeler *et al.* 2008).

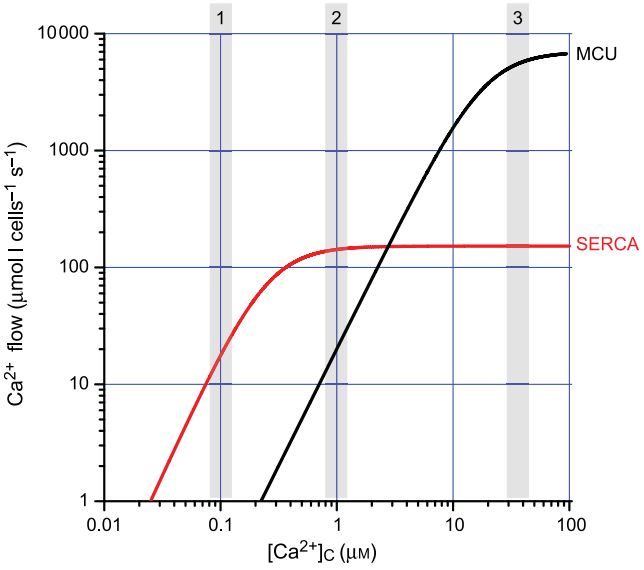

Figure 4. Comparison of intracellular calcium stores refilling at different $[Ca^{2+}]_C$
The curves shown have been generated with the equation:

$$v = (V_{max} \cdot [Ca^{2+}]^n)/(K_m^n + [Ca^{2+}]^n),$$

and are approximately valid for both chromaffin cells and HEK293 cells. The values used for V_{max} and K_m are given in the table below. The shaded boxes correspond to $[Ca^{2+}]_C$ values at rest (box 1) and small (box 2) and large (box 3) high Ca^{2+} microdomains. MCU, mitochondrial calcium uniporter.

	MCU	SERCA
V_{max}	7000	170
K_m	15	0.25
n	2	2

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Additional information

Competing interests

None.

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