Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion

Mayte Montero*, Maria Teresa Alonso*, Estela Carnicero*, Inmaculada Cuchillo-Ibáñez†, Almudena Albillos†, Antonio G. García†, Javier García-Sancho* and Javier Alvarez*‡

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

†Instituto de Farmacología Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, E-28029 Madrid, Spain

‡e-mail: jalvarez@ibgm.uva.es

Activation of calcium-ion (Ca^{2+}) channels on the plasma membrane and on intracellular Ca^{2+} stores, such as the endoplasmic reticulum, generates local transient increases in the cytosolic Ca^{2+} concentration that induce Ca^{2+} uptake by neighbouring mitochondria. Here, by using mitochondrially targeted aequorin proteins with different Ca^{2+} affinities, we show that half of the chromaffin-cell mitochondria exhibit surprisingly rapid millimolar Ca^{2+} transients upon stimulation of cells with acetylcholine, caffeine or high concentrations of potassium ions. Our results show a tight functional coupling of voltage-dependent Ca^{2+} channels on the plasma membrane, ryanodine receptors on the endoplasmic reticulum, and mitochondria. Cell stimulation generates localized Ca^{2+} transients, with Ca^{2+} concentrations above 20–40 μM, at these functional units. Protonophores abolish mitochondrial Ca^{2+} uptake and increase stimulated secretion of catecholamines by three- to fivefold. These results indicate that mitochondria modulate secretion by controlling the availability of Ca^{2+} for exocytosis.

During cell activation, some mitochondria take up Ca^{2+} from cytosolic microdomains of high Ca^{2+} concentration that are generated by activation of nearby Ca^{2+} channels[1–3]. Changes in the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_M$) are therefore heterogeneous at the subcellular level, and the $[Ca^{2+}]_M$ transient in a particular mitochondrion depends on its proximity to a Ca^{2+} source[1–3]. Increases in $[Ca^{2+}]_M$ activate dehydrogenases[2,4], coupling ATP production to energy demands. In addition, increasing amounts of evidence indicate that mitochondria may also modulate homeostasis of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). For example, in neurons and chromaffin cells, mitochondria act as rapid and reversible Ca^{2+} buffers during cell stimulation[5–9] and in the clearance of large Ca^{2+} loads[10]. Moreover, overloading of mitochondria with Ca^{2+} may be involved in the development of several pathological conditions, including ischaemia-reperfusion lesions, neurotoxicity and neurodegenerative diseases, where ATP depletion, overproduction of oxidative radicals and release of apoptotic factors lead to cell damage[11–14]. However, $[Ca^{2+}]_M$ measurements during cell stimulation, obtained by a variety of different techniques, have provided values only in the low micromolar range[1–4,9]. Although these changes are enough to activate mitochondrial metabolism, their physiological significance for cellular Ca^{2+} homeostasis remains unclear. Similarly, the actual relevance of mitochondrial Ca^{2+} overload is obscure. We show here that $[Ca^{2+}]_M$ can in fact reach the millimolar range during stimulation of chromaffin cells, and that this large mitochondrial Ca^{2+} uptake regulates the availability of Ca^{2+} for the secretory machinery.

Results

We used three types of mitochondrially targeted aequorin with different Ca^{2+} sensitivities. This allowed us to measure $[Ca^{2+}]_M$ at a wide range of concentrations, from the submicromolar to the millimolar. Wild-type aequorin reconstituted with native coelenterazine (AEQ1) shows affinity for Ca^{2+} in the concentration range 0.3–8 μM; wild-type aequorin reconstituted with coelenterazine *n* (AEQ2) covers the range 1–40 μM Ca^{2+}; and mutated low-Ca^{2+}-affinity aequorin[15] reconstituted with coelenterazine *n* (AEQ3) can measure Ca^{2+} in the concentration range 20 μM to 1 mM[16]. A further decrease in the Ca^{2+} sensitivity of aequorin was obtained by using AEQ3 at 22 °C (ref. 17). Another property of aequorin, its irreversible consumption as it emits light, enables it to be used in detecting subcellular heterogeneities in $[Ca^{2+}]$ (ref. 18). Aequorin with high Ca^{2+} affinity is rapidly consumed in areas of high $[Ca^{2+}]$. After that, measurements reflect only the behaviour of low-$[Ca^{2+}]$ areas. Instead, when low-Ca^{2+}-affinity aequorin is used, only regions with high $[Ca^{2+}]$ contribute significantly to the luminescence. In each case, the relative fraction of aequorin consumed provides an estimation of the size of the different $[Ca^{2+}]$ pools.

Heterogeneity of the $[Ca^{2+}]_M$ response. With regard to their response to $[Ca^{2+}]$, we found two main populations of mitochondria in chromaffin cells. Figure 1a, b show similar experiments performed with either AEQ1 or AEQ2. Cell depolarization using a medium with a high potassium-ion (K^+) concentration triggered a rapid consumption of 40–50% aequorin in both cases (dotted line). Calibrated in $[Ca^{2+}]$ (continuous line), we estimated peaks of 5 μM and 30 μM Ca^{2+} when using AEQ1 and AEQ2, respectively. Subsequent depolarizations produced smaller $[Ca^{2+}]_M$ peaks of similar magnitude (about 2 μM) when calculated using both types of aequorin. Figure 1c, d show similar experiments but using caffeine instead of high $[K^+]$. Caffeine activates ryanodine receptors (Ca^{2+} channels found on the endoplasmic reticulum), inducing Ca^{2+} release from the endoplasmic reticulum[19]. The first addition of caffeine resulted in the consumption of ~30% of AEQ1 or AEQ2, a value that converts to estimated $[Ca^{2+}]_M$ peaks of 5 μM and 40 μM, respectively. Subsequent additions of caffeine produced much smaller $[Ca^{2+}]_M$ peaks that gave similar calibrated results with both aequorins ($[Ca^{2+}]_M$ 2 μM). The $[Ca^{2+}]_c$ response to the same pattern of stimuli was very different, with consecutive additions of high K^+ concentrations or caffeine producing similar $[Ca^{2+}]_c$ peaks[19]. The discrepancy can be explained only if ~50% of chromaffin-cell mitochondria respond to stimulation with 2 μM $[Ca^{2+}]_M$ peaks while the rest undergo a much larger increase in $[Ca^{2+}]_M$. Aequorin in the latter pool of mitochondria is consumed during the first stimulus, and subsequent stimuli evoke only the smaller response from the other mitochondria. In contrast to HeLa

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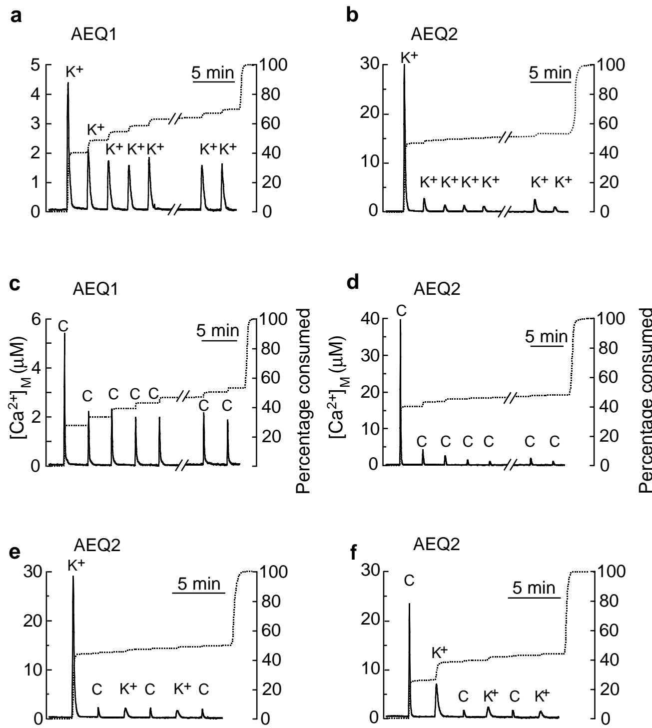

Figure 1 **Effects of high K$^+$ concentrations or caffeine on $[Ca^{2+}]_M$, measured with AEQ1 or AEQ2.** The effects of 10-s pulses with medium containing 70 mM K$^+$ (K$^+$) and/or 30-s pulses with 50 mM caffeine (C) on $[Ca^{2+}]_M$ (continuous line) and the aequorin consumption (values shown are the percentage of aequorin consumed; dotted line), measured either with AEQ1 (**a**, **c**) or with AEQ2 (**b**, **d–f**). The total amount of aequorin consumed was measured at the end of every experiment by perfusion of 100 μM digitonin plus 10 mM Ca^{2+}. The breaks in **a–d** indicate 15 min without stimulation. The temperature was 37 °C.

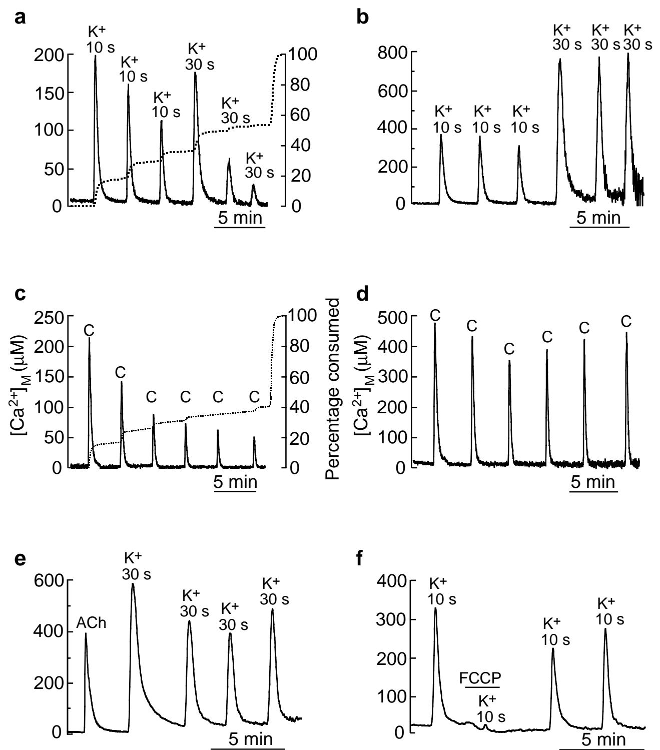

Figure 2 **Effects of high K$^+$ concentrations or caffeine on $[Ca^{2+}]_M$ measured with AEQ3.** **a**, **c**, The effects of 10-s or 30-s pulses with a high K$^+$ concentration (K$^+$) or 30-s pulses with 50 mM caffeine (C) on $[Ca^{2+}]_M$, measured with AEQ3 (continuous line), and on the percentage of aequorin consumption (dotted line). **b**, **d**, Results of experiments that were the same as those in **a**, **c** but recalibrated by assuming that $[Ca^{2+}]_M$ changes occurred in only 58% and 48%, respectively, of the total space. **e**, The effect of a 10-s pulse with 10 mM acetylcholine (ACh) followed by 30-s K$^+$ pulses. **f**, 2 μM FCCP and 10-s K$^+$ pulses were perfused as indicated. Temperature was 37 °C.

cells, for which extensive mitochondrial lumenal diffusion has been reported[20], large $[Ca^{2+}]_M$ peaks were not restored in chromaffin cells after 20 min without stimulation (Fig. 1).

Co-localization of Ca^{2+}-entry and -release sites. We used the pattern of AEQ2 consumption during the consecutive addition of high K$^+$ levels and caffeine to study the degree of overlapping among the mitochondrial pools that respond to each stimulus with large $[Ca^{2+}]_M$ peaks. Caffeine was unable to induce any large $[Ca^{2+}]_M$ response after treatment of cells with high [K$^+$]; however, caffeine applied before K$^+$ resulted in the consumption of ~60% of the aequorin that was able to respond to high [K$^+$] with large $[Ca^{2+}]_M$ peaks (Fig. 1e, f). If we consider the amount of AEQ2 consumption after two consecutive pulses of high [K$^+$] and caffeine to be 100%, then the initial pulse of high [K$^+$] resulted in the consumption of 96.5± 0.4% (mean±s.e.m.; n=10) of this aequorin. However, if caffeine were added before K$^+$, the initial caffeine pulse resulted in the consumption of only 59±2% of AEQ2 (n=16). We conclude that about 30% of mitochondria co-localize with both plasma-membrane Ca^{2+} channels (which are activated by high [K$^+$]) and ryanodine receptors (which are activated by caffeine). An additional 20% of mitochondria co-localize with plasma-membrane Ca^{2+} channels, but not with ryanodine receptors. The remaining 50% of the mitochondria are apparently located far away from these Ca^{2+} hotspots.

Millimolar $[Ca^{2+}]_M$ transients. Peak $[Ca^{2+}]_M$ values calculated using AEQ2 (30–40 μM) are underestimated because the aequorin is consumed. Quantification of the real values of the large $[Ca^{2+}]_M$ peaks required the use of the lower-Ca^{2+}-affinity AEQ3. Figure 2a shows the $[Ca^{2+}]_M$ changes induced by a series of high-[K$^+$] pulses. The initial $[Ca^{2+}]_M$ peak reached 200 μM, and subsequent peaks decreased in magnitude as soon as aequorin consumption approached 50–60%. These values are still underestimates, because large $[Ca^{2+}]_M$ changes take place in only half of the mitochondria. Taking this fact into account (Fig. 2b), we estimate $[Ca^{2+}]_M$ peaks of similar magnitude for equal repetitive stimuli, with peaks being ~300 μM (340±20 μM, n= 15) for 10-s pulses and ~700 μM (670±30 μM, n=11) for 30-s pulses. Figure 2c shows the effect of caffeine, which produced a first $[Ca^{2+}]_M$ peak of 200 μM and then decreasing peaks as consumption approached 40%. Again, if data are recalibrated considering only the active mitochondrial space (Fig. 2d), we obtain a series of similar fast $[Ca^{2+}]_M$ transients reaching peak values of near 500 μM (430±30 μM, n=8). The effect of caffeine resulted from Ca^{2+} release from intracellular stores, because it produced the same $[Ca^{2+}]_M$ transient in Ca^{2+}-free medium (data not shown).The physiological agonist acetylcholine also produced a large $[Ca^{2+}]_M$ peak (280±50 μM, n=4; Fig. 2e). Finally, the high-[K$^+$]-induced $[Ca^{2+}]_M$ transient was reversibly abolished by the protonophore carbonyl cyanide p-trifluoromethoxy phenyl hydrazone (FCCP) (Fig. 2f).

Return of $[Ca^{2+}]_M$ to resting levels after stimulation was fast, reaching background values within 1–2 min. CGP37157, an inhibitor of the mitochondrial Na$^+$/Ca^{2+} exchanger, reduced the rate of $[Ca^{2+}]_M$ decrease by 60% (half-time 62±3 s, n=6; control, half-time 25±1 s, n=9; Fig. 3a). The $[Ca^{2+}]_M$ and $[Ca^{2+}]_c$ transients induced by high [K$^+$] follow very similar time courses (Fig. 3b). This means that $[Ca^{2+}]_M$ is able to follow rapid oscillations in $[Ca^{2+}]_c$, but at concentrations more than two orders of magnitude higher. Ca^{2+} exit from mitochondria and Ca^{2+} uptake by mitochondria differed in sensitivity to temperature. The rate of $[Ca^{2+}]_M$ increase was very similar at 22 °C (67±2 μM s^{-1}, n=17) and 37 °C (58±3 μM s^{-1}, n=15). The

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kinetics of the $[Ca^{2+}]_c$ transient were also similar at both temperatures (Fig. 3c). However, the rate of Ca^{2+} exit from mitochondria was nearly fourfold slower at 22 °C than at 37 °C (half-times 92±4 s, n=11, and 25±1 s, n=9, at 22 °C and 37 °C, respectively). Therefore, at 22 °C, the $[Ca^{2+}]_M$ transient lasted for much longer than the cytosolic one. The height of the $[Ca^{2+}]_M$ peaks induced by the stimuli was also larger at 22 °C than at 37 °C (10-s high-[K+] pulse, $[Ca^{2+}]_M$ peak = 550 ± 30 μM, n = 10; 30-s high-[K+] pulse, $[Ca^{2+}]_M$ peak = 870±30 μM, n=8; caffeine, $[Ca^{2+}]_M$ peak=520±50 μM, n=4; acetylcholine, $[Ca^{2+}]_M$ peak=670±40 μM, n=6). These findings are consistent with the reported ability of mitochondria to prolong the $[Ca^{2+}]_c$ peaks by sequestering Ca^{2+} during the stimulation and releasing it afterwards, in experiments performed at room temperature[5–9]. Our results indicate that the ability of mitochondria to prolong $[Ca^{2+}]_c$ transients should be much less at 37 °C. Figure 3c also shows the inhibition by CGP37157 of mitochondrial Ca^{2+} release at 22 °C (half-time 380±60 s, n=3; 76% inhibition).

Mitochondria as biosensors of local $[Ca^{2+}]_c$. The large $[Ca^{2+}]_M$ peaks that we have observed can be generated only if mitochondria are exposed to very high cytosolic Ca^{2+} concentrations. As the rate of Ca^{2+} uptake by mitochondria is proportional to $[Ca^{2+}]_c$ (refs 1, 10, 21, 22), we can estimate the size of the local $[Ca^{2+}]_c$ transients by comparing the rates of $[Ca^{2+}]_M$ increase observed in intact cells with those obtained in permeabilized cells perfused with known Ca^{2+} concentrations (Fig. 4a). A similar approach has been used in permeabilized RBL mucosal mast cells to show that inositol-1,4,5-trisphosphate produces local $[Ca^{2+}]_c$ transients above 16 μM (ref. 21). We found that the consumption of aequorin by permeabilized cells exposed to Ca^{2+} buffers followed a monotonic rate, as would be expected for a single mitochondrial pool with a homogeneous rate of Ca^{2+} uptake. This result indicates that the heterogeneous responses shown in Fig. 1 are due to the subcellular localization of each mitochondrial pool and not to differences in Ca^{2+}-uptake properties. The relationship between the rate of $[Ca^{2+}]_M$ increase and $[Ca^{2+}]_c$ is plotted in Fig. 4b. The rate of $[Ca^{2+}]_M$ increase was negligible below 4 μM $[Ca^{2+}]_c$, but increased steeply above these values and was fully blocked by ruthenium red, a blocker of the mitochondrial Ca^{2+} uniporter. Fitted values for the maximal rate of uptake and for the $[Ca^{2+}]_c$ that gives a half-maximal uptake rate were 225 μM s^{-1} and 43 μM, respectively. This K_M value is consistent with previous measurements made in chromaffin cells[10,22]. The rate of increase of $[Ca^{2+}]_M$ obtained in cells stimulated with high [K+] corresponded to ~20 μM $[Ca^{2+}]_c$. The rates of $[Ca^{2+}]_M$ increase observed in intact cells stimulated with caffeine or acetylcholine were, respectively, 110±9 μM s^{-1} (n=11) and 94±7 μM s^{-1} (n=6), which correspond to cytosolic Ca^{2+} concentrations of 40 μM and 30 μM, respectively.

In those mitochondria undergoing small (1–2 μM) $[Ca^{2+}]_M$ transients on stimulation (Fig. 1), the rate of high-[K+]-induced $[Ca^{2+}]_M$ increase was only 0.29±0.02 μM s^{-1} (n=35). This rate corresponds to a $[Ca^{2+}]_c$ of 2–3 μM, close to the mean $[Ca^{2+}]_c$ values measured using Fura-2 (Fig. 3b).

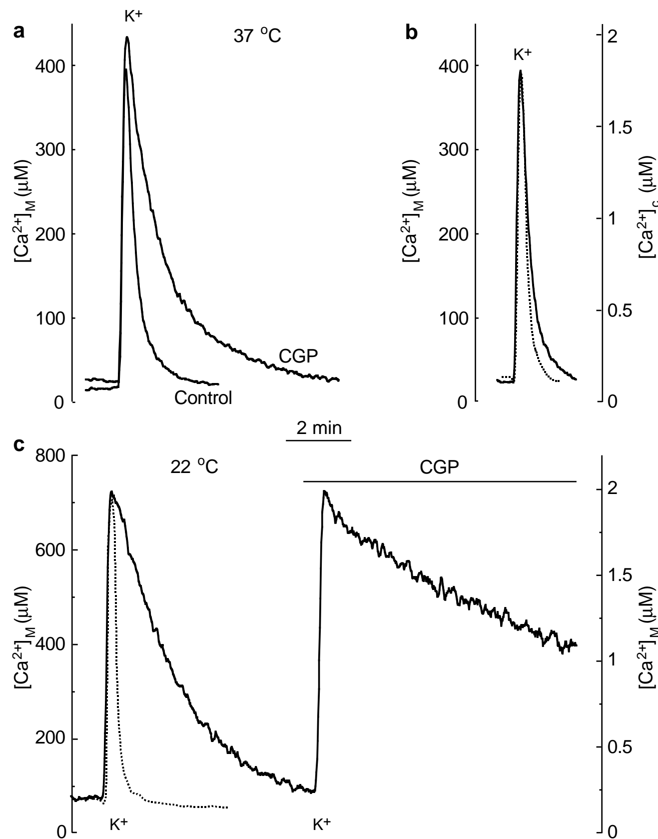

Figure 3 **Effects of CGP37157, an inhibitor of the mitochondrial Na+/Ca^{2+} exchanger, and temperature on the rate of $[Ca^{2+}]_M$ decrease after stimulation. a**, Control cells and cells incubated at 37 °C for 2 min with 20 μM CGP37157 (CGP) were stimulated with a 10-s pulse of high [K+] (K+). The inhibitor was also present after the stimulus. **b**, Comparison of the kinetics of the $[Ca^{2+}]_M$ (continuous line) and $[Ca^{2+}]_c$ (dotted line) transients at 37 °C after stimulation with a 10-s pulse of high [K+]. **c**, 10-s pulses of high [K+] were given at 22 °C in the presence or absence of 20 μM CGP37157, as indicated. The kinetics of the $[Ca^{2+}]_c$ transient at 22 °C is also shown (dotted line). Experiments were performed using AEQ3 and calibrated using the assumption that $[Ca^{2+}]_M$ changes occurred in only 55% of the total space.

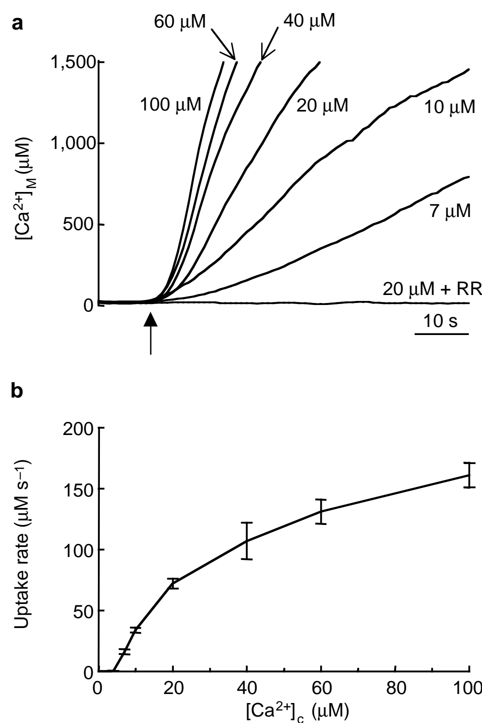

Figure 4 **Dependence of the rate of mitochondrial Ca^{2+} uptake on $[Ca^{2+}]_c$. a**, An increase in $[Ca^{2+}]_M$ was induced by perfusion of permeabilized cells with buffers of known Ca^{2+} concentration, as indicated. In the curve labelled '20 μM+RR', the buffer containing 20 μM Ca^{2+} was added in the presence of 4 μM ruthenium red. These experiments were carried out using AEQ3 at 22 °C, to increase the sensitivity of the measurements at high uptake rates. **b**, Plot of the maximum rates of uptake obtained at every Ca^{2+} concentration. Data at 2 μM and 4 μM $[Ca^{2+}]_c$ (rates of uptake 0.2 and 0.45 μM s^{-1}, respectively) were obtained using AEQ2. Error bars show s.e.m. of four determinations made for each point.

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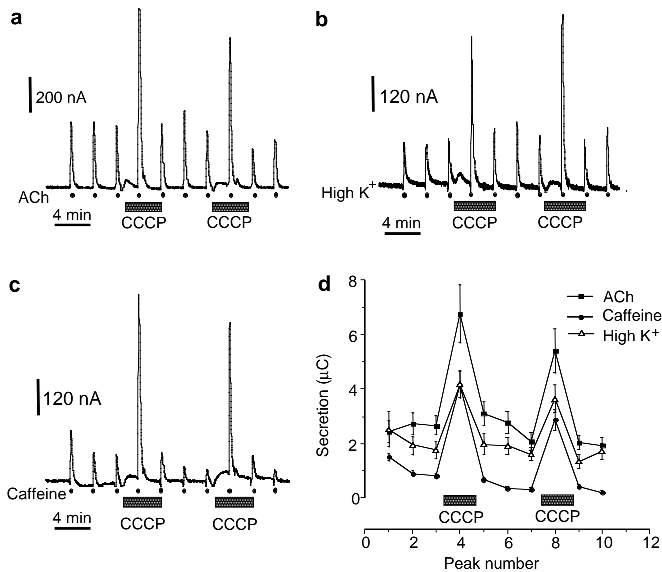

Figure 5 **CCCP enhances secretory responses evoked by acetylcholine, high K^+ concentration or caffeine. a–c**, Cells were superfused with Krebs–HEPES medium. After a 10-min equilibration period, to get a stable baseline secretion, cells were challenged at 2-min intervals with 2-s pulses of **a**, 100 µM acetylcholine (ACh), **b**, medium with a high K^+ concentration (70 mM, with iso-osmotic reduction of Na^+), or **c**, caffeine (50 mM). The dots at the bottom of the panels each represent one challenge with one of these secretagogues. In addition, 2 µM CCCP was applied in two phases, each of 90 s, as indicated by the bars. Graphs represent original traces from three typical experiments using pulses of acetylcholine (**a**), high [K^+] (**b**) or caffeine (**c**). The amplitudes of secretion peaks are expressed as oxidation currents (measured in nA). **d**, Averaged results obtained from ten repetitions each of the experiments described in **a–c**. Total secretion was calculated by integration of the areas of the secretory peaks, and is expressed as µC (µA × s).

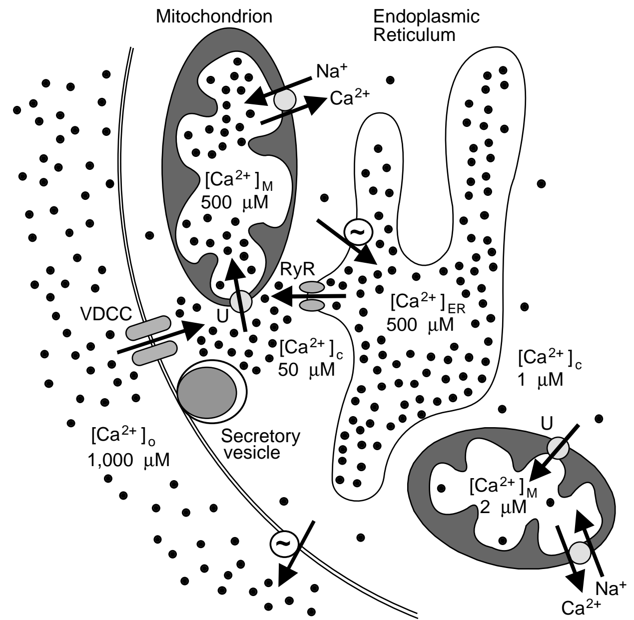

Figure 6 **The complex functional unit responsible for the generation of local high $[Ca^{2+}]_c$ transients and catecholamine secretion in a bovine chromaffin cell.** The voltage-dependent Ca^{2+} channel (VDCC), the Ca^{2+} uniporter (U) of the upper mitochondria, the ryanodine receptor (RyR) from the endoplasmic reticulum (ER) and the secretory vesicle are all strategically located close together beneath the plasma membrane. Ca^{2+} entry through VDCCs triggers Ca^{2+}-induced Ca^{2+}-release from the ER through RyRs and generates a local $[Ca^{2+}]_c$ hotspot of about 50 µM. In the rest of the cell, the $[Ca^{2+}]_c$ increase is much less, the resulting $[Ca^{2+}]_c$ being 1–2 µM. Mitochondria take up Ca^{2+} through the uniporter, with uptake depending on the surrounding $[Ca^{2+}]_c$. The uniporter located close to the $[Ca^{2+}]_c$ hotspot in this diagram will take up Ca^{2+} very quickly and the mitochondrial $[Ca^{2+}]$ will reach near millimolar levels. This uptake effectively reduces the amount of Ca^{2+} available for the secretory vesicle, allowing a tight control of local $[Ca^{2+}]_c$ and hence of the secretory response. Ca^{2+} is then released through the Na^+/Ca^{2+} exchanger. The other mitochondrion, placed far away from the region of high $[Ca^{2+}]_c$, increases its $[Ca^{2+}]_M$ to only about 2 µM. $[Ca^{2+}]_{ER}$, the ER Ca^{2+} concentration; $[Ca^{2+}]_o$, the extracellular Ca^{2+} concentration.

Modulation of secretion by mitochondria. We analysed the physiological relevance of mitochondrial Ca^{2+} uptake by comparing catecholamine secretion induced by stimulation with high [K^+], caffeine or acetylcholine, both in the presence and in the absence of the protonophore carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). We expected that CCCP, by preventing Ca^{2+} sequestration by mitochondria, would allow a larger local increase in subplasmalemmal $[Ca^{2+}]_c$ and hence potentiate secretion. This was the case (Fig. 5). In response to cellular challenge with acetylcholine (100 µM for 2 s, at 2-min intervals), fairly reproducible secretory peaks of around 300 nA were produced; CCCP (2 µM) reversibly increased the secretory response to ~1,000 nA (Fig. 5a). Similar results were obtained when cells were challenged with high [K^+] (70 mM K^+, 2 s; Fig. 5b) or caffeine (50 mM, 2 s; Fig. 5c). Figure 5d shows the averaged total secretion obtained from ten repetitions of each of these experiments. The small effect on secretion of CCCP alone disappeared when the interval between stimuli was increased to 5 min, but potentiation of secretion remained unchanged.

Potentiation of secretion by CCCP was not due to increased Ca^{2+} uptake from extracellular sources. Experiments in which the uptake of $^{45}Ca^{2+}$ was measured showed that CCCP did not increase the net Ca^{2+} uptake induced by any of the stimuli (data not shown). Neither was the effect of CCCP mediated by a possible Ca^{2+} release from the endoplasmic reticulum. When cells were treated with a combination of 10 mM caffeine, 10 µM ryanodine and 1 µM thapsigargin in Ca^{2+}-free medium for 30 min, caffeine produced no secretory response, but the potentiation by CCCP of K^+-induced catecholamine secretion was unmodified (data not shown). Finally, mitochondrial inhibitors produced the same effects as CCCP. We stimulated cells with 2-s high-[K^+] pulses every 2 min. Oligomycin (3 µM, preincubation 90 s), which blocks ATP production but should not decrease the mitochondrial membrane potential, had little effect on K^+-stimulated secretion. The mean normalized peak secretion was 100 ± 12% in control pulses, and 139 ± 21% in the presence of oligomycin (mean ± s.e.m, $n = 11$; $P > 0.1$, Student's *t*-test). However, a combination of 4 µM rotenone (which blocks respiration) and 3 µM oligomycin produced a large potentiation of high-K^+-induced secretion. The mean normalized peak secretion in control pulses was 100±6%, and it increased to 204±21% ($n = 11$; $P < 0.001$, *t*-test) in the presence of oligomycin plus rotenone.

Discussion

It was astonishing to find that some mitochondria can undergo, in stimulated intact cells, rapid reversible increases in Ca^{2+} concentration from the submicromolar to the millimolar range. Such high mitochondrial Ca^{2+} concentrations were previously associated only with pathological phenomena related to cell damage or the initial stages of apoptosis[10–13]. These $[Ca^{2+}]_M$ values contrast with those obtained in a similar preparation using the fluorescent dye rhod2 (ref. 9). The discrepancy can be explained by differences in the specificity of targeting and range of measurement of the probes.

Co-localization of those mitochondria undergoing large $[Ca^{2+}]_M$ transients with both plasma-membrane Ca^{2+} channels and ryanodine receptors reveals the presence of functional units of Ca^{2+}-induced

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Ca^{2+} release (CICR). Our data indicate that most of the ryanodine receptors in chromaffin cells are either located within these CICR functional units or have no mitochondria placed near them. A precise spatial relationship between ryanodine receptors and mitochondria occurs only at these functional CICR units. The large size of the mitochondrial pool close to the Ca^{2+}-entry and -release sites (50%) indicated that it might be important for rapid buffering of Ca^{2+} in local subplasmalemmal regions. We confirmed this by showing that catecholamine secretion induced by several stimuli depended markedly on mitochondrial Ca^{2+} uptake. When this uptake was blocked, either by collapsing the mitochondrial transmembrane H^+ gradient with CCCP or by blocking respiration with rotenone and preventing reversal of the mitochondrial ATPase with oligomycin, the secretory response increased several-fold. These results indicate that mitochondrial Ca^{2+} uptake controls the increase of $[Ca^{2+}]_c$ at exocytic sites. In addition, blockade of mitochondrial Ca^{2+} uptake could allow spreading of the Ca^{2+} wave to deeper cytosolic areas, thus favouring mobilization of the reserve pool of secretory vesicles[23]. In any case, our results support the idea that chromaffin cells possess complex functional units (Fig. 6) that cluster together all the elements needed to control the subplasmalemmal $[Ca^{2+}]_c$ transients near exocytic sites. The interplay between Ca^{2+}-channel activation, mitochondrial Ca^{2+} uptake and CICR will thus modulate the rate and extent of exocytosis and vesicle transport to exocytic sites.

We also used mitochondria as biosensors to quantify the localized $[Ca^{2+}]_c$ transients generated during cell stimulation. We found that about 50% of cell mitochondria become exposed to a cytosolic Ca^{2+} concentration of 20–40 µM. These values are consistent with the upper reported estimates of the local $[Ca^{2+}]_c$ reached during physiological stimulation[24]. We cannot estimate the size of these hotspots. However, half of the mitochondria in the same cells detected only 2–3 µM $[Ca^{2+}]_c$ during stimulation, indicating the presence of a steep subcellular $[Ca^{2+}]_c$ gradient.

Finally, we speculate that if either the location or the Ca^{2+}-uptake properties of those mitochondria located in the functional units could be modulated, this would be an effective mechanism by which to regulate secretion. If similar phenomena were applicable to neurons, it would be also a very simple mechanism by which to generate synaptic plasticity. Under pathological conditions or during ageing, mitochondrial damage may reduce the ability of mitochondria to take up Ca^{2+}. This would lead to increased secretion of neurotransmitters and increased cell activation, a vicious cycle that may trigger processes leading to necrosis or apoptosis. □

Methods

Cell culture and expression of aequorin.

Bovine adrenal medulla chromaffin cells were obtained as described[19] and used within 1–3 days. The mitochondrial aequorin[1,2] was a gift from T. Pozzan. Mutated (Asp 119 → Ala) mitochondrial aequorin was obtained by replacing, in-frame, complementary DNA encoding the wild-type aequorin with that encoding the mutated aequorin[15]. Expression in chromaffin cells was achieved by infecting the cells with a defective herpes simplex virus type 1 containing the mitochondrial aequorin gene (pHSVmitAEQ). Virus packaging and titring have been described[25]. Chromaffin-cell cultures (5×10^5 cells per 0.5 ml) were routinely infected with 2×10^3 infectious virus units 12–24 h before measurements. FCCP and CCCP were obtained from Sigma. CGP37157 was from Tocris (Bristol, UK).

$[Ca^{2+}]_M$ and $[Ca^{2+}]_c$ measurements.

To reconstitute aequorins, cells expressing mitochondrial aequorin were incubated for 1–2 h at room temperature with 1 µM of either wild-type coelenterazine or coelenterazine *n*, in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Cells were then placed in the perfusion chamber of a purpose-built thermostatic luminometer at 22 °C or 37 °C. For experiments with permeabilized cells, reconstitution was performed as above and cells were placed in the luminometer. Standard medium containing 0.5 mM EGTA instead of $CaCl_2$ was then perfused for 1 min, followed by a 1-min perfusion of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.2 mM EGTA, 1 mM ATP, 20 µM ADP, 2 mM succinate, 20 mM HEPES, pH 7) containing 20 µM digitonin. Intracellular medium without digitonin was perfused for 1 min, followed by buffers of known $[Ca^{2+}]$, between 2 and 100 µM, prepared using HEDTA/Ca^{2+}/Mg^{2+} mixtures. Calibration was done using the calibration curves corresponding to each aequorin type. Calibration curves for AEQ3 at both 22 °C and 37 °C have been described[16,17]. For AEQ1 and AEQ2, new calibration curves at 37 °C were generated as described[16]. Aequorin consumption was calculated as the integral of the luminescence measured during the experiment, normalized as a percentage. $[Ca^{2+}]_c$ measurements were obtained using Fura-2 as described[19]. Statistical values are given as means ± s.e.m.

On-line measurements of catecholamine release.

Cells were scraped off carefully from the bottom of the Petri dish with a rubber policeman, and centrifuged at 800 r.p.m. (Heraeus centrifuge) for 10 min. The cell pellet was resuspended in 200 µl Krebs-HEPES medium (144 mM NaCl, 5.9 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose, 10 mM HEPES, pH 7.4). Cells were introduced in a jacketed microchamber for superfusion at 37 °C. The superfusion rate was 2 ml min^{-1}. The liquid flowing from the superfusion chamber reached an electrochemical detector (model Metrohn AG CH-9100 Hersau), placed just at the outlet of the microchamber, that monitors 'on-line', under the amperometric mode, the amount of catecholamines secreted[26]. Cells were stimulated to secrete with short pulses (2 s) of Krebs-HEPES solution containing the stimuli.

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Correspondence and requests for materials should be addressed to J.A.

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