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Modulation of secretion by the endoplasmic reticulum in mouse chromaffin cells

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Abstract

The endoplasmic reticulum (ER) has been suggested to modulate secretion either behaving as a Ca^{2+} sink or as a Ca^{2+} source in neuronal cells. Working as a Ca^{2+} sink, through ER-Ca^{2+} pumping, it may reduce secretion induced by different stimuli. Instead, working as a Ca^{2+} source through the Ca^{2+} induced Ca^{2+} release (CICR) phenomenon, it may potentiate secretion triggered by activation of plasma membrane Ca^{2+} channels. We have previously demonstrated the presence of CICR in bovine chromaffin cells, but we now find that mouse chromaffin cells almost lack functional caffeine-sensitive ryanodine receptors in the ER and, consistently, no CICR from the ER could be observed. In addition, inhibition of ER Ca^{2+} pumping with ciclopiazonic acid or thapsigargin strongly stimulated high-K^+-evoked catecholamine secretion and cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) transients. Surprisingly, 5 mM caffeine reduced high-K^+-induced $[Ca^{2+}]_c$ peaks but considerably potentiated secretion induced by high-K^+ stimulation. However, this potentiation was insensitive to ryanodine and additive to that induced by emptying the ER of Ca^{2+} with thapsigargin, suggesting that it is unrelated to the activation of ryanodine receptors. We conclude that, in mouse chromaffin cells, CICR is not functional and the ER strongly inhibits secretion by acting as a damper of the $[Ca^{2+}]_c$ signal.

Introduction

Ca^{2+} binding to ryanodine receptors (RyR) present in the endoplasmic reticulum (ER) may lead to the opening of these receptors, triggering Ca^{2+} release into the cytosol (Berridge, 1998). This phenomenon, known as Ca^{2+} induced Ca^{2+} release (CICR), is a well-characterized mechanism in heart cells, but its presence and functional role in other excitable cells, in particular neuronal cells, is unclear. Three isoforms of RyR are expressed in mammalian tissues. RyR1 and RyR2 are expressed predominantly in sarcoplasmic reticulum of skeletal muscle and heart, respectively (Sutko & Airey, 1996; Zucchi & Ronca-Testoni, 1997), but all three RyR have been also found in brain (McPherson & Campbell, 1993; Sorrentino & Volpe, 1993; Giannini *et al.*, 1995; Martin *et al.*, 1998; Mori *et al.*, 2000; Faure *et al.*, 2001) and other tissues, including the adrenal gland (Giannini *et al.*, 1995; Mackrill *et al.*, 1997; Jiménez & Hernández-Cruz, 2001). The presence of RyR in different neuronal tissues, together with the demonstration of a functional role of caffeine- or ryanodine-sensitive Ca^{2+} stores in neuronal Ca^{2+} transients or synaptic transmission (Peng, 1996; Smith & Cunnane, 1996; Mothet *et al.*, 1998; Narita *et al.*, 1998, 2000; Llano *et al.*, 2000; Emptage *et al.*, 2001; Carter *et al.*, 2002) has led to suggest an important role for CICR in Ca^{2+} signalling, Ca^{2+} homeostasis and activity-dependent synaptic plasticity in neurons (Rose & Konnerth, 2001).

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Measuring CICR in small neuronal cells has proven elusive because of the difficulty of distinguishing Ca^{2+} release from Ca^{2+} entry when only cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) is monitored. In bovine chromaffin cells, we have shown unequivocally the presence of CICR by measuring directly the $[Ca^{2+}]$ in the ER ($[Ca^{2+}]_{ER}$). Stimulation with high-K^+ medium produced a large increase in $[Ca^{2+}]_c$ in parallel with a rapid decrease of $[Ca^{2+}]_{ER}$ mediated by RyR (Alonso *et al.*, 1999). However, the importance of this phenomenon for catecholamine secretion is unclear. Capacitance measurements in bovine chromaffin cells have shown a depression in the secretory response after ER Ca^{2+} depletion with thapsigargin (Mollard *et al.*, 1995; Pan & Fox, 2000). In the same cells, measurements in cell populations have suggested that the ER could behave either as a Ca^{2+} source (enhancing secretion) when it was full or as a Ca^{2+} sink (reducing secretion) when it was fully emptied by caffeine (Lara *et al.*, 1997). In contrast, it has been shown recently (Cuchillo-Ibáñez *et al.*, 2002) that irreversible emptying of the ER with thapsigargin modifies little the secretory response induced by high-K^+. Although most of these data suggest a role for the ER in the modulation of the secretory response, the precise mechanism(s) of this modulation remain obscure. We have used here mouse chromaffin cells to study this phenomenon and show that they behave in a very different way regarding the CICR phenomenon. They almost lack functional RyR in the ER and both the $[Ca^{2+}]_c$ increase and the secretory responses induced by high-K^+ were strongly potentiated by inhibiting ER Ca^{2+} pumping. Interestingly, the secretory response was also potentiated by low concentrations of caffeine, but this effect was unrelated to CICR, as it was insensitive to ryanodine and additive to that obtained after ER Ca^{2+} depletion.

Materials and methods

Animal and surgical procedures

Experiments were performed using adrenal medulla from mice of 6–8 weeks. Mice were anaesthetized with sodium pentobarbitone administered intraperitoneally (60 mg/kg body weight). After a longitudinal incision in the abdomen, adrenal glands were removed and placed in a lucite chamber filled with ice cold Tyrode-bicarbonate solution (116 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$; 1.1 mM $MgCl_2$, 5 mM glucose, 23 mM $NaHCO_3$) equilibrated with 95%O_2 : 5%CO_2. Under a dissecting microscope, adrenal glands were gently decapsulated with fine forceps (Dumont n°5) and stored in fresh ice cold Tyrode until tissues were processed for measuring catecholamine content, catecholamine secretion or monitoring calcium levels in dissociated cells from primary tissue cultures using recombinant aequorin.

Measurement of catecholamine content

Adrenal medulla were homogenized (glass/glass) in ice-cold (200 µL) perchloric acid 0.2 M containing 0.01% EDTA and centrifuged (10 000 × *g* for 10 min). Aliquots from supernatant (25 µL) were injected directly into a high-performance liquid chromatography-electrochemical detection (HPLC-ED) system. HPLC-ED system was composed of a Milton Roy CM 400 pump (Riviera, FL, USA), a Water c18 (particle size 4 µm) column, a Water U6K injector (Milford, MA, USA) and a Bioanalitical System (West Lafayette, IN, USA) LC-4 electrochemical detector (holding potential of 0.65 V). Identification and quantification of endogenous content were performed against external standards (PeakSimple Chromatography Data System; SRI instruments, East Norwalk, CT, USA).

Amperometric measurements of catecholamine secretion

Adrenal medulla were incubated in a diluted enzymatic solution for 20 min at 37 °C with gentle agitation in order to facilitate penetration of the carbon fibre electrode into the tissues. The diluted enzymatic Tyrode-bicarbonate solution equilibrated with 95%O_2 : 5%CO_2 contains collagenase (0.1%, Worthington type I, Lakewood, NJ, USA) and trypsin (0.01% Sigma grade IX). After washing in fresh ice-cold Tyrode, tissues previously subjected to the diluted enzymatic solution, were transferred to a thermostatized lucite recording chamber (37 °C, volume 200 µL) and superfused by gravity with Tyrode bicarbonate solution equilibrated with 20% O_2 : 5% CO_2 : 75% N_2 (3–4 mL/min). The perfusion system had several lines and switching from one to another was made by an electronic valve system. Both saline reservoir bottles and the superfusion tube lines were immersed in a circulating water bath at 37 °C.

Free catecholamines were measured using carbon fibre electrodes composed of a single 5-µm carbon fibre insulated, except for the tip, with a polyethylene tube (ProCFE, DAGAN Instruments, Minneapolis, MN, USA). The electrodes were attached to an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN, USA). Recordings were undertaken with a fixed voltage (0.6 V amperometric mode) against an AgCl reference electrode. This voltage is optimum for measuring norepinephrine and epinephrine (the main catecholamine contained in mice adrenal medulla), as was demonstrated by the HPLC-ED measurements of the catecholamine content in mice adrenal medulla. Currents corresponding to free catecholamine measurements were sampled at 4 Hz, recorded by computer (Data sponge, WPI Instruments, Sarasota, FL, USA) and later analyzed offline (Microcal Origin, Northampton, MA, USA).

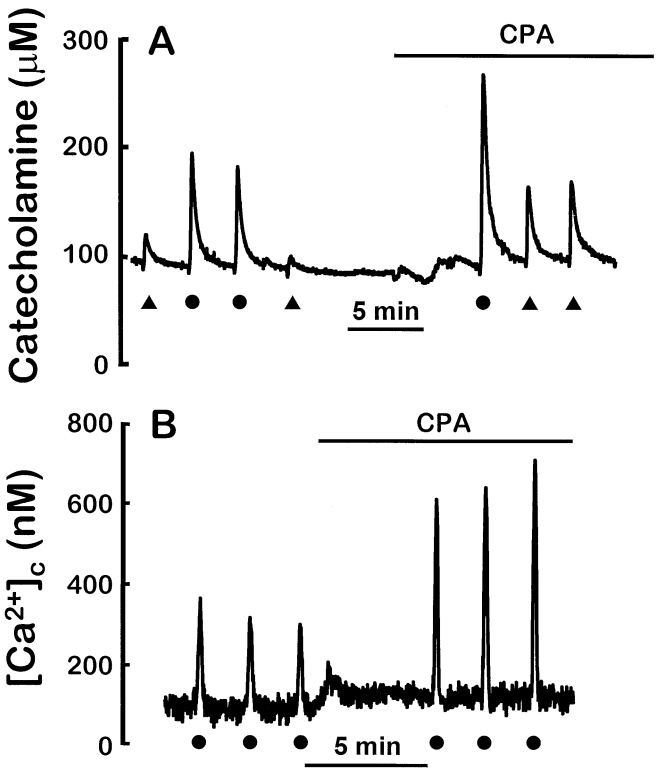

FIG. 1. Effects of ciclopiazonic acid on catecholamine secretion and $[Ca^{2+}]_c$ increase induced by high K^+. In panel A, catecholamine secretion induced by either 5 s or 10 s pulses (marked by ▲ or ● symbols at the bottom) of medium containing 35 mM KCl was measured in a whole mouse adrenal medulla. When indicated, 10 µM ciclopiazonic acid (CPA) was perfused. In panel B, $[Ca^{2+}]_c$ peaks induced by 10 s pulses (●) of medium containing 35 mM KCl were measured in dissociated mouse chromaffin cells expressing cytosolic aequorin. When indicated, 10 µM CPA was perfused.

Tissues were impaled with a single 5-µm carbon fibre under microscope. After a variable period (60–90 min) of superfusion in control conditions, the amperometric recordings became stable and preparations were subjected to different experimental protocols, as detailed in the Results section. In most cases, prior to recording from the tissues the carbon fibre electrode was advanced into the bath chamber and calibrated by switching between Tyrode solution with or without 10 µM epinephrine. At the end of the experiment, the electrode was withdrawn from the tissue and calibrated again. Comparison of the calibration before and following the study was used to assess whether the electrode sensitivity changed over the recording period. As caffeine was used at high concentration (5 mM), the possible effect of the drug on our amperometric recording system was checked. It was found that caffeine, under our conditions, neither generates any signal nor interferes in the measurement of the catecholamine secretory response.

Measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$. Decapsulated adrenal glands were dissociated as described previously (Hernández-Guijo *et al.*, 1998) by incubation for 40 min at 37 °C in Locke's medium (154 mM NaCl, 5.6 mM KCl; 3.6 mM $NaHCO_3$; 5.6 mM glucose, 10 mM Hepes, pH 7.2) containing 1.3 mg/mL collagenase (Hoffmann-La Roche, Basel, Switzerland) and 3 mg/mL bovine serum albumin, with gentle periodic agitation. The suspension was centrifuged for 10 min at 100 × *g* and resuspended in Locke's medium. Cells were then plated onto 13 mm glass poly D-lysine-

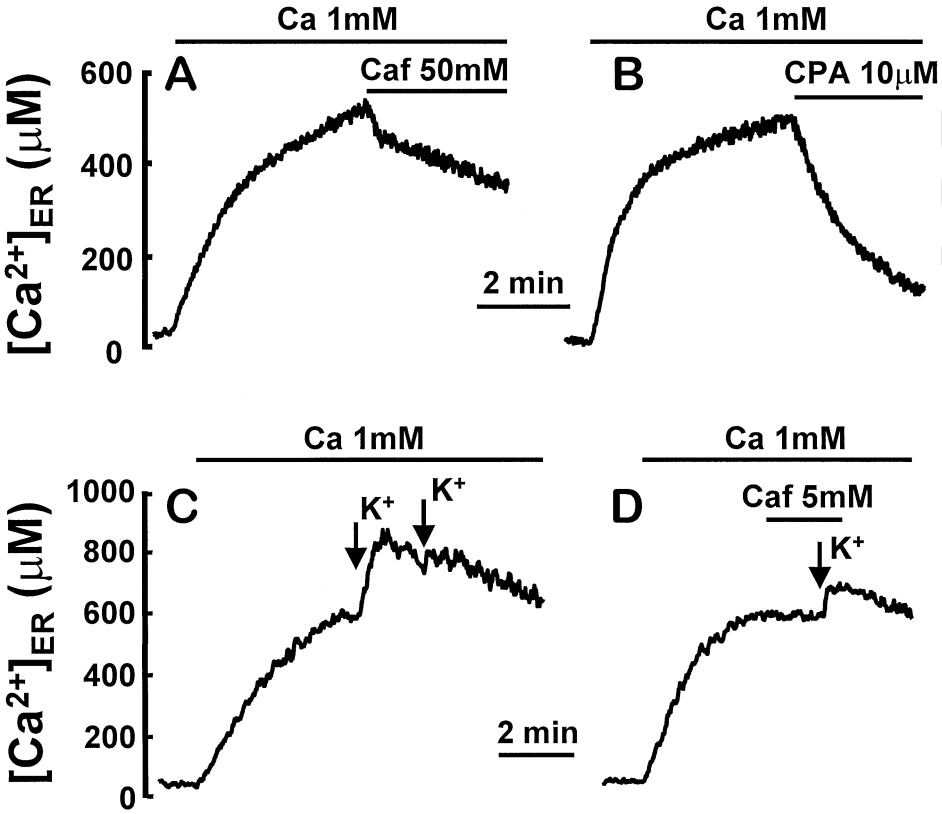

FIG. 2. Effect of caffeine, CPA and high K^+ on $[Ca^{2+}]_{ER}$. Dissociated mouse chromaffin cells expressing ER-targeted aequorin were depleted of Ca^{2+} and reconstituted with coelenterazine n. Then, when indicated in the panels, medium containing 1 mM Ca^{2+} was perfused to refill the ER with Ca^{2+}. Once $[Ca^{2+}]_{ER}$ was almost at the steady-state level, either 50 mM caffeine (panel A), 10 μM CPA (panel B), 10 s pulses of medium containing 70 mM KCl (panels C and D) or 5 mM caffeine (panel D) were perfused as indicated.

coated coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 50 IU/mL penicillin and 50 μg/mL streptomycin. After allowing 6 h for complete cell attachment, they were infected with defective herpes simplex viruses type 1 carrying the corresponding construct for either the cytosolic- or the ER-targeted aequorin chimera. Preparation of mutated aequorin targeted to the endoplasmic reticulum has been described before (Montero *et al.*, 1995). Cytosolic aequorin cDNA was obtained from Molecular Probes and cloned in the pHSVpUC plasmid. Virus packaging and titreing have been previously described (Alonso *et al.*, 1998). Chromaffin cell cultures were routinely infected with 2×10^3 infectious virus units 12–24 h prior to measurements.

For aequorin reconstitution, cells expressing wild-type aequorin targeted to the cytosol were incubated for 1–2 h at room temperature with 1 μM of wild-type coelenterazine in 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 luminometer thermostatized at 37 °C. In the case of aequorin targeted to the ER, Ca^{2+} depletion of the ER was required prior to reconstitution. For this purpose, cells were incubated for 10 min in medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μM 2,5-di-tert-buthyl-hydroquinone (an inhibitor of ER Ca^{2+} ATPase), 10 mM glucose, and 10 mM HEPES, pH 7.4. Then, after washing once, cells were placed in the same medium in the presence of 1 μM coelenterazine n for 1–2 h. Before starting the experiment, cells were placed in the perfusion chamber of a luminometer (composed of a 9789-A photomultiplier connected through an amplifier-discriminator AD 2 to a PCB CT1 card in the computer, all components from Electron Tubes, Ruislip, UK) and perfused with the same medium without 2,5-di-tert-buthyl-hydroquinone for at least 5 min. Experiments with ER-targeted aequorin were performed at 22 °C to reduce the rate of aequorin consumption.

Materials

Wild-type coelenterazine and coelenterazine n were obtained from Molecular Probes Europe BV, Leiden, The Netherlands. Ciclopiazonic acid and ryanodine were from Sigma, Madrid. Thapsigargin was from Alomone Laboratories, Israel. Other reagents were from Sigma, Madrid or Merck, Darmstadt.

Results

Characterization of the superfused in vitro *preparation of mice adrenal medulla*

A new *in vitro* superfused preparation of adrenal medulla from mice is reported in this work. This preparation is useful to study the secretion of catecholamines from the adrenal medulla as it is easy to obtain and allows monitoring the secretory responses evoked by different stimuli in the intact organ with a good time resolution. Recordings of the catecholamine efflux from the glands were initiated after a 60–90-min period to allow stabilization of the preparation. As shown below, the responses evoked by short pulses (5–10 s) of depolarizing stimuli were quite fast, had a short latency and remained constant without significant attenuation for several hours. Another aspect that we have characterized in our preparation was the content

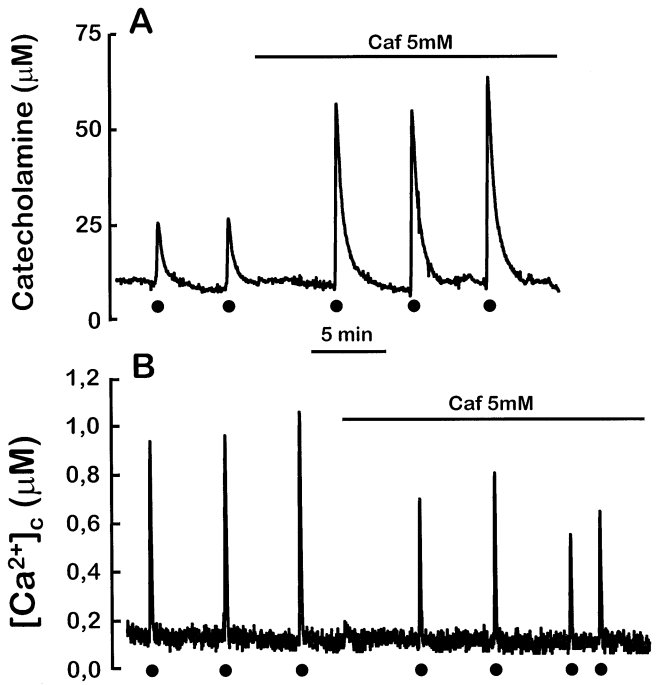

FIG. 3. Effects of 5 mM caffeine on high-K^+-induced catecholamine secretion and $[Ca^{2+}]_c$ peaks. Panel A shows a record of catecholamine secretion obtained from a whole mouse adrenal medulla. Secretion was stimulated by pulses of 7 s of medium containing 35 mM KCl (●). When indicated, 5 mM caffeine ('Caf 5 mM') was perfused. Panel B shows a record of $[Ca^{2+}]_c$ in dissociated mouse chromaffin cells expressing cytosolic aequorin. Cells were stimulated by 5 s pulses of medium containing 35 mM KCl (●). When indicated, 5 mM caffeine was perfused ('Caf 5 mM').

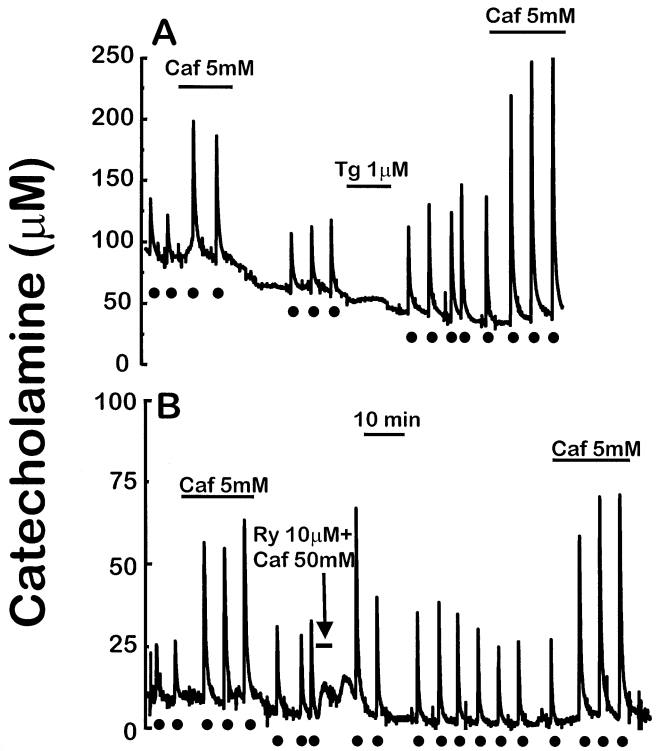

FIG. 4. Efects of caffeine, thapsigargin and ryanodine on high-K^+-induced catecholamine secretion. The figure shows catecholamine secretion records obtained from whole mouse adrenal medulla. Secretion was stimulated by either 10 s (panel A) or 7 s (panel B) pulses of medium containing 35 mM KCl (●). When indicated, either 5 mM caffeine ('Caf 5 mM'), 1 μM thapsigargin ('Tg 1 μM') or 10 μM ryanodine + 50 mM caffeine ('Ry 10 μM + Caf 50 mM') were perfused.

of catecholamines. Using HPLC-ED, the values obtained from these measurements, expressed in nmol/gland (mean ± SEM, $n = 6$), were 1.08 ± 0.30, 2.16 ± 0.65 and 0.60 ± 0.10 for norepinephrine, epinephrine and dopamine, respectively. These results are quite similar to those previously reported by other authors (Bornstein *et al.*, 1999; Bland *et al.*, 2000). All of these properties make the *in vitro* preparation of adrenal medulla from mice a good model to study the modulation of the release response evoked by different stimuli.

Cyclopiazonic acid potentiates both secretion and $[Ca^{2+}]_c$ peaks evoked by high-K^+

Using the preparation described above, we have first studied the contribution of calcium stores to the catecholamine release response evoked by depolarizing stimuli. First, we emptied the ER-Ca^{2+} store by inhibiting the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) with cyclopiazonic acid (CPA). In this way both CICR and Ca^{2+} uptake by the ER were abolished. Figure 1a shows that perfusion of CPA 10 μM modified little the resting catecholamine release but produced a large increase in the secretory response evoked when 5 or 10 s pulses of medium containing 35 mM KCl were applied. The release responses were increased to 148 ± 5% of the control value (mean ± SEM, $n = 9$) when 10 s depolarizing pulses were used and much more (273 ± 36%, mean ± SEM, $n = 6$) for shorter depolarizing pulses. This effect was not due to saturation of the secretory response, as stronger stimulation (i.e. 60 mM K^+, 10 s) produced a much larger secretion peak (up to 6-fold higher). The same effects were obtained using thapsigargin to empty the ER of Ca^{2+} (Fig. 4a). These results suggest that CICR is not functional in these cells and that the ER behaves more as a sink of Ca^{2+} than as a source under resting conditions (with the ER full of Ca^{2+}). If this is the case, inhibition of Ca^{2+} pumping to the ER would be expected to increase the $[Ca^{2+}]_c$ levels obtained after the depolarizing stimulus. Figure 1b shows that this was the case. Perfusion of CPA produced a small increase in the resting $[Ca^{2+}]_c$ level, from 102 ± 6 nM to 157 ± 15 nM (mean ± SEM, $n = 7$), but considerably increased the $[Ca^{2+}]_c$ peaks induced by high-K^+ treatment. In several similar experiments, high-K^+-induced $[Ca^{2+}]_c$ peaks were increased to 150 ± 14% (mean ± SEM, $n = 9$) of the control value in the presence of CPA.

Absence of CICR induced by high-K^+ and poor Ca^{2+} release response to caffeine

Another pharmacological manoeuvre usually employed to activated RyR is caffeine. In bovine chromaffin cells, stimulation with 50 mM caffeine produces a very fast and full emptying of the ER ($t_{\frac{1}{2}} < 10$ s, Alonso *et al.*, 1999), and a significant release response (Montero *et al.*, 2000). Half-maximal effect was obtained with 5 mM caffeine, and lower concentrations (1–2 mM) produced little effect by themselves, but potentiated CICR induced by high-K^+ stimulation (Alonso *et al.*, 1999). In contrast, 50 mM caffeine produced a very small Ca^{2+} release from the ER in mouse chromaffin cells. Figure 2a shows that 50 mM caffeine produced only a small initial Ca^{2+} release that reduced the $[Ca^{2+}]_{ER}$ by approximately 50 μM (50 ± 7 μM, mean ± SEM, $n = 8$), followed by a much slower decrease in the ER Ca^{2+} content. Consistently, 50 mM caffeine produced only a small secretory response (data not shown). Figure 2b shows the effect of

CPA on $[Ca^{2+}]_{ER}$ for comparison. Inhibiting SERCA produces a relatively slow release of Ca^{2+} from the ER, that was in this case much faster than that induced by caffeine, and complete. Given the poor effect of caffeine on ER-Ca^{2+} release, we expected that CICR could not be observed in these cells. Figure 2c shows that this was the case. In contrast to the results obtained in bovine chromaffin cells (Alonso *et al.*, 1999), stimulation with high-K^+ of mouse chromaffin cells produced a rapid increase in $[Ca^{2+}]_{ER}$, probably because of the activation of SERCA after the high-K^+-induced $[Ca^{2+}]_c$ increase. The presence of low concentrations of caffeine (2–5 mM) did not modify the effect of high-K^+ on $[Ca^{2+}]_{ER}$ (Fig. 2d). This panel shows also that a concentration of caffeine of 5 mM, that produced a half-maximal Ca^{2+} release response in bovine chromaffin cells (Alonso *et al.*, 1999) had no effect on $[Ca^{2+}]_{ER}$ in mouse chromaffin cells.

Caffeine potentiates the secretory response evoked by depolarizing stimuli by a mechanism unrelated to ER-Ca^{2+} release

The results of Figs 1 and 2 clearly suggest that CICR is not operative in mouse chromaffin cells. It was therefore a surprise to find that low concentrations of caffeine, which do not affect the basal catecholamine response, strongly potentiates the release response promoted by 35 mM KCl. Figure 3a shows that perfusion of 5 mM caffeine did not modify the secretory response by itself, but largely increased that induced by high-K^+ stimulation. In several similar experiments, high-K^+-evoked catecholamine secretion was increased to 224 ± 15% (mean ± SEM, $n = 12$) of the control value in the presence of 5 mM caffeine. However, this effect was not due to an increase in the high-K^+-induced $[Ca^{2+}]_c$ peaks. Figure 3b shows that, instead, the $[Ca^{2+}]_c$ transients induced by depolarization were reduced in the presence of caffeine 5 mM. In several similar experiments, $[Ca^{2+}]_c$ peaks evoked by high-K^+ in the presence of caffeine were reduced to 66 ± 3% (mean ± SEM, $n = 19$) with respect to the controls.

Further evidence in favour of the suggestion that the effect of caffeine was unrelated to CICR was obtained by studying its effect after emptying the ER of Ca^{2+}. Figure 4a shows that caffeine still potentiated secretion after full and irreversible depletion of Ca^{2+} from the ER. Taking advantage of the long period of time that the *in vitro* preparation of the mice adrenal medulla remains stable, we tested whether caffeine still potentiates the secretory response evoked by 10 s pulses of 35 mM KCl once the ER had been completely emptied of Ca^{2+} with 1 μM thapsigargin, a powerful, selective and irreversible inhibitor of SERCA. In the experiment shown in Fig. 4a, the initial part demonstrates again the potentiation by caffeine of the secretory response evoked by 35 mM KCl. After washing away caffeine, the control secretory responses to high-K^+ stimulation were recovered. Then, a 5 min superfusion with thapsigargin was applied to irreversibly empty the ER of Ca^{2+}. It can be observed that the responses to high-K^+ became potentiated after this treatment (to 166 ± 13% of the control, mean ± SEM, $n = 6$), probably by the same mechanism that in the case of CPA (Fig. 1). Finally, perfusion of 5 mM caffeine strongly potentiated again the secretory responses to high-K^+ (to 269 ± 27% of the response in the presence of thapsigargin, mean ± SEM, $n = 6$). After treatment with thapsigargin, the response evoked by the depolarizing stimulus in the presence of caffeine was approximately the sum of the individual responses obtained in the presence of either thapsigargin alone or caffeine alone. The potentiating effect of caffeine was therefore additive to that of emptying the ER of Ca^{2+}, indicating that it is not due to activation of CICR from the ER.

The presence of RyR in subcellular locations other than the ER is controversial. It has been suggested, for example, that RyR may be present in the membrane of the secretory vesicles, and that it may mediate Ca^{2+} release from the vesicle (Gerasimenko *et al.*, 1996; Mitchell *et al.*, 2001). In this way, these RyR could contribute to the release of the Ca^{2+} required for the secretion of that vesicle. To investigate if the effects of caffeine could be due to stimulation of RyR located outside the ER, we decided to use ryanodine to block RyR responses independently of its subcellular location. Figure 4b shows a secretion experiment in which we first demonstrate the reversible potentiating effect of 5 mM caffeine. Once the high-K^+-induced secretory peaks had almost returned to the control levels, we superfused the mice adrenal medulla with ryanodine, the irreversible antagonist of ryanodine receptors. Ryanodine (10 μM) was applied in the presence of high doses of caffeine to assure the irreversible antagonist effect of ryanodine that requires the channel to be open in order to block it (the so-called use-dependence of ryanodine, Cheek *et al.*, 1994; Ehrlich *et al.*, 1994; Alonso *et al.*, 1999). A long period of washing was allowed after that, during which the response to high-K^+ stimulation slowly decreased, probably as a result of the slow washing of the potentiating effect of caffeine. After that, perfusion of 5 mM caffeine increased again the secretory response evoked by the depolarizing stimulus. Overall, these results strongly argue against the involvement of RyR in the potentiation of secretion by caffeine. The actual mechanism of this potentiation may then rely on a different property of caffeine, e.g. the inhibition of phosphodiesterase. In fact, perfusion of 1 mM 3-isobutyl-1-methylxanthine, a compound that inhibits phosphodiesterase without activating RyR, stimulated secretion induced by high-K^+ to a similar extent as caffeine (data not shown).

Discussion

In this paper we provide new evidence for the involvement of the ER in the modulation of catecholamine secretion in chromaffin cells. Blocking Ca^{2+} pumping into the ER, either with CPA or with thapsigargin, produced a strong stimulation of catecholamine secretion, which was more evident when short high-K^+ stimulations were used. Consistently, high-K^+ stimulation produced a fast increase in $[Ca^{2+}]_{ER}$. These findings suggest that, after cell depolarization, the ER takes up a significant proportion of the Ca^{2+} entering the cell through voltage-operated Ca^{2+} channels, damping the secretory response. This modulation appears to be particularly important for stimuli of small intensity. This seems quite reasonable, because when the stimulus is too intense, the large increase in $[Ca^{2+}]_c$ probably saturates ER Ca^{2+} uptake, so that the amount of Ca^{2+} taken up by the ER becomes a small fraction of the total Ca^{2+} entry. Under these conditions, Ca^{2+} uptake by the ER cannot be effective to modulate secretion. Instead, Ca^{2+} uptake by the ER appears to be prepared to play a role in the control of secretion induced by low-intensity, more physiological, stimuli. In this case, ER regions close to the plasma membrane Ca^{2+} channels may be able to sequester a significant proportion of the Ca^{2+} entering the cell through those channels. Of course, Ca^{2+} uptake by the ER is not the only mechanism responsible of cytosolic Ca^{2+} clearance. Mitochondria and plasma membrane Ca^{2+} pump and Na^+/Ca^{2+} exchange also contribute to damp the $[Ca^{2+}]_c$ signal. On the other hand, we should note that depletion of Ca^{2+} from the ER may have additional effects, such as activation of store-operated Ca^{2+} channels, which may contribute to the facilitation of exocytosis (Fomina *et al.*, 1999). However, blocking ER-Ca^{2+} uptake with CPA or thapsigargin produced only a small and transient increase in $[Ca^{2+}]_c$. This suggests that the different Ca^{2+} clearance

mechanisms rapidly extrude the Ca^{2+} released from the ER and also that store-operated Ca^{2+} channels are not very active in these cells.

The absence of CICR and the small effect of caffeine on $[Ca^{2+}]_{ER}$ in mouse chromaffin cells suggest that these cells possess only a scarce number of functional RyR in the ER. This came as a surprise, given the high activity observed in bovine chromaffin cells (Alonso *et al.*, 1999), where they clearly participate in CICR induced by high-K^+. Chromaffin cells obtained from 19 to 31-day-old-rats are also very sensitive to caffeine, both in terms of secretion and $[Ca^{2+}]_c$ response (Finnegan *et al.*, 1996; Guo *et al.*, 1996). Interestingly, cultures obtained from 10-day-old rats lack a significant response to caffeine, and only recover it after 15 days in culture in the presence of nerve growth factor, in parallel with an increased expression of RyR type 2 (Jiménez & Hernández-Cruz, 2001). CICR appears therefore not to be an essential mechanism for the development of the secretory response in chromaffin cells, although it may have physiological significance when it is present. Regarding the modulation by ER Ca^{2+} uptake, depletion of Ca^{2+} from the ER in bovine chromaffin cells produced little effect (or perhaps a slight enhancement) on the secretory response induced by high-K^+ (Cuchillo-Ibáñez *et al.*, 2002), but reduced secretion induced by cell depolarization (Pan & Fox, 2000) and that induced by activation of nicotinic receptors (Mollard *et al.*, 1995; Cuchillo-Ibáñez *et al.*, 2002). Perhaps the mixture of ER-Ca^{2+} uptake and CICR in these cells may produce compensating effects, that could be variable depending on the stimulus and even heterogeneous at the single-cell level. This could also explain the controversial findings mentioned above. In the mouse, the absence of CICR helps to observe uncontaminated the effects of ER-Ca^{2+} uptake on the secretory response. We should mention also that inositol 1,4,5-trisphosphate receptors might participate as well in CICR phenomena. Mouse chromaffin cells possess this kind of receptors, which can be activated by agonists such as carbachol producing large $[Ca^{2+}]_c$ peaks (1.70 ± 0.14 μM, mean ± SEM, $n = 6$). However, this response requires the presence of inositol 1,4,5-trisphosphate, and is not expected to occur after high-K^+ stimulation.

In the absence of functional RyR in the ER, the potentiating effect of low caffeine concentrations on catecholamine secretion triggered by cell depolarization was puzzling. This effect persisted after full ER-Ca^{2+} depletion with thapsigargin and also after treatment with ryanodine. Therefore, we can conclude that RyR are not involved in that potentiation. Additional effects of caffeine, such as phosphodiesterase inhibition and the subsequent increase of cyclic nucleotide levels, may be responsible for the enhancement of the secretory response. Potentiation of catecholamine secretion by an increase in the levels of cyclic nucleotides has been reported previously (Morita *et al.*, 1985; Starke *et al.*, 1989), and our finding that an inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine, stimulates secretion similarly to caffeine, argues also in favour of this hypothesis. On the other hand, the reduction observed in the size of the high-K^+-induced $[Ca^{2+}]_c$ peaks in the presence of caffeine may be attributed to the inhibition of voltage-operated Ca^{2+} channels by this compound (Villalobos & García-Sancho, 1996). Moreover, these findings indicate that the effects of caffeine should not be taken as unequivocally due to activation of RyR or as an equivalent of CICR.

The differential behaviour regarding CICR of mouse and bovine chromaffin cells is rather surprising. It has been reported previously that chromaffin cells from different species (cow, pig, cat, rat or mouse) show large differences in the relative expression of plasma membrane high-threshold Ca^{2+} channel subtypes (Hernández-Guijo *et al.*, 1998). We show here that extreme differences (all-or-none) in expression among different species can also be found regarding intracellular Ca^{2+} channels. Knowledge of these properties of mouse chromaffin cells is interesting, because of the possibility of generating transgenic mice to study specific problems of Ca^{2+} homeostasis and secretion. On the other hand, interspecies differences observed in this study raise questions as to the physiological importance of mechanisms such as CICR on the secretory response of adrenal cells, if it is not observed in all species.

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Abbreviations

$[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_{ER}$, endoplasmic reticulum $[Ca^{2+}]$; ER, endoplasmic reticulum; CICR, Ca^{2+} induced Ca^{2+} release; RyR, ryanodine receptors; SERCA, sarcoendoplasmic reticulum Ca^{2+} ATPase; CPA, ciclopiazonic acid; DMEM, Dulbecco's modified Eagle medium; HPLC-ED, high-performance liquid chromatography-electrochemical detection.

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