Ca^{2+}-induced Ca^{2+} Release in Chromaffin Cells Seen from inside the ER with Targeted Aequorin

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Abstract. The presence and physiological role of Ca^{2+}-induced Ca^{2+} release (CICR) in nonmuscle excitable cells has been investigated only indirectly through measurements of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$). Using targeted aequorin, we have directly monitored $[Ca^{2+}]$ changes inside the ER ($[Ca^{2+}]_{ER}$) in bovine adrenal chromaffin cells. Ca^{2+} entry induced by cell depolarization triggered a transient Ca^{2+} release from the ER that was highly dependent on $[Ca^{2+}]_{ER}$ and sensitized by low concentrations of caffeine. Caffeine-induced Ca^{2+} release was quantal in nature due to modulation by $[Ca^{2+}]_{ER}$. Whereas caffeine released essentially all the Ca^{2+} from the ER, inositol 1,4,5-trisphosphate ($InsP_3$)-producing agonists released only 60–80%. Both $InsP_3$ and caffeine emptied completely the ER in digitonin-permeabilized cells whereas cyclic ADP-ribose had no effect. Ryanodine induced permanent emptying of the Ca^{2+} stores in a use-dependent manner after activation by caffeine. Fast confocal $[Ca^{2+}]_c$ measurements showed that the wave of $[Ca^{2+}]_c$ induced by 100-ms depolarizing pulses in voltage-clamped cells was delayed and reduced in intensity in ryanodine-treated cells. Our results indicate that the ER of chromaffin cells behaves mostly as a single homogeneous thapsigargin-sensitive Ca^{2+} pool that can release Ca^{2+} both via $InsP_3$ receptors or CICR.

Key words: endoplasmic reticulum • aequorin • chromaffin cells • calcium • ryanodine

At present, the role played by ryanodine receptors (RyR)[1] in the homeostasis of intracellular Ca^{2+} in nonmuscle cells is unclear. Mammalian tissues express three isoforms, RyR1, RyR2, and RyR3, encoded by different genes. RyR1 and RyR2 are expressed predominantly in sarcoplasmic reticulum of skeletal muscle and heart, respectively, where they have an essential role to trigger muscle contraction (Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). RyR3 was originally identified in brain (Hakamata et al., 1992), but in fact all three isoforms are actually expressed in brain, and the major brain isoform appears to be RyR2 (McPherson and Campbell, 1993; Sorrentino and Volpe, 1993; Giannini et al., 1995). RyRs are widely distributed in many other different tissues (Giannini et al., 1995; Mackrill et al., 1997), including the adrenal gland, and they are in many cases coexpressed with one or more isoforms of inositol 1,4,5-trisphosphate receptors ($InsP_3R$) (Walton et al., 1991; Poulsen et al., 1995).

The reasons for the presence of multiple Ca^{2+} release mechanisms in the same cell are not clear (for discussion see Newton et al., 1994; Sutko and Airey, 1996). A possible reason for the coexistence of both $InsP_3R$ and RyR in the same cells could be that they might release Ca^{2+} from different compartments, and with a different physiological significance. They may be modulated by different second messengers, such as $InsP_3$ in the case of $InsP_3R$ or cyclic adenosine diphosphate ribose (cADPR) for the RyR (Lee, 1998). However, although $InsP_3$ is a well-established physiological activator of the $InsP_3R$, the role of cADPR as activator or modulator of RyR in the presence of physiologi-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_{ER}$, ER $[Ca^{2+}]$; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; cADPR, cyclic adenosine diphosphate ribose; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, ciclopiazonic acid; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; HSV-1, herpes simplex virus type 1; $InsP_3R$, $InsP_3$ receptor; $InsP_3$, inositol 1,4,5-trisphosphate; ivu, infectious virus units; RyR, ryanodine receptor.

cal concentrations of ATP remains controversial (Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). In neuronal cells, RyR could be activated via the classical Ca^{2+}-induced Ca^{2+} release (CICR) mechanism after Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Studies in some neuronal preparations have shown that depolarizing stimuli produce an increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) that may be due in part to Ca^{2+} release from intracellular stores (Verkhratsky and Shmigol, 1996). However, little direct evidence has been presented for CICR in nonmuscle cells; this is due to the difficulties in separating the contribution of Ca^{2+} entry and Ca^{2+} release to the $[Ca^{2+}]_c$ signal and to the nonspecificity of caffeine and other pharmacological agents used to activate RyR.

To study CICR in neuronal cells, it would be greatly advantageous to measure $[Ca^{2+}]$ specifically inside the Ca^{2+} stores. We have recently reported a method to measure $[Ca^{2+}]$ in the lumen of the endoplasmic reticulum ($[Ca^{2+}]_{ER}$) of intact cells, the main intracellular Ca^{2+} store, by using an ER-targeted aequorin (Montero et al., 1995, 1997a,b; Barrero et al., 1997), which can be expressed in different types of cells using a viral vector (Alonso et al., 1998). This technique is ideal to study directly CICR, because Ca^{2+} release can be measured independently of the variations in $[Ca^{2+}]_c$. Here we have used this technique to monitor $[Ca^{2+}]_{ER}$ in chromaffin cells. These neuroendocrine cells have a potent caffeine-sensitive Ca^{2+} release mechanism (Cheek et al., 1990), and Ca^{2+} entry through several types of voltage-dependent Ca^{2+} channels can be induced by K^+ depolarization or more physiologically, using nicotinic agonists (Núñez et al., 1995; Lara et al., 1998). Direct evidence for a CICR mechanism working under physiological conditions in these cells has not been provided. However, we have reported recently that the caffeine-sensitive Ca^{2+} stores may modulate catecholamine secretion induced by depolarization with high K^+ in these cells. Catecholamine secretion was reduced after store emptying with caffeine, and recovered as Ca^{2+} stores refilled during consecutive K^+/Ca^{2+} pulses. The main conclusion from that work was that Ca^{2+} stores could have a double role, acting either as a sink or as a source of Ca^{2+}, depending of their state of filling (Lara et al., 1997).

Additionally, several questions regarding the function of RyR in chromaffin cells remain unanswered. For instance, chromaffin cells have also $InsP_3R$, which are at least in part colocalized with RyR2 in the ER (Poulsen et al., 1995). The presence of separate or overlapping Ca^{2+} pools responsive to either $InsP_3$, caffeine, or cADPR, their differential sensitivity to inhibitors of the ER Ca^{2+}-pump such as thapsigargin, and the physiological significance of the different Ca^{2+} release mechanisms, has been a subject of debate for many years (Cheek et al., 1991; Liu et al., 1991; Robinson and Burgoyne, 1991; Stauderman et al., 1991; Morita et al., 1997). On the other hand, the mechanism of Ca^{2+} release induced by caffeine is quite particular because increasing concentrations of caffeine release Ca^{2+} in a quantal manner, a phenomenon that has been suggested to indicate that the caffeine-sensitive Ca^{2+} pool is composed of functionally discrete stores with heterogeneous sensitivities to caffeine (Cheek et al., 1993, 1994a).

Here we have monitored $[Ca^{2+}]_{ER}$ in chromaffin cells to investigate the mechanism of quantal Ca^{2+} release by caffeine, its relationship in terms of Ca^{2+} pools with $InsP_3$-mediated Ca^{2+} release, and the presence of CICR triggered by Ca^{2+} entry. In brief, our results indicate that the ER Ca^{2+} pools responding to caffeine and $InsP_3$ mostly overlap. The response to caffeine was also quantal when studied from inside the ER, but this quantal response could be explained by the control of caffeine-induced Ca^{2+} release by $[Ca^{2+}]_{ER}$, with no need for separate ER compartments with heterogeneous sensitivities to caffeine, as proposed previously (Cheek et al., 1993, 1994a). We show that CICR can be induced by Ca^{2+} entry elicited either by high K^+ depolarization or by stimulation with nicotinic agonists. This is consistent with our previously proposed model for the Ca^{2+} store as a modulator of secretion (Lara et al., 1997). Additionally, using fast confocal $[Ca^{2+}]$ measurements, we show that CICR participates in the generation and propagation of the Ca^{2+} wave induced by cell depolarization.

Materials and Methods

Preparation and Culture of Bovine Chromaffin Cells

Bovine adrenal medulla chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro et al., 1990). Cells were suspended in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 IU ml^{-1} streptomycin. For secretion experiments, cells were plated in 5-cm-diam Petri dishes (5×10^6 cells per 5 ml of DME). For aequorin experiments, cells were plated on 12- or 13-mm glass poly-D-lysine–coated coverslips (0.5×10^6 cells per 1 ml of DME). For measurements of $[Ca^{2+}]_c$ transients by confocal microscopy and ionic currents, cells were plated on 2.5-cm-diam glass coverslips at a density of 5×10^4 cells per ml. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of Viral Stock and Infection of Cultures

Construction, packaging, and titering of the pHSVerAEQ amplicon vector and expression in chromaffin cells has been previously described (Alonso et al., 1998). The EcoRI fragment of the erAEQmut cDNA was subcloned into the pHSVpuc vector to generate the pHSVerAEQ. As a helper virus, the herpes simplex virus type 1 (HSV-1) IE2 deletion mutant 5dl1.2 was used with a titer of 2×10^7 infectious virus units (ivu)/ml (Lim et al., 1996). Titers of viral stocks were determined by immunocytochemistry on PC12 cells. Infected cells were visualized by using a rabbit anti–HSV-1 particle antibody (1:10,000 dilution; Dako) or a mouse anti-HA1 primary antibody (1:200 dilution; Boehringer Mannheim) followed by an alkaline phosphatase-conjugated anti-mouse IgG antibody (1:200 dilution; Sigma). The titers of the vector stock were 1.1×10^6 ivu/ml pHSVerAEQ and 4.1×10^6 ivu/ml 5dl1.2. Chromaffin cell cultures (5×10^5 cells/0.5 ml) were routinely infected with 1.2×10^4 ivu 1 d before measurements. The percentage of cells expressing ER-targeted aequorin was usually ~20%. Immunofluorescence revealed a typical nonnuclear reticular pattern (data not shown), similar to that previously seen in HeLa cells (Montero et al., 1995). This pattern was not modified by the 1-h period of ER Ca^{2+} depletion required for ER aequorin measurements.

Measurements of $[Ca^{2+}]_{ER}$ with Aequorin

For $[Ca^{2+}]_{ER}$ studies, cells were infected after 1 d in culture with HSV-1 carrying the ER-targeted aequorin construct as described previously (Alonso et al., 1998). Measurements of $[Ca^{2+}]_{ER}$ were started ~16 h after infection that were required to allow adequate expression of the targeted photoprotein. Aequorin photoluminescence measurements were performed essentially as previously described (Barrero et al., 1997). In brief, cells were depleted of Ca^{2+} by incubation for 5–10 min at 37°C with the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor 2,5-di-tert-butyl-benzohydroquinone (BHQ) 10 μM in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes,

pH 7.4, supplemented with 3 mM EGTA. Cells were then incubated for 1 h at room temperature in standard medium containing 0.5 mM EGTA, 10 μM BHQ, and 1 μM coelenterazine n. The coverslip was then placed in the perfusion chamber of a purpose-built thermostatized luminometer and standard medium containing 1 mM Ca^{2+} was perfused to refill the ER with Ca^{2+}. Measurements were performed at 22°C and $[Ca^{2+}]_{ER}$ values were calculated from the luminescence records using a computer algorithm (Brini et al., 1995) which follows the calibration curve reported before (Barrero et al., 1997). In the experiments carried out with permeabilized cells, cells were placed in the luminometer as described above and perfused for 1 min with intracellular-like medium (10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 2 mM ATP, 20 mM Hepes, pH 7) containing 2 mM EGTA and 20 μM digitonin. Then, intracellular medium without digitonin and containing 100 nM EGTA-buffered Ca^{2+} was perfused for 3–5 min to refill the ER with Ca^{2+}. The total number of counts obtained ranged between 0.3 and 2 million.

Measurements of Single-cell $[Ca^{2+}]_c$

Single-cell measurements of $[Ca^{2+}]_c$ were performed at room temperature in fura-2–loaded cells as described previously (Núñez et al., 1995). Cells were epi-illuminated alternatively at 340 and 380 nm and light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science) and analyzed using an Applied Imaging Magical image processor (Sunderland). 16 frames excited at every wavelength were averaged by hardware, with a time resolution of ~7 s for each pair of images, and $[Ca^{2+}]_c$ was estimated from the ratio F_{340}/F_{380} by comparison with fura-2 standards. Field electric stimulation (McIlwain and Rodnight, 1962) was performed through a pair of silver electrodes placed 7 mm apart and 1.5 mm above the cells. Alternating positive-negative square pulses of 50-ms duration and 60-V intensity were applied at 10 Hz. The peak current was 100 mA.

Confocal $[Ca^{2+}]_c$ Measurements and Electrophysiological Recordings

Electrical measurements and $[Ca^{2+}]_c$ were recorded by using the whole-cell patch-clamp technique (Hamill et al., 1981) in combination with fluo-3 based microfluorometry. Cells were placed in an experimental chamber that was mounted on the stage of an inverted microscope (Diaphot 200; Nikon). Cells were loaded via the patch pipette with the pentaammonium salt form of the fluorescent dye fluo-3 (100 μM). The dye was excited with a Kr-Ar laser light at 488 nm and emission was detected at 522 nm (32-nm band width). Cells were dialyzed with an intracellular solution containing 135 mM $CsCl_2$, 8 mM NaCl; 1 mM $MgCl_2$, 20 mM Hepes, 2 mM ATP, and 0.3 mM GTP, pH 7.3. The chamber was continuously perfused with Krebs-Hepes medium. Line-scan images (0.33-μm width) of the intracellular Ca^{2+} distribution were acquired every 2 ms with a confocal microscope (MRC 1024; Bio-Rad), using an oil immersion, planapochromatic 60× objective (NA = 1.4 [Nikon]). Changes in $[Ca^{2+}]_c$ were inferred from the intensity of fluo-3 fluorescence normalized to that in resting conditions (F/F_0). Whole-cell currents were monitored with a DAGAN PC-ONE patch-clamp amplifier. Data were recorded and analyzed with Igor Pro 3.02 (Wave Metrics).

Chemicals

Coelenterazine n, fura-2AM, and fluo-3 were obtained from Molecular Probes. $InsP_3$ was from Research Biochemicals International. cADPR was obtained from Sigma and from Calbiochem-Novabiochem. Other reagents were of the highest quality available from Sigma or Merck.

Results

Overlap between Caffeine-, Ryanodine-, Histamine-, and Thapsigargin-sensitive Components of $[Ca^{2+}]_{ER}$

After aequorin reconstitution with coelenterazine, with the ER completely depleted of Ca^{2+}, the experiments were started by perfusing the cells with medium containing 1 mM Ca^{2+} to refill the ER (Fig. 1 a). As in other cells studied previously (Montero et al., 1995, 1997a; Barrero et al., 1997; Alonso et al., 1998), full refilling of the ER required 3–5 min and the steady-state $[Ca^{2+}]_{ER}$ reached was 500–800 μM (Fig. 1 a). Addition of histamine produced a rapid but partial (60–80%) Ca^{2+} emptying of the ER, a new $[Ca^{2+}]_{ER}$ steady-state being reached at ~200–300 μM. Subsequent addition of caffeine (50 mM) induced a further emptying to near background aequorin luminescence. The effects were reversible by washing, this allowing refilling of the ER that was completed within 3–5 min. Addition of caffeine at that point, when the stores were completely refilled, triggered a rapid and complete emptying of the ER. Histamine was then unable to produce any further effect. These results suggest that essentially the whole ER Ca^{2+} pool is sensitive to caffeine and a large part of it is also sensitive to $InsP_3$ producing agonists such as hista-

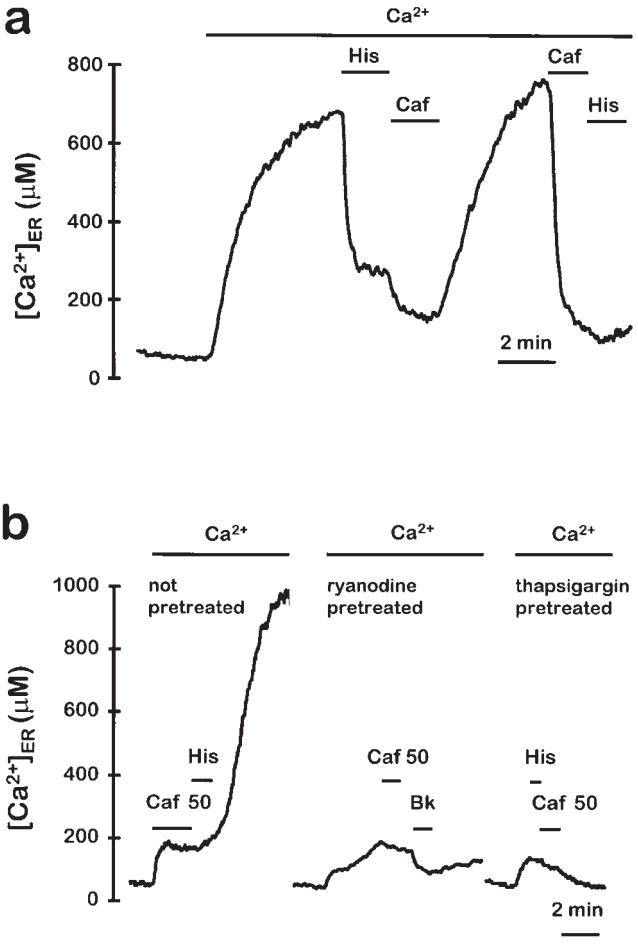

Figure 1. Effects of histamine and caffeine and pretreatment with ryanodine or thapsigargin on $[Ca^{2+}]_{ER}$. HSV-1–infected chromaffin cells were depleted of Ca^{2+} and reconstituted with coelenterazine n. (a) The ER was refilled by incubation with medium containing 1 mM Ca^{2+}, then either 10 μM histamine or 50 mM caffeine were perfused as indicated. (b) Where indicated, ryanodine-pretreated cells were treated before aequorin reconstitution with medium containing 50 mM caffeine and 10 μM ryanodine for 2 min. Cells were then washed and the same treatment was repeated four times at 2-min intervals. Where indicated, thapsigargin-pretreated cells were incubated with 1 μM thapsigargin for 10 min before starting the record. During the experiments, medium containing 1 mM Ca^{2+} and either 50 mM caffeine, 1 μM bradykinin, or 10 μM histamine was perfused as indicated.

mine. Similar results were obtained using 1 μM bradykinin instead of histamine (data not shown).

To obtain more information about the nature of the caffeine-sensitive Ca^{2+} pool, we studied the ability of the ER to refill in the presence of caffeine, ryanodine, or the Ca^{2+}-ATPase inhibitor thapsigargin. Fig. 1 b, left panel shows that when Ca^{2+}-depleted cells were incubated with Ca^{2+}-containing medium but in the presence of 50 mM caffeine, refilling was almost abolished until caffeine was washed away. The small increase observed in $[Ca^{2+}]_{ER}$ in the presence of caffeine was insensitive to histamine. Ryanodine has been reported to lock open irreversibly the RyR in a use-dependent manner, that is, when ryanodine is present while the channels have been opened by caffeine (Ehrlich et al., 1994). If all the ER had functional caffeine and ryanodine-sensitive RyR, we would then expect that pretreatment of the cells with caffeine and ryanodine would inhibit also refilling of the ER with Ca^{2+}. Fig. 1 b, middle panel, shows that this is the case. Cells were treated with five pulses of 50 mM caffeine and 10 μM ryanodine, and then the drugs were removed before aequorin reconstitution. Addition of 1 mM Ca^{2+} to these cells produced only an small increase in $[Ca^{2+}]_{ER}$, that was little sensitive to caffeine or bradykinin. The same results were obtained (Fig. 1 b, right panel) if the cells were pretreated with the SERCA inhibitor thapsigargin (1 μM). Similar effects were obtained using lower (20 nM) thapsigargin concentrations (data not shown). Therefore, the whole ER has caffeine- and ryanodine-sensitive RyRs, and refills with Ca^{2+} via thapsigargin-sensitive Ca^{2+} pumps.

Fig. 2 illustrates the time course of the use-dependent effect of ryanodine on RyR. Fig. 2 a shows that consecutive additions of 50 mM caffeine produced comparable decreases in $[Ca^{2+}]_{ER}$ if an interval of 3–5 min was left between two consecutive additions to allow refilling with Ca^{2+} of the ER. If 10 μM ryanodine was present during the caffeine pulses (Fig. 2 b), the first pulse was identical to the control but then the ER became progressively unable to refill. After four pulses, the ER remained at near background $[Ca^{2+}]_{ER}$ levels, and only the increase in $[Ca^{2+}]_c$ elicited by depolarization with 70 mM K^+ was able to activate the Ca^{2+} pump and produce a small and transient increase in $[Ca^{2+}]_{ER}$. The effect of caffeine was not inhibited by incubation with 20 μM dantrolene (data not shown), an inhibitor of RyR that is particularly effective on the skeletal muscle RyR1 (Van Winkle, 1976).

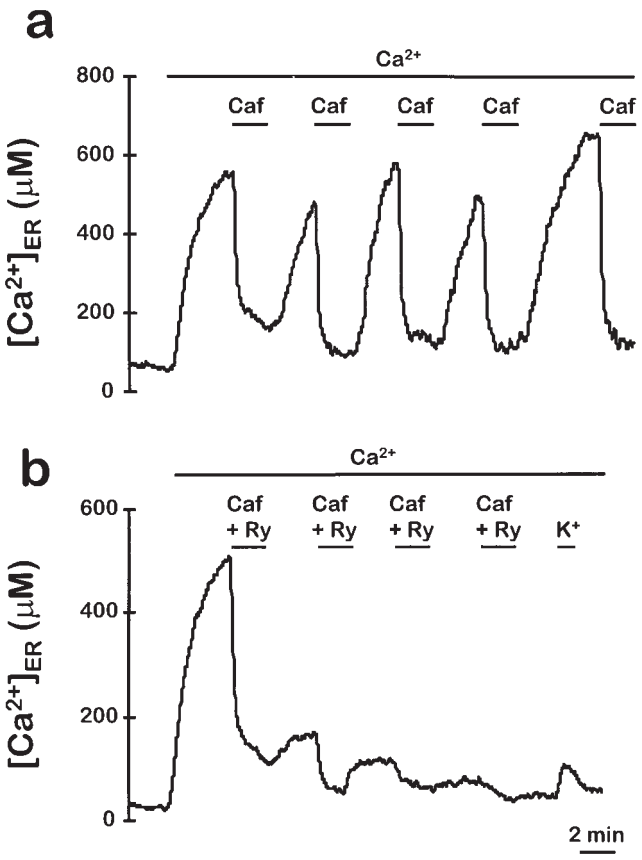

Figure 2. Use-dependent inhibition of ER refilling by ryanodine. The ER was refilled by perfusing with medium containing 1 mM Ca^{2+}. Then, several consecutive stimulations with 50 mM caffeine were performed as indicated, either in the absence (a) or in the presence (b) of 10 μM ryanodine. In b, standard medium containing 70 mM KCl (replacing an equimolar amount of NaCl) was perfused when indicated (K^+). Other details are as in Fig. 1.

Quantal Effect of Caffeine

The effect of caffeine has been reported to be quantal (Cheek et al., 1993, 1994a), meaning that low caffeine concentrations release only part of the caffeine-sensitive pool. The same phenomenon was observed here. Fig. 3 a shows that addition of submaximal caffeine concentrations induced a rapid but partial emptying of the ER, leading within 30 s to a new lower steady-state of $[Ca^{2+}]_{ER}$. At that point, only the addition of a higher caffeine concentration was able to produce further emptying of the ER. Similar quantal effects were also observed during refilling of the ER when it was carried out in the presence of caffeine. Fig. 3 b shows that the ER did not refill in the presence of 50 mM caffeine, but refilled about halfway once the caffeine concentration was dropped to 5 mM, and completely when caffeine was washed away. Subsequent addition of 5 and 50 mM caffeine released Ca^{2+} and reached the same $[Ca^{2+}]_{ER}$ levels obtained during refilling in the presence of these caffeine concentrations. The degree of emptying induced by a particular caffeine concentration was quite reproducible in consecutive additions. Fig 3 c shows that consecutive additions of 5 mM caffeine produced always ~50% emptying of the ER, and only the addition of a higher caffeine concentration was able to produce further emptying. Fig. 3 d shows the effect of ryanodine added in the presence of a submaximal dose of caffeine. We can see that the first pulses were identical to the control, but again here the ER refilled progressively more slowly after each new caffeine addition. In this case, in contrast to the experiment shown in Fig. 2, finally a $[Ca^{2+}]_{ER}$ steady-state corresponding to about half-filling was reached, in fact, the same $[Ca^{2+}]_{ER}$ obtained initially after addition of 5 mM caffeine. Once at this point, addition of a maximal dose of caffeine was required to induce emptying of the remaining portion of the ER.

The experiments of Fig. 3 are consistent with previous results obtained looking at $[Ca^{2+}]_c$ in the same cell prepa-

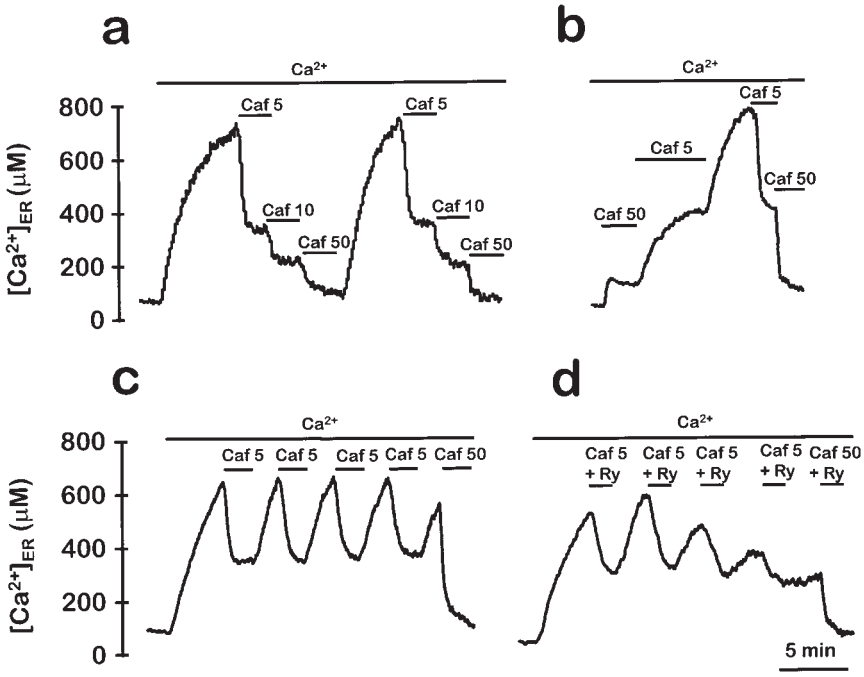

Figure 3. Quantal response to submaximal concentrations of caffeine. The ER was refilled by perfusing with medium containing 1 mM Ca^{2+} either in the presence (b) or in the absence of caffeine. Then, different concentrations of caffeine were added as indicated, either in the presence or in the absence of 10 μM ryanodine. Other details are as in Fig. 1.

ration by Cheek et al., 1994a. Those experiments led the authors to suggest that there should be different compartments within the ER having different sensitivities to caffeine. This hypothesis would explain why ryanodine only empties the pool sensitive to 5 mM caffeine but leaves the rest of the pool untouched, which could be only released with a higher caffeine dose. However, there is an alternative explanation for these results, based on the regulation of caffeine-induced Ca^{2+} release by the lumenal $[Ca^{2+}]$. In this hypothesis, the results can be explained with only one ER compartment if we assume that submaximal caffeine concentrations can only release Ca^{2+} until $[Ca^{2+}]_{ER}$ is reduced to a certain level. The higher the caffeine concentration, the lower the $[Ca^{2+}]_{ER}$ level attained. Both alternative hypotheses lead to different predictions under some experimental conditions. In particular, if we obtain half-filled stores by different procedures, e.g., by emptying them with an agonist acting via $InsP_3$ production or by refilling the stores only halfway, the hypothesis of several compartments predicts that we should have all of them half-filled. Therefore, a submaximal dose of caffeine should still release Ca^{2+} from half of them. On the contrary, if the release was directly controlled by $[Ca^{2+}]_{ER}$, we would expect the effect of 5 mM caffeine to be independent of the procedure used to reach that half-filling. The experiments shown in Fig. 4 indicate that the last hypothesis is the correct one. In Fig. 4 a, half-filling was obtained by emptying the ER with histamine. After that, 5 mM caffeine had no effect. Instead, if 5 mM caffeine was added with the ER full of Ca^{2+}, it was able to empty it exactly down to the same point. Fig. 4 b shows the effect of half-filling the ER by reducing the time of refilling. Again, the effect of 5 mM caffeine was strictly dependent on the level of $[Ca^{2+}]_{ER}$ reached at the point it was added. It had no effect at half-filling, but released 50% of the pool when the Ca^{2+} stores were completely filled. Fig. 4 c shows a similar approach but made in cells preloaded with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) to slow the refilling. We can observe again that the effect of 5 mM caffeine was strictly dependent on the $[Ca^{2+}]_{ER}$ at the moment of addition. In addition, this experiment also shows that Ca^{2+} release induced by caffeine requires only resting $[Ca^{2+}]_c$. Fura-2 measurements performed in parallel showed that in cells loaded with BAPTA, the $[Ca^{2+}]_c$ changes induced by caffeine were almost abolished (data not shown). This result points out also that quantal Ca^{2+} release by caffeine is due to the regulation of Ca^{2+} release by the lumenal $[Ca^{2+}]$, and suggests that changes in $[Ca^{2+}]_c$ do not play a major role in the development of the quantal effect.

Ca^{2+} Entry Activates CICR

The next step in this study was to investigate the presence of CICR activated by Ca^{2+} entry through the plasma membrane Ca^{2+} channels. Depolarization with high K^+ medium or by stimulation with nicotinic acetylcholine agonists such as 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), produced large $[Ca^{2+}]_c$ peaks (Núñez et al., 1995), that were not significantly modified by previous Ca^{2+} depletion of the ER with caffeine or with the ER Ca^{2+} pump inhibitor thapsigargin (see below). Therefore, the possible contribution of CICR to these $[Ca^{2+}]_c$ peaks cannot be estimated from conventional $[Ca^{2+}]_c$ studies, and direct measurement of $[Ca^{2+}]_{ER}$ becomes essential. Fig. 5 a shows that 10-s pulses of depolarization with high K^+ medium induced a transient Ca^{2+} release from the ER, which could be triggered repetitively by consecutive pulses. The $[Ca^{2+}]_{ER}$ decrease was of 60–100 μM (10–15% of the steady-state $[Ca^{2+}]_{ER}$). Therefore, in spite of the large increase in $[Ca^{2+}]_c$, the activation of CICR produced a much smaller $[Ca^{2+}]_{ER}$ decrease than treatment with caf-

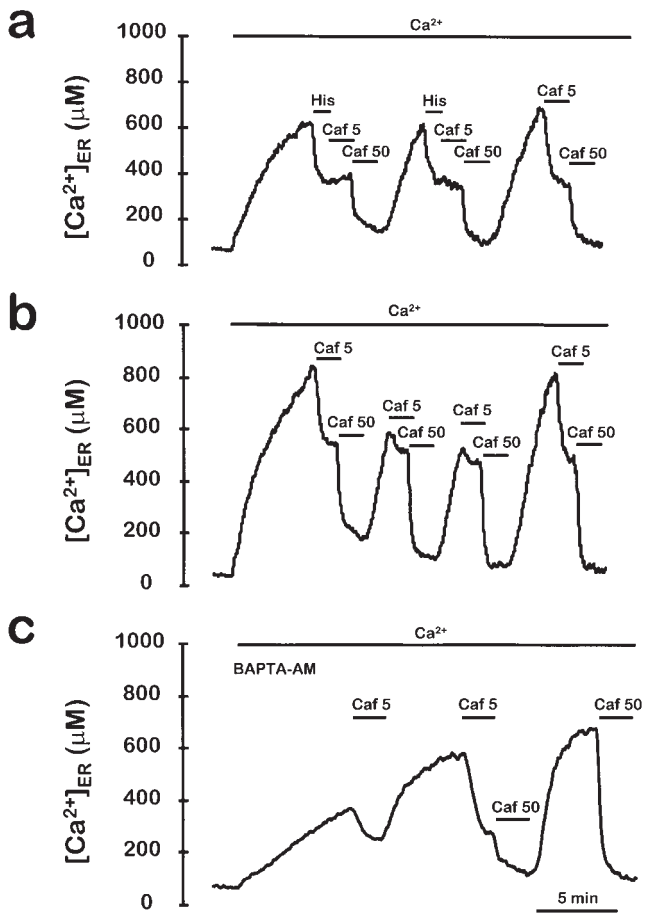

Figure 4. Regulation of caffeine-induced Ca^{2+} release by $[Ca^{2+}]_{ER}$. The ER was refilled by perfusing with medium containing 1 mM Ca^{2+} and then 1 μM histamine or different concentrations of caffeine were added, as indicated. In the experiment shown in c, cells were incubated with 10 μM BAPTA-AM during aequorin reconstitution in order to load the cytosol with this Ca^{2+} chelator. Other details are as in Fig. 1.

feine. Increasing the duration of the high K^+ pulse increased the magnitude of Ca^{2+} release little. Fig. 5 b illustrates the effect of depolarization with longer (1 min) high K^+ pulses. In this case, the first high K^+ pulse produced the same effect as in Fig. 5 a, and then $[Ca^{2+}]_{ER}$ increased more rapidly, probably as a result of the prolonged stimulation of the ER Ca^{2+} pump by the sustained high $[Ca^{2+}]_c$ levels. This led to a new $[Ca^{2+}]_{ER}$ steady-state at ~800–900 μM. After that, subsequent K^+ pulses induced somewhat larger $[Ca^{2+}]_{ER}$ decreases of 150–200 μM, but corresponding still to only 20% of the steady-state $[Ca^{2+}]_{ER}$. To investigate if increased Ca^{2+} pumping could be responsible for the incomplete Ca^{2+} release, the effect of K^+ depolarization was tested in the presence of the ER Ca^{2+} pump inhibitor ciclopiazonic acid (CPA). Fig. 5 c shows that CPA itself induces a slow Ca^{2+} release from the ER, and that simultaneous addition of high K^+ medium induced a fast initial Ca^{2+} release of ~20% of the $[Ca^{2+}]_{ER}$, followed by a slower release at a rate comparable to that induced by CPA alone. This suggests that CICR induced by a maximal K^+ depolarization is able to produce only a decrease of ~20% of the $[Ca^{2+}]_{ER}$, even in the absence of Ca^{2+} pumping. CICR, however, was potentiated by simultaneous addition of a low caffeine concentration. Fig. 5 d shows that addition of 1 mM caffeine produced little effect by itself, but strongly potentiated the effect of K^+ depolarization, that was now able to release rapidly ~50% of the stored Ca^{2+}. Finally, CICR could also be triggered in a more physiological way using an agonist for the nicotinic acetylcholine receptor. Addition of DMPP induced a rapid and partial Ca^{2+} release from the ER, very similar to that shown above for K^+ depolarization, and which was also potentiated by low concentrations of caffeine (data not shown).

The effect of a low caffeine concentration as positive modulator of CICR might reproduce the action of a physiological modulator of this phenomenon. Phosphorylation by cAMP-dependent protein kinase and production of the β-NAD^+ metabolite cyclic ADP ribose (cADPR) have been reported to act as physiological modulators for RyR in bovine chromaffin cells (Morita et al., 1997). However, incubation for 3–5 min with the adenylate cyclase activator forskolin (20 μM) had neither any significant effect on the sensitivity to caffeine of Ca^{2+} release nor on the magnitude of high K^+ depolarization-induced CICR (data not shown). Regarding cADPR, it has been reported that acetylcholine, high K^+ depolarization, and forskolin all stimulate its synthesis by ADP ribosyl cyclase in bovine chromaffin cells (Morita et al., 1997). Therefore, production of this mediator should already be stimulated in our CICR experiments. Nevertheless, to study directly the effect of cADPR on Ca^{2+} release from the ER, we performed experiments in permeabilized cells. Cells were depleted of Ca^{2+} and reconstituted with coelenterazine as usual. Recording of luminescence was started and the cells were permeabilized by perfusion with intracellular-like medium containing 20 μM digitonin and 2 mM EGTA for 1 min. Then, intracellular-like medium containing 100 nM Ca^{2+} (buffered with EGTA) and 2 mM ATP-Mg was perfused. Fig. 6 a shows that $[Ca^{2+}]_{ER}$ increased in digitonin-permeabilized cells with very similar kinetics to that found in intact cells after addition of 1 mM extracellular Ca^{2+}. The steady-state $[Ca^{2+}]_{ER}$ reached was also similar (compare with Fig. 1 a). Fig. 6 a also shows that 2 μM $InsP_3$ and 50 mM caffeine produced a rapid and near complete release of Ca^{2+} from the ER whereas 5 μM cADPR had no effect. Two different commercial sources of cADPR were tested with the same results. In some experiments, cADPR was added in the presence of 1 μM calmodulin and no effect was found either. A possible explanation for the discrepancy among our results and those of Morita et al. (1997) would be that cADPR releases Ca^{2+} from a different (non-ER) Ca^{2+} pool. In fact, these authors report that $InsP_3$ releases Ca^{2+} from a pool sensitive to 20 nM thapsigargin, whereas cADPR and caffeine release Ca^{2+} from a pool only sensitive to >200 nM thapsigargin. In our hands, refilling of the ER was completely inhibited by either 20 nM or 1 μM thapsigargin in both intact (see above) and permeabilized cells (data not shown). Therefore, in order to make compatible our own results and those of Morita et al. (1997), cADPR and caffeine should be able to release Ca^{2+} from an additional non-ER Ca^{2+} pool in the presence of 20 nM thapsigargin. We then performed single-cell

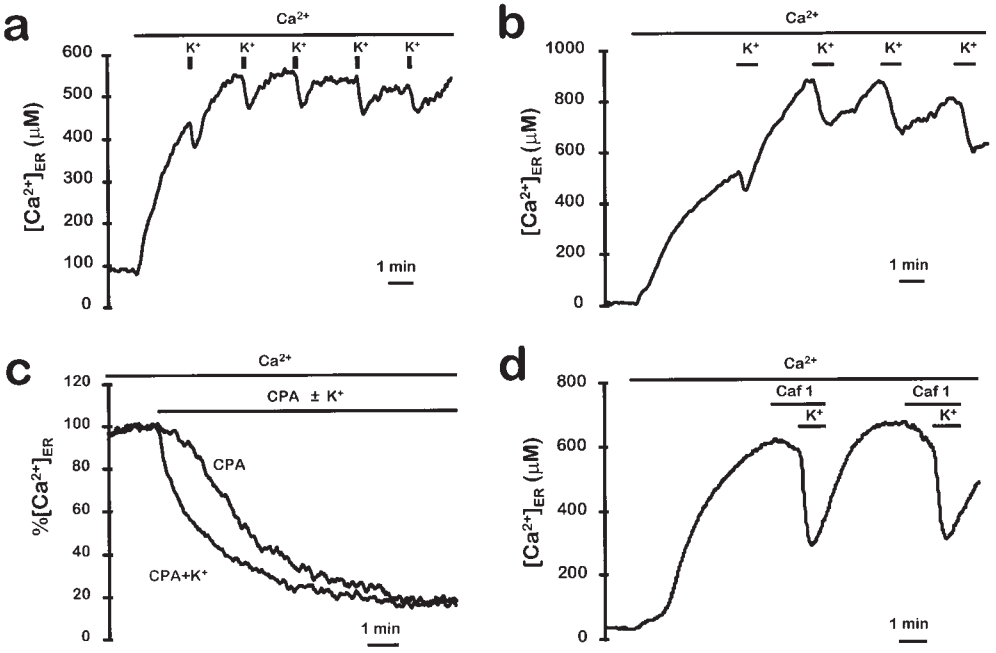

Figure 5. Activation of CICR by the Ca^{2+} entry elicited by high K^+-induced cell depolarization. The ER was refilled by perfusing medium containing 1 mM Ca^{2+}. Then, standard medium containing 70 mM KCl was perfused as indicated. In c, 10 μM CPA was added as indicated with or without high K^+ medium. In this panel, the $[Ca^{2+}]_{ER}$ scale has been normalized as percentage of the maximum $[Ca^{2+}]_{ER}$ level in order to facilitate comparison. In the presence of CPA and high K^+ medium the initial rate of $[Ca^{2+}]_{ER}$ decrease is much higher that with CPA alone, but once $[Ca^{2+}]_{ER}$ is below 80% of the initial level, the rates of release in both cases turn similar and can be nearly superimposed. In d, 1 mM caffeine was also added as indicated. Other details are as in Fig. 1.

fura-2 imaging experiments in intact cells looking at the effect of caffeine on $[Ca^{2+}]_c$ in cells pretreated with 20 nM thapsigargin. Fig. 6 b shows that addition of caffeine or histamine in Ca^{2+}-free medium produced no increase in $[Ca^{2+}]_c$ under these conditions (the observed decrease is due to the perfusion of Ca^{2+}-free medium), whereas K^+ depolarization still produced the usual $[Ca^{2+}]_c$ peak due to Ca^{2+} entry.

Effect of Ryanodine on CICR

On the basis of the use-sensitive action of ryanodine, illustrated here for caffeine stimulation (Figs. 2 and 3), we should expect also to find a use-sensitive inhibition of ER refilling after repeated stimulation with high K^+ pulses inducing CICR. Under this rationale, experiments similar to those shown in Fig. 5, but in the presence of 10 μM ryanodine, were performed. In these experiments we were not able to detect any significant effect of ryanodine on the $[Ca^{2+}]_{ER}$ decrease induced by four or five consecutive K^+ pulses (data not shown). However, as aequorin consumption limits the sensitivity of the measurement at the end of these experiments, we also decided to perform single-cell imaging experiments looking at the magnitude of the $[Ca^{2+}]_c$ peak induced by caffeine after several K^+ pulses in the presence of ryanodine. Fig 7 a shows that the $[Ca^{2+}]_c$ increase induced by caffeine was little modified after five consecutive 30-s K^+ pulses given in the presence of ryanodine (compare with the initial three caffeine additions). It is interesting to note that the second caffeine addition after the K^+ + ryanodine pulses produced no $[Ca^{2+}]_c$ increase, even though caffeine was always added in the absence of ryanodine. In fact, perfusion with ryanodine for a short period (30–60 s) before any caffeine addition was effective in promoting the typical use-dependent inhibition of ER refilling on application of caffeine (without ryanodine) 30–60 min later. This means that, even though ryanodine by itself does not produce any apparent effect on ER filling, it remains within the cells after washing and acts later, on stimulation of Ca^{2+} release (see below). Depolarization can also be produced using the nicotinic acetylcholine agonist DMPP instead of K^+. Again here, consecutive pulses of 10 μM DMPP in the presence of ryanodine did not have any effect on a later $[Ca^{2+}]_c$ peak induced by caffeine (data not shown). An alternative and perhaps more physiological depolarizing maneuver is field electric stimulation. Fig. 7 b shows the effect of several consecutive 10-s pulses at 10 Hz, before and after the addition of ryanodine. Again, we can observe that 5 mM caffeine produced the same $[Ca^{2+}]_c$ peak after and before field electric stimulation. As in Fig. 7 a, consecutive additions of caffeine produced inhibition long after washing of ryanodine. The lack of effect of ryanodine in these experiments may be attributed to the much smaller activation of Ca^{2+} release by high K^+-induced Ca^{2+} entry compared with that induced by caffeine.

An alternative possibility to activate RyR by Ca^{2+} would be to release Ca^{2+} directly from the ER using an $InsP_3$-producing agonist in order to produce a big increase in $[Ca^{2+}]_c$ just besides the RyR. In fact, it has been reported that histamine-induced Ca^{2+} release could be inhibited partially with ryanodine after five consecutive stimulation pulses (Stauderman and Murawsky, 1991). In our hands, stimulation by five consecutive pulses of histamine and ryanodine had little effect on the first subsequent $[Ca^{2+}]_c$ peak obtained by caffeine stimulation (Fig. 7 c). Histamine was always added in the absence of extracellular Ca^{2+}, as it also activates Ca^{2+} entry (Cheek et al., 1994b).

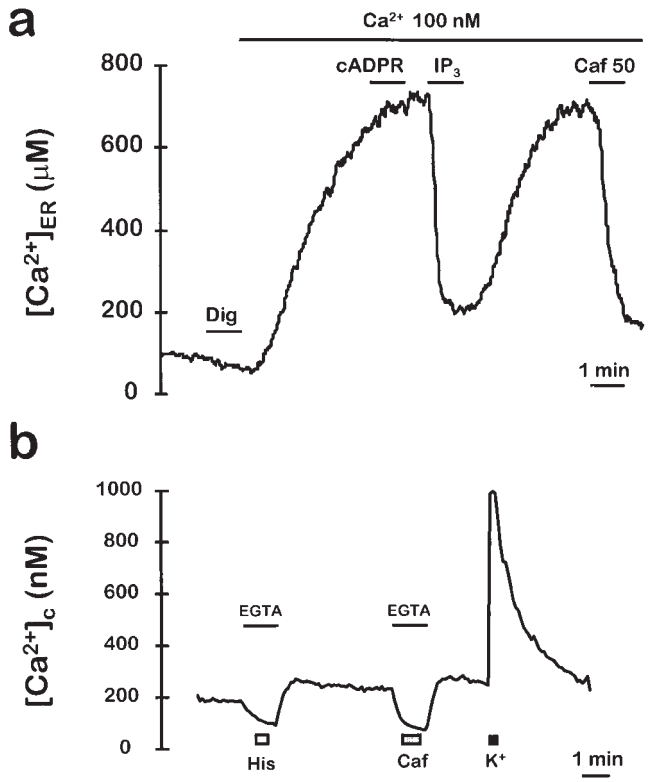

Figure 6. (a) Effects of cADPR, $InsP_3$, and caffeine on $[Ca^{2+}]_{ER}$ in permeabilized cells. Cells were permeabilized by perfusion with 20 μM digitonin for 1 min as indicated. Then the ER was refilled by perfusion with medium containing 100 nM Ca^{2+} (buffered with EGTA). Finally, either 5 μM cADPR, 2 μM $InsP_3$, or 50 mM caffeine were perfused as indicated. (b) Effect of preincubation with 20 nM thapsigargin on the $[Ca^{2+}]_c$ responses to histamine, caffeine, and high K^+ medium. Cells were loaded with fura-2, preincubated for 20 min with 20 nM thapsigargin, and then suspended in standard medium containing 1 mM $CaCl_2$. Then, either 10 μM histamine, 50 mM caffeine, or standard medium containing 1 mM $CaCl_2$ and 70 mM KCl were perfused as indicated. Both histamine and caffeine were perfused in Ca^{2+}-free medium (containing 100 μM EGTA) to avoid Ca^{2+} entry. Perfusion with Ca^{2+}-free medium was started 15 s before and continued for 15 s after stimulation with histamine or caffeine. The trace shown corresponds to the average of 42 cells present in the microscope field. Other details are as in Fig. 1.

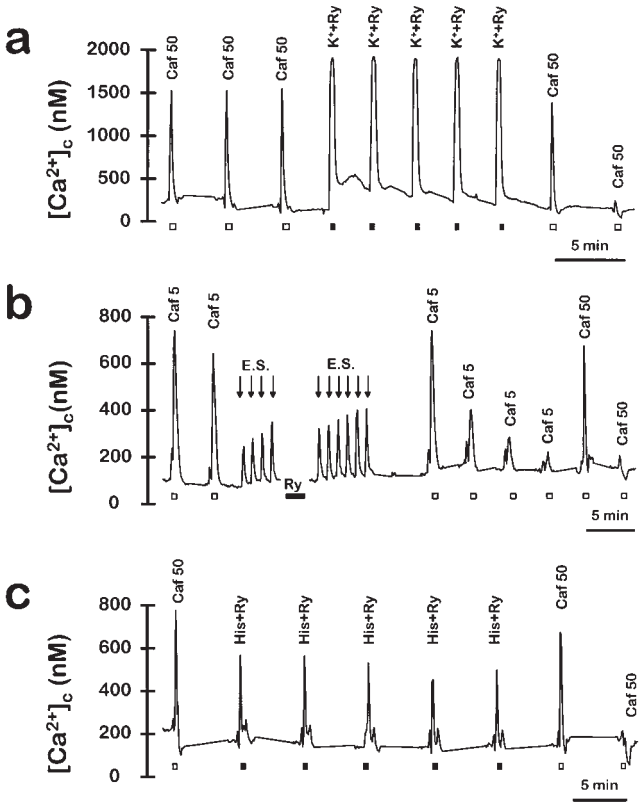

Figure 7. Effects of ryanodine and either depolarization with high K^+ medium (a), field electric stimulation (b), or histamine (c) on the $[Ca^{2+}]_c$ responses induced by caffeine. Cells were loaded with fura-2 and placed under the microscope in standard medium containing 1 mM $CaCl_2$. Then, different stimuli were given as indicated: different concentrations of caffeine (Caf, in mM), medium containing 70 mM KCl (K^+), 10 μM histamine (His), 10 μM ryanodine (Ry), or field electric stimulation (E.S. 10 Hz for 10 s, arrows). Caffeine and histamine were added in Ca^{2+}-free medium (containing 100 μM EGTA). Perfusion with Ca^{2+} free medium was started 15 s before and continued for 15 s after the stimuli. Transition from Ca^{2+}-containing to Ca^{2+}-free medium sometimes produced a small $[Ca^{2+}]_c$ peak. The traces shown are the average of 36 (a), 49 (b), and 61 (c) cells present in the microscope field.

Similarly, caffeine was added also in Ca^{2+}-free medium to avoid the Ca^{2+} entry induced by caffeine, similar to that reported in GH_3 pituitary cells (Villalobos and García-Sancho, 1996). As before, the second caffeine stimulation after histamine and ryanodine pulses was completely abolished.

Effects of $[Ca^{2+}]_{ER}$ on CICR

In spite of the lack of inhibition by ryanodine, the potentiation by caffeine of Ca^{2+} release induced by K^+ depolarization (as shown in Fig. 5 d) suggests that CICR may take place through the same RyR activated by caffeine. An important property of the RyR activated by caffeine is the regulation by lumenal $[Ca^{2+}]$ shown in Figs. 3 and 4. In the case of CICR, data of Fig. 5 b also suggest that Ca^{2+} release may be stronger at higher lumenal $[Ca^{2+}]$ since the first stimulation with high K^+, carried out when the ER was only half-filled, was less efficient than the subsequent ones. To obtain more evidence on this point, we investigated the effects of 10-s pulses of high K^+/Ca^{2+}-containing medium at different $[Ca^{2+}]_{ER}$ levels (Fig. 8). At the beginning of the experiment the ER was completely depleted of Ca^{2+} and the first two K^+ pulses induced an increase in $[Ca^{2+}]_{ER}$. Since the cells were kept in EGTA-containing medium during the intervals between the K^+/Ca^{2+} pulses, refilling of the ER took place only from Ca^{2+} entering into the cells during the pulses. Subsequent K^+ pulses produced also $[Ca^{2+}]_{ER}$ increase, although progressively smaller, reaching finally a steady-state $[Ca^{2+}]_{ER}$ at ~300 μM, where the K^+/Ca^{2+} pulses had almost no effect. We then completely refilled the ER with Ca^{2+} by perfusing the cells with Ca^{2+}-containing medium, thus reaching the usual

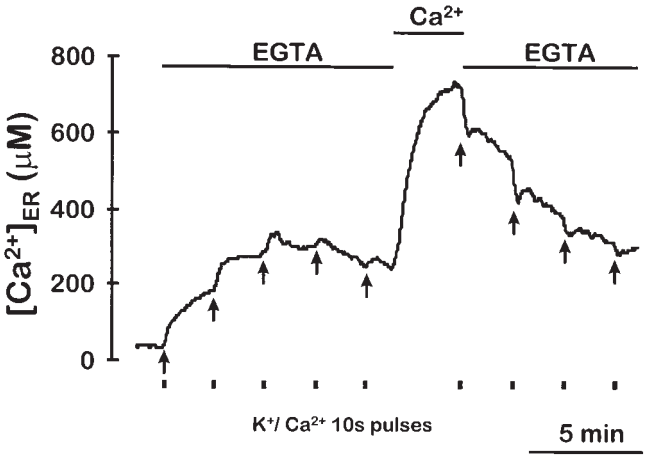

Figure 8. Effect of $[Ca^{2+}]_{ER}$ on CICR induced by depolarization with high K^+. Cells depleted of Ca^{2+} and reconstituted with coelenterazine n were placed in the luminometer in 0.5 mM EGTA containing standard medium. Then, 10-s pulses of medium containing 70 mM KCl and 2 mM $CaCl_2$ were given as indicated and 0.5 mM EGTA containing standard medium was perfused during the intervals. After five pulses, Ca^{2+}-containing (1 mM) medium was perfused for 3 min to refill the ER, and then the previous protocol was started again.

$[Ca^{2+}]_{ER}$ steady-state levels at ~700 μM. At that point, we restarted the protocol of repeated stimulation with 10-s high K^+/Ca^{2+} pulses, using EGTA-containing medium for the intervals. The pulses produced now a rapid Ca^{2+} release that could be clearly distinguished from the slower $[Ca^{2+}]_{ER}$ decrease induced by the lack of extracellular Ca^{2+}. After three K^+ pulses, $[Ca^{2+}]_{ER}$ was reduced again to ~300 μM, and at that point the last pulse produced little effect. This result shows clearly that Ca^{2+} release induced by K^+ depolarization, similarly to that induced by caffeine, is strictly dependent on the $[Ca^{2+}]_{ER}$.

Differential Inhibition of CICR by Type-specific Ca^{2+} Channel Inhibitors

We have tested the effect of several inhibitors of Ca^{2+} entry on both refilling of the ER and high K^+ depolarization-induced CICR. A combination of inhibitors of all the voltage-dependent Ca^{2+} channels including 1 or 2 h of preincubation with ω-conotoxin GVIA (1 μM), ω-conotoxin MVIIC (3 μM), and ω-agatoxin IVA (1 μM), together with preincubation and perfusion along the experiment with 3 μM nisoldipine, had little effect on the rate of refilling of the ER with Ca^{2+}. Instead, refilling was almost completely blocked by perfusion of 100 μM Cd^{2+} (data not shown). Next, the effects of the inhibitors of voltage-gated Ca^{2+} channels on CICR were tested. For these purposes, we stimulated the cells with high K^+ medium in the presence of 1 mM caffeine (as in the experiments shown in Fig. 5 d) in order to potentiate the mechanism and increase the sensitivity of the measurements. The decreases in $[Ca^{2+}]_{ER}$ observed, normalized as percentage of those obtained in the controls, were (mean ± SEM): control, 100 ± 11 (n = 6); 3 μM nisoldipine, 103 ± 6 (n = 5); 1 μM ω-conotoxin GVIA, 93 ± 6 (n = 6); 3 μM ω-conotoxin MVIIC, 42 ± 6 (n = 7); 1 μM ω-agatoxin IVA, 41 ± 9 (n = 5); a combination of all three toxins and nisoldipine, 20 ± 6 (n = 5). Toxins and nisoldipine were preincubated with the cells for 1 or 2 h before the measurements. When the effect of nisoldipine was tested, this inhibitor was also perfused along the experiment.

Ryanodine Treatment Modifies the $[Ca^{2+}]_c$ Wave Induced by Short Depolarizations as Visualized by Confocal Microscopy

Under physiological conditions, cell stimulation is triggered by short depolarizations lasting a few milliseconds. To estimate the contribution of CICR to the Ca^{2+} transient under these conditions, we have compared the rate of diffusion of the Ca^{2+} wave induced by a short (100 ms) cell depolarization both in control cells or in cells in which the Ca^{2+} stores had been blocked by previous treatment with caffeine and ryanodine. We combined the whole-cell patch-clamp technique with fluo-3–based microfluorimetry using a confocal microscope. Cells were line-scanned along 100-ms square depolarizing pulses from a holding potential of −70 to +10 mV. The recorded inward currents showed two typical components: a initial transient peak (I_{Na}) followed by a slow inactivating phase (I_{Ca}) (data not shown). The ryanodine treatment did not affect the total stimulated Ca^{2+} entry, calculated as the integral of the last 90 ms of the recorded inward current (mean ± SEM: control cells, 7.15 ± 0.42 pC [n = 34]; ryanodine-treated cells, 6.68 ± 1.03 pC [n = 21]). In spite of this, line scan images representing $[Ca^{2+}]_c$ showed clear differences between control and ryanodine-treated cells. Fig. 9 a shows the spatiotemporal pattern of $[Ca^{2+}]_c$ increase in control cells, codified in pseudocolor. $[Ca^{2+}]_c$ increased first near the plasma membrane and then the Ca^{2+} wave propagated intracellularly. Fig. 9 b shows the results obtained in cells with the Ca^{2+} stores previously emptied by treatment with caffeine and ryanodine. In this case, the $[Ca^{2+}]_c$ increase was smaller and the propagation of the Ca^{2+} wave delayed. Fig. 9, panels c–e detail the behavior of several parameters that quantify the phenomenon described above in terms of peak $[Ca^{2+}]_c$ rise (Fig. 9 c), maximum rate of $[Ca^{2+}]_c$ increase (Fig. 9 d), and time required to increase fluorescence by 10% (Fig. 9 e) at different intracellular locations. Fig. 9 c shows that the maximum fluo-3 fluorescence (indicating the maximum $[Ca^{2+}]$ peak) was reached near the plasma membrane. An 80% increase was found in control cells compared with only a 40% increase in ryanodine-treated cells. The fluorescence peaks were smaller as we move deep inside the cell, but the difference among control and ryanodine-treated cells was maintained. Fig. 9 d shows that the maximum rate of fluorescence increase was located near the plasma membrane and decreased steeply as we move into the cell. Again here, the rates were two to three times faster in the control cells than in the ryanodine-treated ones. Fig. 9 e shows the time required for the fluorescence to be increased by 10% at different locations. This parameter is very sensitive to the intracellular propagation of the $[Ca^{2+}]_c$ wave. We find that the $[Ca^{2+}]_c$ wave propagates about twice as fast in control cells than in cells treated with ryanodine. These results indicate that CICR significantly contributes to the Ca^{2+} signal induced by cell

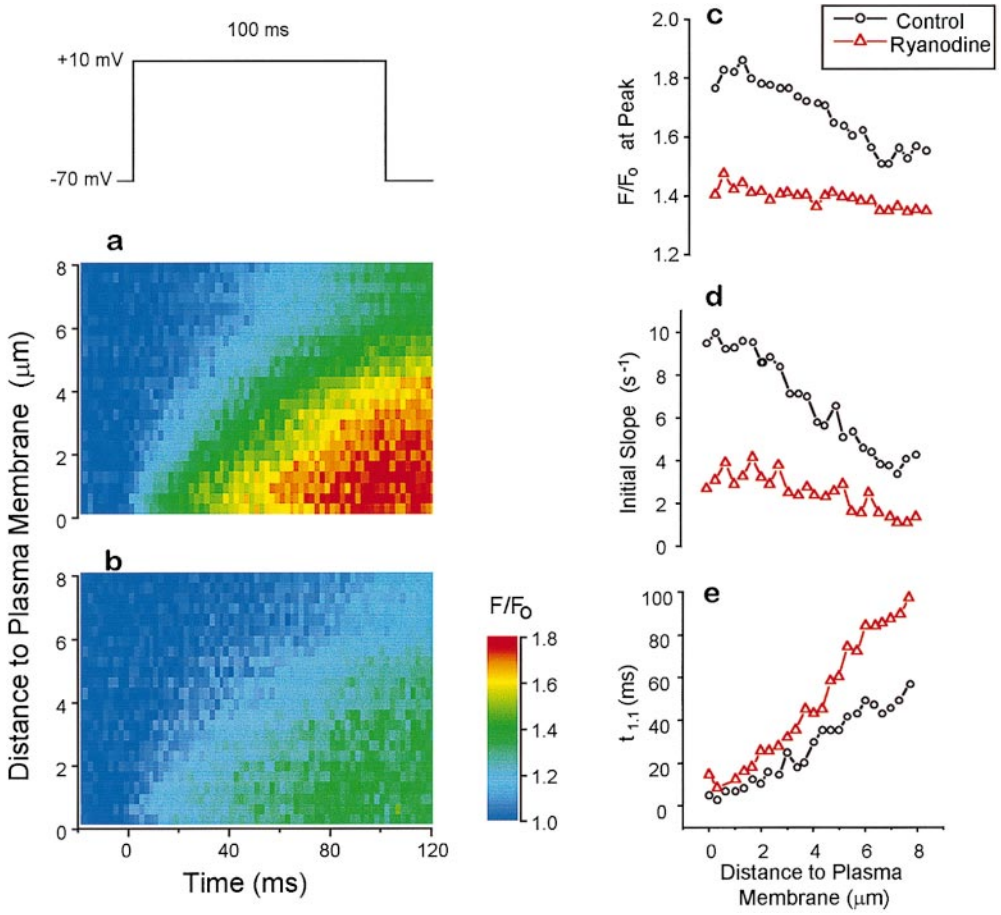

Figure 9. Confocal imaging of the propagation of the $[Ca^{2+}]_c$ signal induced by cell depolarization. (a) Mean image from 34 line-scan fluorescence images representing F/F_0 (ratio between fluo-3 fluorescence at a certain time and before stimulation, an index for $[Ca^{2+}]_c$) in control cells stimulated by a 100-ms depolarizing pulse from a holding potential of −70 mV to 10 mV (top). (b) Mean image from 21 records of ryanodine-treated cells displayed as in panel a. Before starting the experiment, cells were exposed three times to a 10 mM caffeine + 10 μM ryanodine-containing Krebs-Hepes solution. After that, cells were maintained in 10 μM ryanodine during the whole experiment. c–e show the distribution with the distance to the plasma membrane of the maximum F/F_0 levels (c), the rate of rise of fluo-3 fluorescence, measured as the slopes calculated from the first 10 ms of the signal rising (d), and the time ($t_{1.1}$) from the initiation of the pulse to the moment in which a value $F/F_0 \geq 1.1$ (taken as an arbitrary threshold) was reached (e).

depolarization during a short, more physiological stimulation.

Discussion

Most of previous research on CICR has been performed by inferring the changes in $[Ca^{2+}]_{ER}$ from the evolutions of $[Ca^{2+}]_c$. Much of the value of the present study comes from the fact that, for the first time, CICR has been looked at from the inside of the ER using targeted aequorin. Such an approach provides unambiguous evidence for the existence of CICR in adrenal chromaffin cells and reveals several previously unrecognized features on the mechanisms and the regulation of this important phenomenon. We find that essentially all the stored Ca^{2+} can be released by stimulation with a maximal dose of caffeine and that refilling was almost completely prevented by both caffeine and ryanodine. This means that RyR must be homogeneously distributed within the ER or, alternatively, Ca^{2+} release at some specific points is able to empty rapidly subcompartments lacking RyRs, that is, the ER behaves as a continuum. We can not distinguish at present between these two possibilities. $InsP_3$-producing agonists such as histamine or bradykinin also produced a major release of Ca^{2+} from the ER, although smaller than caffeine. This may suggest that the $InsP_3$-sensitive pool is smaller than the caffeine-sensitive one, as previously suggested (Liu et al., 1991). In permeabilized cells, however, direct addition of $InsP_3$ produced Ca^{2+} release as big as that induced by caffeine. This suggests that the incomplete releasing effect of histamine or bradykinin in intact cells may be due to desensitization phenomena, either at the plasma membrane receptors or at the $InsP_3$ receptor, or to other mechanisms that may limit $InsP_3$-mediated Ca^{2+} release such as inhibition by $[Ca^{2+}]_c$ (Bezprozvanny et al., 1991; Finch et al., 1991; Barrero et al., 1997; Montero et al., 1997a). In conclusion, our results support the idea that the bulk of the ER is able to release Ca^{2+} either via $InsP_3$ receptors or via RyR. In addition, the whole ER was also sensitive to inhibition of the ER Ca^{2+} pump with thapsigargin, even at low concentrations (20 nM).

These results are consistent with those of Poulsen et al. (1995), showing that the distribution of thapsigargin-sensitive Ca^{2+} pumps was parallel to that of $InsP_3R$ and RyR in subcellular fractions of bovine chromaffin cells. On the other hand, our results contrast with those of several authors working also with bovine chromaffin cells. Cheek et al. (1991) concluded using permeabilized cells that the caffeine-sensitive Ca^{2+} store is largely distinct from the $InsP_3$-sensitive one. The presence of separate pools exclusively sensitive to $InsP_3$ or to caffeine, or sensitive to both has also been reported from studies with permeabilized

cells by Stauderman et al. (1991), although in the same study the caffeine and the $InsP_3$-sensitive pools overlapped more than 90% in intact cells. Robinson and Burgoyne (1991), also using permeabilized cells, suggested that there are two distinct nonoverlapping Ca^{2+} stores sensitive to $InsP_3$ or to caffeine, and only the $InsP_3$-sensitive one was emptied by thapsigargin. Finally, Morita et al. (1997) have shown recently that in permeabilized cells, cADPR and caffeine release Ca^{2+} from a compartment insensitive to 20 nM thapsigargin, a concentration that, however, abolished $InsP_3$-mediated Ca^{2+} release. We have no explanation for the discrepancies among the different studies. Nevertheless, we should make clear that, in the aequorin experiments, we are measuring $[Ca^{2+}]$ specifically inside the ER, whereas in all the other studies there may be a contribution to Ca^{2+} release from other Ca^{2+}-storing organelles. Using mitochondrially targeted aequorin in chromaffin cells, we have seen that mitochondria have a resting $[Ca^{2+}]$ similar to that in the cytosol (our unpublished observations), as it has been also reported in other cell types (Rizzuto et al., 1993); therefore they cannot release Ca^{2+} under resting conditions. An alternative possibility would be the secretory granules, which can accumulate large amounts of Ca^{2+} by a mechanism not requiring Ca^{2+}-ATPase (Pozzan et al., 1994). It has been claimed that $InsP_3$ may release Ca^{2+} from chromaffin granules (Yoo and Albanesi, 1991), but evidence against the presence of $InsP_3R$ in the secretory granules of the closely related PC12 pheochromocytoma cells has also been reported (Fasolato et al., 1991; Zachetti et al., 1991). Pancreatic acinar secretory granules have also been shown to release Ca^{2+} on stimulation with either $InsP_3$ or cADPR (Gerasimenko et al., 1996), but these results have been also questioned later (Yule et al., 1997). Moreover, Pouli et al. (1998), have just reported in PC12 cells, using an aequorin chimera targeted to the outer side of the granule membrane, that both the agonist- or the K^+-induced increases in $[Ca^{2+}]$ are identical in the cytosol and in the outer side of the granule membrane, suggesting that there is no Ca^{2+} release from the granules during physiological stimulation. Our results are consistent with the last view since even low concentrations of thapsigargin (20 nM) were able to abolish the Ca^{2+} release induced by both histamine or caffeine in fura-2 single-cell imaging experiments (Fig. 6 b). Perhaps some of the discrepancies among the different studies may come from the modifications generated by permeabilization in the organellar structure. In our experiments with permeabilized cells (Fig. 6 a), we have attempted to minimize this problem by performing a very fast (1 min) on-line permeabilization procedure.

Regarding Ca^{2+} release from the ER by caffeine, our experiments confirm previous results by Cheek et al. (1993 and 1994a), showing that Ca^{2+} release by increasing concentrations of caffeine is quantal in nature. We can add a new insight to this effect, since our results suggest that this quantal effect is due to the control of Ca^{2+} release by $[Ca^{2+}]_{ER}$ rather than to the existence in the ER of different Ca^{2+} pools with varying sensitivity to caffeine. Fig. 4 shows that when $[Ca^{2+}]_{ER}$ was at the half-filling level, obtained either using an $InsP_3$-producing agonist (Fig. 4 a) or after incomplete refilling of the ER (Fig. 4 b), 5 mM caffeine had no effect. These results could be compatible with the hypothesis of heterogeneous Ca^{2+} pools only if we assume that (a) half-maximal quantal release induced by $InsP_3$ involves the same compartments than that induced by 5 mM caffeine, and (b) refilling of the ER is sequential and starts first by those compartments having the lowest sensitivity to caffeine. The first condition cannot be ruled out conclusively, given that it is not known if both receptors may share the same mechanism for quantal release. However, we consider the second one highly improbable because the distribution of Ca^{2+}-ATPases in chromaffin cells has been reported to be parallel to that of $InsP_3$ receptors and RyR (Poulsen et al., 1995). In addition, Fig. 3 b shows that the rate of refilling is slower in the presence of 5 mM caffeine than in the absence of caffeine, suggesting that compartments sensitive to 5 mM caffeine refill early in control cells. Therefore, our results clearly suggest that 5 mM caffeine does not release Ca^{2+} from one half of the stores, but releases Ca^{2+} from all of them until $[Ca^{2+}]_{ER}$ reaches half-filling. The same type of control of Ca^{2+} release by $[Ca^{2+}]_{ER}$ was evidenced when it was triggered by Ca^{2+} entry. This suggests that Ca^{2+} release through RyR is similarly regulated in both cases, stimulation by caffeine or CICR. Control of Ca^{2+} release through both $InsP_3R$ and RyR by the lumenal $[Ca^{2+}]$ has been suggested previously (Missiaen et al., 1992; Berridge, 1993; Hidalgo and Donoso, 1995; Tanimura and Turner, 1996) and we provide here the first direct demonstration in intact cells, obtained by measuring $[Ca^{2+}]$ specifically into the ER. The mechanism for this regulation remains unknown. The fact that the same quantal phenomena were observed in BAPTA-loaded cells suggests that $[Ca^{2+}]_c$ is not involved in the regulation. Since the intralumenal portion of RyR is quite small and it has no apparent Ca^{2+}-binding sites, it is likely that intralumenal low affinity Ca^{2+}-binding proteins participate in the modulation of Ca^{2+} release by $[Ca^{2+}]_{ER}$ (Hidalgo and Donoso, 1995).

One of the main aims of this study was to investigate the presence of CICR elicited by the activation of plasma membrane Ca^{2+} channels. Results in Fig. 5 demonstrate for the first time that CICR is operative in bovine chromaffin cells, and that it can be potentiated by caffeine. This suggests that CICR takes place through the same RyR activated by caffeine. However, we have been unable to show inhibition of CICR by ryanodine after several pulses of K^+ depolarization, stimulation by DMPP or field electrical stimulation. A possible reason may be that stimulation of RyR by CICR is much smaller than stimulation by caffeine and therefore should require a much longer activation before inhibition is achieved, because of the use dependence. In fact, inhibition by ryanodine was much slower on stimulation with 5 mM caffeine than with 50 mM caffeine (Figs. 3 and 7). Similarly, the fact that in our hands Ca^{2+} release induced by histamine was not sensitive to ryanodine (Fig. 7 c), does not necessarily mean that RyR have not been activated by Ca^{2+} released by $InsP_3R$. Regarding this point, it has been reported that histamine-induced Ca^{2+} spikes are inhibited by ryanodine in bovine chromaffin cells (Stauderman and Murawsky, 1991), although inhibition required at least five consecutive stimuli with histamine and was relatively small and variable. In conclusion, ryanodine locks open RyR Ca^{2+} channels in a use-dependent manner, and both the extent and the rate

of this effect depend on the magnitude of the stimulus (caffeine or K^+). Therefore, a lack of or very slow effect of ryanodine should not be taken as a conclusive proof against the involvement of RyR. We also find remarkable that ryanodine required the presence of caffeine to lock the channels open, but not to bind to the channels. As seen in Fig. 7, ryanodine produced no effect when it was added in the absence of caffeine, but even after extensive washing it was rapidly effective much later provided that caffeine was added to open the channels. This kind of memory could be best explained if ryanodine binds irreversibly to RyR even in a closed state, but produces no effect until the channels open.

Low concentrations of caffeine produce a clear potentiation of CICR and therefore it would be extremely important to see if there may be a physiological modulator able to produce similar effects. Here we have studied the effects of several possible modulators of RyR, such as cAMP-mediated phosphorylation or cADPR. We did not find any effect of forskolin on caffeine- or K^+-induced Ca^{2+} release and cADPR did not release Ca^{2+} from the ER in permeabilized cells. The lack of effect of cADPR contrasts with the effects recently reported by Morita et al. (1997). These authors find mobilization to the extracellular medium, where Ca^{2+} appearance is measured, of ~2 nmole/10^7 digitonin-permeabilized cells upon maximal stimulation with cADPR. However, Morita et al. (1997) report under the same conditions, a catecholamine secretion amounting ~2 μg (120 nmole)/10^7 cells. For a ratio of catecholamine to Ca^{2+} of about 1:20 inside the secretory granules (von Grafenstein and Powis, 1989) the exocytotic mobilization of intragranular Ca^{2+} could amount as much as 6 nmole/10^7 cells, more than enough to explain the reported Ca^{2+} mobilization by cADPR. Therefore, this Ca^{2+} could come from a non-ER Ca^{2+} pool and would not even pass through the cytosol in the intact cells.

CICR may have multiple physiological functions in chromaffin cells. It could amplify the effect of a brief Ca^{2+} entry through the plasma membrane, generating a wave of $[Ca^{2+}]_c$ that might be required at the earlier steps of exocytosis, i.e., vesicle transport to exocytotic sites (von Rüden and Neher, 1993; Neher, 1998). The observed $[Ca^{2+}]_{ER}$ decrease of 100–200 μM could correspond to a mean increase in $[Ca^{2+}]_c$ of 1–2 μM, assuming that the ER constitutes ~10% of the cell volume and that 90% of the ER Ca^{2+} and 99% of the cytosolic Ca^{2+} are bound (Pozzan et al., 1994; Meldolesi and Pozzan, 1998). This $[Ca^{2+}]_c$ increase is well in the range required for stimulation of vesicle transport to exocytotic sites (von Rüden and Neher, 1993; Neher, 1998). Additionally, CICR could also be designed to amplify subcellular Ca^{2+} gradients resulting from agonist-induced Ca^{2+} release via $InsP_3$ receptors (Berridge, 1998). The question now arises as to what may be the importance of the K^+-stimulated Ca^{2+} release observed here for catecholamine secretion. We have reported that, when the Ca^{2+} stores had been depleted with caffeine, a subsequent K^+ depolarization elicits smaller secretion than in cells with full Ca^{2+} stores (Lara et al., 1997). Consistently, we show here (Fig. 8) that after full depletion of the ER Ca^{2+} pool, the first two or three initial depolarizations 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. After several K^+ pulses, the $[Ca^{2+}]_{ER}$ reached the threshold for release, ~300 μM, and was not able to take up more Ca^{2+} upon the following depolarizations. This leads to more Ca^{2+} available for secretion and probably to the increase in catecholamine secretion. On the other hand, when the ER is allowed to refill completely, it would be able to contribute with Ca^{2+} to secretion via CICR. These results are consistent with the model we proposed previously for the caffeine-sensitive store acting as a sink or source of Ca^{2+} that modulates K^+-evoked secretion depending on its filling state (see Fig. 10 in Lara et al., 1997).

The importance of the ER as a possible source of Ca^{2+} for secretion under physiological conditions was investigated in the fast confocal experiments, that showed that CICR contributes very significantly to the generation and propagation of the intracellular Ca^{2+} wave induced by 100-ms depolarizations (Fig. 9). Depolarization-induced Ca^{2+} influx has been reported previously in confocal imaging studies to release Ca^{2+} from ryanodine-sensitive stores in several types of neurons (Hua et al., 1993; Kocsis et al., 1994; Usachev and Thayer, 1997). In experiments performed at low time resolution (2 Hz), Usachev and Thayer (1997) have shown that CICR facilitates all-or-none inward propagation of the $[Ca^{2+}]_c$ signal from the plasmalemma to the nucleus in rat sensory neurons. However, in these experiments CICR had to be facilitated with 5 mM caffeine to be evidenced. Here we present records of $[Ca^{2+}]_c$ at high-time resolution in order to study whether CICR affects Ca^{2+} signaling during more physiological brief stimulations and in the absence of caffeine. It is remarkable that the presence of functional Ca^{2+} stores increased not only the rate of $[Ca^{2+}]_c$ increase at points deep inside the cell, but also doubled the increase in $[Ca^{2+}]_c$ near the plasma membrane (Fig. 9 c). This may indicate the presence of RyRs coupled to voltage-dependent Ca^{2+} channels, a mechanism that reminds the behavior of cardiac RyR (Cheng et al., 1996) and would make possible that stimuli as short as the action potentials could induce CICR. The existence of local subplasmalemmal CICR has been proposed previously from the ryanodine sensitivity of Ca^{2+}-activated chloride currents (Ivanenko et al., 1993). The rise of $[Ca^{2+}]_c$ was also faster in the control than in the ryanodine-treated cells (Fig. 9 d) and, probably as a consequence of this, the Ca^{2+} wave moved into the interior of the cells at greater speed in the control cells (Fig. 9 e). Thus CICR makes the Ca^{2+} signal larger in magnitude and faster to reach the inner parts of the cells. CICR may therefore play an important role in catecholamine release under physiological conditions, both by providing directly the Ca^{2+} required to trigger exocytosis and by facilitating Ca^{2+}-dependent early steps such as vesicle transport to the exocytotic sites. Additionally, CICR could have functional implications not directly related to secretion but important for the cell physiology such as gene expression or vesicle transport (Berridge, 1998).

Finally, additional evidence for the physiological role of CICR in secretion came from the finding that the pattern of inhibition of both CICR and K^+-induced secretion by several Ca^{2+} channel inhibitors was similar. In bovine chromaffin cells, Ca^{2+} channels are expressed in the following proportions: 10% P-type, 20% L-type, 30% N-type,

and 40% Q-type (Lopez et al., 1994; Albillos et al., 1996). The L- and Q-type Ca^{2+}-channels appear to be more closely coupled to the exocytotic machinery than the N- or P-type ones (Lopez et al., 1994; Lomax et al., 1997). In addition, functional evidence suggests that the Q-type Ca^{2+} channels are located closer to the secretory sites than the L-type ones (Lara et al., 1998). We find here that a combination of inhibitors of all voltage-dependent Ca^{2+} channels inhibited CICR by 80%, even though the initial rate of refilling of the ER was little modified. The lack of effect on the rate of refilling suggests that the small fraction of Ca^{2+} entry that takes place through voltage-independent channels is enough to sustain a normal refilling. This is not surprising since, as shown in Fig. 4 c, the ER can refill even without noticeable change of $[Ca^{2+}]_c$ in BAPTA-loaded cells. A similar behavior has been reported in non-excitable cells (Montero et al., 1997a; Hofer et al., 1998). In contrast, CICR appears to require rapid Ca^{2+} entry through voltage-dependent Ca^{2+} channels, particularly P-/Q-type, as it was inhibited ~60% by ω-conotoxin MVIIC (N-/P-/Q-type Ca^{2+} channel inhibitor) and ω-agatoxin IVA (P-/Q-type Ca^{2+} channel inhibitor at the concentration used here). On the other hand, ω-conotoxin GVIA (N-type Ca^{2+} channel inhibitor) and nisoldipine (L-type Ca^{2+} channel inhibitor) had no significant effect. The fact that the same channels that are closer to the secretory sites (Q-type, Lara et al., 1998) are also those responsible for triggering CICR suggests that CICR may occur preferentially near the secretory sites. This again suggests that CICR may be important for the secretory response under physiological conditions.

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