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\(^{2+}\)]\(_c\) ([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) 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\(_3\)R) (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\(_3\)R 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\(_3\)R or cyclic adenosine diphosphate ribose (cADPR) for the RyR (Lee, 1998). However, although InsP\(_3\) is a well-established physiological activator of the InsP\(_3\)R, the role of cADPR as activator or modulator of RyR in the presence of physiologi-
tial concentrations of ATP remains controversial (Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). In neuneral cells, RyR could be activated via the classical Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) mechanism after Ca<sup>2+</sup> entry through voltage-dependent Ca<sup>2+</sup> channels. Studies in some neuneral preparations have shown that depolarizing stimulii produce an increase in cytosolic [Ca<sup>2+</sup>] ([(Ca<sup>2+</sup>)<sub>c</sub>]) that may be due in part to Ca<sup>2+</sup> release from intracellular stores (Verkhovsky and Shigli, 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<sup>2+</sup> entry and Ca<sup>2+</sup> release to the [Ca<sup>2+</sup>]<sub>c</sub> 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<sup>2+</sup>] specifically inside the Ca<sup>2+</sup>-stores. We have recently reported a method to measure [Ca<sup>2+</sup>] in the lumen of the endoplasmic reticulum ([Ca<sup>2+</sup>]<sub>ER</sub>) of intact cells, the main intracellular Ca<sup>2+</sup> 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<sup>2+</sup> release can be measured independently of the variations in [Ca<sup>2+</sup>]<sub>c</sub>. Here we have used this technique to monitor [Ca<sup>2+</sup>]<sub>ER</sub> in chromaffin cells. These neuroendocrine cells have a potent caffeine-sensitive Ca<sup>2+</sup> release mechanism (Cheek et al., 1990), and Ca<sup>2+</sup> entry through several types of voltage-dependent Ca<sup>2+</sup> channels can be induced by K<sup>+</sup> 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<sup>2+</sup>-stores may modulate catecholamine secretion induced by depolarization with high K<sup>+</sup> in these cells. Catecholamine secretion was reduced after store emptying with caffeine, and recovered as Ca<sup>2+</sup>-stores refilled during consecutive K<sup>+</sup>/Ca<sup>2+</sup>-pulses. The main conclusion from that work was that Ca<sup>2+</sup>-stores could have a double role, acting either as a sink or as a source of Ca<sup>2+</sup>, 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<sub>3</sub>R, which are at least in part colocalized with RyR2 in the ER (Poulsen et al., 1995). The presence of separate or overlapping Ca<sup>2+</sup>-pools responsive to either InsP<sub>3</sub>, caffeine, or cADPR, their differential sensitivity to inhibitors of the ER Ca<sup>2+</sup>-pump such as thapsigargin, and the physiological significance of the different Ca<sup>2+</sup>-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<sup>2+</sup> release induced by caffeine is quite particular because increasing concentrations of caffeine release Ca<sup>2+</sup> in a quanmtal manner, a phenomenon that has been suggested to indicate that the caffeine-sensitive Ca<sup>2+</sup>-pool is composed of functionally discrete stores with heterogeneous sensitivities to caffeine (Cheek et al., 1993, 1994a).

Here we have monitored [Ca<sup>2+</sup>]<sub>ER</sub> in chromaffin cells to investigate the mechanism of quantal Ca<sup>2+</sup>-release by ca-

**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 (Moror 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 fluoro-

**Preparation of Viral Stock and Infection of Cultures**

Construction, packaging, and titering of the pHSeVærAEQ ampiclon 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 pHSeVpc vector to generate the pHSeVerAEQ. 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<sup>6</sup> infectious virus units (i.u.)/ml (Lim et al., 1996). Titers of viral stocks were determined by immunocytochemisty on PC12 cells. Infected cells were visualized by using a rabbit anti-

**Measurements of [Ca<sup>2+</sup>]<sub>ER</sub> with Aequorin**

For [Ca<sup>2+</sup>]<sub>ER</sub> 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<sup>2+</sup>]<sub>ER</sub> 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<sup>2+</sup> by incubation for 5–10 min at 37°C with the sarco-endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) inhibitor 2.5-di-tet-

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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 lumino- meter 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 (Birni et al., 1995) which follows the calibration curve reported before (Brini et al. 1997). In the experiments carried out with permeabilized cells, cells were placed in the lumino- meter 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\(_2\)PO\(_4\), 2 mM ATP, 20 mM Hepes, pH 7) contain- ing 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 (Nübel 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 Sci- ence) 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 dura- tion 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, 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 intracel- lular [Ca\(^{2+}\)]\(_c\) distribution were acquired every 2 ms with a confocal micro- scope (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 condi- tions (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 re- agents 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 contain- ing 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 re- quired 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 com- pletely 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 histo-

![Figure 1. Effects of histamine and caffeine and pretreatment with ryanodine or thapsigargin on [Ca\(^{2+}\)]\(_{ER}\).](image)

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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 a 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\(^{2+}\)]\(_{ER}\) 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).

**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\(^{2+}\)], in the same cell prepa-
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\(^{\prime}\)-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\(^{2+}\)]\(_{E}\). Fura-2 measurements performed in parallel showed that in cells loaded with BAPTA, the [Ca\(^{2+}\)]\(_{E}\) 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\(^{2+}\)]\(_{E}\) 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\(^{2+}\)]\(_{E}\) 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\(^{2+}\)]\(_{E}\) peaks cannot be estimated from conventional [Ca\(^{2+}\)]\(_{E}\) 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 \(\mu\)M (10–15% of the steady-state [Ca\(^{2+}\)]\(_{ER}\)). Therefore, in spite of the large increase in [Ca\(^{2+}\)]\(_{E}\), the activation of CICR produced a much smaller [Ca\(^{2+}\)]\(_{ER}\) decrease than treatment with caf-
Figure 4. Regulation of caffeine-induced Ca\textsuperscript{2+} release by [Ca\textsuperscript{2+}]\textsubscript{i}. The ER was refilled by perfusing with medium containing 1 mM Ca\textsuperscript{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\textsuperscript{2+} chelator. Other details are as in Fig. 1.

Increasing the duration of the high K\textsuperscript{+} pulse increased the magnitude of Ca\textsuperscript{2+} release little. Fig. 5 b illustrates the effect of depolarization with longer (1 min) high K\textsuperscript{+} pulses. In this case, the first high K\textsuperscript{+} pulse produced the same effect as in Fig. 5 a, and then [Ca\textsuperscript{2+}]\textsubscript{ER} increased more rapidly, probably as a result of the prolonged stimulation of the ER Ca\textsuperscript{2+} pump by the sustained high [Ca\textsuperscript{2+}]\textsubscript{i} levels. This led to a new [Ca\textsuperscript{2+}]\textsubscript{ER} steady-state at ~800–900 μM. After that, subsequent K\textsuperscript{+} pulses induced somewhat larger [Ca\textsuperscript{2+}]\textsubscript{ER} decreases of 150–200 μM, but corresponding still to only 20% of the steady-state [Ca\textsuperscript{2+}]\textsubscript{ER}. To investigate if increased Ca\textsuperscript{2+} release could be responsible for the incomplete Ca\textsuperscript{2+} release, the effect of K\textsuperscript{+} depolarization was tested in the presence of the ER Ca\textsuperscript{2+} pump inhibitor cyclopiazonic acid (CPA). Fig. 5 c shows that CPA itself induces a slow Ca\textsuperscript{2+} release from the ER, and that simultaneous addition of high K\textsuperscript{+} medium induced a fast initial Ca\textsuperscript{2+} release of ~20% of the [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{+} depolarization is able to produce only a decrease of ~20% of the [Ca\textsuperscript{2+}]\textsubscript{ER}, even in the absence of Ca\textsuperscript{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\textsuperscript{+} depolarization, that was now able to release rapidly ~50% of the stored Ca\textsuperscript{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\textsuperscript{2+} release from the ER, very similar to that shown above for K\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{2+} release nor on the magnitude of high K\textsuperscript{+} depolarization-induced CICR (data not shown). Regarding cADPR, it has been reported that acetylcholine, high K\textsuperscript{+} 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\textsuperscript{2+} release from the ER, we performed experiments in permeabilized cells. Cells were depleted of Ca\textsuperscript{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\textsuperscript{2+} (buffered with EGTA) and 2 mM ATP-Mg was perfused. Fig. 6 a shows that [Ca\textsuperscript{2+}]\textsubscript{ER} increased in digitonin-permeabilized cells with very similar kinetics to that found in intact cells after addition of 1 mM extracellular Ca\textsuperscript{2+}. The steady-state [Ca\textsuperscript{2+}]\textsubscript{ER} reached was also similar (compare with Fig. 1 a). Fig. 6 a also shows that 2 μM InsP\textsubscript{3} and 50 mM caffeine produced a rapid and near complete release of Ca\textsuperscript{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\textsuperscript{2+} from a different (non-ER) Ca\textsuperscript{2+} pool. In fact, these authors report that InsP\textsubscript{3} releases Ca\textsuperscript{2+} from a pool sensitive to 20 nM thapsigargin, whereas cADPR and caffeine release Ca\textsuperscript{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\textsuperscript{2+} from an additional non-ER Ca\textsuperscript{2+} pool in the presence of 20 nM thapsigargin. We then performed single-cell
fura-2 imaging experiments in intact cells looking at the effect of caffeine on $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ under these conditions (the observed decrease is due to the perfusion of Ca$^{2+}$-free medium), whereas K$^+$ depolarization still produced the usual $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ peak induced by caffeine after several K$^+$ pulses in the presence of ryanodine. Fig 7 a shows that the $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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$_7$-producing agonist in order to produce a big increase in $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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).
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$\text{3}$ pituitary cells (Villalobos and García-Sancho, 1996). As before, the second caffeine stimulation after histamine and ryanodine pulses was completely abolished.

**Effects of [Ca$^{2+}$]$\text{ER}$ 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 luminal [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$^+$-medium (containing 70 mM KCl) (K$^+$), 10 mM histamine (His), 10 mM 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 mM EGTA). 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.
[Ca\(^{2+}\)]_\text{ER} steady-state levels at \(\sim 700\) \(\mu\)M. 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\(^{2+}\)]_\text{ER} decrease induced by the lack of extracellular Ca\(^{2+}\). After three K\(^+\) pulses, [Ca\(^{2+}\)]_\text{ER} was reduced again to \(\sim 300\) \(\mu\)M, 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\(^{2+}\)]_\text{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 \(\omega\)-conotoxin GVIA (1 \(\mu\)M), \(\omega\)-conotoxin MVIIIC (3 \(\mu\)M), and \(\omega\)-agatoxin IVA (1 \(\mu\)M), together with preincubation and perfusion along the experiment with 3 \(\mu\)M 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 \(\mu\)M 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\(^{2+}\)]_\text{ER} observed, normalized as percentage of those obtained in the controls, were (mean \pm SEM): control, 100 \pm 11 (\(n = 6\)); 3 \(\mu\)M nisoldipine, 103 \pm 6 (\(n = 5\)); 1 \(\mu\)M \(\omega\)-conotoxin GVIA, 93 \pm 6 (\(n = 6\)); 3 \(\mu\)M \(\omega\)-conotoxin MVIIIC, 42 \pm 6 (\(n = 7\)); 1 \(\mu\)M \(\omega\)-agatoxin IVA, 41 \pm 9 (\(n = 5\)); a combination of all three toxins and nisoldipine, 20 \pm 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\(^{2+}\)]_\text{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\(_{\text{trans}}\)) followed by a slow inactivating phase (I\(_{\text{slow}}\)) (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 \pm SEM: control cells, 7.15 \pm 0.42 pC [\(n = 34\)]; ryanodine-treated cells, 6.68 \pm 1.03 pC [\(n = 21\)]). In spite of this, line scan images representing [Ca\(^{2+}\)]_\text{ER} showed clear differences between control and ryanodine-treated cells. Fig. 9 a shows the spatiotemporal pattern of [Ca\(^{2+}\)]_\text{ER}, increase in control cells, codified in pseudocolor. [Ca\(^{2+}\)]_\text{ER} 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\(^{2+}\)]_\text{ER} 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\(^{2+}\)]_\text{ER}, rise (Fig. 9 c), maximum rate of [Ca\(^{2+}\)]_\text{ER} 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\(^{2+}\)]_\text{ER} 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\(^{2+}\)]_\text{ER} wave. We find that the [Ca\(^{2+}\)]_\text{ER} 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
depolarization during a short, more physiological stimulation.

**Discussion**

Most of previous research on CICR has been performed by inferring the changes in $[Ca^{2+}]_{\text{ER}}$ from the evolutions of $[Ca^{2+}]_{\text{c}}$. 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 $[Ca^{2+}]_{\text{c}}$ (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$_3$R 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

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**Figure 9.** Confocal imaging of the propagation of the $[Ca^{2+}]_{\text{c}}$ 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 $[Ca^{2+}]_{\text{c}}$) 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 + $10 \mu$M ryanodine-containing Krebs-Hepes solution. After that, cells were maintained in 10 $\mu$M 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).
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 acquerin 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$_3$R 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$_3$R and RyR by the luminal [Ca$^{2+}$]$_{l}$ 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$^{2+}$]$_{ER}$ 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 intraluminal 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 chromaf-fin 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$_3$R. 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\(^7\) digitoxin-permeabilized cells upon maximal stimulation with cADPR. However, Morita et al. (1997) report under the same conditions, a catecholamine secretion amounting ~2 \(\mu\)g (120 nmole)/10\(^7\) cells. For a ratio of catecholamine to Ca\(^{2+}\) of about 1:20 inside the secretory granules (von Grafenberg 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\(^{2+}\)]\(_{ER}\) 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 \(\mu\)M could correspond to a mean increase in [Ca\(^{2+}\)]\(_{c}\) of 1–2 \(\mu\)M, assuming that the ER constitutes ~10% of the cell volume and that 90% of the ER Ca\(^{2+}\) and 90% 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 \(\mu\)M, 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; Koësis 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 plasma membrane 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\(^{2+}\)]\(_c\). A critical evaluation.

Other hand, Ca\(^{2+}\)-entry through voltage-dependent Ca\(^{2+}\) channels, particularly P/Q-type, as it was inhibited ~60% by o-conotoxin MVIIIC (N/P/Q-type Ca\(^{2+}\) channel inhibitor) and o-agatoxin IVA (P/Q-type Ca\(^{2+}\) channel inhibitor at the concentration used here). On the other hand, o-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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References

Albillos, A., A.G. Garcia, B. Olivera, and L. Gandia. 1996. Re-evaluation of the P/Q Ca\(^{2+}\) channel components of Ba\(^{2+}\) currents in bovine chromaffin cells superfused with solutions containing low and high Ba\(^{2+}\) concentrations. Pfug. Arch. Eur. J. Physiol. 432:1030–1038.

Alonso, M.T., M.J. Barrero, E. Carnicerio, M. Montero, J. García-Sancho, and J. Alvarez. 1998. Functional measurements of [Ca\(^{2+}\)]\(_c\) in the endoplasmic reticulum using a herpes virus to deliver targeted acini. Cell Calcium 24: 87–96.

Barrero, M.J., M. Montero, and J. Alvarez. 1997. Dynamics of [Ca\(^{2+}\)]\(_c\) in the endoplasmic reticulum and cytoplasm of intact HeLa cells. A comparative study. J. Biol. Chem. 272:27049–27059.

Berridge, M.J. 1993. Inositol triphosphate and calcium signalling. Nature 361: 315–325.

Berridge, M.J. 1998. Neuronal calcium signalling. Neuron 21:13–26.

Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P\(_3\) and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature 351:751–754.

Brimi, M., R. Marsault, C. Bantianutto, J. Alvarez, T. Pozzan, and R. Rizzuto. 1995. Transfection-mediated measurement of the distribution of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)). A critical evaluation. J. Biol. Chem. 270:9896–9903.

Cheek, T.R., A.J. O’Sullivan, R.B. Moreton, M.J. Berridge, and R.D. Burgoine. 1990. The caffeine-sensitive Ca\(^{2+}\) store in bovine adrenal chromaffin cells; an examination of its role in triggering secretion and Ca\(^{2+}\) homeostasis.
Meldolesi, J., and T. Pozzan. 1998. The endoplasmic reticulum Ca\(^{2+}\) store: a view from the lumen. *Trends Biochem. Sci.* 23:10–14.

Missiaen, L., H. De Smet, G. Droogmans, and R. Casteels. 1992. Ca\(^{2+}\) release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca\(^{2+}\) in permeabilized cells. *Nature.* 357:599–602.

Montero, M., M. Brini, R. Marsault, J. Alvarez, R. Sitta, T. Pozzan, and R. Rizzuto. 1995. Monitoring dynamic changes in free Ca\(^{2+}\) concentration in the endoplasmic reticulum of intact cells. *EMBO (Eur. Mol. Biol. Organ.)* 14: 5467–5475.

Montero, M., M.J. Barrero, and J. Alvarez. 1997a. [Ca\(^{2+}\)]\(_{\text{mic}}\) microdomains control agonist-induced Ca\(^{2+}\) release in intact HeLa cells. *FASEB (Fed. Am Soc. Exp. Biol.)* 11: 881–885.

Montero, M., J. Alvarez, W.J. Scheenen, R. Rizzuto, J. Meldolesi, and T. Pozzan. 1997b. Ca\(^{2+}\) homeostasis in the endoplasmic reticulum: coexistence of high and low [Ca\(^{2+}\)]\(_{\text{mic}}\) subcompartments in intact HeLa cells. *J. Cell Biol.* 139: 601–611.

Monta, K., S. Kitayama, and T. Dohi. 1997. Stimulation of cyclic ADP-ribose synthesis by acetylcholine and its role in catecholamine release in bovine adrenal chromaffin cells. *J. Biol. Chem.* 272:21002–21009.

Morita, K., S. Kitayama, and T. Dohi. 1997. Stimulation of cyclic ADP-ribose synthesis by acetylcholine and its role in catecholamine release in bovine adrenal chromaffin cells. *J. Biol. Chem.* 272:21002–21009.

Neher, E. 1998. Vesicle pools and Ca\(^{2+}\) microdomains: New tools for understanding their roles in neurotransmitter release. *Neuron.* 20:389–399.

Newton, C.L., G.A. Mignery, and T.C. Sudhof. 1994. Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP\(_3\)) receptors with distinct affinities for InoP\(_3\)g. *J. Biol. Chem.* 269:28613–28619.

Núñez, L., M.T. De La Fuente, A.G. Garcia, and J. Garcia-Sancho. 1995. Differential Ca\(^{2+}\) responses of adrenergic and noradrenergic chromaffin cells to various secretagogues. *Am. J. Physiol.* 269:C1540–C1546.

Poulsen, J.C., C. Caspersen, D. Mathiasen, J.M. East, R.E. Tunwell, F.A. Lai, N. Maeda, K. Mikoshiba, and M. Treiman. 1995. Thapsigargin-sensitive Ca\(^{2+}\)-ATPases account for Ca\(^{2+}\) uptake to inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive Ca\(^{2+}\) stores in adrenal chromaffin cells. *Biochem. J.* 307:749–758.

Pozzan, T., R. Rizzuto, P. Volpe, and J. Meldolesi. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* 74:395–636.

Rizzuto, R., M. Brini, M. Murgia, and T. Pozzan. 1993. Microdomains with high Ca\(^{2+}\) close to IP\(_3\)-sensitive channels that are sensed by neighboring mitochondria. *Science.* 262:744–747.

Robinson, I.M., and R.D. Burgoyne. 1991. Characterisation of distinct inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive calcium stores in digito- nin-permeabilised adrenal chromaffin cells. *J. Neurochem.* 56:1587–1593.

Sorrentino, V., and P. Volpe. 1993. Ryanodine receptors: how many, where and why? *Trends Pharmacol. Sci.* 14:98–103.

Stauderman, K.A., and M.M. Murawsky. 1991. The inositol 1,4,5-trisphosphate-forming agonist histamine activates a ryanodine-sensitive Ca\(^{2+}\) release mechanism in bovine adrenal chromaffin cells. *J. Biol. Chem.* 266:19150–19153.

Stauderman, K.A., R.A. McKinney, and M.M. Murawsky. 1991. The role of caffeine-sensitive Ca\(^{2+}\) stores in agonist- and inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release from bovine adrenal chromaffin cells. *Biochem. J.* 278: 643–650.

Sutko, J.L., and J.A. Airey. 1996. Ryanodine receptor Ca\(^{2+}\) release channels: does diversity in form equal diversity in function? *Physiol. Rev.* 76:1027–1071.

Tanimura, A., and R.J. Turner. 1996. Calcium release in HSY cells conforms to a steady-state mechanism involving regulation of the inositol 1,4,5-trisphosphate receptor Ca\(^{2+}\) channel by luminal [Ca\(^{2+}\)]\(_{\text{cyt}}\). *J. Cell Biol.* 133:607–616.

Usachev, V.M., and S.A. Thayer. 1997. All-or-none Ca\(^{2+}\) release from intracellular stores triggered by Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels in rat sensory neurons. *J. Neurosci.* 17:7404–7414.

Vann Winkle, W.B. 1976. Calcium release from skeletal muscle sarcoplasmic reticulum: site of action of dantrolene sodium. *Science.* 193:1130–1131.

Verkhratsky, A., and A. Shmigol. 1996. Calcium-induced calcium release in neurons. *Cell Calcium.* 19:1–14.

Villalobos, C., and J. García-Sancho. 1996. Caffeine-induced oscillations of cytosolic Ca\(^{2+}\) in GH\(_3\) pituitary cells are not due to Ca\(^{2+}\) release from intracellular stores but to Ca\(^{2+}\) entry through voltage-gated channels. *Pflug. Arch.* 431:371–378.

von Grafenstein, H.R., and D.A. Powis. 1989. Calcium is released by exocytosis together with catecholamines from bovine adrenal medullary cells. *J. Neurochem.* 53:428–435.

von Ruden, L., and E. Neher. 1993. A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. *Science.* 262:1061–1065.

Walton, P.D., J.A. Airey, J.L. Suitko, C.F. Beck, G.A. Mignery, T.C. Sudhof, T.J. Deerinck, and M.H. Ellisman. 1991. Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons. *J. Cell Biol.* 113:1145–1157.

Yoo, S.H., and J.P. Albanesi. 1990. Inositol 1,4,5-trisphosphate-activated Ca\(^{2+}\) release from bovine adrenal medullary secretory vesicles. *J. Biol. Chem.* 265: 13446–13448.

Yule, D.L., S.A. Ernst, H. Ohnishi, and R.J. Wojcikiewicz. 1997. Evidence that zymogen granules are not a physiologically relevant calcium pool. Defining the distribution of inositol 1,4,5-trisphosphate receptors in pancreatic acinar cells. *J. Biol. Chem.* 272:9093–9098.

Zacchetti, D., E. Clementi, C. Fassolato, P. Lorenzon, M. Zottini, F. Grohovaz, G. Fumagalli, T. Pozzan, and J. Meldolesi. 1991. Intracellular Ca\(^{2+}\) pools in PC12 cells. A unique, rapidly exchanging pool is sensitive to both inositol 1,4,5-trisphosphate and caffeine-ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol. Rev.* 49:1–51.