A Novel Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} Release Mechanism in A7r5 Cells Regulated by Calmodulin-like Proteins*

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Intracellular Ca\textsuperscript{2+} release is involved in setting up Ca\textsuperscript{2+} signals in all eukaryotic cells. Here we report that an increase in free Ca\textsuperscript{2+} concentration triggered the release of up to 41 ± 3% of the intracellular Ca\textsuperscript{2+} stores in permeabilized A7r5 (embryonic rat aorta) cells with an EC\textsubscript{50} of 700 nM. This type of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) was neither mediated by inositol 1,4,5-trisphosphate receptors nor by ryanodine receptors, because it was not blocked by heparin, 2-aminoethoxydiphenyl borate, xestospongion C, ruthenium red, or ryanodine. ATP dose-dependently stimulated the CICR mechanism, whereas 10 mM MgCl\textsubscript{2} abolished it. CICR was not affected by exogenously added calmodulin (CaM), but CaM\textsubscript{234}, a Ca\textsuperscript{2+}-insensitive CaM mutant, strongly inhibited the CICR mechanism. Other proteins of the CaM-like neuronal Ca\textsuperscript{2+}-sensor protein family such as Ca\textsuperscript{2+}-binding protein 1 and neuronal Ca\textsuperscript{2+}-sensor-1 were equally potent for inhibiting the CICR. Removal of endogenous CaM, using a CaM-binding peptide derived from the ryanodine receptor type-1 (amino acids 3614–3643) prevented subsequent activation of the CICR mechanism. A similar CICR mechanism was also found in 16HBE14o- (human bronchial mucosa) cells. We conclude that A7r5 and 16HBE14o-cells express a novel type of CICR mechanism that is silent in normal resting conditions due to inhibition by CaM but becomes activated by a Ca\textsuperscript{2+}-dependent dissociation of CaM. This CICR mechanism, which may be regulated by members of the family of neuronal Ca\textsuperscript{2+}-sensor proteins, may provide an additional route for Ca\textsuperscript{2+} release that could allow amplification of small Ca\textsuperscript{2+} signals.

Changes in cytosolic-free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}) mediate a variety of cellular processes, ranging from fertilization to cell death (1, 2). Cells generate Ca\textsuperscript{2+} signals through both intracellular (mainly the endoplasmic/sarcoplasmic reticulum) and extracellular Ca\textsuperscript{2+} sources. Regulation of these Ca\textsuperscript{2+} signals via a variety of Ca\textsuperscript{2+} channels, expressed either in the plasma membrane or in the membranes of intracellular stores, is thereby essential. Ca\textsuperscript{2+} fluxes from extracellular and intracellular Ca\textsuperscript{2+} sources do not occur independently of each other. For example, the intracellular Ca\textsuperscript{2+} store content regulates Ca\textsuperscript{2+} entry from the extracellular medium via capacitative Ca\textsuperscript{2+} entry (3, 4), whereas Ca\textsuperscript{2+} released by one channel can alter the activity of other channels. These are all well documented mechanisms whereby Ca\textsuperscript{2+} can exert important effects on its own activity. The most important type of regulation is represented by the various mechanisms that may lead to the characteristic bell-shaped dependence of intracellular Ca\textsuperscript{2+} channels on Ca\textsuperscript{2+} itself (5–9). This may in principle be due to direct interaction with Ca\textsuperscript{2+} or indirectly via Ca\textsuperscript{2+}-sensor proteins such as calmodulin (CaM). The inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R) and the ryanodine receptor (RyR) are the two major families of intracellular Ca\textsuperscript{2+} release channels that have been characterized. Both types of intracellular channels are regulated in a complex way by Ca\textsuperscript{2+} and CaM. CaM has been demonstrated to affect the activity of RyRs in both a stimulatory and an inhibitory manner (10, 11) but not by the same mechanism for all three RyR isomers. For the IP\textsubscript{3}R, CaM clearly exerts an inhibitory effect, but the precise mechanism is not yet understood (12, 13). RyRs and IP\textsubscript{3}Rs are stimulated by small increases in [Ca\textsuperscript{2+}]\textsubscript{c}, and inhibited at higher [Ca\textsuperscript{2+}]\textsubscript{c} (14–20). Stimulation is important for the mechanism of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR), which allows amplification and regenerative propagation of intracellular Ca\textsuperscript{2+} signals. CICR seems to be an operational mode of both IP\textsubscript{3}R\textsubscript{s} and RyR\textsubscript{s}, and it is clearly a key feature of intracellular Ca\textsuperscript{2+} signaling (21). Recent studies have emphasized the role of novel types of intracellular Ca\textsuperscript{2+} release channels possibly playing an important role in intracellular Ca\textsuperscript{2+} signaling (22–28). Wissing et al. (26) identified a novel CICR mechanism in permeabilized hepatocytes that responded to modest increases in [Ca\textsuperscript{2+}]\textsubscript{c}. Polycystin-2, the product of the gene mutated in type-2 autosomal dominant polycystic kidney disease and a prototypical member of a subfamily of the transient receptor potential channel superfAMILY (TRP), is expressed abundantly in the endoplasmic reticulum (ER) (24). It was shown recently that polycystin-2 expressed in the ER of epithelial cells is a Ca\textsuperscript{2+}-activated channel that is permeable for divalent cations. Increased levels of intracellular Ca\textsuperscript{2+} activated polycystin-2-mediated release of Ca\textsuperscript{2+}.
Ca$^{2+}$ from intracellular stores. Recent data also suggested that activation of the ER-associated vanilloid receptor 1 (VR1), a member of the TRP family, by capsaicin binding resulted in Ca$^{2+}$ mobilization from intracellular stores. This raises the possibility that VR1 may also function as an intracellular Ca$^{2+}$ release channel (27, 28).

In the present study we have identified a novel CICR mechanism in permeabilized A7r5 cells, a permanent cell line derived from embryonic rat aorta. We identified this CICR mechanism that was mediated by neither the IP$_3$R nor the RyR. Moreover, we found that this CICR mechanism could be inhibited by CaM$_{323}$, a Ca$^{2+}$-insensitive CaM mutant, and by different members of the superfamily of CaM-like Ca$^{2+}$-binding proteins. Our data suggest that the CICR mechanism described here may represent a novel type of release channel, which is silent at low [Ca$^{2+}$] eff, due to inhibition by bound apoCaM and which becomes activated by the Ca$^{2+}$-dependent dissociation of CaM. This CICR mechanism may provide an additional pathway for intracellular Ca$^{2+}$ release and could play an important role in amplifying Ca$^{2+}$ signals generated by other Ca$^{2+}$ release channels.

**EXPERIMENTAL PROCEDURES**

**Ca$^{2+}$ Fluxes—**A7r5 cells, which are derived from embryonic rat aorta, were obtained from the American Tissue Type Culture Collection CRL 1444 (Bethesda, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 3.8 mM l-glutamine, 0.9% (v/v) non-essential amino acids, 85 IU/ml penicillin, 85 µg/ml streptomycin, and 20 mM HEPES (pH 7.4). For 16HBE14o- (human bronchial mucosa) and mouse embryonal fibroblast cells a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium was used and for LLC-PK1, cells minimal essential medium α was used. Ca$^{2+}$ fluxes were performed on saponin-permeabilized cells. The cells were seeded in 12-well clusters (Costar, MA) at a density of 10$^4$ cells/well between the 7th and 9th days after plating. Cells were permeabilized by incubating them for 10 min with a solution containing 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 2 mM MgCl$_2$, 1 mM ATP, 1 mM EGTA, and 20 µg ml$^{-1}$ saponin at 25°C. The non-mitochondrial Ca$^{2+}$ stores were loaded for 45 min at 37°C in 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5 mM MgCl$_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM Na$_2$SO$_4$, and 150 mM free Ca$^{2+}$ (28 µM M$^{-1}$). The cells were then washed twice with 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 1 mM EGTA, and 10 µM thapsigargin. Thapsigargin was added to block the ER Ca$^{2+}$ pumps during subsequent additions of Ca$^{2+}$. The efflux medium was replaced every 2 min during 18 min, and the efflux was performed at 37°C. The additions of 4Ca$^{2+}$ and IP$_3$ are indicated in the legends of the figures. Free [Ca$^{2+}$] was calculated by the Cabuf program (available at ftp://r.ep. kuleuven.ac.be/pub/droogmans/cabuf.zip) and based on the stability constants given by Fabiato and Fabiato (29). At the end of the experiment the 4Ca$^{2+}$ remaining in the stores was released by incubation with 1 ml of a 2% SDS solution for 30 min. Ca$^{2+}$ release is plotted as the fractional loss, i.e. the amount of Ca$^{2+}$ released in 2 min divided by the total store Ca$^{2+}$ content at that time. The latter value was calculated by summing in retrograde order the amount of tracer collected during the successive time intervals. In experiments performed to exclude 4Ca$^{2+}$↔4Ca$^{2+}$ exchange in Fig. 2, cells were loaded during 45 min in loading buffer, containing 4 mM EGTA and 680 µM total CaCl$_2$, resulting in 285 nM free Ca$^{2+}$ and a specific activity for Ca$^{2+}$ of 28 Ci ml$^{-1}$. After 45 min, the loading buffer was replaced for 2 min by a loading buffer with an EGTA concentration of 0.76 mM and supplemented with thapsigargin, to maintain the same 4Ca$^{2+}$↔4Ca$^{2+}$ ratio but resulting in an increase in free Ca$^{2+}$ to 10 µM. Efflux was then further performed in Ca$^{2+}$-free efflux buffer.

**Peptide Synthesis—**RyR1 peptide (amino acids 3614–3643) (30) was synthesized by Eurogentec S.A. (Herstal, Belgium).

**Cloning of CaBP1 and CaBP4—**Mouse CaBP1 cDNA was cloned from mouse cerebellum using cDNA synthesis with reverse transcriptase. Reverse transcription-PCR was performed with forward primer 5’-GGCAGCCCATGGA-CAGGCGAGACATCATCCCGAGC-3’. PCR fragments of both isoforms, the short (sCaBP1) and long (lCaBP1) form, were then cloned into the NdeI site at 45°C of an efflux buffer. The latter value was calculated by summing in retrograde order the amount of tracer collected during the successive time intervals. In experiments performed to exclude 4Ca$^{2+}$↔4Ca$^{2+}$ exchange in Fig. 2, cells were loaded during 45 min in loading buffer, containing 4 mM EGTA and 680 µM total CaCl$_2$, resulting in 285 nM free Ca$^{2+}$ and a specific activity for Ca$^{2+}$ of 28 Ci ml$^{-1}$. After 45 min, the loading buffer was replaced for 2 min by a loading buffer with an EGTA concentration of 0.76 mM and supplemented with thapsigargin, to maintain the same 4Ca$^{2+}$↔4Ca$^{2+}$ ratio but resulting in an increase in free Ca$^{2+}$ to 10 µM. Efflux was then further performed in Ca$^{2+}$-free efflux buffer.

**RESULTS**

**Increase in [Ca$^{2+}$]i, Stimulates Ca$^{2+}$ Release from Intracellular Stores—**In A7r5 cells (embryonic smooth muscle) Ca$^{2+}$ release from internal stores, mainly from the ER, occurs to a large extent via production of the second messenger IP$_3$. In this permeabilized cell system a maximal effective dose of IP$_3$ can release about 95% of the intracellular Ca$^{2+}$ content (33). Here, the non-mitochondrial stores of permeabilized A7r5 cells were loaded to steady state with 4Ca$^{2+}$ and then incubated in a non-labeled efflux medium containing 10 µM thapsigargin. The loss of Ca$^{2+}$ from the stores under these conditions is plotted as the fractional loss in function of time (Fig. 1). After 10 min the cells were challenged with 1 µM IP$_3$ (circles), as indicated by the black bar. As previously documented in detail, using the same 4Ca$^{2+}$ efflux technique (17, 34–37), IP$_3$ increased the rate of Ca$^{2+}$ release (Fig. 1). In the same assay cells with 3 µM

![Fig. 1. Effect of IP$_3$ and 4Ca$^{2+}$ on the fractional loss of 4Ca$^{2+}$ in Ca$^{2+}$-free medium. After loading of permeabilized A7r5 cells during 45 min in 150 mM 4Ca$^{2+}$, efflux was started. 1 µM IP$_3$ (circles), 3 µM 4Ca$^{2+}$ (squares), or 5 µM A23187 (triangles) were added for a 2-min period (black bar), 8 min after starting the efflux. Fractional loss is defined as the amount of 4Ca$^{2+}$ released in 2 min, divided by the total amount of 4Ca$^{2+}$ stored at that moment. Each curve represents the mean ± S.E. for three wells.](http://www.jbc.org/)
measured by treating the cells with 45Ca2+ with thapsigargin together with a lowered EGTA concentration, maintaining the same 45Ca2+/40Ca2+ but resulting in 10 μM free [Ca2+] and 5 μM A23187 (circles). From time 0 onward, cells were incubated in a Ca2+-free efflux medium, and their Ca2+ content was plotted as a function of time. Results represent the means ± S.E. of three independent experiments each performed twice.

free 40Ca2+ (squares) also showed an increase in the rate of Ca2+ release. 3 μM free 40Ca2+ was able to release 25 ± 2% of the stored Ca2+. The total amount of releasable Ca2+ was measured by treating the cells with 5 μM ionophore A23187 (triangles). This activation of Ca2+ release upon elevation of the cytosolic [Ca2+], has also previously been observed by others (15, 38), but it could not be excluded that it reflected 45Ca2+/40Ca2+ exchange without net transport (38).

It is indeed important to emphasize that in this type of experiment the challenge by 3 μM 40Ca2+ could have caused an exchange of 40Ca2+/45Ca2+. To exclude the contribution of 44Ca2+/40Ca2+ exchange we maintained the same 44Ca2+/40Ca2+ ratio during the loading and efflux phases and we changed the [EGTA] to alter the free [Ca2+] (Fig. 2). After incubation in loading buffer during 45 min, the cells were incubated for 2 min in the same loading buffer supplemented with thapsigargin and lowered [EGTA]. This resulted in a complete inhibition of the Ca2+ uptake via the sarcolemmal/endoplasmic reticulum Ca2+-ATPase (SERCA) and in a rise of the free [Ca2+] to 10 μM, while maintaining the 44Ca2+/40Ca2+ ratio constant. Subsequently, the cells were incubated in Ca2+-free efflux medium. The traces in Fig. 2 illustrate how the Ca2+ content of the stores decreased during the 10-min incubation in the Ca2+-free efflux medium and show that the initial Ca2+ content was decreased by the rise in free [Ca2+] during the first 2 min subsequent to the loading. Cells incubated during 2 min in 10 μM free [Ca2+] medium showed a decrease in Ca2+ content of 27 ± 6% compared with cells that were not subjected to a [Ca2+] rise. This finding demonstrates that a decrease in the Ca2+ content was induced by 10 μM free Ca2+ without a change in the 44Ca2+/40Ca2+ ratio thereby excluding passive 44Ca2+/40Ca2+ exchange.

**CICR Is Neither IP3-R- nor RyR-mediated**—The two major classes of intracellular Ca2+ release channels are the IP3Rs and the RyRs. In A7r5 cells both IP3R1 (73%) and IP3R3 (26%) are expressed (39). No evidence has been found for a functional role of the RyR in A7r5 cells (33, 40). IP3R1 and IP3R3 are both known to be regulated by increases in [Ca2+], (9, 14–16). We therefore investigated whether the CICR described here originated from the IP3-sensitive stores. Permeabilized cells were loaded with 45Ca2+ in the presence or absence of a saturating dose of IP3 (300 μM). Efflux was then performed in medium without added Ca2+. After 10 min cells were incubated for 2 min with 10 μM free 40Ca2+. No CICR was observed in cells that were loaded in the presence of IP3 (data not shown). This finding suggested that the CICR mechanism only occurred from the IP3-sensitive stores. Furthermore we looked whether this CICR mechanism was also restricted to the thapsigargin-sensitive stores. In permeabilized A7r5 cells 92% of the total Ca2+ uptake involved a thapsigargin-sensitive SERCA pump, and 8% was mediated by a thapsigargin-insensitive Ca2+-uptake mechanism (41). Cells that were loaded in the presence of 10 μM thapsigargin were challenged with 10 μM free 40Ca2+. Also in this condition no CICR mechanism was observed (data not shown). Taken together, these results suggest that this CICR mode is only occurring from the thapsigargin and IP3-sensitive compartments of the ER.

Heparin, 2-aminoethoxydiphenyl borate (2-APB) and xestospongin C (XeC) are the most used antagonists of the IP3-R. In Fig. 3 it is shown that none of these components affected the fractional loss induced by 10 μM free 40Ca2+, revealing that the IP3-R was not involved in this mechanism. Although there is no evidence for a functional RyR in A7r5 cells, we also used antagonists of the RyR to exclude any role of the RyR in this CICR mechanism. Fig. 3 illustrates that neither ruthenium red (RuRed) (100 μM) nor ryanodine (5 μM) had any effect on the fractional loss induced by 10 μM free 40Ca2+.

Ca2+ release stimulated by sphingosine 1-phosphate (42) and NAADP (43) has been observed in a number of cell types. However, it is unlikely that one of these mechanisms mediated CICR in A7r5 cells, because NAADP-stimulated Ca2+ release was not modulated by Ca2+ (44) and no sphingosine 1-phosphate or NAADP-stimulated Ca2+ release was observed in A7r5 cells under our assay conditions (data not shown).

**Characteristics of the Observed CICR**—To further characterize the CICR mechanism in A7r5 cells, we measured its [Ca2+] dependence. The Ca2+ release as a function of increasing free [40Ca2+] was plotted in Fig. 4a. A maximally effective [40Ca2+] of 10 μM stimulated release of 27 ± 4% of the stored 44Ca2+. The activation by Ca2+ occurred with an EC50 of 700 ± 30 μM and had a positive cooperativity, with a Hill coefficient of 1.9 ± 0.2. This means that a steep activation occurs within the
physiological range of cytosolic Ca\(^{2+}\) levels (0.1–10 \(\mu M\)). Fig. 4b illustrates that the CICR mechanism was controlled by the level of store loading. Ca\(^{2+}\) stores from permeabilized A7r5 cells loaded to steady state with \(^{45}\text{Ca}^{2+}\) were incubated in Ca\(^{2+}\)-free efflux medium, and their Ca\(^{2+}\) content was plotted as a function of time. \(^{40}\text{Ca}^{2+}\) (10 \(\mu M\)) was added either after 2 min (circles, full stores) or after 20 min (triangles, less filled stores). Application of 10 \(\mu M\) free \(^{40}\text{Ca}^{2+}\) was clearly less efficient to release \(^{45}\text{Ca}^{2+}\) from less filled stores. These results indicate that the CICR mechanism was controlled by the luminal [Ca\(^{2+}\)]. In this respect the CICR mechanism shows the same dependence on the luminal Ca\(^{2+}\) content as described for IP\(_3\)-induced Ca\(^{2+}\) release in those cells (34).

Fig. 5a illustrates that Mg\(^{2+}\) dose-dependently blocked the CICR in A7r5 cells. The EC\(_{50}\) for this inhibition was 0.59 ± 0.04 mM. The inhibitory effect of Mg\(^{2+}\) was not due to the increase in osmolarity of the medium, because a similar increase in osmolarity by addition of 15 mM KCl instead of 10 mM MgCl\(_2\), did not inhibit the CICR (data not shown).

Furthermore, CICR was stimulated by increasing the [ATP] in the absence of Mg\(^{2+}\) (Fig. 5b). Stimulation occurred with an EC\(_{50}\) of 320 ± 23 \(\mu M\). By adding 1 mM ATP together with 10 \(\mu M\) free \(^{40}\text{Ca}^{2+}\), maximal Ca\(^{2+}\) release was increased from 27 ± 4% to 41 ± 3%. This indicates that in physiological conditions this CICR can release a significant fraction of the intracellular stores.

**Regulation of CICR by CaM-like Proteins—**CaM is a ubiquitous regulator of most if not all types of Ca\(^{2+}\) channels, including the intracellular Ca\(^{2+}\) release channels. We therefore investigated the effect of CaM and CaM mutants on the CICR mechanism in A7r5 cells. CaM\(_{1234}\), which is CaM rendered Ca\(^{2+}\)-insensitive by point mutations (45), has the ability to associate with apoCaM-binding sites on Ca\(^{2+}\) release channels (46). In this way CaM\(_{1234}\) can prevent access to Ca\(^{2+}\)/CaM effector sites, thereby eliminating Ca\(^{2+}\) regulation via CaM as the Ca\(^{2+}\) sensor. Recombinant CaM (10 \(\mu M\)) or CaM\(_{1234}\) (10 \(\mu M\)) was added together with 3 \(\mu M\) free \(^{40}\text{Ca}^{2+}\) to permeabilized cells loaded with \(^{45}\text{Ca}^{2+}\). Fig. 6a shows that exogenously added...
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Fig. 6. Effects of CaM, CaM1, and CaM1234 on CICR. a, Ca\(^{2+}\) release from permeabilized cells incubated during 45 min in loading buffer, expressed as fractional loss. After incubation during 8 min in Ca\(^{2+}\)-free efflux buffer, cells were challenged with 3 \(\mu\)M free Ca\(^{2+}\) (squares), 3 \(\mu\)M free Ca\(^{2+}\) and 10 \(\mu\)M CaM (circles), 3 \(\mu\)M free Ca\(^{2+}\) and 10 \(\mu\)M CaM1 (diamonds), or 3 \(\mu\)M free Ca\(^{2+}\) and 10 \(\mu\)M CaM1234 (triangles) during 2 min as indicated by the bar. Results represent the means ± S.E. for three wells. b, Ca\(^{2+}\) release induced by 10 \(\mu\)M CaM was measured in the presence of increasing concentrations of CaM (circles), CaM1 (diamonds), or CaM1234 (triangles). 100% represents the release induced by 10 \(\mu\)M Ca\(^{2+}\) alone. Results represent the means ± S.E. of three independent experiments each performed twice.

EC\(_{50}\) for CaM1 inhibition was lower and inhibition was not complete (Fig. 6b). These data suggest that only wild type CaM is capable to fulfill the activation of the CICR by sensing the increase in free Ca\(^{2+}\), whereas mutated CaMs act as inhibitors of this mechanism.

CaM is the most ubiquitous mediator of cellular Ca\(^{2+}\) functions, but it has also become clear in recent studies that there is a large number of other EF-hand-containing Ca\(^{2+}\)-binding proteins belonging to the CaM superfamily. Particularly the Ca\(^{2+}\)-binding protein (CaBP) subfamily and the neuronal Ca\(^{2+}\)-sensor (NCS-1) subfamilies that are primarily expressed in neurons may be important for Ca\(^{2+}\) signaling.

Both members of the CaM superfamily are small proteins (about 20 kDa) that share with CaM the basic structure of two N-terminal and two C-terminal EF-hands. However, only three of their EF-hands can bind Ca\(^{2+}\) (Fig. 7a). Hence, we investigated whether two of these Ca\(^{2+}\)-binding proteins, CaBP1 and NCS-1 protein, could also alter the activity of this CICR mechanism in A7r5 cells, because they both have one inactive EF-hand. CICR was measured as described above. For CaBP1, both short (sCaBP1) or long (lCaBP1) isoforms were added for 2 min together with a maximally effective free \([40Ca^{2+}]\) of 10 \(\mu\)M. 10 \(\mu\)M of sCaBP1 or lCaBP1 inhibited the CICR by more than 80% (Fig. 7b). Under the same conditions 10 \(\mu\)M GST-NCS-1 equally inhibited the CICR mechanism (Fig. 7c). GST (10 \(\mu\)M) by itself, however, did not affect the CICR mechanism in our system, indicating a specific effect of NCS-1 (data not shown). To exclude that the remaining Ca\(^{2+}\)-binding sites of NCS-1 could contribute to the inhibitory effect on CICR through simple Ca\(^{2+}\) chelation, the same experiments were conducted using a mutant of NCS-1. NCS-1\(_{E120Q}\) with its third EF-hand disrupted, showed impaired Ca\(^{2+}\)-dependent conformational changes (47). This mutant was still able to inhibit the CICR mechanism to the same extent as wild type NCS-1 (Fig. 7c), thereby excluding a Ca\(^{2+}\)-chelation effect.

To test the hypothesis that the CICR mechanism is activated by a Ca\(^{2+}\)-dependent dissociation or dislocation from an apoCaM-binding site, we performed experiments in which we trapped the endogenous CaM with a high affinity CaM-binding peptide derived from the RyR1 (amino acids 3614–3643) (30). Fig. 8a shows that in cells incubated during the loading phase with 10 \(\mu\)M of the CaM-binding peptide the CICR mechanism was nearly abolished. However, the RyR1 peptide had no effect on the extent of 45Ca\(^{2+}\) loading of the cells (data not shown). To strengthen the argument regarding the specific effects of the RyR1 peptide, CaM and CaM1234 were re-added for a 2-min period after stripping the cells with the RyR1 peptide. Re-addition of 10 \(\mu\)M CaM almost completely restored CICR activation by 3 \(\mu\)M free \([40Ca^{2+}]\), whereas 10 \(\mu\)M CaM1234 was unable to restore CICR activation (Fig. 8b). Therefore, it is likely that, in permeabilized A7r5 cells, Ca\(^{2+}\) activates a Ca\(^{2+}\) release mechanism by binding to endogenously bound apoCaM.

CICR in Different Cell Types—To verify whether a similar CICR mechanism is also expressed in other cell types we have screened 16HBE140- (human bronchial mucosa), LLC-PK1 (porcine kidney cell line), COS-1, and mouse embryonal fibroblast cells in the same conditions as described above for A7r5 cells. Permeabilized cells loaded with 45Ca\(^{2+}\) were challenged with 10 \(\mu\)M free \([40Ca^{2+}]\). A significant CICR response was only found in 16HBE140- cells, although the fraction of released Ca\(^{2+}\) was smaller (15 ± 3%) than for A7r5 cells. This response in 16HBE140- cells was also inhibited by CaM1234, sCaBP1, lCaBP1, and NCS-1 (data not shown), suggesting that this same CICR mechanism is not only expressed in A7r5 cells but that it could be more widespread.
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**FIG. 7.** Effects of EF-hand containing Ca\(^{2+}\)-binding proteins on CICR. *a,* schematic representation of the Ca\(^{2+}\)-binding proteins used in this study. *Dark bars* represent functionally active EF-hands, and *white bars* represent inactive EF-hands. *Circles* represent the myristoylation sites at the N terminus. Two splice variants of CaBP1, both long (lCaBP1) and short (sCaBP1) are represented. *b,* inhibitory effects of sCaBP1 (*squares*) and lCaBP1 (*triangles*) on Ca\(^{2+}\) release induced by 10 \(\mu\)M free \(^{45}\)Ca\(^{2+}\). 100% represents the Ca\(^{2+}\) release in the absence of sCaBP1 or lCaBP1. *c,* inhibitory effects of GST-NCS-1 (*squares*) and GST-NCS-1E120Q (*triangles*) on Ca\(^{2+}\) release induced by 10 \(\mu\)M free Ca\(^{2+}\). 100% represents the Ca\(^{2+}\) release in the absence of GST-NCS-1 or GST-NCS-1E120Q. Results represent the means ± S.E. of three independent experiments each performed twice.

**FIG. 8.** Effect of RyR1 (amino acids 3614–3643) derived peptide on CICR. *a,* permeabilized A7r5 cells were loaded for 45 min in the absence (*squares*) or presence of 10 \(\mu\)M RyR1 peptide (3614–3643) (*triangles*). After a 6-min incubation in Ca\(^{2+}\)-free efflux buffer, cells were challenged with 3 \(\mu\)M free Ca\(^{2+}\) during a 2-min time period as indicated by the bar. In cells preincubated with the peptide CICR was abolished. Results represent the means ± S.E. for three wells. *b,* permeabilized A7r5 cells were loaded for 45 min in the presence of 10 \(\mu\)M RyR1 peptide (3614–3643). Before challenging the cells with 3 \(\mu\)M free Ca\(^{2+}\) during a 2-min time period as indicated by the dark bar, cells were incubated with 10 \(\mu\)M CaM (*squares*) or 10 \(\mu\)M CaM1234 (*triangles*) during a 2-min period as indicated by the white bar. Cells that were incubated with CaM responded to 3 \(\mu\)M free Ca\(^{2+}\), whereas cells incubated with CaM1234 did not. Results represent the means ± S.E. for three wells.

**DISCUSSION**

**Ca\(^{2+}\)** release from the intracellular stores can be triggered by either IP\(_3\) or by a CICR mechanism. Here we report that in permeabilized A7r5 cells an increase in the free [Ca\(^{2+}\)]\(_i\) stimulated a Ca\(^{2+}\) release of up to 41% of the intracellular stores with an EC\(_{50}\) of 700 nM and a Hill coefficient of about 2. This type of CICR mechanism was not mediated by IP\(_3\)Rs nor by RyRs, because it was not blocked by ruthenium red, ryanodine, heparin, 2-APB, or xestospongin C. ATP dose-dependently stimulated the CICR mechanism, whereas 10 mM MgCl\(_2\) completely abolished it. All these results suggested a novel type of CICR from the non-mitochondrial intracellular stores in permeabilized A7r5 cells. This CICR mechanism did not simply reflect passive \(^{45}\)Ca\(^{2+}/^{40}\)Ca\(^{2+}\) exchange and did not result from the SERCA pumps running in reverse, because thapsigargin was present during the efflux phase.

Recently a similar CICR pathway was identified in hepatocytes (26) suggesting that it may be more ubiquitously expressed in different cell types. We identified a similar type of CICR pathway in 16HBE14o- cells, confirming this idea. Al-
though these CICR pathways appear to be quite similar, there are also striking differences between the observations made in the present study as compared with those described for hepatocytes. The CICR mechanism in hepatocytes appeared to be more sensitive, with an EC of 170 nM compared with 700 nM in the present study and was reported to be ATP-independent. This may suggest different types of transporters or at least differences in their regulation.

Polycystin-2 was recently identified as a new Ca\(^{2+}\) release channel. Polycystin-2 is a member of the TRP channel superfamily. Polycystin-2 behaved as a Ca\(^{2+}\)-activated, high conductance ER channel that is permeable to divalent ions and exhibited channel behavior reminiscent of RyRs and IP\(_{3}\)Rs (22–24). It remains to be established if the CICR mechanism described in our study could be related to polycystin-2. The observation that LLC-PK\(_1\) cells that endogenously express polycystin-2 did not show the CICR mechanism, however, seems to disprove this hypothesis. Another member of the TRP family, the VR1, was also recently found to act as an intracellular Ca\(^{2+}\) release channel. Capsaicin binding to the VR1 resulted in Ca\(^{2+}\) mobilization from the intracellular Ca\(^{2+}\) stores, and it was found to localize with the ER (27, 28). These data suggest that different members of the TRP family can act as intracellular Ca\(^{2+}\) release channels.

The presence of a CICR mechanism could be important for the propagation and amplification of Ca\(^{2+}\) signals initiated by other Ca\(^{2+}\) release channels. Indeed, CICR mediated by RyRs and IP\(_{3}\)Rs plays a crucial role in amplifying the Ca\(^{2+}\) signals provided by Ca\(^{2+}\) entry in cells such as cardiac myocytes (48), neurons (49, 50), astrocytes (51), and pancreatic \(\beta\)-cells (52). For example the nature of long-term changes in synaptic activity in the hippocampus depends on whether Ca\(^{2+}\) entry triggers CICR via RyRs or IP\(_{3}\)Rs (50). It became clear that CICR is an important feature of intracellular signaling. The available data strongly suggest the presence of additional CICR pathways different from the well documented IP\(_{3}\)R and RyR.

A new finding in our study is that the CICR mechanism described here was inhibited by CaM\(_{1234}\) and by members of the family of CaM-like Ca\(^{2+}\)-sensor proteins. It became clear from recent work that most of the Ca\(^{2+}\) channels, both situated in the plasma membrane or in intracellular stores, are regulated by CaM, apoCaM, or members of the CaM superfamily. This has recently been well documented for the RyR and the IP\(_{3}\)R. The skeletal-muscle Ca\(^{2+}\) release channel, RyR1, is activated by apoCaM and inhibited by Ca\(^{2+}\)-bound CaM (10, 11, 30). For the IP\(_{3}\)R the functional significance of CaM is not clear (12, 13). Other Ca\(^{2+}\) channels, like the voltage-dependent Ca\(^{2+}\) channels (53–56), as well as members of the TRP family (57–61), have CaM- and apoCaM-binding sites. We found that the CICR mechanism described in this study is regulated by CaM. The CICR mechanism was not affected by CaM itself, but CaM\(_{1}\) and CaM\(_{1234}\) inhibited it. Using CaM\(_{1234}\) as a negative dominant already revealed the role of CaM in K\(^{+}\) channels (62), L-type Ca\(^{2+}\) channels (45, 55), P/Q-type Ca\(^{2+}\) channels (56), store-operated channels (63), and the RyR (10, 11, 30). Our data indicate the presence of an inhibitory CaM-binding site in the absence of Ca\(^{2+}\) (apoCaM-binding site). CaM tethered to this position could then act as a Ca\(^{2+}\)-sensor, and CICR could be interpreted as a Ca\(^{2+}\)-dependent dissociation or delocalization of CaM from its inhibitory binding site. The dominant negative effect of CaM\(_{1234}\) results from its inability to perform a Ca\(^{2+}\)-dependent interaction. Further evidence supporting this hypothesis was obtained by preincubation of permeabilized cells with a high-affinity peptide for CaM, derived from RyR1. Endogenous CaM could be trapped by this peptide, and therefore the Ca\(^{2+}\) sensor for the CICR mechanism would be removed. In agreement with our hypothesis the preincubation with the RyR1 peptide indeed abolished a subsequent CICR mechanism. Moreover, re-addition of CaM, but not of CaM\(_{1234}\), could restore CICR after preincubation with the RyR1 peptide. Because preincubation with the RyR1 peptide during the loading phase did not interfere with "64Ca\(^{2+}\)" loading of the cells, stripping of CaM per se seems not to be sufficient for CICR. The data rather support a mechanism where a Ca\(^{2+}\)-dependent delocalization of CaM to another binding site is responsible for CICR activation. Results obtained with other members of the CaBP family can also be explained by this hypothesis. These CaM-like proteins apparently all show binding affinity in the absence of Ca\(^{2+}\). By binding to the apoCaM-binding site they may prevent the role of CaM as a Ca\(^{2+}\) sensor. The C termini of CaBPs are highly homologous to the corresponding region in CaM, whereas the N termini are longer and have more variation, including the myristoylation sites or alternative exons. CaBPs also have an extended 32-amino acid-long flexible central \(\alpha\)-helical segment, versus 28 amino acids in CaM. These differences together with a disabled EF-hand 2 could explain the different binding characteristics as compared with CaM.

Indeed, the sequential binding of the highly homologous C-terminal domain with further binding of the N-terminal domain could tether CaBPs to the effector molecules at all [Ca\(^{2+}\)] (64). Such changes in binding properties of CaM have also been observed when EF-hand 2 was disabled by mutations (65, 66). Furthermore, NCS-1 bound in a Ca\(^{2+}\)-independent manner to rat brain membranes (67). The more restricted expression and subcellular localization of the CaM-like Ca\(^{2+}\)-sensor proteins could thereby provide a physiological mechanism to inhibit CICR in specific areas of neurons.

In summary, we found a novel CICR mechanism in A7r5 and 16HBE14o- cells. Although we have not yet established the molecular identity of this novel Ca\(^{2+}\) release pathway, we found that its activation is mediated by CaM. The data suggest that CaM tethered to an inhibitory apoCaM site may act as the Ca\(^{2+}\) sensor for activation of CICR. A possible candidate for this pathway could be a member of the TRP-channel superfamily, like the polycystin-2 channel, but there is as yet no evidence to support this. The apoCaM-binding property described here may offer a practical tool for the future identification of the transport protein involved. In addition, this novel CICR mechanism may provide an additional pathway in Ca\(^{2+}\) release and could play an important role in amplifying Ca\(^{2+}\) signals generated by other Ca\(^{2+}\) release channels.

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A Novel Ca$^{2+}$-induced Ca$^{2+}$ Release Mechanism in A7r5 Cells Regulated by Calmodulin-like Proteins

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