A novel Ca^{2+}-induced Ca^{2+} release mechanism mediated by neither inositol trisphosphate nor ryanodine receptors

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Members of both major families of intracellular Ca^{2+} channels, ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors, are stimulated by substantial increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). They thereby mediate Ca^{2+}-induced Ca^{2+} release (CICR), which allows amplification and regenerative propagation of intracellular Ca^{2+} signals. In permeabilized hepatocytes, increasing $[Ca^{2+}]_c$ to 10 μM stimulated release of 30 ± 1 % of the intracellular stores within 60 s; the EC_{50} occurred with a free $[Ca^{2+}]$ of 170 ± 29 nM. This CICR was abolished at 2 °C. The same fraction of the stores was released by CICR before and after depletion of the IP_3-sensitive stores, and CICR was not blocked by antagonists of IP_3 receptors. Ryanodine, Ruthenium Red and tetracaine affected neither the Ca^{2+} content of the stores nor the CICR response. Sr^{2+} and Ba^{2+} (EC_{50} = 166 nM and 28 μM respectively) mimicked the effects of increased $[Ca^{2+}]$ on the intracellular stores, but Ni^{2+} blocked the passive leak of Ca^{2+} without blocking CICR. In rapid superfusion experiments, maximal concentrations of IP_3 or Ca^{2+} stimulated Ca^{2+} release within 80 ms. The response to IP_3 was complete within 2 s, but CICR continued for tens of seconds despite a slow [half-time ($t_{1/2}$) = 3.54 ± 0.07 s] partial inactivation. CICR reversed rapidly ($t_{1/2} = 529 \pm 17$ ms) and completely when the $[Ca^{2+}]$ was reduced. We conclude that hepatocytes express a novel temperature-sensitive, ATP-independent CICR mechanism that is reversibly activated by modest increases in $[Ca^{2+}]$, and does not require IP_3 or ryanodine receptors or reversal of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase. This mechanism may both regulate the Ca^{2+} content of the intracellular stores of unstimulated cells and allow even small intracellular Ca^{2+} signals to be amplified by CICR.

Key words: Ca^{2+} stores, Ca^{2+} release kinetics, CICR, hepatocytes.

INTRODUCTION

Increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) regulate almost every aspect of cellular activity [1]. Regulated opening of Ca^{2+} channels in either the plasma membrane or the membranes of intracellular stores, primarily within the endoplasmic reticulum, is the most common means whereby extracellular stimuli evoke such increases in $[Ca^{2+}]_c$. It is, however, clear that the intracellular and extracellular sources do not behave independently. The Ca^{2+} content of the intracellular Ca^{2+} stores regulates Ca^{2+} entry via the capacitative Ca^{2+} entry pathway [2], and Ca^{2+} passing through one channel may regulate the activity of other Ca^{2+} channels. The latter occurs because most Ca^{2+} channels are themselves regulated by cytosolic Ca^{2+}. The increase in $[Ca^{2+}]_c$ that follows opening of a channel often provides negative feedback, but for each of the major families of intracellular Ca^{2+} channels, ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors, Ca^{2+} can both stimulate and inhibit channel opening [3,4]. Stimulation of intracellular Ca^{2+} channels by Ca^{2+} is important because it allows them, via Ca^{2+}-induced Ca^{2+} release (CICR), to amplify the Ca^{2+} signal provided by other channels in either the plasma membrane [5] or the membranes of the intracellular stores [6]. CICR mediated by ryanodine [5,7,8] or IP_3 [8,9] receptors is clearly important for amplifying the Ca^{2+} signals provided by Ca^{2+} entry in cells such as cardiac myocytes [5], neurones [8,9], astrocytes [10] and pancreatic β-cells [7]. Indeed, the nature of the long-term changes in synaptic activity in the hippocampus depends on whether Ca^{2+} entry triggers CICR via ryanodine or IP_3 receptors [8]. Both ryanodine [11] and IP_3 [1,11] receptors are also known to amplify the Ca^{2+} release mediated by neighbouring intracellular Ca^{2+} channels and to thereby generate a hierarchy of Ca^{2+} release events that may culminate in global Ca^{2+} waves. CICR is clearly a key feature of intracellular Ca^{2+} signalling [12].

The sensitivity of CICR to drugs that selectively inhibit IP_3 or ryanodine receptors has provided persuasive evidence for the involvement of these intracellular Ca^{2+} channels in intact cells, but there are examples of CICR that appear not to be inhibited by these antagonists. In pancreatic β-cells, for example, although CICR appears to be mediated largely by ryanodine receptors, other unidentified channels may also contribute [7].

In the present study, we have identified a novel CICR mechanism in permeabilized hepatocytes. We conclude that a Ca^{2+} efflux pathway that is neither an IP_3 nor a ryanodine receptor mediates reversible release of Ca^{2+} from intracellular stores in response to modest increases in $[Ca^{2+}]_c$. We suggest that this sensitive CICR mechanism may both determine the Ca^{2+} content of the stores in unstimulated cells and provide an additional means of amplifying even small Ca^{2+} signals generated by other regulated Ca^{2+} channels.

MATERIALS AND METHODS

Materials

IP_3 was purchased from American Radiolabeled Chemicals (St Louis, MO, U.S.A.). Xestospongin C and ionomycin were obtained from Calbiochem (Nottingham, U.K.). Ryanodine and thapsigargin were from Alamone Laboratories (Jerusalem, Israel), and $^{45}Ca^{2+}$ was purchased from ICN (Thame, Oxon, U.K.). Sphingosine-1-phosphate, 2-aminoethoxydiphenyl

Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borane; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; CLM, cytosol-like medium; IP_3, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid–adenine dinucleotide phosphate; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; $t_{1/2}$, half-time.

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borane (2-APB), tetracaine, nicotinic acid–adenine dinucleotide phosphate (NAADP), Ruthenium Red and all other reagents were obtained from Sigma (Poole, Dorset, U.K.).

Measurements of $^{45}Ca^{2+}$ efflux

Hepatocytes were prepared from the livers of male Wistar rats [13], resuspended in cytosol-like medium (CLM; 140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Pipes, pH 7.0 at 37 °C), and permeabilized by incubation with saponin (10 μg/ml for 8 min). The cells were then washed and resuspended (10^7 cells/ml) in CLM supplemented with 344 μM $CaCl_2$ (free $[Ca^{2+}] = 200$ nM), ATP (7.5 mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ('FCCP'; 10 μM) and $^{45}Ca^{2+}$ (10 μCi/ml). After 7 min at 37 °C, the $^{45}Ca^{2+}$ content of the intracellular stores reached a steady state (1–2 nmol of $Ca^{2+}/10^6$ cells) and the cells were used for unidirectional efflux experiments [13].

For most experiments, cells loaded with $^{45}Ca^{2+}$ were diluted 5-fold into CLM containing an appropriate free $[Ca^{2+}]$ and thapsigargin (1 μM), in order to inhibit further Ca^{2+} uptake. Variations of this procedure are described in the text. At appropriate intervals, the $^{45}Ca^{2+}$ contents of the stores were then determined by rapidly quenching the incubations with cold medium (310 mM sucrose/1 mM trisodium citrate), followed by filtration (Whatman glass-fibre C filters) using a Brandel receptor binding harvester (SEMAT, St Albans, Herts., U.K.). Active $^{45}Ca^{2+}$ uptake was defined as that which could be released by the addition of 1 μM ionomycin.

To resolve the rapid kinetics of $^{45}Ca^{2+}$ efflux, permeabilized hepatocytes loaded to steady state with $^{45}Ca^{2+}$ (15 μCi/ml) were immobilized within a filter sandwich held within a rapid superfusion apparatus. A complete description of the apparatus has been described previously [14]. Briefly, the equipment allowed $^{45}Ca^{2+}$ release from the immobilized cells to be measured with a temporal resolution of up to 9 ms, as CLM flowed continuously (2 ml/s) over the cells and (with the $^{45}Ca^{2+}$ released) into a circular fraction collector. Addition of a trace of [^{3}H]inulin to some of the media allowed changes of media to be precisely related to changes in $^{45}Ca^{2+}$ efflux. All superfusion experiments were carried out at 20 °C, and the half-time ($t_{1/2}$) for exchange of media bathing the cells was 36 ± 3 ms ($n = 3$).

The free $[Ca^{2+}]$ of CLM was measured using fura 2 for Ca^{2+} concentrations less than 1 μM and a Ca^{2+}-sensitive electrode (Russel, Auchtermuchly, Scotland, U.K.) for higher Ca^{2+} concentrations, as previously described [3]. Free $[Ba^{2+}]$ and $[Sr^{2+}]$ were computed using the computer program, MaxChelator (http://www.stanford.edu/~cpatton/maxc.html).

RESULTS AND DISCUSSION

Modest increases in $[Ca^{2+}]_c$ stimulate Ca^{2+} release from intracellular stores

Increasing $[Ca^{2+}]_c$ stimulated $^{45}Ca^{2+}$ release from the intracellular stores of hepatocytes that had been loaded to steady state with $^{45}Ca^{2+}$. During an incubation lasting 60 s, a maximally effective concentration of Ca^{2+} ($[Ca^{2+}]_c = 10$ μM) stimulated release of 30 ± 1 % ($n = 6$) of the intracellular stores, and the EC_{50} occurred when the $[Ca^{2+}]_c$ was 170 ± 29 nM (Figure 1). It is noteworthy that the amount of $^{45}Ca^{2+}$ released from the stores by increasing $[Ca^{2+}]_c$ (30 %) is comparable with the response evoked by a maximal concentration of IP_3 (28 %). The effect of Ca^{2+} on the time course of unidirectional $^{45}Ca^{2+}$ efflux from the intracellular stores is shown in Figure 2(A). At 37 °C, the $t_{1/2}$ for $^{45}Ca^{2+}$ efflux

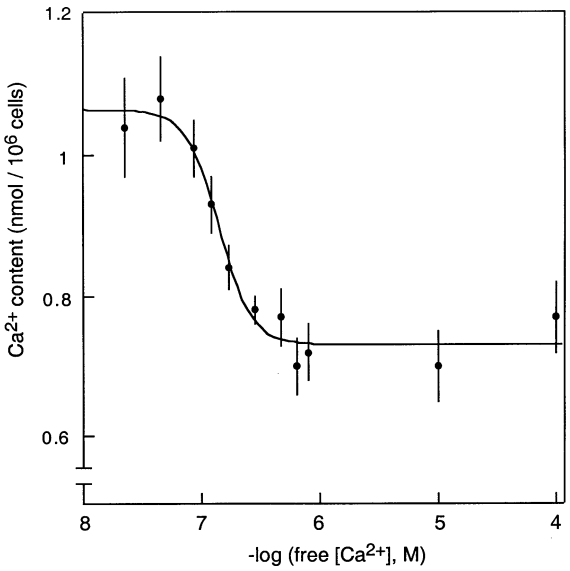

Figure 1 CICR from the intracellular stores of permeabilized hepatocytes

Permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ in CLM containing a free $[Ca^{2+}]$ of 200 nM were diluted into CLM at 37 °C containing thapsigargin (1 μM) and sufficient Ca^{2+} to give the indicated final free $[Ca^{2+}]$. After 60 s, the incubations were terminated and the $^{45}Ca^{2+}$ contents of the stores were measured. Results are presented as means $\pm$ S.E.M. from six independent experiments.

was 140 ± 4 s ($n = 3$) when the free $[Ca^{2+}]$ was approx. 20 nM, but in paired experiments the $t_{1/2}$ decreased to 69 ± 12 s when the free $[Ca^{2+}]$ was 10 μM. The mono-exponential curves fitted to the data shown in Figure 2(A) demonstrate that the only effect of increasing the $[Ca^{2+}]$ was to decrease the $t_{1/2}$ for Ca^{2+} efflux. The extrapolated initial Ca^{2+} contents of the stores were similar for cells in low and high Ca^{2+} (108 ± 1 % and 108 ± 9 % respectively of their contents measured at 30 s) as were the Ca^{2+} contents extrapolated to infinite time (22 ± 2 % and 20 ± 5 %). The incomplete loss of $^{45}Ca^{2+}$ from preloaded stores after sustained inhibition of Ca^{2+} uptake is consistent with previous work showing that luminal Ca^{2+} regulates the basal leak of Ca^{2+} from the intracellular stores of hepatocytes [15].

It is important to emphasize that in each of these experiments, changes in the free $[Ca^{2+}]$ were accompanied by changes in the specific activity of the $^{45}Ca^{2+}$. We therefore sought to eliminate the possibility that the responses might simply reflect an exchange of $^{45}Ca^{2+}$ for $^{40}Ca^{2+}$ with no net change in the Ca^{2+} content of the stores. For practical reasons, it proved impossible to reliably measure the effects of substantially increasing the $[Ca^{2+}]$ while preserving the specific activity of $^{45}Ca^{2+}$. Instead, we argued that if $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange contributed significantly to the loss of $^{45}Ca^{2+}$ from the intracellular stores, we would expect the rate of $^{45}Ca^{2+}$ efflux to be faster if addition of thapsigargin was accompanied by dilution of the specific activity of the $^{45}Ca^{2+}$ in the CLM. The results demonstrate that when $^{45}Ca^{2+}$ efflux was measured under conditions that maintained the free $[Ca^{2+}]$ of the CLM, the rate of $^{45}Ca^{2+}$ efflux after addition of thapsigargin was similar whether measured with no change in the specific activity of $^{45}Ca^{2+}$ ($t_{1/2}$ for efflux $= 173 \pm 4$ s, $n = 3$) or following a 5-fold reduction in the specific activity of $^{45}Ca^{2+}$ ($t_{1/2} = 152 \pm 5$ s, $n = 3$). We conclude that the release of $^{45}Ca^{2+}$ evoked by increasing the free $[Ca^{2+}]$ is unlikely to result from $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange.

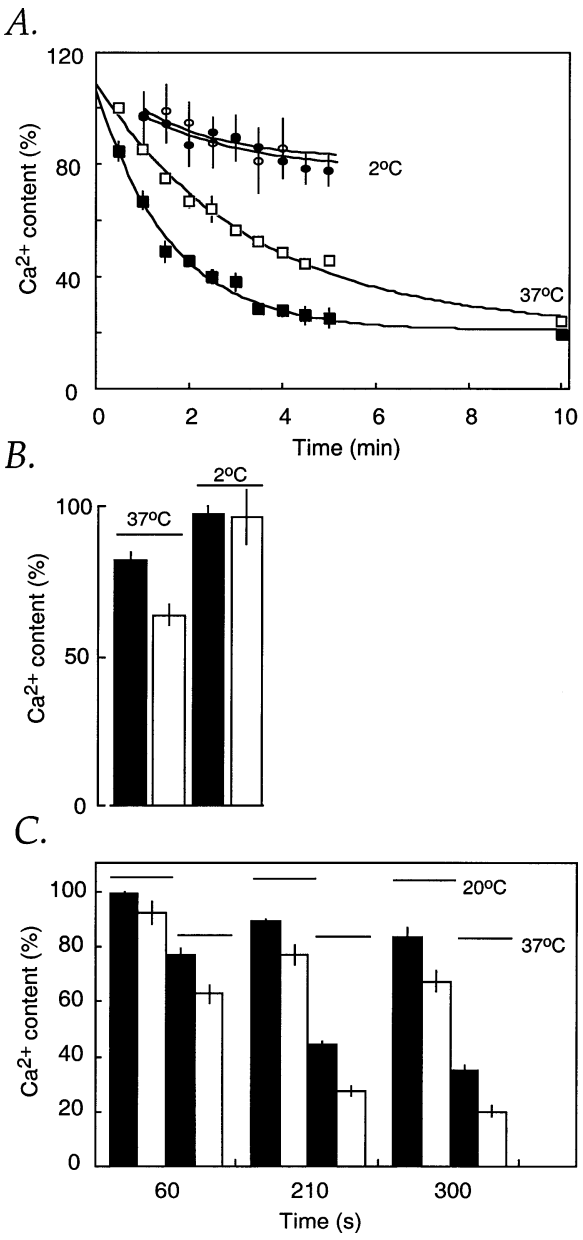

Figure 2 Ca^{2+} stimulates temperature-sensitive $^{45}Ca^{2+}$ efflux from the intracellular stores

(**A**) Permeabilized cells loaded with $^{45}Ca^{2+}$ in normal CLM were rapidly diluted into CLM containing thapsigargin (1 μM) and either nominally Ca^{2+}-free CLM (free $[Ca^{2+}]$ of approx. 20 nM; ○, □) or CLM containing 10 μM free Ca^{2+} (■, ●). The incubations at either 37 °C (■, □) or 2 °C (●, ○) were then terminated at the indicated times. Lines are fitted to mono-exponential equations. (**B** and **C**) The Ca^{2+} contents of the stores are shown after incubations lasting 60 s (**B**) or for the times shown (**C**) at the indicated temperatures in nominally Ca^{2+}-free CLM (■) or CLM with 10 μM free Ca^{2+} (□). Results (percentages of Ca^{2+} content after 30 s in nominally Ca^{2+}-free CLM) are presented as the means ± S.E.M. from six (37 °C) or three (2 °C and 20 °C) independent experiments. Note that the final free $[Ca^{2+}]$ of the low-Ca^{2+} CLM varies between different experimental protocols depending on the incubation temperature (the K_d^{Ca} of EGTA is temperature-dependent) and according to whether the reduced free $[Ca^{2+}]$ is achieved by dilution of cells (most experiments, and all the results shown in this Figure) or complete replacement of the incubation medium by superfusion (Figure 6).

Collectively, these results suggest that an increase in $[Ca^{2+}]_c$ stimulates a slow leak of Ca^{2+} from all of the intracellular Ca^{2+} stores of permeabilized hepatocytes. Because thapsigargin prevents Ca^{2+} from leaking from the endoplasmic reticulum through the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) [16], the CICR response observed in the presence of thapsigargin cannot result from the SERCA running in reverse. Nor is it likely that the CICR response simply reflects an increased rate of $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange.

At 2 °C, the rate of $^{45}Ca^{2+}$ efflux was much slower than at 37 °C, in keeping with previous work [15], and the rates were similar in CLM containing a $[Ca^{2+}]_c$ of approx. 40 nM or 100 μM (Figures 2A and 2B). Because both IP_3 receptors [17] and ryanodine receptors [18] respond to their agonists at 2 °C, the inhibition of CICR in hepatocytes at 2 °C provides the first indication that the response may be mediated by neither ryanodine nor IP_3 receptors. At 20 °C (the only practicable temperature for rapid superfusion experiments, see below), the rate of $^{45}Ca^{2+}$ efflux was slower than at 37 °C ($t_{1/2} = 474 \pm 72$ s in CLM containing approx. 30 nM free $[Ca^{2+}]$), but Ca^{2+} efflux was stimulated by Ca^{2+} (Figure 2C). These results establish that under conditions where the SERCA has been completely inhibited [15], concentrations of Ca^{2+} similar to those found in unstimulated cells, stimulate temperature-sensitive Ca^{2+} efflux from the intracellular stores.

Neither ryanodine nor IP_3 receptors mediate the CICR

Each of the three isoforms of IP_3 and ryanodine receptors is stimulated by increases in $[Ca^{2+}]_c$ [3,4,19]. We therefore sought to establish whether either of these families of intracellular Ca^{2+} channels might be responsible for the CICR. In CLM without added Ca^{2+} ($[Ca^{2+}]_c$ of approx. 20 nM), a maximally effective concentration of IP_3 (10 μM) released 28 ± 2 % of the Ca^{2+} stores within 30 s. Subsequent addition of Ca^{2+}, to increase the free $[Ca^{2+}]$ to 10 μM, released 29 ± 2 % of the remaining stores during a 60 s incubation. The same fraction of the Ca^{2+} stores (29 ± 1 %) was released by 10 μM Ca^{2+} when it was added without prior stimulation by IP_3 (Figure 3A). These results establish that CICR persists after complete depletion of the IP_3-sensitive Ca^{2+} stores. Because the same fraction of the stores was released before and after depletion of the IP_3-sensitive stores, this suggests further that the CICR mechanism is expressed similarly in IP_3-sensitive and IP_3-insensitive Ca^{2+} stores, consistent with our measurements of the effects of Ca^{2+} on rates of $^{45}Ca^{2+}$ efflux (see above; Figure 2A).

Heparin, 2-APB and xestospongin C [20] have each been reported to be antagonists of IP_3 receptors. Heparin (100 μg/ml) blocked the release of $^{45}Ca^{2+}$ evoked by a submaximal concentration (200 nM) of IP_3 (results not shown), consistent with its well documented ability to compete with IP_3 for binding to the IP_3 receptor [21]. However, 2-APB which has been reported to block IP_3-evoked Ca^{2+} release without affecting IP_3 binding [22], and which has been extensively used to address the physiological roles of IP_3 receptors [23,24], proved not to be a useful antagonist of IP_3 receptors in hepatocytes. 2-APB (75 μM) directly stimulated Ca^{2+} efflux, while only modestly increasing the EC_{50} for IP_3-evoked Ca^{2+} release (from 156 ± 8 nM to 324 ± 35 nM) and modestly decreasing the response to a maximal concentration of IP_3 (from 47 ± 1 % to 43 ± 1 % release) (Figure 4). Indeed, the inhibition of the Ca^{2+} release evoked by a submaximal concentration of IP_3 ($IC_{50} = 39$ μM) is only slightly more sensitive to 2-APB than is the stimulation of Ca^{2+} efflux ($EC_{50} = 63$ μM) (Figure 4). We conclude, in keeping with other recent reports [7,25], that 2-APB cannot be used to selectively block IP_3 receptors without causing substantial stimulation of Ca^{2+} release.

Incubation of cells with heparin (⩽ 1 mg/ml), 2-APB (⩽ 100 μM) or xestospongin C (5 μM), a non-competitive antagonist

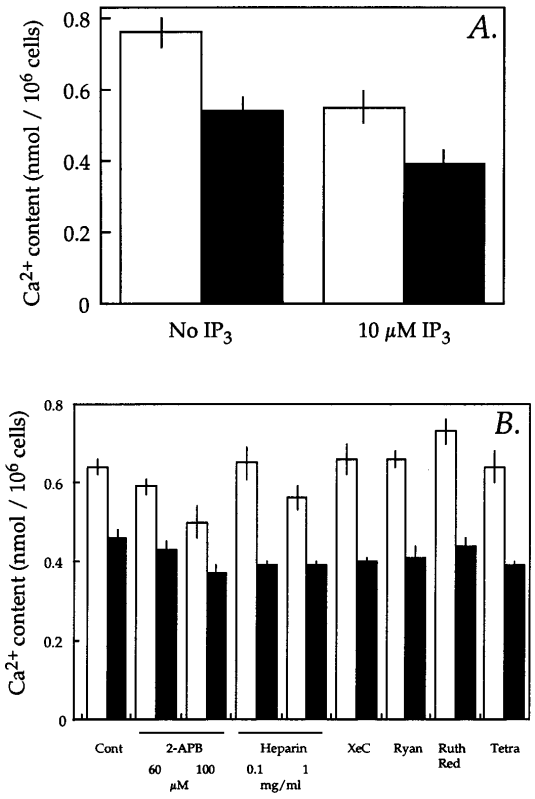

Figure 3 Neither IP_3 nor ryanodine receptors mediate the CICR

(**A**) Cells were stimulated for 30 s with IP_3 (10 μM) in CLM with a free $[Ca^{2+}]$ of approx. 20 nM, before either continuing the incubation in the same CLM (□) or increasing the free $[Ca^{2+}]$ to 10 μM (■). The $^{45}Ca^{2+}$ contents of the stores (means ± S.E.M., $n \geqslant 3$) were measured 60 s later. The results demonstrate that Ca^{2+} stimulates release of a similar fraction of the Ca^{2+} stores whether (right-hand bars) or not (left-hand bars) the IP_3-sensitive stores have been emptied. (**B**) Cells were pre-treated with the indicated drugs for 30 s in CLM with a free $[Ca^{2+}]$ of approx. 20 nM before increasing the free $[Ca^{2+}]$ to 10 μM and then measuring the $^{45}Ca^{2+}$ contents of the stores after a further 60 s. Results (means ± S.E.M., $n = 3$) are expressed as percentages of the Ca^{2+} content of control cells in CLM with low Ca^{2+}. White bars represent responses in CLM with a free $[Ca^{2+}]$ of approx. 20 nM, and black bars those from cells in CLM with a free $[Ca^{2+}]$ of 10 μM. The following concentrations of the drugs were used: 2-APB, 60 or 100 μM; heparin, 0.1 or 1 mg/ml; xestospongin C (XeC), 5 μM; ryanodine (Ryan), 100 μM; Ruthenium Red (Ruth Red), 100 μM; and tetracaine (Tetra), 1 mM.

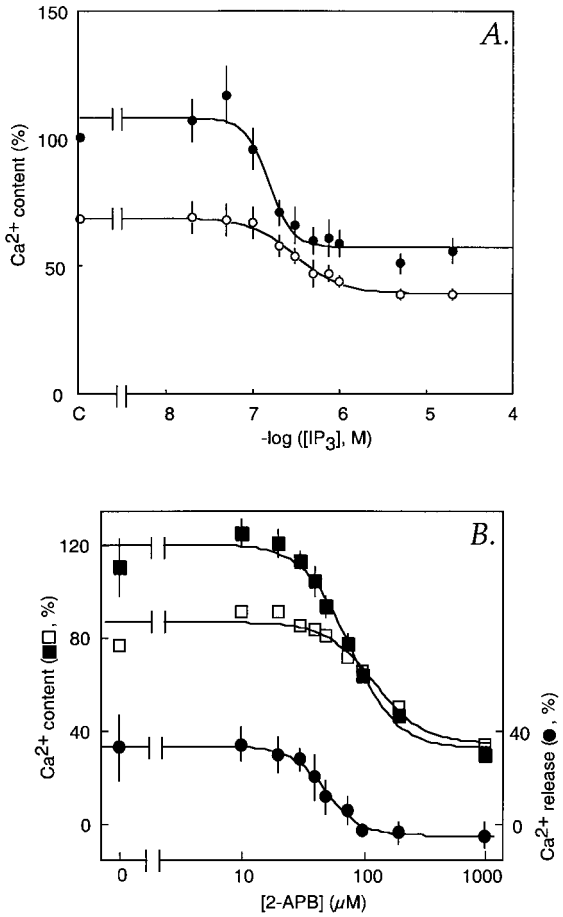

Figure 4 Effects of 2-APB on intracellular Ca^{2+} stores

(**A**) Concentration-dependent effect of IP_3 in the absence (●) or presence (○) of 75 μM 2-APB. (**B**) Concentration-dependent effect of 2-APB on the Ca^{2+} content of unstimulated cells (■) and cells stimulated with 200 nM IP_3 (□). The bottom trace (●, control minus IP_3) shows the direct effect of 2-APB on responses to IP_3. Results (percentages of control) are presented as means ± S.E.M. ($n \geqslant 3$).

of IP_3 receptors [20], did not prevent CICR (Figure 3B). We conclude that the CICR response is not mediated by IP_3 receptors.

It is unclear whether hepatocytes express functional ryanodine receptors. Specific [^{3}H]ryanodine-binding sites have been detected in hepatocytes [26], but neither their properties nor the conflicting reports of the effects of ryanodine on intracellular Ca^{2+} stores [27–29] are entirely consistent with the properties of known ryanodine receptors. There are conflicting reports too of the ability of cADP-ribose to stimulate Ca^{2+} release from the intracellular stores of hepatocytes [29,30]. Finally, liver appears not to express mRNA for any of the three ryanodine receptor subtypes [31] and nor do antisera detect them [30].

All three known subtypes of ryanodine receptor are inhibited by appropriate concentrations of ryanodine or Ruthenium Red [32]. Preincubation of permeabilized hepatocytes with ryanodine (100 μM), Ruthenium Red (100 μM) or tetracaine (1 mM), another antagonist of ryanodine receptors (and perhaps also of IP_3 receptors [33]), did not affect their Ca^{2+} content in nominally Ca^{2+}-free CLM, and nor did it affect the subsequent CICR (Figure 3B). We chose not to use caffeine to address the possible involvement of ryanodine receptors, because earlier work suggested that it stimulated Ca^{2+} release from hepatocytes by a mechanism that did not involve ryanodine receptors [28].

Sphingosine-1-phosphate [34] and NAADP [35] also stimulate release of Ca^{2+} from the intracellular stores of some cells. Responses to NAADP are not modulated by Ca^{2+} [36], but the effects of Ca^{2+} on responses to sphingosine-1-phosphate appear not to have been investigated. Neither of these mechanisms is likely to mediate CICR in hepatocytes, because we have consistently failed to detect Ca^{2+} mobilization after stimulation of permeabilized hepatocytes with NAADP ($\leqslant 25$ μM) or sphingosine-1-phosphate ($\leqslant 50$ μM) (results not shown).

Because Ni^{2+} blocks many Ca^{2+} channels, we examined its effect on CICR. Addition of Ni^{2+} (free $[Ni^{2+}]$ of approx. 100 μM) almost completely blocked the passive efflux of $^{45}Ca^{2+}$ recorded after addition of thapsigargin, without blocking the Ca^{2+} release evoked by increasing the free $[Ca^{2+}]$ (Figure 5A). These results indicate that the pathways through which Ca^{2+} normally leaks from intracellular stores appear to be completely inhibited by Ni^{2+} (Figure 5A), but the mechanism that mediates CICR is insensitive to Ni^{2+}. For both IP_3 and ryanodine receptors, the stimulatory effect of Ca^{2+} is mimicked by Sr^{2+} but not by Ba^{2+}

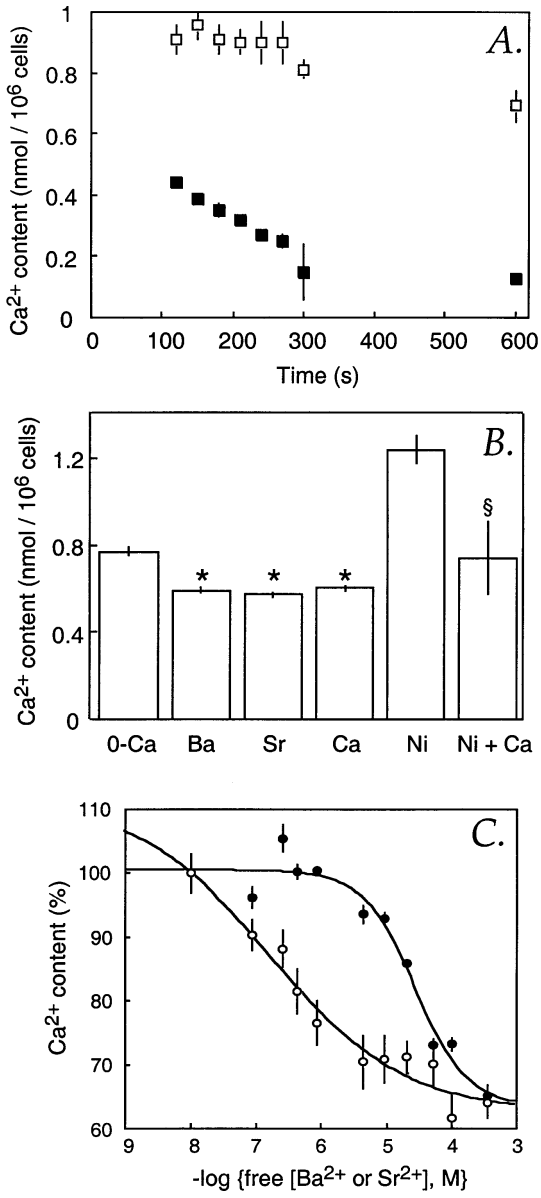

Figure 5 Effects of other bivalent cations on CICR

(**A**) Permeabilized cells loaded with $^{45}Ca^{2+}$ in normal CLM were diluted into CLM containing a free $[Ca^{2+}]$ of approx. 20 nM (□) or 100 μM (■), each containing 100 μM Ni^{2+}. The time courses of the subsequent change in the $^{45}Ca^{2+}$ contents of the stores are shown. (**B**) Cells loaded with $^{45}Ca^{2+}$ were diluted 5-fold into nominally Ca^{2+}-free CLM (0-Ca) or into CLM supplemented with the indicated bivalent cations, with final free concentrations of approx. 350 μM (Sr^{2+} and Ba^{2+}) or 100 μM (Ca^{2+} and Ni^{2+}). After 60 s, the $^{45}Ca^{2+}$ contents of the stores were determined. Results are shown as means ± S.E.M. ($n = 3$). *Significantly different from control of 0-Ca ($P < 0.05$). §Significantly different from Ni^{2+} alone ($P < 0.05$). (**C**) Cells loaded with $^{45}Ca^{2+}$ were diluted into CLM containing 1 μM thapsigargin and the indicated free concentrations of Sr^{2+} (○) or Ba^{2+} (●). Incubations were terminated after 2 min and the $^{45}Ca^{2+}$ contents of the stores are expressed as percentages of that observed for cells diluted into nominally Ca^{2+}-free CLM (means ± S.E.M., $n = 3$).

[37,38]. A similar pattern is evident for the CICR response. During an incubation lasting 60 s, a high concentration (free concentration of approx. 350 μM) of either Ba^{2+} or Sr^{2+} mimicked the effect of Ca^{2+} by stimulating release of approx. 25 % of the intracellular Ca^{2+} stores (Figure 5B). However, whereas Sr^{2+} (EC_{50} of approx. 166 nM) and Ca^{2+} ($EC_{50} = 170$ nM; Figure 1) were similarly potent in stimulating $^{45}Ca^{2+}$ release, Ba^{2+} ($EC_{50} = 28$ μM) was more than 150-fold less potent (Figure 5C). This pattern of regulation ($Ca^{2+} \approx Sr^{2+} \gg Ba^{2+}$) is consistent with the effects of these bivalent cations in regulating many Ca^{2+}-sensitive processes (see [37]).

Sustained, reversible activation of CICR

To explore the kinetics of CICR, we used rapid superfusion methods. For these experiments, permeabilized cells pre-loaded with $^{45}Ca^{2+}$ were immobilized on a filter array. This allows $^{45}Ca^{2+}$ efflux to be measured at 20 °C in the absence of ATP and without addition of thapsigargin (both of which were present in the earlier experiments), and under conditions where the free $[Ca^{2+}]$ could be rapidly increased from approx. 5 nM to 10 μM. Figure 6(A) shows that when the free $[Ca^{2+}]$ of the superfusing CLM was rapidly increased from approx. 5 nM to 10 μM, there was a substantial increase in the rate of $^{45}Ca^{2+}$ release, which then decayed ($t_{1/2} = 3.54 \pm 0.07$ s, $n = 3$) to a level (0.85 ± 0.05 %/s at infinite time) that remained significantly faster than the rate of $^{45}Ca^{2+}$ release from cells in nominally Ca^{2+}-free medium (0.43 ± 0.03 %/s at 30 s) (Figure 6A). Because the $^{45}Ca^{2+}$ content of the stores declines as $^{45}Ca^{2+}$ is released, we expect the amount of $^{45}Ca^{2+}$ released into each fraction to fall as the response progresses. However, during the 10 s following addition of 100 μM Ca^{2+}, the stimulated rate of CICR falls by approx. 70 % from its peak rate (Figure 6A), whereas the $^{45}Ca^{2+}$ content of the stores falls by less than 18 %; the declining rate of CICR is not therefore a simple consequence of the declining $^{45}Ca^{2+}$ content of the intracellular stores. We conclude that CICR responses slowly inactivate ($t_{1/2} = 3.54 \pm 0.07$ s), but that the inactivation is incomplete.

Figure 6(B) shows CICR results obtained at greater temporal resolution (80 ms) and with the free $[Ca^{2+}]$ of the CLM increased to 100 μM Ca^{2+} for only 500 ms before rapidly restoring it to approx. 5 nM. The results show that CICR is triggered within 80 ms of increasing the free $[Ca^{2+}]$ of the superfusing CLM and that when the free $[Ca^{2+}]$ is subsequently reduced, the rate of $^{45}Ca^{2+}$ release rapidly falls ($t_{1/2} = 529 \pm 17$ ms) to a rate (1.36 ± 0.04 %/s, $n = 3$) indistinguishable from that recorded before the Ca^{2+} stimulus (1.37 ± 0.06 %/s). These results establish that CICR is rapidly activated by an increase in free $[Ca^{2+}]$ and is rapidly and fully reversed when the $[Ca^{2+}]$ is reduced. The superfusion experiments are performed in the absence of ATP; neither activation of CICR nor its reversal can therefore require ATP. Furthermore, the rapid flow of medium around the cells during superfusion experiments (2 ml/s), together with our earlier demonstration that within the apparatus the intracellular stores are freely accessible to even very large molecules [14], effectively eliminate the possibility that any additional soluble messenger is required for the CICR.

Although prolonged (60 s) stimulation with maximally effective concentrations of IP_3 or Ca^{2+} release similar fractions of the intracellular Ca^{2+} stores (approx. 30 %; Figure 3A), the time courses of the responses are very different for the two stimuli. The peak rate of stimulated Ca^{2+} release evoked by 10 μM IP_3 (32.8 ± 1.4 %/s) is approx. ten times greater than that evoked by 10 μM Ca^{2+} (3.71 ± 0.21 %/s) (Figure 6C). But whereas the response to IP_3 is complete within 2 s (Figure 3C), the Ca^{2+} release evoked by increased $[Ca^{2+}]_c$ continues, despite a partial inactivation, for tens of seconds (Figure 6A). The inset to Figure 6(C) compares the responses to sustained stimulation with IP_3 and Ca^{2+} recorded under similar conditions and at the same temporal resolution.

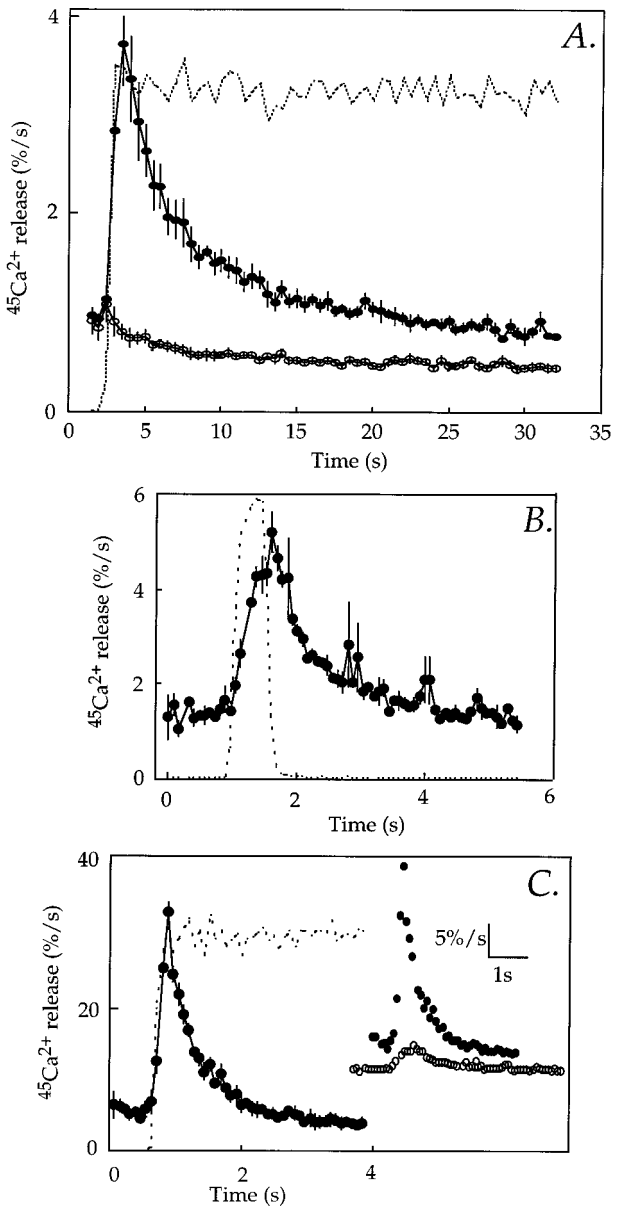

Figure 6 Kinetics of CICR

Permeabilized cells loaded with $^{45}Ca^{2+}$ in normal CLM were immobilized on the filters of a rapid superfusion apparatus and then continuously superfused with CLM (2 ml/s) while the effluent was collected into vials arranged around the circumference of a turntable. (**A**) Cells were superfused for 5 s with CLM containing a free $[Ca^{2+}]$ of approx. 5 nM and then the medium was switched to CLM with a free $[Ca^{2+}]$ of either approx. 5 nM (○) or 10 μM (●). The broken line denotes the arrival in the superfusate of [^{3}H]inulin, included as an inert marker in the CLM containing 10 μM Ca^{2+}. Fractions were collected at 500 ms intervals and the results are shown as percentages of the entire $^{45}Ca^{2+}$ content of the intracellular Ca^{2+} stores. (**B**) $^{45}Ca^{2+}$ release from cells stimulated for only 500 ms with CLM containing 100 μM Ca^{2+} (denoted by the broken line showing the [^{3}H]inulin marker). Fractions were collected at 80 ms intervals. (**C**) The main panel shows $^{45}Ca^{2+}$ release from cells continuously stimulated with 10 μM IP_3 (denoted by the broken line showing the [^{3}H]inulin marker). Fractions were collected at 80 ms intervals. The inset shows responses to IP_3 (10 μM; ●) and Ca^{2+} (10 μM; ○) collected under identical conditions and drawn to the same scale ($n = 3$, but error bars are omitted for clarity). All results (**A**–**C**) are presented as means ± S.E.M. ($n = 3$).

Conclusions

We conclude that the intracellular Ca^{2+} stores of hepatocytes express a temperature-sensitive, ATP-independent CICR mechanism that is reversibly activated by modest increases in $[Ca^{2+}]$. This Ca^{2+} release mechanism requires neither IP_3 nor ryanodine receptors, it is not mediated by the SERCA running in reverse, and it is not likely to be mediated by the intracellular Ca^{2+} channels that respond to NAADP, which are Ca^{2+}-insensitive [35,36], or sphingosine-1-phosphate. We considered the possibility that the $^{45}Ca^{2+}$ release detected might simply result from an increased rate of $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange, but that seems very unlikely. The CICR detected in our experiments is abolished at 2 °C (Figure 1A), it is activated by relatively low $[Ca^{2+}]_c$ (Figure 1), $^{45}Ca^{2+}$ efflux in normal CLM occurs at similar rates before and after 5-fold dilution of $^{45}Ca^{2+}$ specific acivity, CICR is mimicked by Sr^{2+} and with lesser potency by Ba^{2+} (Figures 5B and 5C), Ni^{2+} prevents the basal $^{45}Ca^{2+}$ leak without affecting CICR (Figure 5A), and CICR partially inactivates (Figures 6A and 6B). Finally, after rapid removal of Ca^{2+}, the free $[Ca^{2+}]$ of the superfusing medium falls below that required to evoke CICR within approx. 100 ms, yet CICR reverses more slowly ($t_{1/2} = 529 \pm 17$ ms) (Figure 6B). None of these characteristics are consistent with the effects of increased $[Ca^{2+}]_c$ resulting simply from enhanced $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange. Instead, they suggest that modest increases in $[Ca^{2+}]_c$ stimulate the opening of a channel that allows Ca^{2+} to leak from the intracellular stores. We have not established the identity of this novel Ca^{2+} release pathway, but it is likely to be directly regulated by Ca^{2+} with no further requirement for additional soluble messengers. This CICR mechanism is more sensitive to cytosolic Ca^{2+} than either ryanodine or IP_3 receptors; unlike these receptors it is not inhibited by further increases in $[Ca^{2+}]$, and, furthermore, it remains active during sustained increases in $[Ca^{2+}]$. We suggest that although the properties of IP_3 and ryanodine receptors are well suited to allowing brisk responses to local increases in $[Ca^{2+}]_c$, the characteristics of this novel CICR pathway may allow it to regulate the Ca^{2+} content of the intracellular stores of unstimulated cells. Furthermore, although the effects of modest increases in $[Ca^{2+}]_c$ must depend on the balance between stimulation of Ca^{2+} uptake by the endoplasmic reticulum and stimulation of Ca^{2+} release processes [12], the novel CICR mechanism we have described may provide an additional route for Ca^{2+} release that could allow even small Ca^{2+} signals to be amplified by CICR.

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