Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states

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Cytosolic Ca^{2+} biphasically regulates $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization in liver [Marshall and Taylor (1993) *J. Biol. Chem.* 268, 13214–13220]. We have investigated the mechanisms underlying this biphasic control of Ca^{2+} mobilization in permeabilized hepatocytes by comparing the effects of Sr^{2+} , Ba^{2+} and Ca^{2+} on the liver $\text{Ins}(1,4,5)\text{P}_3$ receptor. Both Ca^{2+} and Sr^{2+} increased the binding of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to liver membranes by converting receptors from a low-affinity ($K_D \sim 35 \text{ nM}$) to a high-affinity ($K_D \sim 5 \text{ nM}$) state. Ba^{2+} ($\leq 20 \mu\text{M}$) did not affect $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding. At concentrations similar to those that caused an enhancement of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding, Sr^{2+} ($\text{EC}_{50} = 570 \text{ nM}$) and Ca^{2+} ($\text{EC}_{50} = 200 \text{ nM}$) increased the sensitivity of the intracellular Ca^{2+} stores to $\text{Ins}(1,4,5)\text{P}_3$. Further modest elevations in $[\text{Ca}^{2+}]$ ($\text{EC}_{50} = 1.5 \mu\text{M}$) inhibited $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization, whereas Sr^{2+} caused

inhibition only when its concentration was very substantially increased ($\text{EC}_{50} \sim 900 \mu\text{M}$). Sr^{2+} is therefore only 3-fold less potent than Ca^{2+} in causing sensitization of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release, but 600-fold less potent in causing inhibition. Ba^{2+} neither sensitized ($[\text{Ba}^{2+}] \leq 20 \mu\text{M}$) nor inhibited ($[\text{Ba}^{2+}] \leq 1 \text{ mM}$) $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release, and did not inhibit either the sensitization of Ca^{2+} release evoked by Sr^{2+} or the inhibition of Ca^{2+} release evoked by Ca^{2+} . Our results suggest that two distinct Ca^{2+} -binding sites, which differ in their selectivities for bivalent cations, mediate the interconversion of $\text{Ins}(1,4,5)\text{P}_3$ receptors between at least three different conformational states. These two Ca^{2+} -binding sites, which may reside either on the $\text{Ins}(1,4,5)\text{P}_3$ receptor itself or on distinct regulatory proteins, can be distinguished by their different selectivities for bivalent cations.

INTRODUCTION

In liver, hormones that stimulate production of the Ca^{2+} -mobilizing second messenger, inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], induce repetitive Ca^{2+} waves which propagate through the cytosol from a fixed origin (Rooney et al., 1990). The propagation of Ca^{2+} waves in *Xenopus* oocytes (DeLisle and Welsh, 1992) and fertilized hamster eggs (Miyazaki et al., 1992) has been shown to be dependent on $\text{Ins}(1,4,5)\text{P}_3$, suggesting that these waves reflect the progressive mobilization of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores. While the underlying mechanisms are strongly debated, a popular model suggests that Ca^{2+} -evoked changes in the sensitivities of $\text{Ins}(1,4,5)\text{P}_3$ receptors may contribute to the propagation of these Ca^{2+} waves (see Taylor and Marshall, 1992).

In hepatocytes, cytosolic Ca^{2+} regulates $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization in a biphasic manner (Marshall and Taylor, 1993; Combettes et al., 1993). Small elevations in cytosolic $[\text{Ca}^{2+}]$ sensitize the intracellular stores to $\text{Ins}(1,4,5)\text{P}_3$, whereas larger elevations are inhibitory. These two effects combine to biphasically regulate Ca^{2+} release in response to low concentrations of $\text{Ins}(1,4,5)\text{P}_3$ (Marshall and Taylor, 1993). Similar biphasic regulation of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization has been reported in a number of other tissues (Iino, 1990; Suarez-Isla et al., 1991; Bezprozvanny et al., 1991; Finch et al., 1991; Zhang et al., 1993), although the sites through which Ca^{2+} regulates $\text{Ins}(1,4,5)\text{P}_3$ -receptor activity are unknown.

The bivalent cations, Sr^{2+} and Ba^{2+} , substitute for Ca^{2+} in eliciting a number of cellular processes (Uvelius et al., 1974; Baba et al., 1985; Schilling et al., 1989). Both Sr^{2+} and Ba^{2+}

mimic Ca^{2+} in their ability to pass through $\text{Ins}(1,4,5)\text{P}_3$ receptors (Bezprozvanny and Ehrlich, 1993) and cardiac ryanodine receptors (Tinker et al., 1992); the rank order of conductances of these cations is the same for each type of Ca^{2+} channel ($\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+}$). Also, both Sr^{2+} and Ba^{2+} substitute for Ca^{2+} in stimulating Ca^{2+} release from the sarcoplasmic reticulum of frog skeletal muscle (Potreau and Raymond, 1980; Cognard and Raymond, 1985). In general, Sr^{2+} is a better surrogate for Ca^{2+} than is Ba^{2+} : Sr^{2+} , but not Ba^{2+} , substitutes for Ca^{2+} in activating Ca^{2+} -sensitive K^+ channels (Haddas et al., 1979); Sr^{2+} and Ca^{2+} , but not Ba^{2+} , are transported by the Ca^{2+} -ATPases of both the sarcoplasmic (Somlyo and Somlyo, 1971; Fujimori and Jencks, 1992) and endoplasmic (Kwan and Putney, 1990) reticula; and refilling of intracellular stores with Sr^{2+} or Ca^{2+} , but not with Ba^{2+} , is reported to switch off the capacitative Ca^{2+} entry pathway (Ozaki et al., 1992).

In the present study, we have investigated the mechanisms which underlie the biphasic effects of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization in permeabilized hepatocytes using Sr^{2+} and Ba^{2+} as surrogates for Ca^{2+} . Our results suggest that the biphasic effects of cytosolic Ca^{2+} result from Ca^{2+} -regulated interconversion between at least three conformational states of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. These effects are mediated via two distinct Ca^{2+} -binding sites.

EXPERIMENTAL

Materials

ATP, calmidazolium (R24571), collagenase, creatine phosphokinase and creatine phosphate were from Boehringer. BaCl_2 ,

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; $[\text{Ca}^{2+}]_m$, medium free Ca^{2+} concentration; CICR, Ca^{2+} -induced Ca^{2+} release; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone; n_H , Hill coefficient; λ_{ex} , fluorescence excitation wavelength; λ_{em} , fluorescence emission wavelength; $[\text{X}^{2+}]$, free bivalent cation concentration; *F*, Fura-2 fluorescence.

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BSA, Eagle's minimal essential medium, EGTA, carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone (FCCP), saponin, SrCl_2 , thapsigargin and trifluoperazine were from Sigma. $\text{Ins}(1,4,5)\text{P}_3$ was from Dr. R. F. Irvine (Babraham, Cambs., U.K.). Ionomycin and thapsigargin were from Calbiochem. Fura-2 pentapotassium salt was from Molecular Probes. Percoll ($d = 1.13 \text{ g/ml}$) was from Pharmacia. All other reagents were from BDH.

Preparation of liver membranes

Membranes were prepared from rat liver by Percoll gradient centrifugation (Prpic et al., 1984), as previously described (Dasso and Taylor, 1991). Briefly, the liver of a male Wistar rat (250–300 g) was perfused via the portal vein with cold buffered saline (118 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 0.96 mM NaH_2PO_4 , 25 mM NaHCO_3 , 1 mM EGTA, 95:5 O_2 : CO_2 , pH 7.4 at 4 °C). The liver was then homogenized in Hepes-buffered sucrose medium (250 mM sucrose, 5 mM Hepes, 1 mM EGTA, pH 7.4 at 4 °C) with a Dounce homogenizer (ten passes with a loose-fitting plunger and three passes with a tight-fitting plunger). The homogenate was centrifuged (2500 g, 10 min) and the pellet resuspended in the buffered sucrose medium (to 24 ml) supplemented with Percoll (3.2 ml). After centrifugation (35000 g, 30 min) the membranes were collected as a discrete fluffy layer close to the top of the tube. The membranes were resuspended in hypo-osmotic medium (5 mM Hepes, 1 mM EGTA, pH 7.4, at 4 °C) in order to lyse membrane vesicles. After centrifugation (48000 g, 10 min), membranes were resuspended (approx. 2 mg of protein/ml) in buffered saline (20 mM Hepes, 1 mM EGTA, 10 mM MgCl_2 , pH 7.4, at 4 °C) and stored at –70 °C.

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to liver membranes

To allow comparison of the effects of bivalent cations on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization and on $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to liver membranes, binding studies were conducted, as far as possible, under the same conditions as Ca^{2+} -flux assays. Membranes were resuspended in cytosol-like medium (140 mM KCl, 20 mM NaCl, 1 mM MgCl_2 , 10 mM EGTA, 20 mM Pipes, pH 7) supplemented with Ca^{2+} , Sr^{2+} or Ba^{2+} to give the required concentration of free bivalent cations in the medium ($[\text{X}^{2+}]_m$). For each solution, $[\text{X}^{2+}]_m$ was calculated from the stability constants for X^{2+} binding to EGTA (Martell and Smith, 1974) under the appropriate conditions of temperature, pH and ionic concentration using the computer program Maxchelator (Dr. C. Patton, Stanford, CA, U.S.A.). $[\text{X}^{2+}]_m$ was checked using Fura-2 (see below) and the $[\text{X}^{2+}]_m$ obtained from these measurements are shown in the Results section (unless otherwise indicated). The membranes were pre-incubated for 15 min at 37 °C, and then incubated for 5 min at 4 °C with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (0.5 nM; 40 Ci/mmol) and unlabelled $\text{Ins}(1,4,5)\text{P}_3$. A preincubation period at 37 °C was necessary to allow the complete conversion of $\text{Ins}(1,4,5)\text{P}_3$ receptors into a low-affinity conformation in low $[\text{X}^{2+}]_m$ (Pietri et al., 1990), whereas a temperature of 4 °C was necessary to successfully measure binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$. This reduction in temperature reduced $[\text{X}^{2+}]_m$ by ~20% according to results calculated using Maxchelator (based on the temperature dependence of EGTA association constants reported by Harrison and Bers, 1989). Earlier work established that equilibrium binding was attained within 5 min. Incubations were terminated by rapid dilution with ice-cold sucrose (310 mM) and filtration through Whatman GF/B filters using a Brandel receptor-binding har-

vester. Non-specific binding was determined in the presence of 10 μM $\text{Ins}(1,4,5)\text{P}_3$.

Preparation of permeabilized hepatocytes

Hepatocytes were isolated from male Wistar rats (200–400 g) by collagenase digestion (Nunn and Taylor, 1990). Cells were stored for up to 24 h at 4 °C in Eagle's minimal essential medium gassed with O_2 : CO_2 (95:5) and supplemented with NaHCO_3 (26 mM) and 2% (w/v) BSA. Shortly before use, cells were resuspended in Ca^{2+} -free cytosol-like medium (140 mM KCl, 20 mM NaCl, 1 mM MgCl_2 , 1 mM EGTA, 20 mM Pipes, pH 7) and permeabilized by incubating with 10 $\mu\text{g/ml}$ saponin (10 min, 37 °C). Permeabilized cells (>98%) were washed once by centrifugation (600 g, 3 min) before use.

$^{45}\text{Ca}^{2+}$ flux measurements

Permeabilized hepatocytes (10^7 cells/ml) were resuspended in the cytosol-like medium supplemented with $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci/ml}$), the mitochondrial inhibitor FCCP (10 μM), and enough CaCl_2 (390 μM) to give a final free $[\text{Ca}^{2+}]$ of 130 nM. ATP (4.5 mM) and an ATP-regenerating system comprising creatine phosphate (15 mM) and creatine phosphokinase (15 units/ml) were added, and after 5 min at 37 °C the non-mitochondrial intracellular stores had loaded to steady state with $^{45}\text{Ca}^{2+}$. After loading to steady state, cells were diluted 5-fold into cytosol-like medium containing FCCP (10 μM), thapsigargin (1 μM), an increased concentration of EGTA (10 mM) and appropriate concentrations of CaCl_2 , SrCl_2 or BaCl_2 (see above). This method allowed $[\text{X}^{2+}]_m$ to be changed simultaneously with the inhibition of further uptake of bivalent cations into intracellular stores. By increasing the total EGTA concentration after loading of cells with Ca^{2+} , the effects of residual free Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization were minimized. Unless otherwise indicated, the free Ca^{2+} concentration in the medium ($[\text{Ca}^{2+}]_m$) remained below 5 nM following the dilution of cells with media supplemented with Sr^{2+} or Ba^{2+} . ATP-dependent $^{45}\text{Ca}^{2+}$ accumulation was assessed following rapid filtration of the cells through Whatman GF/C filters using a Brandel receptor-binding harvester (Nunn and Taylor, 1992).

Fluorimetric measurement of $[\text{X}^{2+}]_m$

For measurements of $[\text{X}^{2+}]_m$ in incubation media, the fluorescent indicator Fura-2 was used. Fura-2 pentapotassium salt (1 μM) was added to media (37 °C), and Fura-2 fluorescence emission was measured ($\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 510 \text{ nm}$). Fura-2 fluorescence (F) was calibrated according to the equation: $[\text{X}^{2+}]_m = K_D(F - F_{\text{min}})/(F_{\text{max}} - F)$ (Gryniewicz et al., 1985) where K_D is the equilibrium dissociation constant for the binding of the bivalent cation X^{2+} to Fura-2 (at 37 °C, K_D for $\text{Ca}^{2+} = 224 \text{ nM}$; K_D for $\text{Sr}^{2+} = 7600 \text{ nM}$; K_D for $\text{Ba}^{2+} = 1360 \text{ nM}$; Kwan and Putney, 1990), F_{max} is the fluorescence of the dye after addition of excess X^{2+} , and F_{min} is the fluorescence of the dye in the absence of X^{2+} . F_{min} was calculated from the equation $F_{\text{min}} = \frac{1}{3}(F_{\text{max}} - F_{\text{Mn}}) + F_{\text{Mn}}$ (Merritt et al., 1990), where F_{Mn} is the Fura-2 fluorescence in the presence of saturating levels of MnCl_2 (10 mM).

Analysis of data

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ displacement curves were analysed by EBDA-LIGAND (BIOSOFT, Cambridge, U.K.) using the experimentally determined value for non-specific binding as an initial estimate for the computer analysis. For each displacement curve, a statistical comparison was made, according to the 'extra sum

of squares' test (Rodbard, 1974), between models consisting of one and two $\text{Ins}(1,4,5)\text{P}_3$ -binding sites. For Ca^{2+} flux assays, concentration-response data were fitted to a four-parameter logistic equation using GraphPAD InPlot 4.0 software (GraphPAD Software, SanDiego, CA, U.S.A.).

RESULTS

Effect of bivalent cations on the binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to liver membranes

Increasing $[\text{Ca}^{2+}]_m$ caused a concentration-dependent increase in the binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to liver membranes ($\text{EC}_{50} \sim 130 \text{ nM}$; Figure 1a); this effect was mimicked by Sr^{2+} ($\text{EC}_{50} \sim 2.2 \mu\text{M}$), but not by Ba^{2+} (Figure 1b). Analysis of equilibrium competition binding curves in media containing different concentrations of free bivalent cations revealed that both Ca^{2+} and Sr^{2+} , but not Ba^{2+} , increased the affinity, but not

Figure 1 Effect of bivalent cations on specific binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to liver membranes

Specific binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (0.5 nM) to liver membranes in the presence of various $[\text{Ca}^{2+}]_m$ (a), $[\text{Sr}^{2+}]_m$ or $[\text{Ba}^{2+}]_m$ (b) is shown. Results are expressed relative to the amount of specifically bound $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ in media with $[\text{Ca}^{2+}]_m < 5 \text{ nM}$. Total $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding was typically 1300 d.p.m./incubation and non-specific binding was $\sim 400 \text{ d.p.m./incubation}$. Results are expressed as means $\pm$ S.E.M. of three to six independent experiments.

Figure 2 Effect of bivalent cations on the displacement of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ from liver membranes

Membranes were incubated with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (0.5 nM) and various concentrations of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ in media containing the indicated $[\text{Ca}^{2+}]_m$ (a), $[\text{Sr}^{2+}]_m$ (b), or $[\text{Ba}^{2+}]_m$ (c). Results are expressed as specific $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ bound to membranes. For each displacement curve, a statistical comparison was made between models consisting of one and two $\text{Ins}(1,4,5)\text{P}_3$ -binding sites (see the Experimental section). The results of single experiments, each reproduced in three independent experiments, are shown.

Table 1 Effects of bivalent cations on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to liver membranes

The table summarizes the results of equilibrium competition binding studies similar to those shown in Figure 2. For each displacement curve, the affinity (K_D) and total number (B_{max}) of binding sites, and the Hill coefficient (n_H) were calculated from curve-fitting using EBDA-LIGAND. The equilibrium dissociation constants (K_D) are expressed as geometrical means and the range of values shown in parentheses are the mean $\pm$ S.E.M. calculated from the logarithms of the values from three independent experiments.

$[\text{X}^{2+}]_m$ (μM)	K_D (nM)	B_{max} (pmol/mg of protein)	n_H
Ca^{2+}			
0.003	37 (36–38)	1.07 ± 0.26	0.82 ± 0.10
0.6	3.3 (1.1–10)	1.56 ± 0.77	1.02 ± 0.07
2.6	7.4 (5.1–11)	2.54 ± 1.90	0.87 ± 0.03
Sr^{2+}			
1	25 (16–38)	1.29 ± 0.76	0.73 ± 0.08
20	5.6 (3.2–9.8)	1.52 ± 0.77	0.95 ± 0.13
Ba^{2+}			
1	32 (30–36)	1.50 ± 0.40	0.74 ± 0.04
20	42 (26–66)	2.15 ± 0.53	0.67 ± 0.04

the total number, of $\text{Ins}(1,4,5)\text{P}_3$ -binding sites (Figure 2 and Table 1). The affinities of the sites determined in medium with $2.6 \mu\text{M}$ $[\text{Ca}^{2+}]_m$ ($K_D = 7.4 \text{ nM}$; see Table 1) and in medium with $20 \mu\text{M}$ $[\text{Sr}^{2+}]_m$ ($K_D = 5.6 \text{ nM}$) were not significantly different. In all cases, individual displacement curves were statistically more consistent with a model containing only a single class of $\text{Ins}(1,4,5)\text{P}_3$ -binding sites, as determined from the 'extra sum of squares' test (see the Experimental section). These results suggest that Ca^{2+} and Sr^{2+} regulate the interconversion of high- and low-affinity conformations of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

Effects of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization

Previously, we reported that cytosolic Ca^{2+} biphasically regulates $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization in hepatocytes (Marshall and Taylor, 1993). In those experiments, changes in $[\text{Ca}^{2+}]_m$ were accompanied by changes in the concentration of

Figure 3 Regulation of $Ins(1,4,5)P_3$ -mediated Ca^{2+} release by $[Ca^{2+}]_m$

Permeabilized hepatocytes were loaded to steady state with $^{45}Ca^{2+}$ and $[Ca^{2+}]_m$ was then adjusted by dilution into medium containing 10 mM EGTA (final EGTA concentration = 8.2 mM) as described in the Experimental section. After 60 s, cells were exposed for 30 s to $Ins(1,4,5)P_3$. (a) Effects of $[Ca^{2+}]_m$ on the EC_{50} for $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization. (b) Effects of $[Ca^{2+}]_m$ on the relative size of the $Ins(1,4,5)P_3$ -sensitive stores. The amount of Ca^{2+} released by 10 μM $Ins(1,4,5)P_3$ at each $[Ca^{2+}]_m$ is expressed relative to the proportion released from cells incubated in medium containing < 5 nM Ca^{2+} . For comparison, dotted lines show results from similar experiments performed with medium containing 1 mM EGTA (Marshall and Taylor, 1993). Results are expressed as means $\pm$ S.E.M. of three independent experiments; error bars smaller than the symbols are not shown.

uncomplexed EGTA, which we have subsequently demonstrated to be a competitive antagonist of the $Ins(1,4,5)P_3$ receptor (Richardson and Taylor, 1993). In the present study, similar experiments were conducted in the presence of a 10-fold higher concentration of EGTA. Elevating $[Ca^{2+}]_m$ still had biphasic effects on $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release: half-maximal sensitization and inhibition of the response occurred when $[Ca^{2+}]_m$ was 200 nM and 1.51 μM respectively (Figure 3). These effects, and their sensitivities to Ca^{2+} , are indistinguishable from our earlier results using media containing only 1 mM EGTA (Marshall and Taylor, 1993; dotted lines in Figure 3), and can therefore be directly attributed to changes in $[Ca^{2+}]_m$. The

Figure 4 Regulation of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release by $[Sr^{2+}]_m$ and $[Ba^{2+}]_m$

Permeabilized hepatocytes were loaded to steady state with $^{45}Ca^{2+}$ in medium containing 130 nM $[Ca^{2+}]_m$, $[Sr^{2+}]_m$ or $[Ba^{2+}]_m$ was then adjusted to the levels shown in the panels (final EGTA concentration = 8.2 mM) and after 60 s cells were exposed to $Ins(1,4,5)P_3$ for 30 s. $[Ca^{2+}]_m$ was always < 5 nM after dilution into the Sr^{2+} - or Ba^{2+} -containing media. (a) Effects of $[Sr^{2+}]_m$ and $[Ba^{2+}]_m$ on the EC_{50} for $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization. (b) Effects of $[Sr^{2+}]_m$ and $[Ba^{2+}]_m$ on the relative size of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} store. The amount of Ca^{2+} released by 10 μM $Ins(1,4,5)P_3$ at each $[Sr^{2+}]_m$ or $[Ba^{2+}]_m$ is expressed relative to the proportion released from cells incubated in medium containing < 5 nM Ca^{2+} and no added Sr^{2+} or Ba^{2+} . Results are expressed as means $\pm$ S.E.M. of three to seven independent experiments; error bars smaller than symbols are not shown.

calmodulin antagonists trifluoperazine (50 μM) and calmidazolium (50 μM) had no effect on either the sensitization of Ca^{2+} release induced by increasing $[Ca^{2+}]_m$ to 600 nM or the inhibition of Ca^{2+} release evoked by increasing $[Ca^{2+}]_m$ to 2.6 μM (results not shown).

Effects of Sr^{2+} and Ba^{2+} on $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization

The sensitivity of the intracellular stores to $Ins(1,4,5)P_3$ increased with increasing $[Sr^{2+}]_m$ (Figure 4a): the EC_{50} for $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization decreased from 299 ± 32 nM

Figure 5 Effects of bivalent cations on Ca^{2+} release in response to a submaximal concentration of $\text{Ins}(1,4,5)\text{P}_3$

The effects of a submaximal concentration of $\text{Ins}(1,4,5)\text{P}_3$ (100 nM) on Ca^{2+} release from permeabilized cells in the presence of various $[\text{Ca}^{2+}]_m$ (a), $[\text{Sr}^{2+}]_m$ or $[\text{Ba}^{2+}]_m$ (b) are shown. The amount of Ca^{2+} released by $\text{Ins}(1,4,5)\text{P}_3$ is expressed as a fraction of the amount of Ca^{2+} released by 10 μM ionomycin under the same conditions. Results are shown as means $\pm$ S.E.M. of three to seven independent experiments.

($n = 9$) to 54 ± 10 nM ($n = 3$) as the $[\text{Sr}^{2+}]_m$ increased from 0 to 20 μM . This effect of Sr^{2+} was half-maximal when $[\text{Sr}^{2+}]_m$ was 570 nM, only ~ 3 -fold higher than the $[\text{Ca}^{2+}]_m$ required for half-maximal sensitization of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization ($\text{EC}_{50} = 200$ nM; Figure 3a). Thus Sr^{2+} mimics Ca^{2+} in sensitizing Ca^{2+} release to $\text{Ins}(1,4,5)\text{P}_3$. The maximal effect of Sr^{2+} in sensitizing Ca^{2+} release was greater [Figure 4a; EC_{50} for $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release with maximally effective $[\text{Sr}^{2+}]_m = 54 \pm 10$ nM] than the maximal effect of Ca^{2+} (Figure 3a; EC_{50} for Ca^{2+} release with maximally effective $[\text{Ca}^{2+}]_m = 98 \pm 19$ nM). This observation may reflect our inability to determine the EC_{50} for Ca^{2+} release in the presence of higher $[\text{Ca}^{2+}]_m$ due to the inhibitory effect of high $[\text{Ca}^{2+}]_m$ (Figure 3). By contrast, $[\text{Sr}^{2+}]_m$ could be more substantially elevated without

causing inhibition of Ca^{2+} release (see below). Although increasing $[\text{Ca}^{2+}]_m$ to > 1 μM inhibited the response to $\text{Ins}(1,4,5)\text{P}_3$ (Figure 3b), increasing $[\text{Sr}^{2+}]_m$ to 20 μM caused no inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization (Figure 4b). When the $[\text{Sr}^{2+}]_m$ was very substantially increased, to ~ 1 mM, the response to a maximal concentration of $\text{Ins}(1,4,5)\text{P}_3$ (10 μM) was inhibited by $64 \pm 11\%$ ($n = 3$). Under these conditions, $[\text{Ca}^{2+}]_m$ was elevated to only 46 nM after addition of Sr^{2+} (as determined from Maxchelator), and therefore could not account for the inhibition of Ca^{2+} release observed. Inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release was therefore approx. 600-fold less sensitive to Sr^{2+} than to Ca^{2+} .

Ba^{2+} (≤ 20 μM) did not significantly affect either the sensitivity of the intracellular Ca^{2+} stores to $\text{Ins}(1,4,5)\text{P}_3$ (Figure 4a) or the amount of Ca^{2+} released by a maximal concentration of $\text{Ins}(1,4,5)\text{P}_3$ (Figure 4b). When $[\text{Ba}^{2+}]_m$ was increased to ~ 1 mM, Ba^{2+} was still unable to inhibit $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization. However, there was an increase in the sensitivity of the stores to $\text{Ins}(1,4,5)\text{P}_3$: the fraction of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores released by a submaximal concentration of $\text{Ins}(1,4,5)\text{P}_3$ (100 nM) increased from $20 \pm 10\%$ to $68 \pm 23\%$ ($n = 3$). Although this may be a direct effect of the increase in $[\text{Ba}^{2+}]_m$, it is more likely to be a consequence of the concomitant increase in $[\text{Ca}^{2+}]_m$ (from ~ 3 nM to ~ 40 nM) as Ba^{2+} displaces Ca^{2+} from EGTA.

Figure 5 compares the effects of different bivalent cations on responses to a submaximal concentration (100 nM) of $\text{Ins}(1,4,5)\text{P}_3$. The two effects of Ca^{2+} combined to biphasically regulate the amount of Ca^{2+} released by 100 nM $\text{Ins}(1,4,5)\text{P}_3$ (Figure 5a; Marshall and Taylor, 1993): the maximum response to 100 nM $\text{Ins}(1,4,5)\text{P}_3$ occurred in the range of 300–600 nM $[\text{Ca}^{2+}]_m$. By contrast, Sr^{2+} (≤ 20 μM) caused a concentration-dependent increase in the amount of Ca^{2+} released by 100 nM $\text{Ins}(1,4,5)\text{P}_3$ (Figure 5b): 100 nM $\text{Ins}(1,4,5)\text{P}_3$ released $5 \pm 1\%$ ($n = 3$) of the total Ca^{2+} store in medium containing no Sr^{2+} , and $23 \pm 5\%$ in medium containing 20 μM Sr^{2+} . Sr^{2+} inhibited the Ca^{2+} release evoked by 100 nM $\text{Ins}(1,4,5)\text{P}_3$ only when $[\text{Sr}^{2+}]_m$ was very substantially elevated: at 1 mM $[\text{Sr}^{2+}]_m$, 100 nM $\text{Ins}(1,4,5)\text{P}_3$ released $4 \pm 2\%$ of the total Ca^{2+} store ($n = 3$). Ba^{2+} (≤ 20 μM) had no effect on the amount of Ca^{2+} released by 100 nM $\text{Ins}(1,4,5)\text{P}_3$ (Figure 5b).

Effect of Ba^{2+} on the ability of Ca^{2+} and Sr^{2+} to regulate $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release

The inability of Ba^{2+} either to sensitize (≤ 20 μM) or to inhibit (≤ 1 mM) Ca^{2+} release induced by $\text{Ins}(1,4,5)\text{P}_3$ could be explained if Ba^{2+} either failed to bind to the sites through which Sr^{2+} and Ca^{2+} regulated $\text{Ins}(1,4,5)\text{P}_3$ -receptor activity, or acted as an antagonist at the Ca^{2+} -binding sites. To distinguish between these possibilities, the ability of Ba^{2+} to antagonize the effects of Ca^{2+} and Sr^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release was assessed. Elevating $[\text{Ba}^{2+}]_m$ to 1 mM (9.2 mM total $[\text{Ba}^{2+}]_m$) was unable to block the inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release induced by exposure of cells to high $[\text{Ca}^{2+}]_m$ (Figure 6).

It was more difficult to determine whether Ba^{2+} antagonized the sensitization of Ca^{2+} release by elevated $[\text{Ca}^{2+}]_m$. If $[\text{Ca}^{2+}]_m$ was elevated to induce sensitization of Ca^{2+} release, then subsequent addition of Ba^{2+} to raise $[\text{Ba}^{2+}]_m$ to 1 mM would result in displacement of sufficient Ca^{2+} from buffers to substantially raise $[\text{Ca}^{2+}]_m$ and indirectly inhibit $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release. It would not therefore be possible to assess whether Ba^{2+} had antagonized the sensitizing effect of Ca^{2+} on Ca^{2+} release. A solution to this problem was provided by the ability of Sr^{2+} to sensitize $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release at much lower

Figure 6 Effect of high $[Ba^{2+}]_m$ on the sensitization and inhibition of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization by bivalent cations

Permeabilized hepatocytes were loaded to steady state with $^{45}Ca^{2+}$, and $[X^{2+}]_m$ was adjusted by addition of Ca^{2+} (7.4 mM total concentration), Sr^{2+} (120 μM) or Ba^{2+} (9.2 mM) (as indicated). After 60 s, cells were exposed to $Ins(1,4,5)P_3$ for 30 s. In control samples, $[Ca^{2+}]_m$ was < 5 nM. (a) Effect of 9.2 mM Ba^{2+} on the sensitization of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization induced by 120 μM total $[Sr^{2+}]_m$ ($[Sr^{2+}]_m$ was 1 μM in the absence of Ba^{2+} and ~12 μM in the presence of Ba^{2+} ; $[Ba^{2+}]_m$ was 1 mM). (b) Effect of 9.2 mM Ba^{2+} on the inhibition of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} mobilization by 7.4 mM total $[Ca^{2+}]_m$ ($[Ca^{2+}]_m$ was 2.6 μM in the absence of Ba^{2+} and 180 μM in the presence of Ba^{2+} ; $[Ba^{2+}]_m$ was ~7.3 mM). Results are expressed as means $\pm$ S.E.M. of three independent experiments.

Table 2 Biphasic regulation of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release shows specificity for different bivalent cations

The concentrations (EC_{50}) of Ca^{2+} , Sr^{2+} and Ba^{2+} required to half-maximally sensitize or inhibit $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release were determined from the experiments described in Figure 3. Parentheses indicate that there was no effect of Ba^{2+} ($\leq 20 \mu M$ $[Ba^{2+}]_m$) on the sensitization of Ca^{2+} release, although the effects of higher $[Ba^{2+}]_m$ on this response could not be determined (see the Results section).

	$[Ca^{2+}]_m$	$[Sr^{2+}]_m$	$[Ba^{2+}]_m$
EC_{50} for sensitization of Ca^{2+} release	200 nM	570 nM	(None)
EC_{50} for inhibition of Ca^{2+} release	1.5 μM	~ 900 μM	None

concentrations than those required to inhibit release (Figure 4; Table 2). Exposure of cells to 1 μM $[Sr^{2+}]_m$ caused a sensitization of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release. When 9.2 mM Ba^{2+} was then added, raising $[Ba^{2+}]_m$ to 1 mM, Sr^{2+} was displaced from buffers resulting in an elevation of $[Sr^{2+}]_m$ to 12 μM : this $[Sr^{2+}]_m$ was insufficient to inhibit $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release (Figure 4). Therefore, the effects of 1 mM $[Ba^{2+}]_m$ on the sensitization of Ca^{2+} release by Sr^{2+} could be assessed independently of any inhibitory effects of Sr^{2+} . Elevating $[Ba^{2+}]_m$ to 1 mM was unable to inhibit the sensitization of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release induced by elevation of $[Sr^{2+}]_m$ from 0 to 1 μM (Figure 6). Indeed, addition of 1 mM $[Ba^{2+}]_m$ significantly reduced the EC_{50} for $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release from 219 ± 9 nM to 116 ± 27 nM ($P < 0.01$; $n = 3$) (Figure 6): it was unclear, though, whether this further sensitization of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release resulted from a direct effect of elevated $[Ba^{2+}]_m$ or from the concomitant elevation in $[Sr^{2+}]_m$.

Figure 7 Two distinct Ca^{2+} -binding sites mediating the regulatory effects of cytosolic Ca^{2+} on liver $Ins(1,4,5)P_3$ receptors

The regulatory effects of cytosolic Ca^{2+} on the liver $Ins(1,4,5)P_3$ receptor are mediated via two distinct Ca^{2+} -binding sites (sites 1 and 2). Site 1, mediating the conversion of $Ins(1,4,5)P_3$ receptors from an active, low-affinity (R_L) state into an active, high-affinity (R_H) state, binds Ca^{2+} with 3-fold higher affinity than it binds Sr^{2+} . Ba^{2+} is either not recognized by this site, or else it binds with very low affinity. Site 2, mediating the conversion of $Ins(1,4,5)P_3$ receptors from an inactive into an active high-affinity state (R_H'), binds Ca^{2+} with ~600-fold higher affinity than it binds Sr^{2+} . Ba^{2+} is not recognized by site 2. Although we have previously shown the complete reversibility of the sensitization phase and substantial reversibility of the inhibitory phase (Marshall and Taylor, 1993), we have not investigated the conformational states through which the receptor passes before returning to the low-affinity state.

DISCUSSION

Consistent with earlier reports (Pietri et al., 1990), both Ca^{2+} and Sr^{2+} increased the binding of $Ins(1,4,5)P_3$ to liver membranes by converting $Ins(1,4,5)P_3$ receptors from a low-affinity into a high-affinity conformation (Figure 1; Table 1). Under similar conditions, Ca^{2+} and Sr^{2+} also sensitized $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release (Figures 3 and 4): these two effects occurred over a similar concentration range for each bivalent cation, suggesting that sensitization of $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release results from the formation of a high-affinity, Ca^{2+} -conducting conformation of the $Ins(1,4,5)P_3$ receptor. At higher concentrations, both Ca^{2+} and Sr^{2+} inhibited Ca^{2+} release with no further change in the affinity of receptors for $Ins(1,4,5)P_3$ (Table 1), indicating the existence of a high-affinity, desensitized state of the $Ins(1,4,5)P_3$ receptor.

Despite the ability of Ca^{2+} and Sr^{2+} to both sensitize and inhibit Ca^{2+} release, the two bivalent cations showed marked differences in their selectivities for these two effects. In the case of Ca^{2+} , the sensitization of Ca^{2+} release occurred at 7.5-fold lower $[Ca^{2+}]_m$ than the inhibition, whereas with Sr^{2+} , inhibition of Ca^{2+} release occurred only when $[Sr^{2+}]_m$ was ~1500-fold higher than that required to sensitize the Ca^{2+} release mechanism (Table 2).

In contrast with Ca^{2+} and Sr^{2+} , Ba^{2+} ($\leq 20 \mu M$) had no effect on either the binding of $Ins(1,4,5)P_3$ to its receptor (Figure 1; Table 1) or $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release (Figure 4). At much higher concentrations, Ba^{2+} apparently sensitized $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release, but this effect was complicated by a concomitant elevation in $[Ca^{2+}]_m$, as described in the Results section.

The most obvious explanation for these results is that the biphasic effects of bivalent cations on the liver $Ins(1,4,5)P_3$ receptor are mediated by two distinct Ca^{2+} -binding sites (see Figure 7). The site to which Ca^{2+} binds to cause the $Ins(1,4,5)P_3$

receptor to switch to a high-affinity, Ca^{2+} -conducting conformation (site 1) binds Sr^{2+} with only 3-fold-lower affinity than it binds Ca^{2+} . However, the site to which Ca^{2+} binds to cause the $\text{Ins}(1,4,5)\text{P}_3$ receptor to switch to a high-affinity, inactivated state (site 2) binds Sr^{2+} approx. 600-fold more weakly than it does Ca^{2+} (Table 2). Ba^{2+} is either not recognized by site 1 or else it binds with very low affinity to the site as it is unable to sensitize Ca^{2+} release at concentrations up to 20 μM (Figure 4), and 1 mM $[\text{Ba}^{2+}]_m$ does not inhibit the sensitization of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} released induced by Sr^{2+} (Figure 6). Ba^{2+} is not recognized by site 2 as it neither mimics (Figure 4) nor antagonizes (Figure 6) the inhibitory effect of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release.

The locations of these regulatory Ca^{2+} -binding sites are unknown. Ca^{2+} reportedly binds, albeit with low affinity ($\sim 50 \mu\text{M}$), to the purified cerebellar $\text{Ins}(1,4,5)\text{P}_3$ receptor (Danoff et al., 1988), and Ca^{2+} -binding domains have been located close to the transmembrane regions of both the type-1 and type-2 $\text{Ins}(1,4,5)\text{P}_3$ receptors (Mignery et al., 1992). Another possibility is that the Ca^{2+} -binding sites reside on separate regulatory proteins. In the AR42J pancreatic acinar cell line, inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release by cytosolic $[\text{Ca}^{2+}]$ was shown to be abolished when cells were preincubated in nominally Ca^{2+} -free medium (Zhao and Muallem, 1990), suggesting that a loosely associated cytosolic factor exists which confers Ca^{2+} -sensitivity on the $\text{Ins}(1,4,5)\text{P}_3$ receptor. However, in the present study, pre-exposure of cells to Ca^{2+} -free medium did not abolish the Ca^{2+} sensitivity of $\text{Ins}(1,4,5)\text{P}_3$ receptors, suggesting that this loosely associated factor is either not present in liver, or is more stably associated with the liver $\text{Ins}(1,4,5)\text{P}_3$ receptor.

Biphasic regulation of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization has now been reported in a number of tissues, including smooth (Iino, 1990) and skeletal (Suarez-Isla et al., 1991) muscle, cerebellum (Bezprozvanny et al., 1991; Finch et al., 1991), A7r5 cells (Missiaen et al., 1992) and liver (Marshall and Taylor, 1993; Combettes et al., 1993). However, although the general features are preserved, there are clear differences in the characteristics of these effects between tissues. $\text{Ins}(1,4,5)\text{P}_3$ binding to liver (Figure 4; Pietri et al., 1990) and cerebellar membranes (Worley et al., 1987) is modulated by similar changes in $[\text{Ca}^{2+}]$ ($\text{EC}_{50} \sim 300 \text{ nM}$), but the effects of Ca^{2+} are different for each tissue: an increase in cytosolic $[\text{Ca}^{2+}]$ increases the affinity of liver $\text{Ins}(1,4,5)\text{P}_3$ receptors for $\text{Ins}(1,4,5)\text{P}_3$ (Table 1), but decreases the affinity of cerebellar $\text{Ins}(1,4,5)\text{P}_3$ receptors (Worley et al., 1987; Joseph et al., 1989). Furthermore, biphasic effects of Ca^{2+} in hepatocytes are manifest only when the cells are stimulated with submaximal $\text{Ins}(1,4,5)\text{P}_3$ concentrations (Figures 3 and 5), but they occur in brain (Bezprozvanny et al., 1991; Finch et al., 1991) and skeletal muscle (Suarez-Isla et al., 1991) even when the intracellular stores are stimulated with maximally effective concentrations of $\text{Ins}(1,4,5)\text{P}_3$. This reflects the inability of $\text{Ins}(1,4,5)\text{P}_3$ receptors in some tissues (e.g. cerebellum; Finch et al., 1991) to respond to $\text{Ins}(1,4,5)\text{P}_3$ when the cytosolic $[\text{Ca}^{2+}]$ is below a critical threshold ($\sim 60 \text{ nM}$; Finch et al., 1991). By contrast, $\text{Ins}(1,4,5)\text{P}_3$ stimulates Ca^{2+} mobilization from permeabilized hepatocytes even when $[\text{Ca}^{2+}]_m$ is reduced to $< 5 \text{ nM}$ (Marshall and Taylor, 1993; Figure 1) and from *Xenopus* oocytes when it is reduced to $\sim 10 \text{ nM}$ (Parys et al., 1992).

Further differences in the characteristics of these regulatory effects of cytosolic Ca^{2+} between tissues are evident when Ca^{2+} is substituted for other bivalent cations. For example, Sr^{2+} mimics Ca^{2+} in potentiating $\text{Ins}(1,4,5)\text{P}_3$ binding to liver $\text{Ins}(1,4,5)\text{P}_3$ receptors (Table 1), but it has no effect on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to cerebellar $\text{Ins}(1,4,5)\text{P}_3$ receptors (Worley et al., 1987). Furthermore, whereas Ba^{2+} does not affect $\text{Ins}(1,4,5)\text{P}_3$ -

stimulated Ca^{2+} release in liver (Figure 4), it reportedly potentiates Ca^{2+} release in *Xenopus* oocytes (Yao and Parker, 1992) and inhibits Ca^{2+} release in thymocytes (Baquero-Leonis and Pintado, 1989). Some of the apparent differences in the effects of bivalent cations on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release, however, may arise from the changes in cytosolic $[\text{Ca}^{2+}]$ that occur as a result of the displacement of Ca^{2+} from buffers following addition of Ba^{2+} or Sr^{2+} . When we examined the effects of Sr^{2+} and Ba^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release in the present study, the contribution from residual Ca^{2+} was minimized by using a high concentration (10 mM) of EGTA (the Experimental section).

Recently, the mechanisms underlying the stimulatory and inhibitory effects of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release from fibroblasts have been ascribed to Ca^{2+} /calmodulin-dependent protein kinase II and Ca^{2+} -dependent protein phosphatase 2B respectively (Zhang et al., 1993). In liver, however, we found no effect of calmodulin antagonists on the sensitization of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release. The role of a calmodulin-independent protein kinase in mediating the regulating effects of cytosolic Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release in liver cannot be discounted. However, a single phosphorylation event, reversible by protein phosphatase 2B, is insufficient to fully account for the regulatory effects of cytosolic Ca^{2+} , because Ca^{2+} appears to convert $\text{Ins}(1,4,5)\text{P}_3$ receptors between at least three distinct conformational states (Figure 7). Furthermore, a different mechanism must mediate the two interconversion steps, because these show different selectivities for bivalent cations (Table 2; Figure 7).

The physiological importance of these effects of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ -receptor activity has yet to be firmly established, although several groups have proposed that they play a central role in regulating the propagation of agonist-evoked Ca^{2+} waves (reviewed in Taylor and Marshall, 1992). Although the mechanisms by which cytosolic Ca^{2+} regulates $\text{Ins}(1,4,5)\text{P}_3$ -receptor activity appear to be cell specific, the conservation of the general features of this regulation suggests that it may play an important role in Ca^{2+} signalling. Such regulation may not be confined to $\text{Ins}(1,4,5)\text{P}_3$ -gated Ca^{2+} channels. The activity of the NAD^+ metabolite, cyclic ADP ribose, which is proposed to be the endogenous ligand triggering Ca^{2+} release through ryanodine receptors in sea urchin eggs (Clapper et al., 1987) and rat pituitary cells (Koshiyama et al., 1991), is also potentiated by Ca^{2+} . Furthermore, the effects of Sr^{2+} and Ba^{2+} on cyclic ADP ribose-mediated Ca^{2+} release (Lee, 1993) are strikingly similar to the effects of the bivalent cations reported here for $\text{Ins}(1,4,5)\text{P}_3$ receptors.

This work was supported by the Medical Research Council and by a Science and Engineering Research Council CASE Studentship with SmithKline Beecham (I.C.B.M.). C.W.T. is a Lister Fellow.

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