RESEARCH COMMUNICATION

Luminal Ca^{2+} increases the affinity of inositol 1,4,5-trisphosphate for its receptor

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Luminal Ca^{2+} has been proposed to regulate the sensitivity of intracellular Ca^{2+} stores to InsP_3 and perhaps thereby to contribute to the mechanisms responsible for regenerative intracellular Ca^{2+} signals. Since Ca^{2+} release from intracellular stores is accompanied by K^+ influx into the stores, InsP_3 -stimulated Ca^{2+} mobilization can be inhibited by substantially reducing the $[\text{K}^+]$ of incubation media. By measuring $[\text{H}^3]\text{InsP}_3$ binding to permeabilized hepatocytes in K^+ -deficient media, thereby preventing InsP_3 -stimulated Ca^{2+} mobilization, we have examined

the effects of Ca^{2+} within the intracellular stores on equilibrium binding of InsP_3 to its receptor. Our results demonstrate a small, but significant ($P < 0.05$, $n = 8-9$), effect of luminal Ca^{2+} on InsP_3 binding; the concentration of InsP_3 causing half-maximal displacement of $[\text{H}^3]\text{InsP}_3$ (1.25 nM) was 3.5 nM for empty stores and 2.1 nM for stores containing Ca^{2+} . Our results suggest that at least part of the stimulatory effect of luminal Ca^{2+} on InsP_3 -stimulated Ca^{2+} mobilization may result from an effect of luminal Ca^{2+} on InsP_3 binding to its receptor.

INTRODUCTION

The InsP_3 receptor is the intracellular Ca^{2+} channel responsible for the initial Ca^{2+} -mobilization phase of the Ca^{2+} signal evoked by receptors linked to polyphosphoinositide hydrolysis (Furuichi et al., 1989; reviewed in Taylor and Richardson, 1991). Although InsP_3 in the intracellular signal linking receptor activation to Ca^{2+} mobilization, the InsP_3 receptor is also regulated by cytosolic $[\text{Ca}^{2+}]$ (Iino and Endo, 1992; reviewed in Taylor and Marshall, 1992) and by the Ca^{2+} content of the intracellular stores (Missiaen et al., 1992a,b,c; Nunn and Taylor, 1992). These effects of cytosolic and luminal Ca^{2+} have been proposed to contribute to the complex Ca^{2+} spiking behaviour triggered by activation of Ca^{2+} -mobilizing receptors (Berridge, 1993).

The effect of InsP_3 on Ca^{2+} efflux from intracellular stores is complex: submaximal InsP_3 concentrations rapidly release a fraction of the InsP_3 -sensitive stores, but there is then either no further Ca^{2+} mobilization (Muallem et al., 1989; Oldershaw et al., 1991) or Ca^{2+} efflux continues at a much reduced rate (Champeil et al., 1989; Missiaen et al., 1992b). In permeabilized hepatocytes, this pattern of response appears to result from InsP_3 stimulating all-or-nothing emptying of intracellular stores that differ in their sensitivities to InsP_3 : a quantal response (Oldershaw et al., 1991). By contrast, in A7r5 smooth-muscle cells, partial emptying of InsP_3 -sensitive Ca^{2+} stores appears largely to reflect an interaction between luminal Ca^{2+} and InsP_3 (Irvine, 1990), such that as stores empty they become less sensitive to InsP_3 and the intrinsic Ca^{2+} channel of the InsP_3 receptor then closes, trapping Ca^{2+} within the stores (Missiaen et al., 1992b).

Although quantal responses to InsP_3 in hepatocytes appear not to be an immediate consequence of the luminal Ca^{2+} effect first suggested by Irvine (1990), we have observed an effect of luminal Ca^{2+} on the sensitivity of the intracellular stores of hepatocytes to InsP_3 ; full stores are most sensitive to InsP_3 (Missiaen et al., 1992a; Nunn and Taylor, 1992). In the present study, we have extended our earlier work by examining the effect

of luminal Ca^{2+} on $[\text{H}^3]\text{InsP}_3$ binding to its receptor in permeabilized hepatocytes.

MATERIALS AND METHODS

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

Hepatocytes were prepared by collagenase digestion of livers from male Wistar rats. Incubation media were more complex than those used in previous work (Nunn and Taylor, 1992) because we needed to optimize conditions for both $[\text{H}^3]\text{InsP}_3$ binding and $^{45}\text{Ca}^{2+}$ -efflux assays, and to manipulate the $[\text{K}^+]$ of the media used for both. Cells were permeabilized with saponin (25 $\mu\text{g}/\text{ml}$) and then resuspended (10⁷ cells/ml) in loading medium {LM: KCl, 100 mM; NaCl, 20 mM; MgCl_2 , 2 mM; sucrose, 80 mM; EGTA, 1 mM; CaCl_2 , 390 μM ; Pipes, 20 mM; pH 7.0; free $[\text{Ca}^{2+}] = 200$ nM, calculated by using the program MaxChelator (C. Patton, Stanford University, Stanford, CA, U.S.A.) and confirmed using fura-2}. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore normally included to inhibit Ca^{2+} uptake into mitochondria (Oldershaw et al., 1992), was omitted from these experiments to minimize the likelihood of protons acting as counterions for Ca^{2+} movements (Muallem et al., 1985); the $^{45}\text{Ca}^{2+}$ content of the intracellular stores at steady state was unaffected by omission of FCCP (results not shown). The permeabilized cells were added to $^{45}\text{Ca}^{2+}$ (10 $\mu\text{Ci}/\text{ml}$), ATP (2.5 mM) and an ATP-regenerating system [creatine phosphate (15 mM) and creatine kinase (10 units/ml)]. After 8 min at 37 °C, the intracellular stores had loaded to steady state with $^{45}\text{Ca}^{2+}$ and the cells were then diluted 10-fold into LM, binding medium (BM: sucrose, 297 mM; EDTA, 1 mM; *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulphonic acid (Epps), 50 mM, pH 8.3 at 4 °C), or release medium {RM: KCl, 100 mM; sucrose, 50 mM; NaCl, 20 mM; MgCl_2 , 2 mM; 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetraacetic acid (BAPTA), 1 mM; CaCl_2 , 651 μM ; Epps, 50 mM,

Abbreviations used: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetraacetic acid; BM, LM and RM, binding, loading and release medium respectively (compositions are given in the Materials and methods section); Epps, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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pH 8.3 at 4 °C; free $[Ca^{2+}] = 200$ nM} in the presence or absence of $InsP_3$. Incubations were terminated by addition of ice-cold wash medium (5 ml; sucrose, 310 mM; EGTA, 1 mM; Hepes, 5 mM, pH 7.0) followed by rapid filtration through Whatman GF/C filters and a further wash. Filters were counted for radioactivity in Emulsifier Safe scintillation cocktail (2 ml; Zinsser Analytic).

$[^3H]InsP_3$ binding to permeabilized cells

Saponin-permeabilized hepatocytes (10^7 cells/ml) suspended in LM were diluted 10-fold into BM containing $[^3H]InsP_3$ (final concn. = 1.25 nM; 0.05 μ Ci/ml). This dilution caused only a minimal change in pH, but the free $[Ca^{2+}]$ was reduced from 200 nM to < 1 nM. After 3 min at 4 °C, during which binding reached equilibrium (Figure 1 below, inset), cells were harvested by filtration through Whatman GF/C filters and washed twice with 2.5 ml of cold medium [sucrose, 297 mM; EDTA, 1 mM; Tris, 50 mM, pH 8.3 (with acetic acid) at 4 °C]. Filters were counted for radioactivity in EcoScint A scintillation cocktail (3 ml; National Diagnostics) with methanol (300 μ l) and HCl (25 μ l of a 0.1 M solution).

For measurements of $[^3H]InsP_3$ binding to stores containing Ca^{2+} , permeabilized cells (10^7 cells/ml) were first loaded with $^{45}Ca^{2+}$ by incubation with ATP (2.5 mM) and the ATP-regenerating system [creatine phosphate (15 mM) and creatine kinase (10 units/ml)] for 8 min at 37 °C. Cells were then diluted 9-fold into BM containing either ionomycin (final concn. 10 μ M) or dimethyl sulphoxide (the vehicle for ionomycin; final concn. 0.1 %). After 30 s, cells were further diluted (to 10^6 cells/ml; final incubation vol. 2.2 ml) by addition to $[^3H]InsP_3$ (1.25 nM, 0.05 μ Ci/ml) and appropriate concentrations of unlabelled $InsP_3$. Incubations were terminated after 3 min at 4 °C by rapid filtration (see above). Specific $[^3H]InsP_3$ binding, which was typically 50 % of total binding, was between 600 and 700 d.p.m./incubation; in all experiments ≤ 1 % of the added $[^3H]InsP_3$ was bound.

Equilibrium-competition-binding curves were analysed by fitting to a logistic equation using the program GraphPad InPlot. Traces of endogenous $InsP_3$ produced while the hepatocytes were incubated at 37 °C (Missiaen et al., 1992a) may contaminate the binding incubation; binding characteristics are therefore reported as the concentrations of $InsP_3$ needed for half-maximal displacement of specific $[^3H]InsP_3$ binding (IC_{50}) and not as equilibrium dissociation constants. The IC_{50} values are presented as geometric means, and statistical tests were performed on the logarithms of the experimental determinations.

Ionomycin was from Calbiochem. $[^3H]InsP_3$ (41 Ci/mmol) and $^{45}CaCl_2$ were from Amersham. $InsP_3$ was from Dr. R. F. Irvine (AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.). Other reagents were of analytical grade and supplied by Sigma.

RESULTS AND DISCUSSION

$InsP_3$ rapidly stimulates Ca^{2+} mobilization from the intracellular stores of permeabilized hepatocytes (Champeil et al., 1989); under normal incubation conditions, the stores are therefore depleted of Ca^{2+} long before the interaction between $InsP_3$ and its receptor has attained equilibrium. Previous studies suggested that $InsP_3$ -stimulated Ca^{2+} efflux from intracellular stores is electrically compensated by an influx of K^+ (Muallem et al., 1985; Shah and Pant, 1988; Palade et al., 1989). We have confirmed that $InsP_3$ -stimulated Ca^{2+} mobilization occurs only when the incubation medium contains sufficient K^+ . This characteristic has allowed us to examine $[^3H]InsP_3$ binding to Ca^{2+} -

Table 1 Depletion of the medium $[K^+]$ reversibly inhibits $InsP_3$ -stimulated Ca^{2+} mobilization

Permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ in LM were first diluted 10-fold (1) into ice-cold K^+ -deficient media (final $[K^+] = 10$ mM) with or without 2 μ M $InsP_3$. After 30 s they were diluted a further 10-fold (2) into media containing K^+ at final concentrations of 1 mM (—) or 91 mM (+), again with or without 2 μ M $InsP_3$. After a further 60 s, the incubations were terminated and the $^{45}Ca^{2+}$ contents of the intracellular stores determined. Results in columns B–D (means $\pm$ SEM, $n = 3$ –4) are expressed as fractions of the mean Ca^{2+} contents of the cells shown in column A.

	A	B	C	D
1 $InsP_3$...	—	+	+	—
K^+ ...	—	—	—	—
2 $InsP_3$...	—	+	+	—
K^+ ...	—	—	+	+
$^{45}Ca^{2+}$ content (%) ...	100 $\pm$ 1	99 $\pm$ 3	75 $\pm$ 2	102 $\pm$ 13

containing stores in the absence of $InsP_3$ -stimulated Ca^{2+} mobilization.

When permeabilized cells loaded to steady state with $^{45}Ca^{2+}$ were diluted 10-fold into ice-cold LM, a maximal concentration of $InsP_3$ (2 μ M) released 30 ± 1 % ($n = 4$) of the intracellular Ca^{2+} stores within 90 s. A very similar response (release of 27 ± 2 % of the stores; $n = 4$) occurred when the cells were diluted into cold RM containing 2 μ M $InsP_3$, indicating that neither the high pH (pH 8.3) nor the Epps used to buffer the binding medium interfered with $InsP_3$ -stimulated Ca^{2+} mobilization. However, when the loaded cells were first diluted into BM for 30 s (final $[K^+] = 10$ mM, cf. $[K^+] = 100$ mM in LM and RM) and then added to $InsP_3$ (2 μ M) for a further 60 s, only 2.8 ± 0.9 % ($n = 13$) of the $^{45}Ca^{2+}$ content of the stores was released. This inhibition of $InsP_3$ -stimulated Ca^{2+} mobilization was not a consequence of a decrease in the affinity of the $InsP_3$ receptor for $InsP_3$, because 20 μ M $InsP_3$ had no more effect than 2 μ M $InsP_3$; release of 4.3 ± 0.6 % and 2.8 ± 0.9 % ($n = 3$) respectively. Nor was the inhibition a consequence of a substantial slowing of the rate of $InsP_3$ -stimulated Ca^{2+} mobilization: similar very small responses to $InsP_3$ (2 μ M) occurred after incubation for 1 min (2.8 ± 0.9 % release), 2 min (1.7 ± 0.3 %) or 4.5 min (2.9 ± 0.2 %) ($n = 3$ –4). To assess the reversibility of this inhibitory effect of K^+ removal, permeabilized cells loaded with $^{45}Ca^{2+}$ were diluted into cold BM (final $[K^+] = 10$ mM) for 30 s in the presence or absence of $InsP_3$ (2 μ M) and then further diluted 10-fold into either RM (final $[K^+] = 91$ mM) or BM. The results (Table 1) demonstrate that the inhibition of $InsP_3$ -stimulated Ca^{2+} mobilization caused by reducing the cytosolic $[K^+]$ to 10 mM was fully reversed when the $[K^+]$ was restored to 91 mM.

These results, which are consistent with earlier work in different media (Muallem et al., 1985), demonstrate that $InsP_3$ -stimulated Ca^{2+} mobilization can be reversibly inhibited by omission of K^+ from media that are optimal for measurements of $InsP_3$ binding. In subsequent experiments we have exploited these observations to examine $[^3H]InsP_3$ binding to the intracellular stores of permeabilized hepatocytes in the absence of Ca^{2+} mobilization.

The effects of luminal Ca^{2+} on the affinity of $InsP_3$ for its receptor were examined by allowing the intracellular stores to load to steady state with Ca^{2+} (in LM) and then diluting them into BM containing $[^3H]InsP_3$ (1.25 nM), various concentrations of unlabelled $InsP_3$ and either ionomycin (5 μ M) or dimethyl sulphoxide (0.1 %, the solvent for ionomycin). Reactions were terminated after 3 min, during which equilibrium binding was attained (Figure 1 inset). Ionomycin (5 μ M) very rapidly empties the intracellular Ca^{2+} stores under these conditions (results not

Figure 1 Luminal Ca^{2+} enhances InsP_3 binding to its receptor

Permeabilized cells (10^7 cells/ml) loaded to steady state with $^{45}\text{Ca}^{2+}$ in LM were diluted 9-fold into ice-cold BM in the presence (O) or absence (●) of $5 \mu\text{M}$ ionomycin. After 30 s, the cells were further diluted by addition of $[^3\text{H}]\text{InsP}_3$ (final concentration, 1.25 nM) and the indicated concentrations of unlabelled InsP_3 (C denotes no added InsP_3). The final cell density was 10^6 cells/ml and the final $[\text{K}^+]$ was 10 mM . Incubations were terminated by rapid filtration after 3 min. The results from a single experiment, representative of eight or nine others, are shown. The inset shows the kinetics of specific $[^3\text{H}]\text{InsP}_3$ (0.5 nM) binding to permeabilized cells. After 5 min (arrow), $1 \mu\text{M}$ unlabelled InsP_3 was added to half the cells to allow the kinetics of $[^3\text{H}]\text{InsP}_3$ dissociation to be followed (O). Results from a single experiment representative of three others are shown.

shown) and therefore allows $[^3\text{H}]\text{InsP}_3$ binding to empty stores to be measured. After inhibition of Ca^{2+} uptake by chilling, unidirectional efflux of Ca^{2+} from the stores occurred with a half-time of $3.0 \pm 0.3 \text{ min}$ ($n = 4$); $[^3\text{H}]\text{InsP}_3$ binding to partially filled stores could therefore be measured during the 3 min needed for $[^3\text{H}]\text{InsP}_3$ binding to reach equilibrium. The results (Figure 1) demonstrate a small, but statistically significant, increase in the affinity of the InsP_3 receptor for InsP_3 in stores containing Ca^{2+} . In empty stores, half-maximal inhibition of $[^3\text{H}]\text{InsP}_3$ binding (IC_{50}) occurred with 3.5 nM InsP_3 (the range encompassed by the geometric mean $\pm$ SEM was $3.0\text{--}4.1 \text{ nM}$, $h = 0.82 \pm 0.06$, $n = 9$), and in stores containing Ca^{2+} the IC_{50} was 2.1 nM ($1.8\text{--}2.4 \text{ nM}$, $h = 1.07 \pm 0.31$, $n = 8$) ($P < 0.05$; two-tailed Student's t test, $n = 8\text{--}9$; $P < 0.05$, Wilcoxon's Signed-Ranks test for paired samples, $n = 8$). Ionomycin ($5 \mu\text{M}$) itself had no effect on $[^3\text{H}]\text{InsP}_3$ binding to permeabilized cells that had not previously been loaded with

Ca^{2+} : specific binding of $[^3\text{H}]\text{InsP}_3$ (1.25 nM) was 687 ± 191 and $648 \pm 125 \text{ d.p.m./incubation}$ in the absence and presence of ionomycin ($5 \mu\text{M}$) respectively. Our results therefore suggest that the decrease in luminal free $[\text{Ca}^{2+}]$, rather than an effect of ionomycin *per se*, causes a decrease in the affinity of the InsP_3 receptor for InsP_3 . Unfortunately it is impracticable with this method to examine the effects of more substantial Ca^{2+} loading of the stores on InsP_3 binding because, during the time taken for binding to reach equilibrium, there is inevitably an appreciable (about 50%) leak of Ca^{2+} from the stores. It is therefore possible that the full effects of luminal Ca^{2+} on InsP_3 binding are more substantial than is apparent from the results of the present study.

Previous studies of hepatocytes (Nunn and Taylor, 1992) and A7r5 cells (Missiaen et al., 1992b,c) demonstrated that stores that had lost much of their Ca^{2+} were 2–5-fold less sensitive to InsP_3 . The modest stimulatory effect of luminal Ca^{2+} on InsP_3 binding may, therefore, underlie the effect of luminal Ca^{2+} on InsP_3 -stimulated Ca^{2+} mobilization.

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REFERENCES

- Berridge, M. J. (1993) *Nature* (London) **361**, 315–325
- Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S. and Claret, M. (1989) *J. Biol. Chem.* **264**, 17665–17673
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) *Nature* (London) **342**, 32–38
- Iino, M. and Endo, M. (1992) *Nature* (London) **360**, 76–78
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Missiaen, L., Taylor, C. W. and Berridge, M. J. (1992a) *J. Physiol.* (London) **455**, 632–640
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992b) *Nature* (London) **357**, 599–602
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992c) *J. Biol. Chem.* **267**, 22961–22966
- Muallem, S., Schoeffield, M., Pandol, S. and Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4433–4437
- Muallem, S., Pandol, S. and Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212
- Nunn, D. L. and Taylor, C. W. (1992) *Mol. Pharmacol.* **41**, 115–119
- Oldershaw, K. A., Nunn, D. L. and Taylor, C. W. (1991) *Biochem. J.* **278**, 705–708
- Oldershaw, K. A., Richardson, A. and Taylor, C. W. (1992) *J. Biol. Chem.* **267**, 16312–16316
- Palade, P., Dettbarn, C., Volpe, P., Alderson, B. and Otero, A. S. (1989) *Mol. Pharmacol.* **36**, 664–672
- Shah, J. and Pant, H. C. (1988) *Biochem. J.* **250**, 617–620
- Taylor, C. W. and Marshall, I. C. B. (1992) *Trends Biochem. Sci.* **17**, 403–407
- Taylor, C. W. and Richardson, A. (1991) *Pharmacol. Ther.* **51**, 97–137