Multiple mechanisms by which protein kinase A potentiates inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization in permeabilized hepatocytes

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The mobilization of Ca^{2+} from intracellular stores by Ins(1,4,5)P_3 in suspensions of permeabilized rat hepatocytes was potentiated by preincubating intact cells with adenosine 3′:5′-cyclic phosphorothioate (cpt-cAMP), or by addition of the catalytic subunit of cyclic-AMP-dependent protein kinase (PKA) after cell permeabilization. This action of PKA involved both an enhancement in Ins(1,4,5)P_3 sensitivity and an increase in the size of the Ins(1,4,5)P_3-releasable Ca^{2+} pool. Inclusion of the protein phosphatase inhibitor okadaic acid in the permeabilization medium augmented the effects of PKA. Treatment with PKA catalytic subunit also increased the rate of ATP-dependent Ca^{2+} sequestration. To determine whether the effects of PKA on the Ca^{2+}-release mechanism were secondary to alterations in the Ca^{2+} load of the Ins(1,4,5)P_3-sensitive stores, a method was developed using Mn^{2+} as a Ca^{2+} surrogate to examine the permeability properties of the Ins(1,4,5)P_3-gated channels independent of Ca^{2+} fluxes. This approach utilized the ability of Mn^{2+} to quench the fluorescence of fura-2 compartmentalized within intracellular Ca^{2+} stores in an Ins(1,4,5)P_3-dependent manner, with thapsigargin added to block the ATP-activated Ca^{2+} pump and to ensure that the Ca^{2+} stores were fully depleted of Ca^{2+}. The initial rate and extent of Mn^{2+} quenching of compartmentalized fura-2 was increased in a dose-dependent manner by Ins(1,4,5)P_3. PKA activation increased both the initial rate and the extent of Mn^{2+} quenching at sub-maximal Ins(1,4,5)P_3 doses, but there was no effect on the quench rate in the presence of saturating Ins(1,4,5)P_3. However, the amount of compartmentalized fura-2 that could be quenched by Mn^{2+} in the presence of maximal Ins(1,4,5)P_3 was increased by PKA. These data suggest two distinct actions of PKA on the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. (1) Modification of the ion-permeability properties of the Ins(1,4,5)P_3 receptor/channel through an increase in the sensitivity to Ins(1,4,5)P_3 for channel opening. (2) A recruitment of Ca^{2+} stores from the Ins(1,4,5)P_3-insensitive pool. Both actions were independent of the Ca^{2+}-loading state of the stores. Imaging studies of single permeabilized hepatocytes showed that the Ins(1,4,5)P_3-sensitive stores were distributed throughout the cell and PKA enhanced the rate of Ins(1,4,5)P_3-stimulated Mn^{2+} quench in individual cells, without modifying the subcellular distribution of Ins(1,4,5)P_3-sensitive stores.

INTRODUCTION

The mobilization of Ca^{2+} in response to sub-maximal levels of Ins(1,4,5)P_3-dependent hormones in liver cells is enhanced by a number of agents that activate cyclic AMP (cAMP)-dependent protein kinase (PKA), including glucagon, β-adrenergic agonists, forskolin and various cell-permeant cAMP analogues. This phenomenon has been observed in studies of $^{45}Ca^{2+}$ fluxes, Ca^{2+}-dependent K^+ channel activation and by direct measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Jenkinson and Koller, 1977; Morgan et al., 1984; Blackmore and Exton, 1986; Poggioli et al., 1986; Burgess et al., 1986, 1991). The $[Ca^{2+}]_i$ responses in single hepatocytes occur as a series of discrete baseline-separated $[Ca^{2+}]_i$ transients, with the frequency, but not the amplitude, being determined by agonist dose (Woods et al., 1986; Rooney et al., 1989; Kawanishi et al., 1989). Activation of PKA results in an increase in frequency or conversion from oscillating to sustained $[Ca^{2+}]_i$ elevation (Hoek et al., 1990; Schofl et al., 1991), similar to that observed with increasing doses of Ins(1,4,5)P_3-dependent agonists. In some studies glucagon or cell-permeant cAMP analogues have been found to cause direct elevation of $[Ca^{2+}]_i$ in the absence of other agonists (Blackmore and Exton, 1986; Poggioli et al., 1986; Staddon and Hansford, 1989), and these $[Ca^{2+}]_i$ increases also have an oscillatory character (Hoek et al., 1990; Capiod et al., 1991).

The role of phospholipase C activation in the actions of PKA on $[Ca^{2+}]_i$ in hepatocytes is unclear. Some studies have described small (10–20%) increases in Ins(1,4,5)P_3 after treatment with glucagon or cAMP analogues (Blackmore and Exton, 1986; Wakelam et al., 1986), whereas others have found no changes in inositol phosphates or the inositol lipid precursors (Creba et al., 1983; Poggioli et al., 1986; Pittner and Fain, 1989, 1990; Burgess et al., 1991). At best, it appears that the Ins(1,4,5)P_3 increases brought about by these agents are insufficient to explain their effects on $[Ca^{2+}]_i$. More marked effects of glucagon and cAMP analogues have been reported in some of the studies where these agents were combined with vasopressin (Blackmore and Exton, 1986; Pittner and Fain, 1989, 1990). However, it has also been reported that activation of PKA can result in a potentiation of the Ca^{2+} response to hormones that act via Ins(1,4,5)P_3 without any additional increment in Ins(1,4,5)P_3 levels (Poggioli et al., 1986; Burgess et al., 1986, 1991). Furthermore, Burgess et al. (1991) showed that isoprenaline or dibutyryl cAMP could elevate $[Ca^{2+}]_i$ after micro-injection of guinea-pig hepatocytes with Ins(2,4,5)P_3 or after photolysis of caged Ins(1,4,5)P_3, but had no effect in the absence of exogenous InsP_3 analogues. In permeabilized hepatocytes, activation of PKA results in a sensitization to the Ca^{2+}-releasing actions of Ins(1,4,5)P_3 and an increase in the amount of Ca^{2+} available for release by maximal Ins(1,4,5)P_3 (Burgess et al., 1991). Thus the ability of the cAMP

Abbreviations used: cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; cpt-cAMP, adenosine 3′:5′-cyclic phosphorothioate; fura-2/AM, fura-2 penta-acetoxymethyl ester; ICM, intracellular medium; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.

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pathway to potentiate Ins(1,4,5)P_3-dependent Ca^{2+} signalling can be explained by enhanced Ca^{2+} release at the level of the Ins(1,4,5)P_3-responsive Ca^{2+} stores. If Ins(1,4,5)P_3 sensitivity is shifted to a point where basal Ins(1,4,5)P_3 levels are sufficient to initiate Ca^{2+} release, this mechanism may also underlie the direct $[Ca^{2+}]_i$ increases in response to glucagon and other agents that activate PKA (Blackmore and Exton, 1986; Poggioli et al., 1986; Staddon and Hansford, 1989; Hoek et al., 1990).

The mechanism by which cAMP increases the efficacy of Ins(1,4,5)P_3 for Ca^{2+} mobilization in hepatocytes remains to be elucidated. Although the Ins(1,4,5)P_3 receptor is a target for phosphorylation by PKA (Grossman et al., 1975; Supattapone et al., 1988; Ferris et al., 1991), the properties of the phosphorylated receptor do not appear to be consistent with the sensitization to Ins(1,4,5)P_3 observed in these cells. Phosphorylation does not appear to affect Ins(1,4,5)P_3 binding (Supattapone et al., 1988; Mauger et al., 1989; Volpe and Alderson-Lang, 1990) and actually causes a decrease in Ins(1,4,5)P_3 sensitivity for Ca^{2+} release in brain microsomes (Supattapone et al., 1988; Volpe and Alderson-Lang, 1990). An alternative mechanism by which PKA could enhance the apparent sensitivity to Ins(1,4,5)P_3 for Ca^{2+} release is by increasing the Ca^{2+}-loading state of Ins(1,4,5)P_3-sensitive Ca^{2+} stores. It has recently been reported that the EC_{50} for Ca^{2+} release by Ins(1,4,5)P_3 in permeabilized hepatocytes is decreased as the Ca^{2+} load of the stores is increased (Nunn and Taylor, 1992). Since the PKA-induced sensitization to Ins(1,4,5)P_3 observed by Burgess et al. (1991) was accompanied by an increase in the Ins(1,4,5)P_3-sensitive Ca^{2+} pool size, it is possible that the sensitization is secondary to alterations in the luminal Ca^{2+} content (Taylor and Richardson, 1991). In the present study we have investigated this possibility by examining the effects of PKA on the permeability of the Ins(1,4,5)P_3-sensitive channel under conditions where the Ca^{2+} stores are fully discharged and Ca^{2+} fluxes have been eliminated. This has been achieved by using Mn^{2+} as a Ca^{2+} surrogate added to the cytosolic phase in fura-2-loaded permeabilized hepatocytes, and examining the Ins(1,4,5)P_3-dependent quenching of compartmentalized fura-2 (Glennon et al., 1992). These experiments demonstrate that PKA increases the permeability of the Ins(1,4,5)P_3-activated Ca^{2+} channels at sub-maximal Ins(1,4,5)P_3 doses, in a manner that is independent of the Ca^{2+}-loading state of the stores. The ability of PKA to increase the size of the Ins(1,4,5)P_3-releasable Ca^{2+} pool may represent an additional site of action, since this can be observed at maximal Ins(1,4,5)P_3 concentrations where there is no measurable effect on the permeability properties of the channels. Dynamic imaging measurements of the Ins(1,4,5)P_3-induced Mn^{2+} quench of compartmentalized fura-2 in single permeabilized hepatocytes indicates that PKA is effective in potentiating the actions of Ins(1,4,5)P_3 in all subcellular regions.

METHODS

Materials

Ins(1,4,5)P_3 and ionomycin were obtained from Calbiochem, calyculin A, okadaic acid and thapsigargin from LC Services, fura-2 and fura-2 acetoxymethyl ester (fura-2/AM) from Molecular Probes, and other chemicals from Sigma Chemical Co.

Preparation and primary culture of hepatocytes

Hepatocytes were prepared from male Sprague–Dawley rats by collagenase perfusion of the liver as described previously (Thomas, 1988). Cell viability was found to be approx. 95% by Trypan Blue exclusion. For experiments using hepatocytes in primary culture, the cells [(4–5) × 10^5] were plated on polylysine-coated glass coverslips in 3 ml of Williams E medium supplemented with 10% fetal-calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin, 0.05 μg/ml gentamycin, 4 μg/ml dexamethasone and 0.02 unit/ml insulin. After 2–3 h incubation at 37 °C the dishes were washed and then incubated in the above buffer with 0.002 unit/ml insulin for a further 15–20 h. Before fura-2 loading, the cells were washed again and then incubated without insulin for at least 2 h.

Measurement of Ca^{2+} fluxes in suspensions of permeabilized hepatocytes

Freshly isolated hepatocytes [(2–3) × 10^6] were washed in a Ca^{2+}-free buffer (120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 20 mM Hepes/Tris, pH 7.4). The cells were then resuspended in 2 ml of intracellular medium (ICM), composed of 120 mM KCl, 1 mM KH_2PO_4, 10 mM NaCl and 20 mM Hepes/Tris, pH 7.2, treated with Chelex to remove contaminating Ca^{2+} and then supplemented with 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM Ruthenium Red, 2 mM Mg-ATP, 5 mM phosphocreatine and 5 units/ml creatine kinase. The cells were permeabilized with 25 μg/ml digitonin, and $[Ca^{2+}]$ in the medium was monitored with 1.5 μM fura-2 (free acid form added to the ICM). Incubations were carried out in the cuvette of a Deltascan spectrofluorimeter (Photon Technology International) at 37 °C with continuous stirring. The excitation wavelength was 340 nm, and the emitted light was measured at 510 nm. At the end of each experiment, $F_{max.}$ and $F_{min.}$ were obtained by addition of 1 mM Ca^{2+} followed by 10 mM EGTA/Tris at pH 8.5. Free $[Ca^{2+}]$ was calculated by using a K_d of 224 nM (Grynkiewicz et al., 1985).

Measurement of Mn^{2+}-induced quench of compartmentalized fura-2 in permeabilized hepatocytes

Freshly isolated hepatocytes in suspension [(3–3.5) × 10^6] were preloaded with fura-2/AM (5 μM) for 40–50 min in 8 ml of cell incubation buffer (121 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose, 5 mM $NaHCO_3$, 10 mM Hepes/NaOH, pH 7.4) supplemented with 2% BSA at 37 °C. After fura-2 loading, the cells were washed and stored on ice. Before permeabilization, the cell suspension was washed with a Ca^{2+}-free buffer (120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 100 μM EGTA, 20 mM Hepes/Tris, pH 7.4) and then resuspended in ICM (2 ml) supplemented with 25 μg/ml digitonin and 2 μM thapsigargin in the fluorimeter cuvette at 37 °C. Quenching of the compartmentalized fura-2 by added Mn^{2+} was monitored by using an excitation wavelength of 360 nm, where dye fluorescence measured with emission at 510 nm was insensitive to Ca^{2+} changes. A high concentration of Mn^{2+} (500 μM) was added in the presence of ionomycin at the end of each experiment to quench all compartments of fura-2; the residual fluorescence signal under these conditions represents the autofluorescence of the cells.

For single-cell studies, hepatocytes attached to coverslips and maintained in primary culture for 24 h were loaded with fura-2/AM essentially as described above. After washing, the coverslip was inserted into a thermostatically regulated chamber (37 °C) and the assembly was placed on the stage of a Zeiss IM-35 inverted microscope with 20× or 40× objective. Permeabilization (3–5 min) was carried out in ICM supplemented with 10 μg/ml digitonin, 30 μM EGTA and 2 μM thapsigargin.

The permeabilization buffer was then exchanged for fresh buffer without digitonin or EGTA (cells did not re-seal under these conditions). Fluorescence images were collected by using 360 nm excitation and 460–600 nm emission. A liquid-N_2-cooled charge-coupled device camera (Photometrics) was used as the imaging device as described previously (Rooney et al., 1989, 1990). Image acquisition was carried out with a Heurikon HK68/M10 computer, and the data were subsequently analysed with a Macintosh Quadra computer running customized image-processing software.

RESULTS

Potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release by PKA

Previous studies carried out using guinea-pig hepatocytes have demonstrated that activation of PKA leads to a sensitization to Ins(1,4,5)P_3 for Ca^{2+} mobilization (decrease in the EC_{50}) and an increase in the maximal amount of Ins(1,4,5)P_3-releasable Ca^{2+} (Burgess et al., 1991). We have also observed a cAMP-dependent decrease in the EC_{50} for Ins(1,4,5)P_3 action in permeabilized rat hepatocytes (J. B. Hoek and T. Nomura, unpublished work). Figure 1 (upper panel) shows how the Ca^{2+}-release responses to sub-maximal and maximal Ins(1,4,5)P_3 doses were affected by preincubation of the hepatocytes with the cell-permeant cAMP analogue adenosine 3′:5′-cyclic phosphorothioate (cpt-cAMP) or addition of the catalytic subunit of PKA (50 units/ml) to the permeabilized hepatocytes. These agents did not affect the final steady-state [Ca^{2+}] in the medium (54 ± 3 nM; n = 14). However, the Ca^{2+} release in response to a low Ins(1,4,5)P_3 dose (75 nM) was increased by about 50 % under these conditions (Figure 1). The lower panel of Figure 1 shows that the effect of PKA catalytic subunit on the Ca^{2+} release response to 75 nM Ins(1,4,5)P_3 was potentiated when the phosphatase inhibitor okadaic acid (10 nM) was also present in the permeabilization medium. This effect of okadaic acid was most pronounced when a threshold concentration of PKA catalytic subunit (8 units/ml) was applied. Okadaic acid had no effect on Ins(1,4,5)P_3-induced Ca^{2+} release in the control cells. Similar data were obtained with the phosphatase inhibitor calyculin A (2 nM) (results not shown). The effects of cpt-cAMP, PKA catalytic subunit and okadaic acid on Ins(1,4,5)P_3-induced Ca^{2+} release are summarized in Table 1.

In addition to the increased efficacy of low Ins(1,4,5)P_3 doses after PKA action, there was also an increase of about 15 % in the size of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool measured with the maximal dose of 5 μM Ins(1,4,5)P_3 in the presence of okadaic acid (Table 1), in agreement with the data obtained by Burgess et al. (1991). This increase in the amount of Ca^{2+} available for release by Ins(1,4,5)P_3 could come about in one of two ways. It might result from a recruitment of additional Ca^{2+} stores to supplement the size of the Ins(1,4,5)P_3-sensitive pool, as has been shown to occur in permeabilized hepatocytes treated with GTP (Thomas, 1988). Alternatively, PKA could increase the Ca^{2+}-loading state of the Ca^{2+} stores by enhancing the Ca^{2+}-uptake pathway or by decreasing the basal leak rate. Previous studies carried out using hepatic microsomes have demonstrated that PKA activates the intracellular ATP-dependent Ca^{2+} pump (Taylor et al., 1980). A similar effect was observed in permeabilized hepatocytes in the present study, where pulses of added Ca^{2+} were sequestered at a faster rate after incubation with PKA catalytic subunit. In a series of such experiments, the initial rate of Ca^{2+} uptake after addition of 0.5 μM Ca^{2+} was increased by 18 ± 7 % compared with the paired controls not treated with PKA (n = 9, $P < 0.05$), although, as noted above, this was not

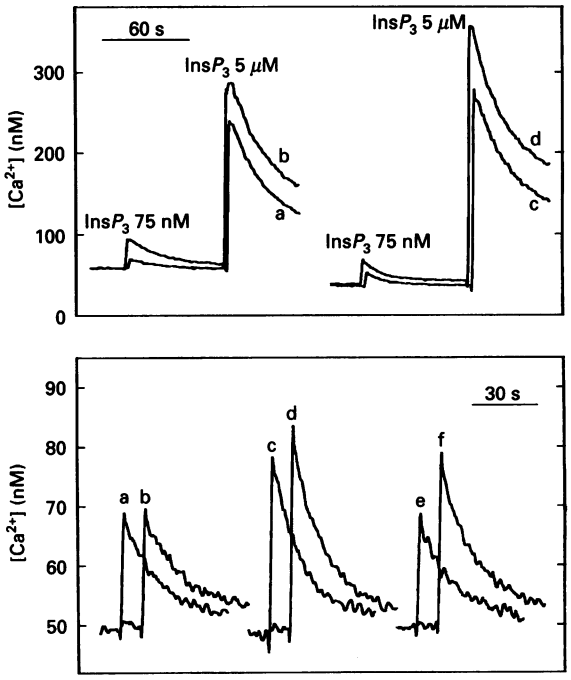

Figure 1 Potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release by PKA in permeabilized hepatocytes

Ins(1,4,5)P_3-induced Ca^{2+} release was monitored fluorimetrically by using fura-2 (free acid form) in the incubation medium of permeabilized hepatocytes maintained in suspension, as described in the Methods section. The cells were permeabilized with digitonin in the presence of 2 mM Mg-ATP and an ATP-regenerating system, and Ca^{2+} uptake was allowed to reach a steady state during 7 min incubation. Sequential additions of 75 nM and 5 μM Ins(1,4,5)P_3 were then made as indicated. Upper panel: For trace (b) the intact hepatocytes were preincubated for 5 min with 2 μM cpt-cAMP before permeabilization; trace (a) is a parallel control incubation. For trace (d) 50 units/ml PKA catalytic subunit was included in the permeabilization medium; trace (c) is a parallel control in the presence of dithiothreitol (90 μg/ml final), since this was present in the catalytic-subunit preparation. Lower panel: Permeabilized hepatocytes were incubated with vehicle (a, b), 50 units/ml PKA catalytic subunit (c, d), or 8 units/ml PKA catalytic subunit (e, f) in the absence (a, c, e) or presence of okadaic acid (b, d, f).Traces show the Ca^{2+}-release response to 75 nM Ins(1,4,5)P_3. Each trace is representative of data obtained in at least three separate experiments.

Table 1 Effect of PKA on Ins(1,4,5)P_3-induced Ca^{2+} mobilization in permeabilized hepatocytes

Fura-2 free acid added to the incubation medium was used to monitor Ca^{2+} uptake and release in permeabilized hepatocytes as described in the Methods section and the legend to Figure 1. PKA catalytic subunit (csPKA), cpt-cAMP and okadaic acid (10 nM) were added to the permeabilization buffer as indicated. Where used, cpt-cAMP was also present during a 5 min preincubation of the intact cells. Amounts of Ca^{2+} release were determined from the fura-2 fluorescence changes by calibration with known Ca^{2+} pulses. To correct for differences in Ca^{2+}-loading state between experiments, values are expressed as a percentage of the control release in the same experiment. The mean control values for Ca^{2+} release were 0.13 ± 0.01 and 1.02 ± 0.05 nmol/10^6 cells for 75 nM and 5 μM Ins(1,4,5)P_3 respectively. Data are presented as means ± S.E.M. for 3–6 cell preparations. Significance of differences from the relevant controls was calculated by Student's t test: $^*P < 0.05$, $^{**}P < 0.01$.

	Ca^{2+} release (%)			
			+ Okadaic acid	
	75 nM InsP_3	5 μM InsP_3	75 nM InsP_3	5 μM InsP_3
Control	100	100	96 ± 7	100 ± 2
csPKA (8 units/ml)	109 ± 3*	108 ± 3*	155 ± 13**	114 ± 3*
csPKA (50 units/ml)	149 ± 6**	115 ± 1*	162 ± 4**	119 ± 3*
cpt-cAMP (2 μM)	133 ± 6**	106 ± 2*	157 ± 5**	114 ± 3**

associated with any difference in the final steady-state free Ca^{2+} level.

Ins(1,4,5)P_3-dependent Mn^{2+} influx

Since luminal Ca^{2+} has been shown to sensitize the Ins(1,4,5)P_3 receptor (Nunn and Taylor, 1992; Missiaen et al., 1992), the ability of PKA to potentiate the Ca^{2+}-releasing effect of Ins(1,4,5)P_3 could be secondary to the activation of the Ca^{2+} uptake. We have therefore examined the effect of PKA on the permeability properties of the Ins(1,4,5)P_3-sensitive channel under conditions where the Ca^{2+} pump is blocked. Our approach is based on a recent study by Glennon et al. (1992) demonstrating that Mn^{2+} can enter Ins(1,4,5)P_3-sensitive Ca^{2+} stores in an Ins(1,4,5)P_3-dependent manner. These authors showed that hepatocytes loaded with fura-2/AM have a substantial fraction of fura-2 compartmentalized within the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. In the presence of Ins(1,4,5)P_3, this dye can be quenched by Mn^{2+} added to the cytosolic phase. Mn^{2+} appears to act as a Ca^{2+} surrogate, passing through the Ins(1,4,5)P_3-gated Ca^{2+} channel. Since Mn^{2+} quenches fura-2 in an essentially stoichiometric manner until the dye becomes saturated, the rate of Ins(1,4,5)P_3-stimulated Mn^{2+} quenching of compartmentalized fura-2 can be used as a measure of the net permeability properties of the stores. In addition, the magnitude of Ins(1,4,5)P_3-stimulated Mn^{2+} quenching reflects the size of the compartment to which the Ins(1,4,5)P_3 receptor/channels have access. Ins(1,4,5)P_3-activated quenching of compartmentalized fura-2 by Mn^{2+} can be measured in the presence of the Ca^{2+}-pump inhibitor thapsigargin, where the Ca^{2+} pump is non-functional and the stores are fully depleted of Ca^{2+}. Thus, this system can be used to distinguish between direct effects of PKA on the permeability properties of the Ins(1,4,5)P_3 receptor/channel and indirect effects due to changes in Ca^{2+}-loading state or Ca^{2+} recycling by the Ins(1,4,5)P_3-sensitive Ca^{2+} stores.

When freshly isolated hepatocytes were loaded with fura-2/AM in suspension, only 30–50 % of the intracellular dye was accessible to be quenched rapidly by added Mn^{2+} (50 μM) after permeabilization with digitonin (Figure 2, trace b). Under these conditions the cell suspension was greater than 99 % permeable, as judged by Trypan Blue exclusion. The residual dye was sequestered within intracellular organelles, where it could be quenched by Mn^{2+} in the presence of ionomycin. In the absence of ionomycin, there was a slow continuous quench of the compartmentalized fura-2 after the rapid initial quench of cytosolic dye by Mn^{2+}. Addition of a maximal dose of Ins(1,4,5)P_3 (5 μM) caused a large stimulation in the quench rate, but this returned close to its basal value after about 35 % of the compartmentalized dye was quenched by Mn^{2+}. Termination of the enhanced rate of quench was not due to Ins(1,4,5)P_3 degradation, since the rate and incremental magnitude of Ins(1,4,5)P_3-activated quench were similar when the Mn^{2+} was added 2 min after Ins(1,4,5)P_3. Thus the increment of Ins(1,4,5)P_3-induced quench presumably reflects the size of the Ins(1,4,5)P_3-sensitive compartments within these cells. Subsequent addition of ionomycin allowed Mn^{2+} to quench fura-2 in the remaining compartments. The Mn^{2+}-quench measurements were carried out by using an excitation wavelength of 360 nm, where fura-2 fluorescence is insensitive to Ca^{2+} changes. Figure 2 (trace a) shows that Ins(1,4,5)P_3, ionomycin and Ca^{2+} had no effect on the fluorescence in the absence of Mn^{2+}. Simultaneous measurements at the Ca^{2+}-sensitive excitation wavelength of 340 nm demonstrated that there were no Ca^{2+} movements under these conditions, although the dye was sensitive to the added Ca^{2+} pulses (results not shown). This is due to the presence of the Ca^{2+}-pump inhibitor thapsigargin, which blocks Ca^{2+} uptake and ensures that the ATP-dependent Ca^{2+} stores are fully depleted of Ca^{2+} due to the presence of constitutive leak pathways.

Figure 3 shows the effect of PKA catalytic subunit on the

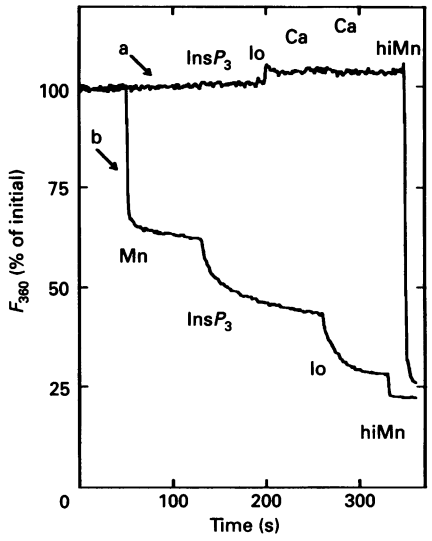

Figure 2 Effect of Ins(1,4,5)P_3 and ionomycin on Mn^{2+} quench of compartmentalized fura-2 in permeabilized hepatocytes

Hepatocytes were loaded with fura-2/AM, washed and then resuspended in permeabilization buffer in the fluorimeter cuvette as described in the Methods section. The cells were permeabilized 5 min before the start of the traces. Additions were made as indicated: Mn, 50 μM $MnCl_2$; InsP_3, 5 μM Ins(1,4,5)P_3; Io, 4 μM ionomycin; Ca, 1 μM $CaCl_2$; hiMn, 500 μM $MnCl_2$. Fluorescence was monitored with 360 nm excitation and 510 nm emission (Ca^{2+}-insensitive wavelength).

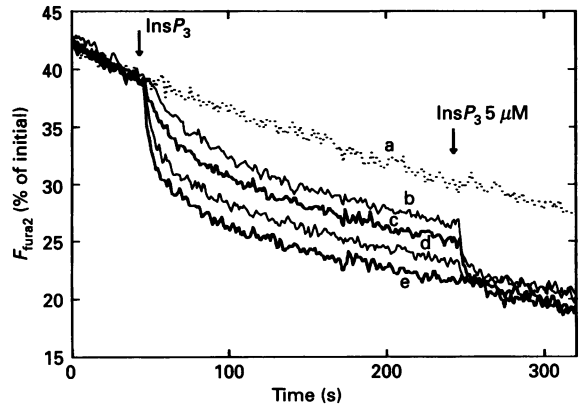

Figure 3 Stimulation by PKA of Ins(1,4,5)P_3-induced Mn^{2+} quench of compartmentalized fura-2

Fura-2-loaded hepatocytes were permeabilized, and the quenching of compartmentalized dye in the presence of 40 μM Mn^{2+} was monitored as described for Figure 2. The traces start 30 s after completion of the fast phase of cytosolic fura-2 quenching. The cells were incubated in the absence (traces a, b, d) or presence of 50 units/ml PKA (traces c, e; thicker lines). At the first arrow Ins(1,4,5)P_3 was added to give 125 nM (b, c) or 250 nM (d, e). Maximal Ins(1,4,5)P_3 (5 μM) was added at the second arrow to all incubations except for (a), which shows the quench rate in the absence of Ins(1,4,5)P_3.

Table 2 Effect of PKA on Ins(1,4,5)P_3-induced Mn^{2+} quench of compartmentalized fura-2

Mn^{2+}-quench measurements in suspensions of permeabilized hepatocytes preloaded with fura-2/AM were carried out as described in the Methods section and the legend to Figure 3. The incubation buffer was supplemented with 100 μM guanosine 5′-[β-thio]diphosphate to prevent any Ins(1,4,5)P_3 formation resulting from activation of phospholipase C by endogenous GTP. Quench responses for each Ins(1,4,5)P_3 dose were measured in separate incubations using 40 μM Mn^{2+}. Initial rates were calculated by linear regression over the linear portion of the quench response occurring immediately after Ins(1,4,5)P_3 addition (arbitrary fluorescence units/s). For measurements of the size of the Ins(1,4,5)P_3-sensitive pool of Mn^{2+}-quenchable fura-2, traces were divided on a point-by-point basis to correct for the basal quench rate (which followed first-order kinetics). This increment is expressed as a percentage of the total compartmentalized fura-2 fluorescence that could be quenched with 500 μM Mn^{2+} in the presence of ionomycin. For PKA treatments, 50 units/ml PKA catalytic subunit was added to the permeabilization medium. 'Percentage Δ with PKA' indicates the percentage increase above the control responses to Ins(1,4,5)P_3 averaged from the individual experiments. Data are presented as means ± S.E.M. of values obtained from 4–5 cell preparations. Significance of differences between the controls and PKA-treated cells was calculated by paired *t* test, and the 'Percentage Δ' values were tested for significance of differences from zero: $^*P < 0.05$, $^{**}P < 0.01$.

	[InsP_3]	Control	PKA	Percentage Δ with PKA
Initial rate (Δ fluorescence/s)	125 nM	507 ± 112	721 ± 153*	46 ± 11*
	5000 nM	3215 ± 517	3195 ± 589	−1 ± 4
Pool size (% of total)	125 nM	17.6 ± 3.8	24.6 ± 3.5**	46 ± 9**
	5000 nM	32.6 ± 3.1	37.6 ± 3.0*	17 ± 8*

Ins(1,4,5)P_3-dependent quench of compartmentalized fura-2 by Mn^{2+} in the presence of thapsigargin. The responses to sequential additions of sub-maximal (125 nM or 250 nM) followed by maximal (5 μM) Ins(1,4,5)P_3 are shown. The quench rate in the absence of Ins(1,4,5)P_3 is also shown for comparison (trace a). For control cells, both the rate and the magnitude of Ins(1,4,5)P_3-stimulated Mn^{2+} quenching increased with Ins(1,4,5)P_3 dose. Incubation of the cells with the catalytic subunit of PKA potentiated the effects of sub-maximal Ins(1,4,5)P_3 doses on both parameters. Table 2 summarizes the effects of PKA on the rate of Ins(1,4,5)P_3-stimulated Mn^{2+} quench of compartmentalized fura-2 and on the size of the Ins(1,4,5)P_3-sensitive compartment of dye for 125 nM and 5 μM Ins(1,4,5)P_3 (added in separate incubations). The last column of Table 2 gives the mean percentage change in response to the treatment with PKA catalytic subunit. The measurements of quench rate, which are determined by the net permeability of the Ins(1,4,5)P_3-sensitive channels to Mn^{2+}, demonstrated that PKA increased the permeability at low Ins(1,4,5)P_3 levels without affecting the permeability at maximal Ins(1,4,5)P_3. These data provide evidence that PKA treatment increases Ins(1,4,5)P_3 sensitivity through a direct modification of channel properties, rather than as a secondary consequence of alterations in Ca^{2+} uptake or loading state. The enhanced sensitivity to Ins(1,4,5)P_3 measured as the rate of quench was also associated with a similar increase in the size of the Mn^{2+}-quenchable pool at 125 nM Ins(1,4,5)P_3 (Table 2). Presumably the shift in Ins(1,4,5)P_3 sensitivity allows a greater fraction of the Ca^{2+} stores to respond at sub-maximal Ins(1,4,5)P_3 doses. Although the Mn^{2+} permeability rate measured with maximal Ins(1,4,5)P_3 was not affected by PKA, Table 2 shows that there was a significant increase in the pool size (17%), which is similar in magnitude to the maximal increase in the size of the Ins(1,4,5)P_3-releasable Ca^{2+} pool (Table 1). These data suggest that PKA treatment may lead to recruitment of additional Ca^{2+} stores into the Ins(1,4,5)P_3-sensitive pool without increasing the number of active channels (reflected in the maximal quench rate).

Effect of PKA on Ins(1,4,5)P_3 action in single cells

Since the fura-2 used to monitor Ins(1,4,5)P_3-induced Mn^{2+} quenching is retained within the intracellular compartments after cell permeabilization, this technique is amenable to measurements of Ins(1,4,5)P_3 action at the single-cell and subcellular levels by using digital imaging fluorescence microscopy. The data described above demonstrate that PKA increases the proportion of Ins(1,4,5)P_3-sensitive stores from which Ca^{2+} can be mobilized by sub-maximal Ins(1,4,5)P_3 doses. This could be a cell-by-cell phenomenon, whereby each cell releases all of its Ca^{2+} at a cell-specific Ins(1,4,5)P_3 threshold, with PKA treatment increasing the number of cells that respond to each sub-maximal Ins(1,4,5)P_3 dose. Alternatively, individual cells could possess a series of Ca^{2+} stores with distinct Ins(1,4,5)P_3 thresholds that are shifted to lower Ins(1,4,5)P_3 levels by PKA. These possibilities can be distinguished by measuring the Mn^{2+}-quench process in individual cells. Furthermore, we have shown that agonist-induced Ca^{2+} transients in intact hepatocytes are organized as waves of $[Ca^{2+}]_i$ initiating at discrete intracellular loci (Rooney et al., 1990, 1991; Thomas et al., 1991). Thus it is possible that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores are spatially heterogeneous within each cell, a suggestion that can also be examined by using the single-cell imaging approach.

For these experiments, hepatocytes were plated on polylysine-

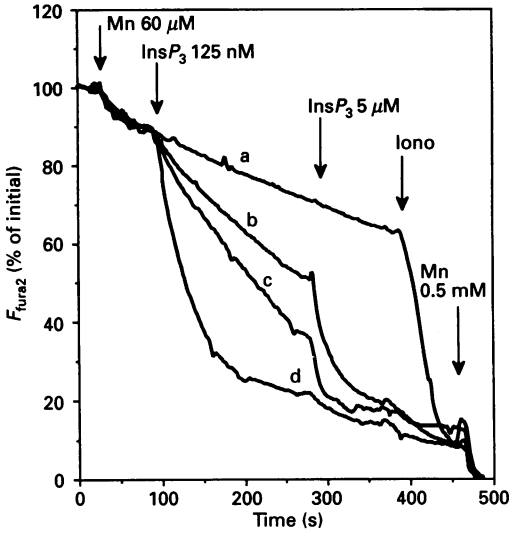

Figure 4 Ins(1,4,5)P_3-induced Mn^{2+} quench of compartmentalized fura-2 in single permeabilized hepatocytes

Hepatocytes attached to glass coverslips (24 h primary culture) were loaded with fura-2/AM and then permeabilized in a thermostatically regulated incubation chamber on the stage of an inverted microscope equipped for low-light-level fluorescence imaging, as described in the Methods section. After completion of cell permeabilization, the cytosolic dye was washed out and the cells were incubated for a further 4 min with (**a**, **b**) no further additions, (**c**) 2 μM cpt-cAMP (also present for 5 min before permeabilization), (**d**) 50 units/ml PKA catalytic subunit. Additions were then made as indicated on the Figure (Mn, 60 μM $MnCl_2$; InsP_3, 125 nM and then 5 μM Ins(1,4,5)P_3; Iono, 4 μM ionomycin], except for trace (**a**), where no Ins(1,4,5)P_3 additions were made. Each trace represents the time course of fura-2 fluorescence quench averaged over a single cell (measured with 360 nm excitation). Traces are representative of data obtained for at least three separate cell preparations, and were selected on the basis of their similarity to mean time courses calculated for all responsive cells in these experiments.

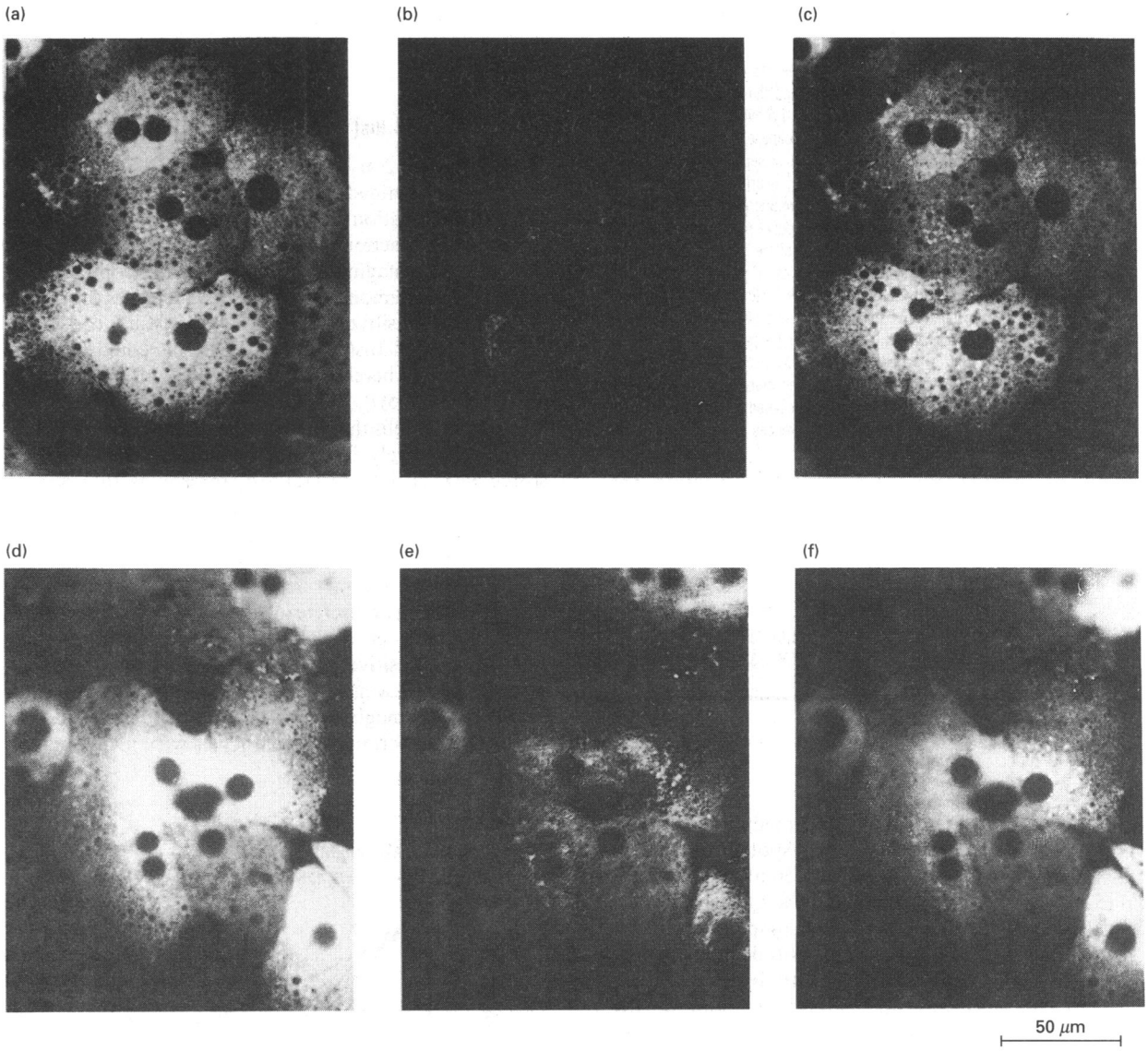

Figure 5 Images of Ins(1,4,5)P_3-activated Mn^{2+} quench of compartmentalized fura-2 in individual hepatocytes

Imaging of Mn^{2+} quench of compartmentalized fura-2 in fura-2/AM-preloaded 24 h primary culture hepatocytes was carried out as described for Figure 4, except that higher magnification and lower time resolution were used to optimize spatial resolution. After cell permeabilization and wash-out of cytosolic fura-2, fluorescence images were accumulated by using 360 nm excitation to monitor the quenching of the compartmentalized dye. Images (**a**)–(**c**) were obtained from control cells, and images (**d**)–(**f**) were obtained from cells preincubated with 50 units/ml PKA catalytic subunit. Panel (**g**) shows the time course of fluorescence quench by Mn^{2+} and the subsequent sequence of additions, for the control (○) and PKA-treated (■) cells. The vertical dashed lines (1–4) indicate the time points used to calculate the images shown in panels (**a**)–(**f**). All images were corrected for autofluorescence (about 10% of signal) by subtracting the residual fluorescence

coated coverslips and loaded with fura-2/AM before carrying out the permeabilization and Mn^{2+} quench experiments in the chamber of a microscope imaging system. After permeabilization, the medium was exchanged for fresh incubation buffer without digitonin, which also resulted in removal of the released cytosolic fura-2. As with the cell-population experiments, Ins(1,4,5)P_3 stimulated the rate of Mn^{2+} quench of the compartmentalized fura-2 in a dose-dependent manner. Figure 4 shows typical single-cell traces demonstrating that cpt-cAMP or PKA catalytic subunit increased the rate of fura-2 quench by Mn^{2+} in the presence of a sub-maximal Ins(1,4,5)P_3 dose (125 nM). Subsequent addition of a maximal dose of Ins(1,4,5)P_3 resulted in a rapid phase of quenching in all cells. Thus it can be concluded that PKA increases the efficacy of Ins(1,4,5)P_3 within individual cells. There was also evidence for an increase in the number of cells responding to the low Ins(1,4,5)P_3 dose. At 125 nM Ins(1,4,5)P_3, the percentage of cells giving a measurable stimulation of Mn^{2+} quench rate increased from 68% in the control cells (67/98 cells in three separate preparations) to 90% in the cells treated with catalytic subunit or with cpt-cAMP (96/106 and 96/107 respectively). However, this apparent shift in threshold for Ins(1,4,5)P_3 action is more difficult to interpret, because the Ins(1,4,5)P_3 responses in the sub-threshold cells may simply have been too small to quantify. Essentially all cells responded to maximal Ins(1,4,5)P_3 in the presence or absence of PKA activation.

The spatial organization of Ins(1,4,5)P_3 action in individual permeabilized hepatocytes is revealed in the images of compartmentalized fura-2 fluorescence shown in Figure 5. The images in Figures 5(a)–5(c) were obtained from a field of control cells, whereas those in Figures 5(d)–5(f) were obtained from a field of hepatocytes pretreated with PKA catalytic subunit. The time course of fluorescence change for cells in the control (○) and PKA-treated (■) preparations is shown in Figure 5(g), which also marks the time points at which the displayed images were obtained. Figures 5(a) and 5(d) show the initial distribution of compartmentalized fura-2 fluorescence after adding Mn^{2+}, just before Ins(1,4,5)P_3 addition (Position 1). Figures 5(b) and 5(e) represent the increment of Mn^{2+} quench of this fura-2 induced by exposure to 125 nM Ins(1,4,5)P_3 for 40 s. These difference images were calculated by subtracting the fluorescence image after Ins(1,4,5)P_3 treatment (Position 2) from the initial fluorescence image (Position 1). The magnitude of Mn^{2+}-quench response at low Ins(1,4,5)P_3 in control cells (Figure 5b) was small, and is barely visible because all of the images of Figure 5 are shown with the same grey scale to allow comparison between the different treatments. For the same Ins(1,4,5)P_3 dose, there was a much larger increment of Mn^{2+} quench in the PKA-treated cells (Figure 5e). Figures 5(c) and 5(f) are difference images (Position 3 minus Position 1) for the response to maximal Ins(1,4,5)P_3. These data demonstrate that maximal Ins(1,4,5)P_3 stimulated Mn^{2+} quench of compartmentalized dye in all parts of the cell, suggesting that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores are distributed throughout the cytoplasm. The only part of the cell where no Ins(1,4,5)P_3-sensitive stores were observed was the nuclear matrix (circular dark areas in Figures 5a and 5d), where there were no fura-2-containing organelles. In the PKA-treated cells, the Mn^{2+} quench induced by sub-maximal Ins(1,4,5)P_3 was distributed similarly to that observed for maximal Ins(1,4,5)P_3 in the same cells. Thus it appears that PKA treatment does not cause a differential sensitization to Ins(1,4,5)P_3 in discrete regions of the cell, and it did not alter the subcellular distribution of Ins(1,4,5)P_3-sensitive stores.

DISCUSSION

Use of Mn^{2+} to monitor Ins(1,4,5)P_3-activated channel function

The stimulation of Mn^{2+} entry into intracellular Ca^{2+} stores by Ins(1,4,5)P_3 was first demonstrated by Glennon et al. (1992). These workers showed that the fluorescence of fura-2 compartmentalized within intracellular organelles could be quenched by Mn^{2+} in the presence of Ins(1,4,5)P_3 or after hormone treatment of the intact cell. We have utilized the Ins(1,4,5)P_3-dependent penetration of Mn^{2+} into intracellular stores as a means to obtain quantitative information on the permeability properties of the Ins(1,4,5)P_3-activated channel. Mn^{2+} is not taken up on the ATP-driven Ca^{2+} pump, and in the absence of Ins(1,4,5)P_3 it quenches compartmentalized fura-2 at a slow rate. Ins(1,4,5)P_3-stimulated Mn^{2+} uptake was examined in the presence of the Ca^{2+}-pump inhibitor thapsigargin, which prevents filling of the Ca^{2+} stores, leaving luminal $[Ca^{2+}]$ in electrochemical equilibrium with the sub-micromolar $[Ca^{2+}]$ in the incubation buffer. Depletion of the intracellular Ca^{2+} stores also minimizes possible competition between Ca^{2+} and Mn^{2+} for passage through the Ins(1,4,5)P_3-activated channel. Mn^{2+} has a very high affinity for fura-2 ($K_d < 3$ nM; Kwan and Putney, 1990), so that it quenches the dye almost stoichiometrically as it enters the intracellular stores. Therefore, the Ins(1,4,5)P_3-stimulated rate of Mn^{2+} quench is directly proportional to the net permeability of the channels. The quench rate is also dependent on $[Mn^{2+}]$. At millimolar Mn^{2+} concentrations, quenching of compartmentalized fura-2 occurs too rapidly to follow, but with 2–10 μM free Mn^{2+} (buffered with ATP) the time course of Ins(1,4,5)P_3-stimulated quenching is extended, allowing ready measurements of the uptake rate over a range of Ins(1,4,5)P_3 doses. There does not appear to be any inactivation of the Ins(1,4,5)P_3-sensitive channels during the period of measurement, since the rate and amplitude of Ins(1,4,5)P_3-dependent Mn^{2+} quench is similar when the Mn^{2+} is added before or several minutes after Ins(1,4,5)P_3.

Compartmentation of fura-2 in intracellular organelles occurs to some extent in almost all cells loaded with the acetoxymethyl ester of the dye (Thomas and Delaville, 1991) and was optimized in the present study by incubating hepatocytes at 37 °C for a prolonged period at very low cell density. It is difficult to determine exactly how the compartmentalized dye is distributed between different organelles in permeabilized hepatocytes. However, the relative sizes of the Ins(1,4,5)P_3- and ionomycin-sensitive fura-2 compartments that can be quenched by Mn^{2+} are similar to the fraction of intracellular Ca^{2+} stores available for release by Ins(1,4,5)P_3 and ionomycin. In the present study we have relied primarily on comparisons of Mn^{2+} permeability rates at low and high Ins(1,4,5)P_3 levels, where other Ins(1,4,5)P_3-insensitive fura-2 compartments are irrelevant. A potential complication of using Mn^{2+} to investigate Ins(1,4,5)P_3-activated channel function is that Mn^{2+} might interact with the Ca^{2+} regulatory sites that are believed to modulate the Ins(1,4,5)P_3 receptor. Any effects of Mn^{2+} on a cytosolic regulatory site would be fixed under our permeabilization conditions, because the $[Mn^{2+}]$ is well buffered by binding to ATP. Mn^{2+} would be unlikely to interact with a luminal Ca^{2+}-binding site during the measurements of initial uptake rate, because it is chelated by the luminal fura-2.

image obtained after addition of ionomycin (position 4). Images (**a**) and (**c**) represent the distribution of total compartmentalized fura-2 20 s after Mn^{2+} addition (position 1). Images (**b**) and (**e**) are difference images representing the Mn^{2+} quench (fluorescence decrease) after 40 s treatment with 100 nM Ins(1,4,5)P_3, calculated by subtracting the image at position 2 from the image at position 1. Images (**c**) and (**f**) are difference images representing the maximal Ins(1,4,5)P_3-induced Mn^{2+} quench, calculated by subtracting position 3 from position 1.

Quantal behaviour of Ins(1,4,5)P_3-stimulated Mn^{2+} quench of compartmentalized fura-2

A number of studies have demonstrated that sub-maximal Ins(1,4,5)P_3 doses release only a fraction of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, with the residual Ca^{2+} being unavailable for release until a higher Ins(1,4,5)P_3 dose is added (Muallem et al., 1989; Taylor and Potter, 1990; Meyer and Stryer, 1990; Oldershaw et al., 1991). This phenomenon is not due to receptor desensitization or Ca^{2+} recycling, and has been referred to as 'quantal' or 'incremental' Ca^{2+} release. Two models have been suggested to explain this behaviour. One is based on the feedback effects of luminal Ca^{2+} on Ins(1,4,5)P_3 sensitivity, giving rise to varying extents of self-limited Ca^{2+} release dependent on Ins(1,4,5)P_3 dose (Missiaen et al., 1992; Taylor and Richardson, 1991). The other model suggests that cells contain a series of discrete Ca^{2+} stores which differ in Ins(1,4,5)P_3 sensitivity, so that the proportion of stores responding depends on the Ins(1,4,5)P_3 dose, with the less sensitive stores being unaffected at lower Ins(1,4,5)P_3 levels (Muallem et al., 1989; Oldershaw et al., 1991; Taylor and Richardson, 1991).

The work described in the present study using suspensions of permeabilized hepatocytes demonstrates that Ins(1,4,5)P_3-stimulated Mn^{2+} quench of compartmentalized fura-2 also occurs in a quantal manner. At sub-maximal Ins(1,4,5)P_3 levels, Mn^{2+} quench occurred in a fraction of the Ins(1,4,5)P_3-sensitive stores that was proportional to Ins(1,4,5)P_3 dose. After the initial phase of Ins(1,4,5)P_3-stimulated quench, the Mn^{2+} quench rate returned to the basal value until a higher Ins(1,4,5)P_3 dose was added. These experiments were carried out under conditions where the Ca^{2+} stores were fully depleted of Ca^{2+} and in the absence of active Ca^{2+} fluxes. Thus the Ins(1,4,5)P_3-sensitive Ca^{2+} stores can behave in a quantal manner independent of regulation by Ca^{2+}. The fact that the residual compartments of fura-2 remained resistant to quenching, despite the sustained open state of the Ins(1,4,5)P_3-activated channels and the high affinity of fura-2 for Mn^{2+}, suggests that the Ins(1,4,5)P_3-gated channels in these residual compartments remained closed throughout the incubation period. Therefore these data support the concept of a series of discrete Ca^{2+} stores with differing Ins(1,4,5)P_3 sensitivities.

In our experiments with single hepatocytes attached to coverslips, Ins(1,4,5)P_3-induced quench of compartmentalized fura-2 did not occur in a clearly quantal manner (although the quench rate was dose-dependent). Furthermore, under these conditions the Ins(1,4,5)P_3-sensitive compartment represented a much larger fraction of the total amount of compartmentalized dye that could be quenched by Mn^{2+} in the presence of ionomycin. This difference in behaviour between the two permeabilized cell preparations appears to result from the maintenance of substantial luminal continuity between intracellular Ca^{2+} stores in the adherent cells, where permeabilization can be controlled more carefully (D. C. Renard-Rooney, G. Hajnóczky, M. B. Seitz and A. P. Thomas, unpublished work). This continuity may be disrupted when cells in suspension are permeabilized, and the fragmented Ca^{2+} stores can then express the quantal Ca^{2+}-release behaviour. This hypothesis is currently under investigation in this laboratory.

PKA effects on Ca^{2+}-pool sizes

A number of studies in a range of different tissues have reported that activation of PKA results in an increase in the size of the Ins(1,4,5)P_3-releasable Ca^{2+} pool. Thus, after treatment with PKA catalytic subunit or cAMP analogues, maximal doses of Ins(1,4,5)P_3 mobilize a larger amount of stored Ca^{2+} from brain microsomes (Supattapone et al., 1988; Volpe and Alderson-Lang, 1990), platelet membrane vesicles (Enouf et al., 1987) and permeabilized hepatocytes (Burgess et al., 1991; the present work). The increase in Ins(1,4,5)P_3-releasable Ca^{2+} is often accompanied by an increase in the total amount of Ca^{2+} accumulated into the Ca^{2+} stores in an ATP-dependent manner (Enouf et al., 1987; Supattapone et al., 1988; Burgess et al., 1991). This suggests that the apparent increase in the Ca^{2+}-pool size available for release by Ins(1,4,5)P_3 could be secondary to alterations in Ca^{2+} sequestration. Direct measurements of the rate of ATP-dependent Ca^{2+} uptake in isolated rat liver microsomes have shown that PKA activation results in an increase in both the initial rate and the maximal extent of Ca^{2+} loading (Taylor et al., 1980). We were able to confirm the stimulation of Ca^{2+}-uptake rate in permeabilized hepatocytes after treatment with PKA catalytic subunit, although this was not accompanied by any change in the final concentration of Ca^{2+} in the medium once a steady state had been attained. Thus, if PKA enhances Ca^{2+} uptake into the Ins(1,4,5)P_3-sensitive stores in permeabilized hepatocytes, it must do so at the expense of other Ins(1,4,5)P_3-insensitive Ca^{2+} stores under the conditions used in the present work.

An alternative mechanism by which PKA could supplement the Ins(1,4,5)P_3-releasable Ca^{2+} pool would be to unmask latent Ins(1,4,5)P_3 receptors in Ca^{2+} stores that were previously insensitive to Ins(1,4,5)P_3. A functionally equivalent effect could also be achieved if PKA induced luminal connections between these Ca^{2+} stores, as appears to occur after GTP treatment (Dawson et al., 1987; Thomas, 1988; Ghosh et al., 1989). These possibilities can be examined by using the Ins(1,4,5)P_3-stimulated Mn^{2+} quenching of compartmentalized fura-2, since this dye is present within both Ins(1,4,5)P_3-sensitive and -insensitive Ca^{2+} stores. Thus, if PKA treatment leads to some form of recruitment of Ca^{2+}-storage organelles, the proportion of compartmentalized fura-2 accessible to be quenched in an Ins(1,4,5)P_3-dependent manner should be increased. The data obtained with suspensions of permeabilized hepatocytes demonstrate just such an increase in the maximal extent of Mn^{2+} quench with saturating Ins(1,4,5)P_3 levels. The increase in the size of the Ins(1,4,5)P_3-sensitive compartment (17%) is similar in magnitude to the enhancement of Ca^{2+} release in response to maximal Ins(1,4,5)P_3 in the presence of PKA catalytic subunit plus okadaic acid (Table 2 versus Table 1). Thus the increase in the Ins(1,4,5)P_3-releasable Ca^{2+} pool size after PKA treatment could be explained by a recruitment of additional stores into the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. The fact that the Mn^{2+}-quenchable pool size increased without any change in the rate of quench at saturating Ins(1,4,5)P_3 levels suggests that the recruitment of stores did not result from an unmasking of latent channels. However, we cannot fully exclude the possibility that a small population of strategically placed latent channels became sensitized to Ins(1,4,5)P_3, causing a disproportionate increase in the size of the Ins(1,4,5)P_3-sensitive store without a measurable effect on the maximal rate of Mn^{2+} permeability. It should also be noted that the data obtained with the Mn^{2+}-quench technique do not exclude an additional role for activation of the Ca^{2+} pump in increasing the amount of Ca^{2+} available for release by Ins(1,4,5)P_3 under normal Ca^{2+} loading conditions.

PKA effects on the sensitivity to Ins(1,4,5)P_3 for Ca^{2+} release

The Ins(1,4,5)P_3 receptor contains consensus phosphorylation sites for PKA, and both the neuronal and non-neuronal receptor types have been shown to be stoichiometrically phosphorylated by PKA (Grossman et al., 1975; Supattapone et al., 1988; Ferris

et al., 1991). Phosphorylation does not appear to alter the binding affinity of the Ins(1,4,5)P_3 receptor from brain (Supattapone et al., 1988; Volpe and Alderson-Lang, 1990) or liver (Mauger et al., 1989). However, the potency of Ins(1,4,5)P_3 for Ca^{2+} release in subcellular systems has been found to be markedly affected after PKA activation or treatment with PKA catalytic subunit. In both rat and dog brain microsomes phosphorylation by PKA results in a decrease in Ins(1,4,5)P_3 sensitivity, yielding a rightward shift in the dose/response curve for Ca^{2+} release by up to 10-fold (Supattapone et al., 1988; Volpe and Alderson-Lang, 1990). By contrast, a 4-fold increase in Ins(1,4,5)P_3 sensitivity was observed in the Ca^{2+}-release studies of Burgess et al. (1991) in permeabilized guinea-pig hepatocytes. The increase in Ins(1,4,5)P_3 sensitivity in this permeabilized hepatocyte system was not due to alterations in metabolism of the added Ins(1,4,5)P_3. The PKA-mediated enhancement in the potency of sub-maximal Ins(1,4,5)P_3 doses for Ca^{2+} release was confirmed for rat hepatocytes in the present study. The contrasting effects of PKA on Ins(1,4,5)P_3 sensitivity in liver and brain could reflect functional differences in Ins(1,4,5)P_3 receptors in these tissues, or may result from distinct pathways of indirect regulation of the Ca^{2+}-release process.

Mechanism of PKA-induced Ins(1,4,5)P_3 sensitization in hepatocytes

A potential indirect mechanism by which PKA could enhance Ins(1,4,5)P_3 sensitivity in hepatocytes would be by increasing the Ca^{2+}-loading state of the Ins(1,4,5)P_3-sensitive stores. Recent studies in permeabilized hepatocytes (Nunn and Taylor, 1992) and A7r5 smooth-muscle cells (Missiaen et al., 1992) have suggested that the Ca^{2+} content of these stores affects their sensitivity to Ins(1,4,5)P_3 for Ca^{2+} release, with higher Ca^{2+} loads being associated with a lower EC_{50} for Ins(1,4,5)P_3 action. Thus PKA could increase the efficacy of sub-maximal Ins(1,4,5)P_3 doses via this luminal Ca^{2+} regulation of Ins(1,4,5)P_3 sensitivity. To examine whether the effect of PKA on Ins(1,4,5)P_3 sensitivity is a secondary consequence of the increased Ca^{2+}-loading state of the Ins(1,4,5)P_3-sensitive stores, we have used the Mn^{2+}-quench technique to measure the permeability properties of the Ins(1,4,5)P_3 receptor/channel in the absence of Ca^{2+} movements. This was achieved by treating the permeabilized hepatocytes with thapsigargin, which resulted in complete depletion of both Ins(1,4,5)P_3- and ionomycin-sensitive Ca^{2+} stores. Therefore thapsigargin treatment should eliminate the luminal effects of Ca^{2+} on Ins(1,4,5)P_3 sensitivity and prevent any alteration in luminal Ca^{2+} content mediated by the effects of PKA on the ATP-dependent Ca^{2+} pumps. Under these conditions, PKA was still able to potentiate the effects of sub-maximal Ins(1,4,5)P_3, indicating that the activation by PKA was not wholly dependent on an altered luminal Ca^{2+} content. The Mn^{2+} used to monitor Ins(1,4,5)P_3-activated channel permeability is unlikely to substitute for luminal Ca^{2+} in mediating the effects of PKA, because the increased Ins(1,4,5)P_3 sensitivity can be observed in the initial rate of Mn^{2+} quench, where the free [Mn^{2+}] within the stores is minimal due to buffering by the fura-2.

The Ins(1,4,5)P_3-induced Mn^{2+} quench of compartmentalized fura-2 is a direct measure of the net permeability of the Ins(1,4,5)P_3-sensitive channels, effectively isolated from other ion uptake and release pathways. Thus the potentiation of Mn^{2+} quench by PKA suggests that the Ins(1,4,5)P_3-receptor protein or an associated regulatory component is the primary target of PKA action. The increase in the rate of Mn^{2+} entry at sub-maximal Ins(1,4,5)P_3 would be consistent with either a shift in the EC_{50} for Ins(1,4,5)P_3 or an enhancement of the overall permeability properties of the channels. The latter could be brought about by an increase in single-channel conductance or by activation of previously latent channels. However, PKA had no effect on the initial rate of Mn^{2+} quench at saturating Ins(1,4,5)P_3 levels, indicating that the effects of PKA were not mediated by an increase in the number of active Ins(1,4,5)P_3-sensitive channels. A PKA-induced increase in the single-channel conductance of the Ins(1,4,5)P_3 receptors would also be expected to result in an enhancement of net permeability at saturating Ins(1,4,5)P_3. Thus the effects of PKA on rates of Mn^{2+} quench at sub-maximal and maximal Ins(1,4,5)P_3 doses suggest that the mechanism of PKA action is associated with a shift in the sensitivity to Ins(1,4,5)P_3 for opening the ion channel, with no change in the number or gating properties of these channels.

Subcellular organization of Ins(1,4,5)P_3-sensitive Ca^{2+} stores

An important benefit of the Ins(1,4,5)P_3-activated Mn^{2+}-quench technique is that the sites of Ins(1,4,5)P_3 action can be resolved spatially in single-cell imaging studies. This is because the signal is derived from essentially immobile Ca^{2+} stores, as opposed to measuring released Ca^{2+}, which rapidly redistributes. Our studies demonstrate that Ins(1,4,5)P_3 stimulates Mn^{2+} quench of compartmentalized dye in all parts of the cell, except the nuclear matrix, which does not contain any membrane-bound compartments of dye (other than the surrounding nuclear envelope). The observation that Ins(1,4,5)P_3-sensitive stores are spread throughout the cell is important in terms of the Ca^{2+} waves that propagate through intact hepatocytes from a fixed origin after hormone treatment (Rooney et al., 1990, 1991; Thomas et al., 1991). We have suggested that the site of Ca^{2+}-wave initiation might reflect a localization of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in this region, with Ca^{2+} waves propagating through the cell by a process of Ca^{2+}-induced Ca^{2+} release from Ins(1,4,5)P_3-insensitive Ca^{2+} stores. However, the subcellular distribution of Ins(1,4,5)P_3-sensitive stores determined in the present study does not support this model. These data are more consistent with models of Ca^{2+}-wave propagation involving regenerative Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} stores located throughout the cell. In the context of the present study, we were interested in determining whether there might be inter- or intra-cellular heterogeneity in PKA responsiveness. However, the data demonstrate that individual cells show graded responses to Ins(1,4,5)P_3 that are enhanced after treatment with PKA catalytic subunit. Furthermore, there is no subcellular heterogeneity in PKA action at the resolution attainable with fluorescence microscopy.

Role of protein phosphatases

Optimal effects of sub-saturating concentrations of PKA catalytic subunit or cAMP analogues on both Ins(1,4,5)P_3-induced Ca^{2+} release and Mn^{2+} quench of compartmentalized fura-2 were only observed when the phosphatase inhibitor okadaic acid was included in the permeabilization medium. This demonstrates that the actions of PKA are actively opposed by endogenous phosphoprotein phosphatases in this preparation (probably protein phosphatase 1 or 2A; Cohen et al., 1990). The presence of an appropriate and effective phosphatase is an important prerequisite for PKA-dependent phosphorylation to control Ins(1,4,5)P_3 sensitivity *in vivo*. The presence of active phosphatases also needs to be taken into account in designing experiments to investigate the regulation of the Ins(1,4,5)P_3 receptor by protein kinases. This may be particularly important for Ins(1,4,5)P_3-binding measurements which are carried out for

prolonged periods at low temperature, usually in the absence of ATP.

Conclusions

The work described here demonstrates that the ability of PKA to potentiate the actions of Ins(1,4,5)P_3-dependent hormones in hepatocytes is mediated, at least in part, by a direct action on the permeability properties of the Ins(1,4,5)P_3 receptor/channel. This action takes the form of an increase in the sensitivity to Ins(1,4,5)P_3 for channel opening, with no apparent change in maximal conductance of these channels. The enhanced sensitivity to Ins(1,4,5)P_3 is not secondary to PKA-induced alterations in Ca^{2+} homoeostasis and can occur in the absence of a Ca^{2+} gradient or Ca^{2+} fluxes across the membrane of the stores. Although the direct effect of PKA on the Ins(1,4,5)P_3 receptor/channels has the most marked effect, two other actions of PKA on the intracellular Ca^{2+} stores have also been identified in permeabilized hepatocytes. PKA treatment leads to a stimulation of the ATP-dependent Ca^{2+} pump, and modifies the functional distribution of Ca^{2+} stores between the Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive Ca^{2+} pools.

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