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Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes

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In permeabilized hepatocytes, inositol 1,4,5-trisphosphate, inositol 2,4,5-trisphosphate and inositol 4,5-bisphosphate induced rapid release of Ca^{2+} from an ATP-dependent, non-mitochondrial vesicular pool, probably endoplasmic reticulum. The order of potency was inositol 1,4,5-trisphosphate > inositol 2,4,5-trisphosphate > inositol 4,5-bisphosphate. The Ca^{2+}-releasing action of inositol 1,4,5-trisphosphate is not inhibited by high [Ca^{2+}], nor is it dependent on [ATP] in the range of 50 μM–1.5 mM. These results suggest a role for inositol 1,4,5-trisphosphate as a second messenger in hormone-induced Ca^{2+} mobilisation, and that a specific receptor is involved in the Ca^{2+}-release mechanism.

A class of receptors exists on hepatocytes and a wide variety of other tissues that, when activated, alter cellular processes by causing an elevation in cytosolic [Ca^{2+}] (Rasmussen, 1981). In liver cells the Ca^{2+} ions involved in this process are believed to enter the cytoplasm mainly from the major Ca^{2+}-sequestering organelles, the endoplasmic reticulum (ER) and mitochondria (Poggioli *et al.*, 1980; Exton, 1981; Williamson *et al.*, 1981; but see also DeWitt & Putney, 1984). Recently we presented evidence that, in agreement with the work in pancreatic acinar cells by Streb *et al.* (1983), suggests that (1,4,5)IP_3 may be the messenger that links the activation of plasma-membrane receptors by Ca^{2+}-mobilizing hormones to intracellular Ca^{2+} release in liver (Burgess *et al.*, 1984). Taking a similar approach, the observations leading to this conclusion have now been confirmed by Joseph *et al.* (1984).

Michell (1975) has put forward a theory according to which activation of Ca^{2+}-mobilizing receptors causes alterations in the metabolism of inositol lipids, which in turn may serve as a signal to mobilize Ca^{2+}. The initial reaction believed to follow receptor activation is the phosphodiesteratic breakdown of the polyphosphoinositides to form diacylglycerol and soluble inositol phosphates (Michell *et al.*, 1981; Kirk *et al.*, 1981; Berridge, 1983; Berridge *et al.*, 1983; Downes & Wusteman, 1983; Aub & Putney, 1984). Previously we have shown that, when applied to permeabilized hepatocytes in concentrations lower than are likely to be formed by receptor activation, and with free [Ca^{2+}] buffered to a level similar to that in cytosol, (1,4,5)IP_3 causes Ca^{2+} release from a pool likely to be a component of the ER (Burgess *et al.*, 1984). The quantity of Ca^{2+} released was similar to that released from the intracellular stores of intact hepatocytes by hormones, and so it was proposed that this soluble low-M_r compound, which is assumed to be formed from the phospholipase C-mediated breakdown of (4,5)PIP_2, could be the link between the plasma-membrane receptors and the intracellular hormone-sensitive Ca^{2+} pool.

We have now examined the ability of (1,4,5)IP_3 to cause $^{45}Ca^{2+}$ release from the intracellular Ca^{2+} stores of permeabilized hepatocytes in more detail and have widened this study to include other inositol phosphates. We have shown that the active inositol phosphates tested are able to release $^{45}Ca^{2+}$ only from an ATP-dependent pool thought to be the ER, and that they are not able to release $^{45}Ca^{2+}$ from mitochondria.

Abbreviations used: ER, endoplasmic reticulum; (4,5)PIP_2, phosphatidylinositol 4,5-bisphosphate; (1,4,5)IP_3, inositol 1,4,5-trisphosphate; (2,4,5)IP_3, inositol 2,4,5-trisphosphate; (4,5)IP_2, inositol 4,5-bisphosphate; (1)IP, inositol 1-phosphate; (1,2)IcP, inositol 1,2-cyclic phosphate; DNP, 2,4-dinitrophenol.

Materials and methods

Preparation of isolated saponin-treated hepatocytes

Isolated hepatocytes were prepared by collagenase digestion of the livers of male Hartley guinea pigs (Burgess *et al.*, 1981). The cells were then exposed to saponin (75 μg/ml) in a medium resembling cytosol, which had the following composition (mM): NaCl, 20.0; KCl, 100; $MgSO_4$, 5.0; NaH_2PO_4, 0.96; $NaHCO_3$, 25; EGTA, 1.0 and 2% albumin at pH 7.2 and 37°C (Burgess *et al.*, 1983) for a period of 10 min. This treatment caused more than 99% of the cells to become permeable to Trypan Blue. The cells were then washed and suspended in a medium of the same composition but without saponin. For most experiments the medium also contained antimycin (10 μM) to prevent substrate oxidation, ATP (usually 1.5 mM) and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase (5 units/ml). Ca–EGTA buffers were used to set free Ca^{2+} concentration as described previously (Burgess *et al.*, 1983).

Measurement of $^{45}Ca^{2+}$ contents of isolated hepatocytes

Cells were incubated with 1 μCi of $^{45}Ca^{2+}$/ml at a density of about 3–5 mg of cellular protein/ml for the times indicated in individual experiments. Contents of $^{45}Ca^{2+}$ were determined by rapidly diluting 100 μl aliquots of the cell suspension in 10 ml of ice-cold iso-osmotic sucrose (310 mM) containing EGTA (4 mM) and tracer amounts (0.5 μCi/ml) of [^{3}H]sucrose or [^{3}H]mannose for determination of trapped volume. The samples were rapidly filtered through GF/C glass-fibre filters and washed with 10 ml of ice-cold iso-osmotic sucrose. The filters were then counted for radioactivity. For most of these experiments the cells were used within 1 h of treatment with saponin because in some preparations a decrease in the responsiveness to (1,4,5)IP_3 was observed with longer incubations.

Preparation of inositol phosphates

(1,4,5)IP_3, (2,4,5)IP_3, (1,4)IP_2 and (4,5)IP_2 were prepared from brain inositol fractions by strong-alkaline hydrolysis and purified by paper chromatography as originally described by Grado & Ballou (1961) and Tomlinson & Ballou (1961), with modifications as given by Irvine *et al.* (1984). (1)IP and (1,2)IcP were prepared enzymically from yeast phosphatidylinositol and purified by ionophoresis (Irvine *et al.*, 1978, 1979).

Materials

Radioactive materials were obtained from New England Nuclear. Collagenase was obtained from Boehringer Mannhein and ionophore A23187 from Calbiochem. All other compounds were obtained from Sigma.

Results and discussion

Release of $^{45}Ca^{2+}$ by inositol phosphates from permeabilized hepatocytes

As it has been established (Burgess *et al.*, 1984) that (1,4,5)IP_3 (5 μM) will release approx. 0.5 nmol of $^{45}Ca^{2+}$/mg of protein from saponin-treated guinea-pig hepatocytes that had previously accumulated this $^{45}Ca^{2+}$ in an ATP-dependent manner, the ability of some other inositol phosphates to initiate $^{45}Ca^{2+}$ release under similar conditions was tested. Permeabilized hepatocytes were incubated in a cytosolic-type medium with Ca^{2+} buffered to 180 nM, which is equivalent to resting cytosolic [Ca^{2+}] in intact hepatocytes (Burgess *et al.*, 1983; Charest *et al.*, 1983). On addition of ATP (1.5 mM, in the presence of a regenerating system), the cells accumulated $^{45}Ca^{2+}$ rapidly to a steady state of approximately 2 nmol/mg of protein. In experiments shown in Fig. 1, various inositol phosphates (5 μM) were applied to these permeable cells. Of the inositol phosphates tested, (1,4,5)IP_3, (1,4)IP_2, (1)IP and (1,2)IcP could possibly be formed in cells after Ca^{2+}-mobilizing-hormone stimulation, either by phosphodiesteratic attack of the polyphosphoinositides or phosphatidylinositol, or by the action of phosphomonoesterases on the inositol phosphates formed by the phospholipase(s) C. (2,4,5)IP_3 and (4,5)IP_2 are less likely to be formed in cells. It can be seen (Fig. 1) that only the inositol trisphosphates (1,4,5)IP_3 and (2,4,5)-IP_3 were able to evoke $^{45}Ca^{2+}$ loss, each releasing about 25% of the total cell $^{45}Ca^{2+}$. The effect was fairly rapid, being maximal in less than 2 min, after which the cells appeared to re-accumulate some of the $^{45}Ca^{2+}$ lost. This latter effect may occur after degradation of the inositol phosphates by cellular enzymes (Joseph *et al.*, 1984). When applied at 5 μM, the inositol bisphosphates [(1,4)IP_2 and (4,5)IP_2], the inositol monophosphates [(1,2)IcP and (1)IP] and *myo*-inositol (not shown) were unable to release any $^{45}Ca^{2+}$.

In order to determine whether some of these latter compounds were simply less potent than (1,4,5)IP_3 and (2,4,5)IP_3, two of them, (1,4)IP_2 and (4,5)IP_2, were tested at higher concentrations. (1,4)IP_2 was ineffective in causing $^{45}Ca^{2+}$ release up to 20 μM, the highest concentration applied, but it was found that (4,5)IP_2 could cause $^{45}Ca^{2+}$ efflux from the permeable hepatocytes in concentrations of 40 μM or greater. Fig. 2 shows the concentration–effect relationship for the three inositol phosphates found to be active in releasing $^{45}Ca^{2+}$ from the permeable guinea-pig hepatocytes. The most

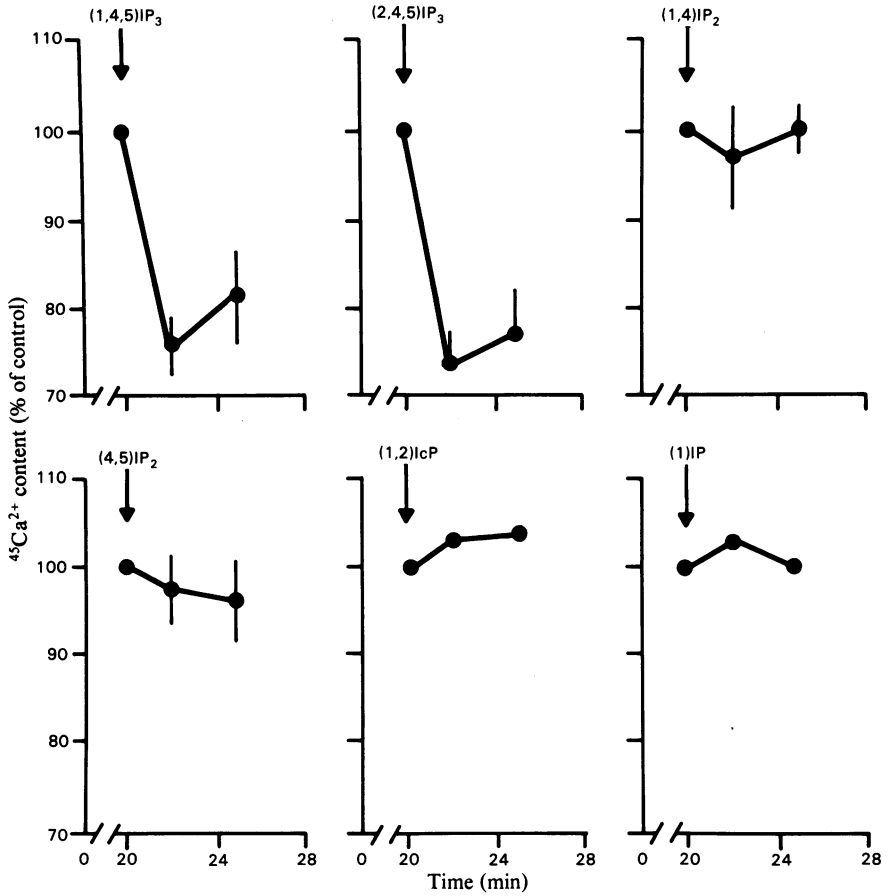

Fig. 1. *Effect of various inositol phosphates on the $^{45}Ca^{2+}$ content of saponin-treated guinea-pig hepatocytes*
At zero time $^{45}Ca^{2+}$ was added to a suspension of saponin-treated hepatocytes with $[Ca^{2+}]$ in the medium set at 180nM. At 10min, ATP [1.5mM, in the presence of creatine phosphate (5mM), creatine phosphokinase (5units/ml) and antimycin (10μM)] was added and there was a rapid uptake of $^{45}Ca^{2+}$ to steady-state of about 1.8nmol of $^{45}Ca^{2+}$/mg of protein. This ATP-dependent uptake was insensitive to a combination of DNP (0.5mM) and oligomycin (10μM) and is believed to be taken up mainly by the ER. At 20min, various inositol phosphates (5μM) were added as indicated by the arrows, and for (1,4,5)IP_3 and (2,4,5)IP_3 there was a loss of 25% of the cell $^{45}Ca^{2+}$ content or about 0.5nmol of $^{45}Ca^{2+}$/mg of protein. The other inositol phosphates had no effect at 5μM. The $^{45}Ca^{2+}$ levels are expressed as percentages of the contents of control cells that were treated with an equivalent volume of water. The bars indicate S.E.M. for three to four observations. The result for (1,2)IcP and (1)IP are from one of two experiments which gave similar results.

potent compound was (1,4,5)IP_3, which had an EC_{50} (concentration causing half-maximal effect) of 0.22μM and was maximal at 5μM. (2,4,5)IP_3 had an EC_{50} of about 1.3μM and was maximal at about 10μM. (4,5)IP_2 was much less potent, releasing approx. 0.4nmol of $^{45}Ca^{2+}$/mg of protein at 100μM. All three compounds had approximately the same efficacy. It is not clear whether, had it been possible to apply them at much higher concentrations, some of the other compounds tested might also have been effective Ca^{2+}-releasing agonists.

The results indicate that although several inositol phosphates are able to release $^{45}Ca^{2+}$ from the permeable cells, the effect was not non-specific, because the 'receptor' on which these compounds act would appear to have strict structural requirements. On the basis of these results, it would seem that an inositol trisphosphate is more potent than a bisphosphate, and that the occupation of positions 1, 4 and 5 appears to be optimal for activity. There is also an indication that the *vicinal* phosphate groups in positions 4 and 5 may confer part of the activity to the molecule. That (1,4,5)IP_3 has the highest potency of those inositol phosphates tested is interesting, since the initial step in Ca^{2+}-mobilizing-hormone action is the breakdown of polyphosphoinositides with the rapid formation of

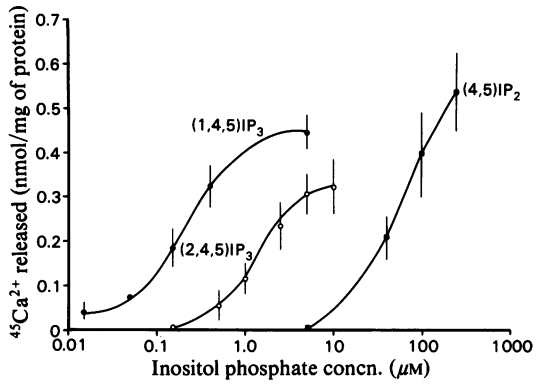

Fig. 2. *Concentration–effect relationship for the ability of some inositol phosphates to release $^{45}Ca^{2+}$ from permeable hepatocytes*

●, (1,4,5)IP$_3$; ○, (2,4,5)IP$_3$; ■, (4,5)IP$_2$. The experiments were carried out as described in Fig. 1, except for those with (4,5)IP$_2$. In this case, the permeable cells were incubated in a buffer containing 2.2 μM-Ca^{2+} (in the presence of 0.5 mM-DNP and 10 μM-oligomycin to prevent ATP-dependent uptake of $^{45}Ca^{2+}$ by the mitochondria). This meant that the ATP-dependent $^{45}Ca^{2+}$ uptake by these cells was greater than that by cells incubated at 180 nM-Ca^{2+}, amounting to 3.0 ± 0.2 nmol of $^{45}Ca^{2+}$/mg of protein ($n = 12$). The higher $^{45}Ca^{2+}$ content may account for the slightly greater inositol phosphate-induced $^{45}Ca^{2+}$ release in these experiments. In all experiments the amount of $^{45}Ca^{2+}$ released was determined 2 min after addition of the inositol phosphates, by which time the effect was maximal. Data were averaged and the bars indicate S.E.M. of three to twelve observations.

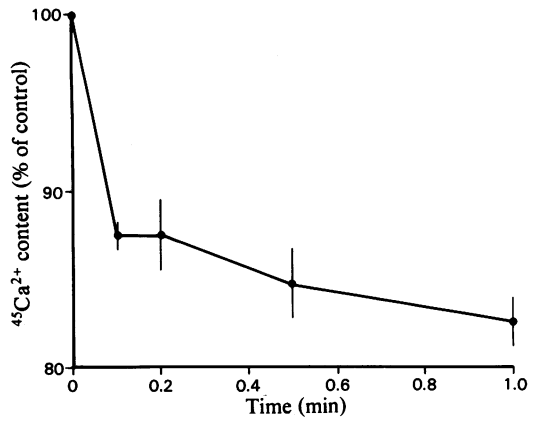

Fig. 3. *Time course of (1,4,5)IP$_3$-induced $^{45}Ca^{2+}$ release from permeable hepatocytes*

Experiments were carried out as described for Fig. 1. Contents of $^{45}Ca^{2+}$ are expressed as percentages of control samples taken simultaneously. The bars indicate the S.E.M. for three observations.

inositol phosphates, one of which is likely to be (1,4,5)IP$_3$ (Michell *et al.*, 1981; Kirk *et al.*, 1981; Downes & Wusteman, 1983; Berridge, 1983; Burgess *et al.*, 1984; Aub & Putney, 1984).

The time course of the effect of (1,4,5)IP$_3$ (5 μM) in causing $^{45}Ca^{2+}$ release from the permeable cells was extremely rapid. Fig. 3 shows that the response was more than 70% complete within 6 s, and could therefore account for the rapid hormone-induced rise in $[Ca^{2+}]_i$ (cytosolic $[Ca^{2+}]$) observed with the Ca^{2+} indicator quin-2 (Charest *et al.*, 1983), and also for the appearance of the various Ca^{2+}-mediated responses of the liver cell (Exton, 1981).

When permeable cells were incubated at 180 nM-Ca^{2+} in the absence of ATP (Fig. 4*a*) they bound approx. 0.3 nmol of $^{45}Ca^{2+}$/mg of protein, and this was not altered on addition of 5 μM-(1,4,5)IP$_3$. Therefore in the absence of a pool of $^{45}Ca^{2+}$ sequestered in an ATP-dependent manner, the (1,4,5)IP$_3$ did not cause $^{45}Ca^{2+}$ release from the cells. This would seem to rule out the possibility that the (1,4,5)IP$_3$ acts by binding or displacing $^{45}Ca^{2+}$ in a non-specific manner.

We have previously shown (Burgess *et al.*, 1983) that, with a very low $[Ca^{2+}]$ in the cytosolic-type medium (20 nM), the saponin-permeabilized hepatocytes will take up about 0.25 nmol of $^{45}Ca^{2+}$/mg of protein in an ATP-dependent manner. This $^{45}Ca^{2+}$ is not, however, released by the Ca^{2+} ionophore A23187 and may be tightly bound, perhaps to the (Ca^{2+} + Mg^{2+})-dependent ATPase of the ER membrane. (In contrast, at 180 nM-Ca^{2+}, the $^{45}Ca^{2+}$ accumulated by the permeable cells in response to ATP, about 1.5 nmol of $^{45}Ca^{2+}$/mg of protein, can be released by the ionophore.) (1,4,5)IP$_3$ could not release the $^{45}Ca^{2+}$ taken up by the permeable cells after addition of ATP (1.5 mM) when the [free Ca^{2+}] in the medium was only 20 nM (Fig. 4*b*). This suggests that IP$_3$ acts by increasing the permeability to Ca^{2+} of a vesicular pool, rather than by displacing or releasing bound Ca^{2+}.

We have made some observations which indicate that the effect of (1,4,5)IP$_3$ in releasing $^{45}Ca^{2+}$ from the saponin-treated cells is independent of the concentration of ATP in the medium within the range of 50 μM–1.5 mM. By setting the [Ca^{2+}] of the cytosolic-type medium at different levels, it is possible to achieve the same amount of ATP-dependent $^{45}Ca^{2+}$ uptake with different concentrations of added ATP. The resulting release of $^{45}Ca^{2+}$ on application of (1,4,5)IP$_3$ is not significantly different at 50 μM or 1.5 mM-ATP, given that an equivalent amount of $^{45}Ca^{2+}$ has been taken up by the permeable hepatocytes. For example, at 180 nM-Ca^{2+} and 1.5 mM-ATP the $^{45}Ca^{2+}$ content of the permeable cells is about 1.8 nmol of $^{45}Ca^{2+}$/mg of protein and (1,4,5)IP$_3$ (5 μM) induced the release of 0.5 nmol of $^{45}Ca^{2+}$/mg of protein. At 760 nM-Ca^{2+} and 50 μM-ATP the $^{45}Ca^{2+}$ content is similarly about 2 nmol of

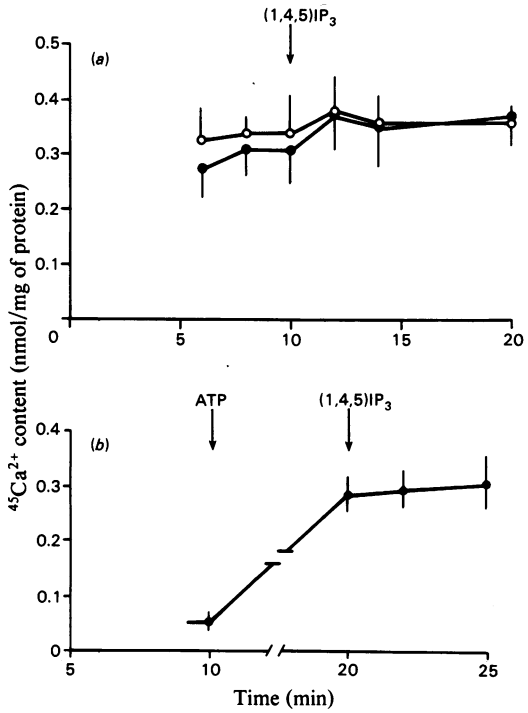

Fig. 4. *Effect of (1,4,5)IP_3 on (a) the $^{45}Ca^{2+}$ associated with the permeabilized cells in the absence of ATP and (b) the ATP-dependent $^{45}Ca^{2+}$ content of permeable cells incubated at low [Ca^{2+}]*

For (*a*) and (*b*), data were averaged and the bars indicate S.E.M. values for three observations. (*a*) Saponin-treated hepatocytes were incubated as described in Fig. 1, with Ca^{2+} set at 180nM, but without any added ATP, creatine phosphate, creatine phosphokinase. Either (1,4,5)IP_3 (5μM, ●) or an equivalent amount of water (○) was added at the arrow. (*b*) Saponin-treated hepatocytes were incubated as described in Fig. 1, but with the [Ca^{2+}] set at 20nM. After addition of ATP there was a small uptake of $^{45}Ca^{2+}$ amounting to 0.3 ± 0.6nmol of $^{45}Ca^{2+}$/mg of protein ($n = 3$). (1,4,5)IP_3 (5μM) had no effect on the $^{45}Ca^{2+}$ content of the cells under these conditions.

$^{45}Ca^{2+}$/mg (this uptake being insensitive to a combination of DNP and oligomycin) and again (1,4,5)IP_3 (5μM) caused 0.5nmol of $^{45}Ca^{2+}$/mg of protein to be released.

The IP_3-sensitive $^{45}Ca^{2+}$ pool in the saponin-permeabilized hepatocytes

With 180nM-Ca^{2+} in the incubation medium (the equivalent of [Ca^{2+}] in the cytosol of an intact unstimulated hepatocyte) the permeable hepatocytes take up Ca^{2+} into an ATP-dependent pool that is insensitive to a combination of the mitochondrial poisons DNP and oligomycin (Burgess *et al.*, 1983). Similar results have been obtained in permeabilized pancreatic acinar cells by Wakasugi *et al.* (1982). This pool is assumed to be a component of the ER. Since (1,4,5)IP_3 [and also (2,4,5)IP_3 and (4,5)IP_2] release ^{45}Ca from this pool, this is strong evidence that the ER may contain the hormone-sensitive pool in the intact cell. Previously (Burgess *et al.*, 1984) we investigated the possibility that (1,4,5)IP_3 might also release $^{45}Ca^{2+}$ from mitochondria by elevating Ca^{2+} in the cytosolic-type medium to 3.3μM, a concentration at which the permeable cells take up very large (10nmol of $^{45}Ca^{2+}$/mg of protein) amounts of $^{45}Ca^{2+}$ in response to ATP (the total cell-associated $^{45}Ca^{2+}$ is 15nmol of $^{45}Ca^{2+}$/mg of protein after ATP). A large proportion of this ATP-dependent $^{45}Ca^{2+}$ is sensitive to the aforementioned mitochondrial poisons and is therefore assumed to be in the mitochondria. No $^{45}Ca^{2+}$ release was observed on addition of (1,4,5)IP_3 (5μM) to these cells, and the 0.5nmol of $^{45}Ca^{2+}$/mg of protein normally released from the ER pool was not detectable, probably because of the higher $^{45}Ca^{2+}$ content. In the experiments shown in Fig. 5, the permeabilized cells were incubated at a [Ca^{2+}] of 2.2μM. At this [Ca^{2+}], roughly similar amounts of Ca^{2+} were accumulated by the ER and mitochondrial pools after addition of ATP (1.5mM). [The amount of $^{45}Ca^{2+}$ in each pool was determined by the degree of sensitivity of the $^{45}Ca^{2+}$ uptake to the mitochondrial poisons DNP and oligomycin (Burgess *et al.*, 1983)]. The ER pool contained 2.8 ± 0.6nmol of $^{45}Ca^{2+}$/mg of protein ($n = 3$) and the mitochondrial pool, 1.5 ± 0.6nmol of $^{45}Ca^{2+}$/mg of protein ($n = 3$). Even so, (1,4,5)IP_3 (5μM) was unable to provoke the release of $^{45}Ca^{2+}$ from the mitochondrial pool (Fig. 5*a*), whereas there was substantial release from the ER pool (Fig. 5*b*), amounting to 1.1 ± 0.04nmol of $^{45}Ca^{2+}$/mg of protein. In the absence of DNP and oligomycin, when both ER and mitochondria were filled, the (1,4,5)IP_3 released 0.75 ± 0.3nmol of $^{45}Ca^{2+}$/mg of protein. In similar experiments (2,4,5)IP_3 up to 50μM and (4,5)IP_2 up to 250μM were unable to induce ^{45}Ca release from the mitochondrial pool.

These latter experiments, which were carried out with a [Ca^{2+}] of 2.2μM and similar experiments where the [Ca^{2+}] in the medium was 3.3μM [either in the presence of DNP and oligomycin, or at low (50μM)ATP] appear to indicate that the response to the inositol phosphates is not antagonized by Ca^{2+}, at least at concentrations up to 3.3μM.

In summary, these results demonstrate that the action of the inositol phosphates in releasing sequestered Ca^{2+} has specific structural requirements and are consistent with the previous suggestion that a specific receptor for IP_3 is involved (Burgess *et al.*, 1984). The most potent compound tested was (1,4,5)IP_3, which is the

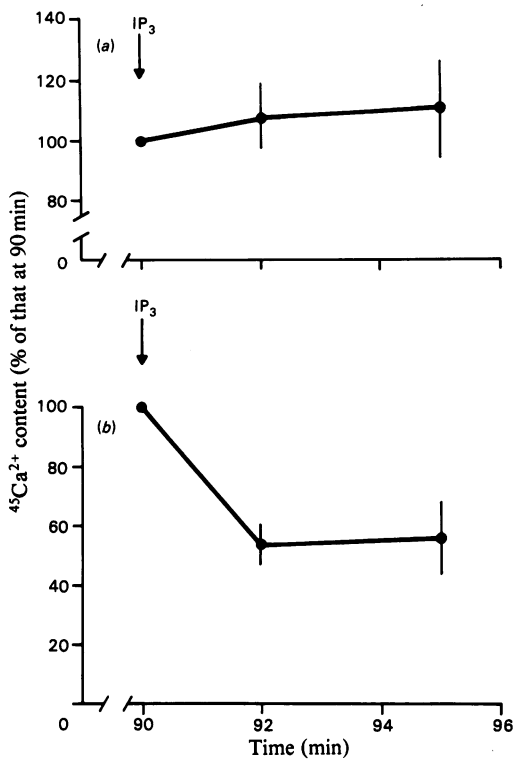

Fig. 5. *Comparison of the effect of (1,4,5)IP_3 on mitochondrial and non-mitochondrial $^{45}Ca^{2+}$ in permeable hepatocytes*

The saponin-treated cells were incubated as in Fig. 1, except that the $[Ca^{2+}]$ of the medium was 2.2 μM and the (1,4,5)IP_3 was added 90 min after application of ATP. At this $[Ca^{2+}]$ there was 2.8 ± 0.6 nmol of $^{45}Ca^{2+}$/mg of protein in the ATP-dependent, DNP- and oligomycin-insensitive pool (ER, *b*), and 1.5 ± 0.6 nmol of $^{45}Ca^{2+}$/mg of protein in the ATP-dependent DNP- and oligomycin-sensitive pool (mitochondria, *a*). When the two pools were separated on the basis of their sensitivity to the mitochondrial poisons (Burgess *et al.*, 1983), (1,4,5)IP_3 released $^{45}Ca^{2+}$ only from the ER. Data are expressed as percentages of the $^{45}Ca^{2+}$ content at 90 min after addition of ATP for each $^{45}Ca^{2+}$ pool. Bars indicate the S.E.M. for three observations.

expected product of phosphodiesteratic breakdown of (4,5)PIP_2. The Ca^{2+} that is released comes from a non-mitochondrial, ATP-dependent vesicular pool and is most probably a component of the ER. The release mechanism is apparently relatively insensitive to the Ca^{2+} concentration and to the concentration of ATP in the incubation medium, as long as sufficient ATP and Ca^{2+} are present to load the relevant pool. These findings confirm and extend the hypothesis that (1,4,5)IP_3 is the messenger that signals the mobilization of Ca^{2+} from the hormone-sensitive Ca^{2+} pool (ER) after stimulation of hepatocytes with Ca^{2+}-mobilizing hormones.

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