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Size of the inositol 1,4,5-trisphosphate-sensitive calcium pool in guinea-pig hepatocytes

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Permeabilized hepatocytes accumulated $^{45}Ca^{2+}$ into a non-mitochondrial pool when provided with ATP. $^{45}Ca^{2+}$ efflux from this pool was revealed by removal of ATP with glucose and hexokinase or by inhibiting uptake with $NaVO_3$. The effect of inositol 1,4,5-trisphosphate (IP_3) on $^{45}Ca^{2+}$ efflux from the pool was investigated. IP_3 (5 μM) evoked a rapid increase in the rate of $^{45}Ca^{2+}$ efflux. Kinetic analysis of the effect of IP_3 indicated the existence of two distinct Ca^{2+} fractions within the pool; only one, accounting for about one-third of the ATP-dependent Ca^{2+} content of the pool, was responsive to IP_3. The effect of IP_3 on $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool does not require ATP, a finding that is inconsistent with a previous suggestion that this effect may be mediated by protein phosphorylation.

INTRODUCTION

An increase in cytosolic $[Ca^{2+}]$ is an early response of many cells to the presence of a hormone or neurotransmitter. In hepatocytes, and in many other cells, the initial increase in cytosolic $[Ca^{2+}]$ is independent of the presence of extracellular Ca^{2+} and is mainly due to mobilization of Ca^{2+} from intracellular stores (Exton, 1980; Aub *et al.*, 1982; DeWitt & Putney, 1984). Phospholipase C-mediated hydrolysis of PIP_2 is believed to be the initial cellular response when receptors bind agonists that mobilize Ca^{2+} (Berridge, 1984). IP_3, the water-soluble product of PIP_2 hydrolysis, is thought to be the link between lipid hydrolysis at the plasma membrane and Ca^{2+} mobilization from intracellular stores (Berridge & Irvine, 1984). In many permeabilized cells, including guinea-pig hepatocytes (Burgess *et al.*, 1984*b*), IP_3 evokes a rapid net release of Ca^{2+} from an intracellular pool. This pool is ATP-dependent; it is vesicular, since Ca^{2+} ionophores discharge it and prevent a subsequent response to IP_3; and it is non-mitochondrial, since normal responses to IP_3 are observed at free $[Ca^{2+}]$ below the threshold for mitochondrial uptake or in the presence of mitochondrial inhibitors (Burgess *et al.*, 1984*b*). These results suggest that endoplasmic reticulum is probably the site of the intracellular IP_3-sensitive Ca^{2+} pool. Cell-fractionation studies further support this conclusion. IP_3 does not evoke Ca^{2+} release from isolated mitochondria, but it does cause release from subcellular fractions enriched in enzyme markers for endoplasmic reticulum (Dawson & Irvine, 1984; Prentki *et al.*, 1984*a*; Streb *et al.*, 1984).

In permeabilized neutrophils, IP_3 mobilizes Ca^{2+} from a non-mitochondrial pool, presumably endoplasmic reticulum, even when Ca^{2+} uptake into that pool is inhibited (Prentki *et al.*, 1984*b*), suggesting that a major site of action of IP_3 is a Ca^{2+}-efflux pathway of the endoplasmic reticulum. Using a different protocol, we report here that IP_3 stimulates Ca^{2+} efflux from a non-mitochondrial pool of permeabilized hepatocytes and that this action is sufficient to account for the quantity of Ca^{2+} released from the intracellular stores of intact hepatocytes by hormones.

Although IP_3 evokes release of Ca^{2+}, maximal concentrations or repeated pulses of IP_3 evoke net release of only part of the accumulated Ca^{2+} (Biden *et al.*, 1984; Burgess *et al.*, 1984*a*; Joseph *et al.*, 1984). This could indicate either complete depletion of a fraction of the pool which is sensitive to IP_3, or partial depletion of a homogeneous Ca^{2+} pool. In the present study, we have examined the effects of IP_3 on the kinetics of Ca^{2+} efflux from the non-mitochondrial Ca^{2+} pool of permeabilized hepatocytes to determine whether all or part of the pool is IP_3-sensitive. We conclude that a distinct pool of Ca^{2+} can be completely emptied by IP_3, but a larger pool is not responsive.

EXPERIMENTAL

Preparation of permeabilized hepatocytes

Hepatocytes were prepared by collagenase digestion of livers isolated from sodium pentabarbitone-anaesthetized male Hartley guinea pigs (200–300 g) (Burgess *et al.*, 1981). The cells were resuspended in Eagle solution supplemented with 2% (w/v) albumin at 37 °C; the pH was maintained at 7.4 by equilibration with O_2/CO_2 (19:1). Samples of cells were resuspended at a cell density of about 1 mg of cellular protein/ml in a Ca^{2+}-free medium whose ionic composition otherwise resembled cytosol (Burgess *et al.*, 1983). This medium had the following composition (mM): KCl, 100; NaCl, 20; $NaHCO_3$, 25; $MgSO_4$, 5; NaH_2PO_4, 0.96; EGTA, 1.0; albumin, 2%, w/v; pH 7.2 at 37 °C; the gas phase was O_2/CO_2 (19:1). Cells were permeabilized by addition of saponin (75 μg/ml) for 7–10 min, after which about 99% of the cells were permeable to Trypan Blue. The permeabilized cells were washed and resuspended in the cytosolic medium without saponin and with $CaCl_2$ added to give a free $[Ca^{2+}]$ of 180 nM, the estimated concentration in an intact unstimulated hepatocyte (Burgess *et al.*, 1983). Antimycin (10 μM) was included to prevent mitochondrial substrate oxidation, and 2,4-dinitrophenol (0.5 mM) and oligomycin (10 μM) were added to prevent Ca^{2+} uptake by mitochondria.

Abbreviations used: IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

Measurement of $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool of permeabilized hepatocytes

Permeabilized cells were incubated in the cytosolic medium (free $[Ca^{2+}] = 180$ nM) with $^{45}Ca^{2+}$ (1 μCi/ml) at a density of 2–3 mg of cellular protein/ml. $^{45}Ca^{2+}$ uptake into the non-mitochondrial pool was initiated by addition of ATP (1.5 mM). The $^{45}Ca^{2+}$ contents of 100 μl samples of cells were determined by rapid dilution into 10 ml of cold iso-osmotic sucrose (310 mM) containing EGTA (4 mM) and [^{3}H]mannose (0.3 μCi/ml) to correct for trapped volume. Samples were rapidly filtered through Whatman GF/C filters, washed with 10 ml of cold iso-osmotic sucrose, and the filters were counted for radioactivity by liquid-scintillation counting. ATP-dependent $^{45}Ca^{2+}$ uptake into the non-mitochondrial pool reaches a steady state within 10 min of the addition of ATP (Burgess *et al.*, 1984*a*). In the present experiments, glucose and hexokinase or $NaVO_3$, at the concentrations given in the Results section, were added to the cells 13 min after addition of ATP. Both procedures inhibit $^{45}Ca^{2+}$ uptake into the pool such that the kinetics of $^{45}Ca^{2+}$ efflux are revealed. The effects of IP_3 on $^{45}Ca^{2+}$ efflux were examined by simultaneously adding it and $NaVO_3$ or glucose and hexokinase. The protein content and $^{45}Ca^{2+}$ specific radioactivity of each incubation were determined, and cell Ca^{2+} contents expressed in nmol of Ca^{2+}/mg of cellular protein.

Analysis of results

ATP-dependent ^{45}Ca uptake into the non-mitochondrial pool was calculated (nmol/mg of cellular protein) and then expressed as a percentage of the uptake at the time of addition of $NaVO_3$ or glucose and hexokinase. Logarithms of these percentages were used to calculate arithmetic means, standard errors and least-squares linear-regression lines. The statistics were then back-transformed to percentages (Sokal & Rohlf, 1981) and plotted semi-logarithmically.

Materials

IP_3 was prepared from ox brain (Irvine *et al.*, 1984). $^{45}CaCl_2$ and [^{3}H]mannose were supplied by NEN. Collagenase was obtained from Boehringer Mannheim and A23187 from Calbiochem. Hexokinase (type V from baker's yeast) and all other reagents were from Sigma.

RESULTS AND DISCUSSION

^{45}Ca uptake into the non-mitochondrial pool of permeabilized cells was increased from 0.36 ± 0.05 nmol/mg of protein (mean ± S.E.M., $n = 18$) to 3.04 ± 0.23 nmol/mg by addition of ATP. Subsequent addition of glucose (10 mM) and hexokinase (0.1–50 units/ml) to deplete the medium of ATP decreased the $^{45}Ca^{2+}$ content of this pool. The maximal rate of $^{45}Ca^{2+}$ loss from the pool was observed after addition of 10 or 50 units of hexokinase/ml, implying that these additions depleted ATP sufficiently rapidly to allow $^{45}Ca^{2+}$ efflux to be observed free of remaining ATP-dependent uptake. In all later experiments, we used 50 units of hexokinase/ml and 10 mM-glucose to deplete ATP rapidly and thereby to reveal the kinetics of $^{45}Ca^{2+}$ efflux from pre-loaded pools.

The kinetics of $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool after addition of glucose and hexokinase are shown in Fig. 1. A single, rapid, component accounts for most of the efflux; slower components have not been further analysed. Subsequent experiments have examined the effects of IP_3 during the time when the rapid component accounts for most ^{45}Ca efflux from the pool.

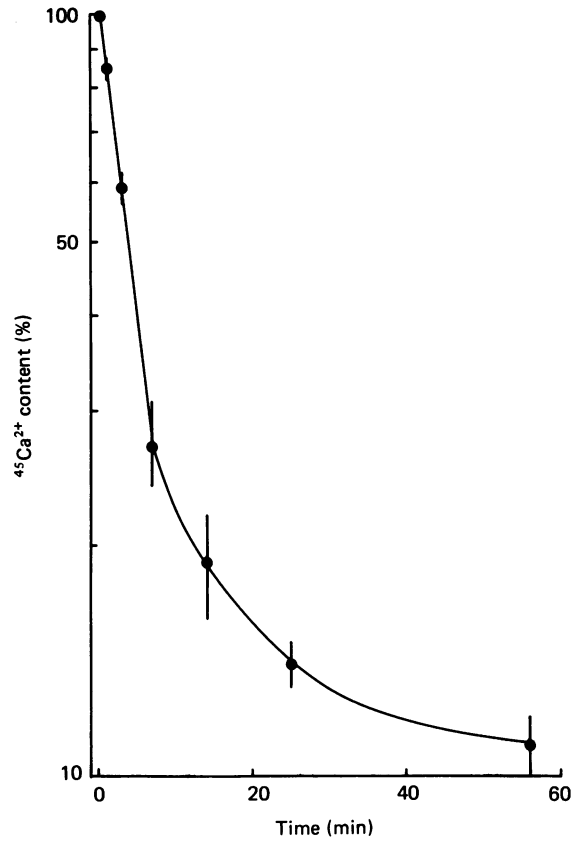

Fig. 1. Kinetics of $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool of permeabilized hepatocytes

At 13 min after addition of ATP to permeabilized cells, glucose (10 mM) and hexokinase (50 units/ml) were added (zero time in this Figure). $^{45}Ca^{2+}$ contents of the cells are shown as a percentage of the ATP-dependent $^{45}Ca^{2+}$ uptake at the time of addition of glucose and hexokinase. Results are means ± S.E.M. for seven observations.

Addition of 5 μM-IP_3 with glucose and hexokinase evoked a more rapid $^{45}Ca^{2+}$ efflux than did glucose and hexokinase alone (Fig. 2). The increased efflux was complete within 30 s and half-complete within 6 s (the shortest time of sampling). After 30 s, the rate constants for efflux were similar irrespective of the presence of IP_3. Similar results were obtained when $NaVO_3$ (1 mM, a concentration that totally inhibited ATP-dependent uptake into the non-mitochondrial pool of permeabilized hepatocytes; results not shown) was used to inhibit Ca^{2+} uptake into the pool. This indicates that $NaVO_3$ does not affect the efflux pathway that is regulated by IP_3. Several lines of evidence indicate that the transient stimulation of $^{45}Ca^{2+}$ efflux is attributable to complete depletion of an IP_3-sensitive Ca^{2+} pool rather than to rapid degradation of IP_3. Firstly, IP_3 is degraded with a half-time of several minutes by permeabilized guinea-pig hepatocytes under these conditions (B. A. Leslie, unpublished work). Secondly, the effects of addition of 5 μM-or 15 μM-IP_3 were not statistically different (results not shown). Thirdly, a second addition of IP_3 (5 μM) did not evoke a second

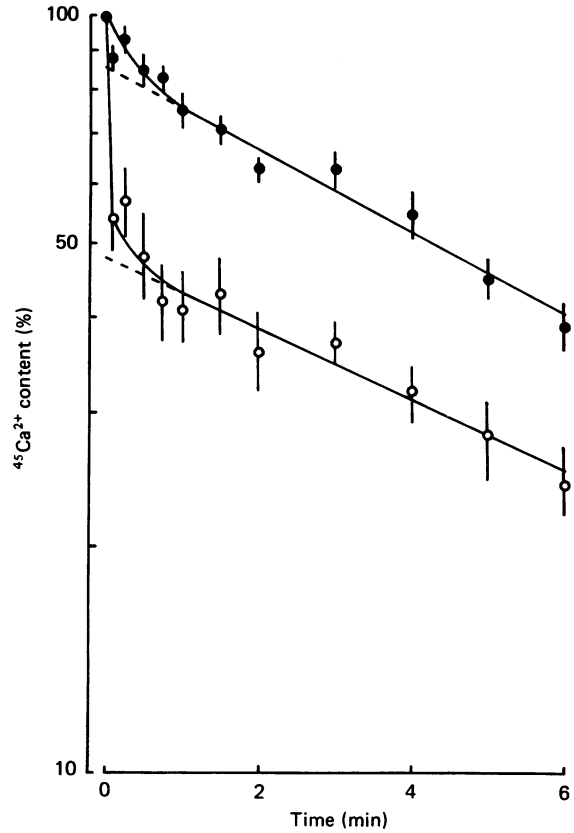

Fig. 2. Effects of IP_3 on $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool of permeabilized hepatocytes

Glucose (10 mM) and hexokinase (50 units/ml) were added alone (●) or with 5 μM-IP_3 (○) to permeabilized cells 13 min after addition of ATP. $^{45}Ca^{2+}$ contents are shown as percentages of the ATP-dependent $^{45}Ca^{2+}$ contents at the time of the additions (zero time in the Figure). Results are means ± S.E.M. for nine observations.

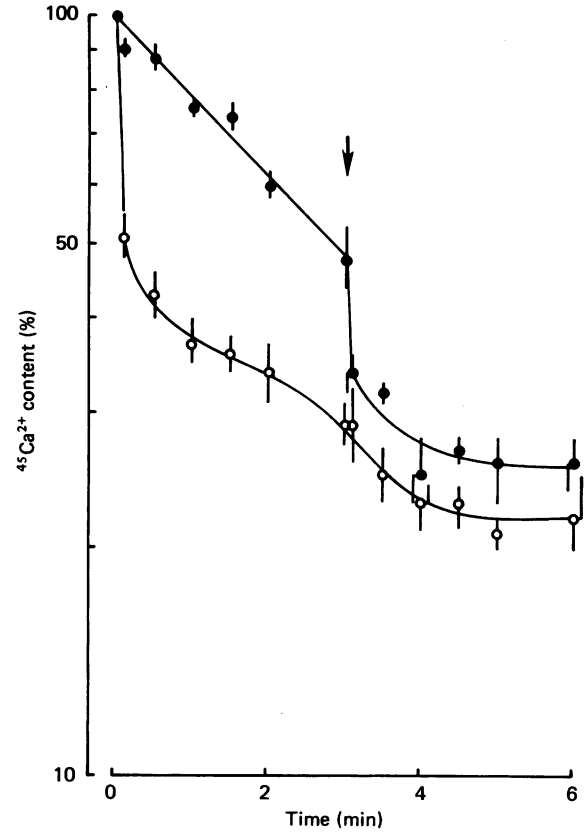

Fig. 3. Effects of two additions of IP_3 on $^{45}Ca^{2+}$ efflux from the non-mitochondrial pool of permeabilized hepatocytes

Permeabilized cells were incubated with ATP for 13 min before addition of glucose (10 mM) and hexokinase (50 units/ml) either alone (●) or with 5 μM-IP_3 (○) (additions made at zero time as shown in the Figure). Then 3 min later, 5 μM-IP_3 was added to both incubations (arrow). $^{45}Ca^{2+}$ contents are shown as percentages of the ATP-dependent $^{45}Ca^{2+}$ content at the time of the first addition. Results are means ± S.E.M. for six observations.

release of $^{45}Ca^{2+}$, whereas addition of IP_3 to the control cells caused a rapid efflux of $^{45}Ca^{2+}$ such that their $^{45}Ca^{2+}$ content fell to about the same value as the cells that had received two pulses of IP_3 (Fig. 3).

After the transient IP_3-induced $^{45}Ca^{2+}$ efflux, the rate constants for $^{45}Ca^{2+}$ efflux were similar irrespective of the presence of IP_3 (Fig. 2), indicating that IP_3 had not affected the Ca^{2+} pool from which this slower efflux occurs. Ionophore A23187 (10 μM) completely discharges all of the $^{45}Ca^{2+}$ in the ATP-dependent non-mitochondrial pool within 90 s ($t_{\frac{1}{2}} = 20$ s), indicating that the inability of IP_3 to discharge this pool totally is not a consequence of the presence of a large bound fraction of Ca^{2+}. Rather, there must be two physically distinct Ca^{2+} fractions within the non-mitochondrial pool, one sensitive to IP_3 and another insensitive. Extrapolation of the lines describing the IP_3-insensitive efflux to the time of addition of glucose and hexokinase or of glucose, hexokinase and IP_3 allows the size of the IP_3-sensitive Ca^{2+} pool to be calculated (Fig. 2). This calculation suggests that, under the conditions of these experiments, with Ca^{2+} buffered at the concentration observed in intact unstimulated cells, about one-third of the ATP-dependent $^{45}Ca^{2+}$ uptake by the non-mitochondrial pool is sequestered in a pool that is IP_3-sensitive.

Permeabilized hepatocytes incubated in the presence of ATP release 25–35% of their $^{45}Ca^{2+}$ in response to maximal concentrations of IP_3 (Burgess *et al.*, 1984*a*; Joseph *et al.*, 1984). Furthermore, similar amounts of Ca^{2+} are released by intact hepatocytes stimulated by adrenaline and by permeabilized hepatocytes stimulated by IP_3 at concentrations estimated to occur in intact cells after stimulation by adrenaline (Burgess *et al.*, 1984*a*). The results of the present study demonstrate that, when Ca^{2+} uptake into the non-mitochondrial pool is inhibited, IP_3 evokes Ca^{2+} release from a pool of comparable size and with a time course comparable with that observed in permeabilized cells in which Ca^{2+} uptake into the intracellular pool is not inhibited. Together, these findings confirm that the effects of agonists on the intracellular Ca^{2+} pools of hepatocytes are adequately explained by stimulation of Ca^{2+} efflux from a fraction of a non-mitochondrial pool that is IP_3-sensitive.

IP_3, at physiological concentrations, has been reported to activate a kinase that phosphorylates a 62kDa protein in a Ca^{2+}-independent manner in cell lysates of cultured monkey fibroblasts and bovine brain (Whitman *et al.*, 1984). An attractive hypothesis, in light of the established role of protein phosphorylation in regulating sarco-

plasmic-reticulum Ca^{2+} fluxes (Le Peuch *et al.*, 1979), is that IP_3-stimulated phosphorylation of this protein may be an early step in the sequence of events whereby IP_3 evokes Ca^{2+} release. In a previous study, Ca^{2+} release by IP_3 was insensitive to changes in [ATP] in the range 50 μM–1.5 mM (Burgess *et al.*, 1984*b*). In the present study, IP_3 evoked Ca^{2+} efflux from the non-mitochondrial pool despite the absence (Fig. 2), even the prolonged absence (Fig. 3), of ATP; and the stimulation of Ca^{2+} efflux from the pool was similar whether uptake had been inhibited by $NaVO_3$ or by removal of ATP. It is therefore very unlikely that protein phosphorylation is a necessary step in the action of IP_3 on intracellular Ca^{2+} pools. Rather, protein phosphorylation may be another, parallel, intracellular effect of IP_3.

The present study and those by Prentki *et al.* (1984*b*) and Dawson & Irvine (1984) provide evidence that IP_3 stimulates Ca^{2+} release from a functionally, and presumably anatomically, distinct fraction of the endoplasmic reticulum. The last authors found that IP_3 evoked Ca^{2+} release from rat liver microsomal fractions, the Ca^{2+} was then re-accumulated, but a second pulse of IP_3 failed to elicit Ca^{2+} release. This they explained by proposing that IP_3 evoked release only from sensitive vesicles, but, in the continued presence of IP_3, the Ca^{2+} is then accumulated into an insensitive pool.

At present, the morphological correlate of the IP_3-sensitive Ca^{2+} pool is unknown. However, it is tempting to speculate that the pool may be within endoplasmic reticulum that is closely associated with the plasma membrane. Such a location could explain the otherwise enigmatic finding that, once the hormone-sensitive intracellular Ca^{2+} pool has been depleted by stimulation of intact cells with agonists in Ca^{2+}-free media, subsequent restoration of extracellular Ca^{2+} allows the pool to refill without changes in cytosolic Ca^{2+} activity (Aub *et al.*, 1982; Poggioli & Putney, 1982).

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