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myo-Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from isolated adipocyte endoplasmic reticulum but not from plasma membranes

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The effects of *myo*-inositol 1,4,5-trisphosphate (IP_3) on Ca^{2+} uptake and release from isolated adipocyte endoplasmic reticulum and plasma membrane vesicles were investigated. Effects of IP_3 were initially characterized using an endoplasmic reticulum preparation with cytosol present (S_1-ER). Maximal and half-maximal effects of IP_3 on Ca^{2+} release from S_1-ER vesicles occurred at 20 μM- and 7 μM-IP_3, respectively, in the presence of vanadate which prevents the re-uptake of released Ca^{2+} via the endoplasmic reticulum Ca^{2+} pump. At saturating IP_3 concentrations, Ca^{2+} release in the presence of vanadate was 20% of the exchangeable Ca^{2+} pool. IP_3-induced release of Ca^{2+} from S_1-ER was dependent on extravesicular free Ca^{2+} concentration with maximal release occurring at 0.13 μM free Ca^{2+}. At 20 μM-IP_3 there was no effect on the initial rate of Ca^{2+} uptake by S_1-ER. IP_3 promoted Ca^{2+} release from isolated endoplasmic reticulum vesicles (cytosol not present) to a similar level as compared with S_1-ER. Addition of cytosol to isolated endoplasmic reticulum vesicles did not affect IP_3-induced Ca^{2+} release. The endoplasmic reticulum preparation was further fractionated into heavy and light vesicles by differential centrifugation. Interestingly, the heavy fraction, but not the light fraction, released Ca^{2+} when challenged with IP_3. IP_3 (20 μM) did not promote Ca^{2+} release from plasma membrane vesicles and had no effect on the $(Ca^{2+}+Mg^{2+})$-ATPase activity or on the initial rate of ATP-dependent Ca^{2+} uptake by these vesicles. These results support the concept that IP_3 acts exclusively at the endoplasmic reticulum to promote Ca^{2+} release.

INTRODUCTION

It has been proposed that the wide variety of hormones and neurotransmitters which exert their effects via Ca^{2+}-dependent mechanisms do so by triggering the mobilization of intracellular Ca^{2+}. One proposed mechanism for intracellular Ca^{2+} mobilization involves the production of IP_3 by rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate following agonist-induced activation of phospholipase C at the plasma membrane. IP_3 then interacts with the endoplasmic reticulum to initiate the release of Ca^{2+} stored in this organelle (Creba *et al.*, 1983). In support of this hypothesis, IP_3 has been shown to induce Ca^{2+} efflux from non-mitochondrial stores (presumably endoplasmic reticulum) of a variety of different permeabilized cells types (Streb *et al.*, 1983; Joseph *et al.*, 1984*a*,*b*; Burgess *et al.*, 1984; Suematsu *et al.*, 1984; Hirata *et al.*, 1984; Gershengorn *et al.*, 1984; Wolf *et al.*, 1985; Yamamoto & Breemen, 1985). A growing number of studies have demonstrated that IP_3 also causes Ca^{2+} to be released from isolated endoplasmic reticulum. These studies include endoplasmic reticulum preparations from insulinoma (Prentki *et al.*, 1984), skeletal muscle (Volpe *et al.*, 1985), platelets (O'Rourke *et al.*, 1985), liver (Dawson & Irvine, 1984) and pancreas (Streb *et al.*, 1984). Nevertheless, the mechanism responsible for IP_3-induced Ca^{2+} release from endoplasmic reticulum is unknown.

Although phosphoinositide regulation of Ca^{2+} homoeostasis in the adipocyte has not yet been reported, there is reason to believe that IP_3 may promote Ca^{2+} release from their endoplasmic reticulum in response to hormonal stimulation. Activation of α_1-adrenergic receptors is one mechanism by which the breakdown of phosphatidylinositol 4,5-bisphosphate occurs in the plasma membrane to produce IP_3 (Exton, 1985). Recently, Cheng & Larner (1985) demonstrated, using adipocytes, that α_1-agonists regulate the activity of enzymes (glycogen synthase, glycogen phosphorylase, pyruvate dehydrogenase) that require Ca^{2+} for either activation or inactivation and that this regulation was dependent on intracellular and not extracellular Ca^{2+}. The adipocyte is also a target cell for insulin and several lines of indirect evidence suggest that intracellular Ca^{2+} may play a part in the mechanism of insulin action (for review see Pershadsingh & McDonald, 1984). A possible role for IP_3 as a mediator of insulin action can be hypothesised, based on the observations that insulin activates phospholipase C in fat cells (Koepfer-Hobelsberger & Wieland, 1984) and that the protein kinase actvity associated with the insulin receptor from human placenta can act as a phosphatidylinositol kinase (Machicao & Wieland, 1984).

In order to determine if IP_3 may play a role in mobilizing Ca^{2+} from intracellular stores in the adipocyte we investigated the effects of IP_3 on Ca^{2+} uptake and

Abbreviations used: $(Ca^{2+}+Mg^{2+})$-ATPase, calcium-stimulated, magnesium-dependent adenosine triphosphatase; IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_2, *myo*-inositol 1,4-bisphosphate; S_1-ER, endoplasmic reticulum preparation with cytosol present.

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release from isolated endoplasmic reticulum vesicles. Although the endoplasmic reticulum is considered to be the primary target site for IP_3-induced Ca^{2+} release, it is possible that IP_3 could also modulate intracellular Ca^{2+} by regulating the plasma membrane Ca^{2+}-transport system and/or the permeability of the plasma membrane to Ca^{2+}. Therefore, the effects of IP_3 on Ca^{2+} uptake and release from plasma membrane vesicles were studied as well. Our results show that, while IP_3 caused Ca^{2+} release from isolated endoplasmic reticulum, it had no effect on the endoplasmic reticulum Ca^{2+} pump, the plasma membrane Ca^{2+} pump and $(Ca^{2+}+Mg^{2+})$-ATPase, or on Ca^{2+} release from isolated plasma membrane vesicles.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats, weighing 120–150 g, were purchased from Chappel Breeders, Bel Ridge, MO, U.S.A. Collagenase (type I) from *Clostridium histolyticum* was from Worthington Biochemical (Freehold, NJ, U.S.A.). Bovine serum albumin (fraction V), Tris, Pipes, EGTA, ATP and GTP (disodium salts), and poly(ethylene glycol) (average M_r 8000) were from Sigma Chemical Co., St. Louis, MO, U.S.A. A23187 was purchased from Calbiochem, La Jolla, CA, U.S.A. [γ-^{32}P]ATP was from Amersham, Arlington Heights, IL, U.S.A. and $^{45}CaCl_2$ was from New England Nuclear, Boston, MA, U.S.A. Stock solutions of sodium orthovanadate (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.) were prepared as described previously (Delfert & McDonald, 1985). All other reagents were from Fisher Scientific.

Adipocyte fractionation

Adipocytes were obtained by collagenase digestion of rat epididymal fat pads as described by Rodbell (1964). A subcellular fraction enriched in plasma membrane was prepared by a modification (Jarett, 1974) of the method of McKeel & Jarett (1970). Centrifugation of the cellular homogenate at 20000 *g* for 15 min yields a supernatant containing cytosol and membranes which are morphologically and biochemically characteristic of endoplasmic reticulum (S_1-ER) (Bruns *et al.*, 1976). A subcellular fraction enriched in endoplasmic reticulum was obtained by rapid centrifugation of the 20000 *g* supernatant at 160000 *g* for 10 min after addition of ATP and $MgCl_2$ to final concentrations of 0.5 mM and 0.25 mM, respectively. It has been previously demonstrated that this method is required for preparing endoplasmic reticulum which retain full activity of the Ca^{2+}-transport system (Black *et al.*, 1980). Further fractionation of the endoplasmic reticulum into heavy and light fractions was accomplished by first centrifuging the S_1-ER at 50000 *g* for 20 min to obtain a membrane pellet labelled 'heavy fraction' and then centrifuging the post-50000 *g* supernatant for 70 min at 212000 *g* to yield a membrane pellet labelled 'light fraction'. Simpson *et al.* (1983) have demonstrated that subcellular fractionation of adipocytes in this manner yielded a heavy fraction that was enriched in endoplasmic reticulum and a light fraction that was enriched in Golgi. Membrane pellets were resuspended by gentle homogenization in 10 mM-Tris/HCl/0.25 M-Sucrose. The plasma membrane and endoplasmic reticulum fractions were frozen immediately after preparation, stored at −70 °C, and used within 7 days of preparation. Protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of Ca^{2+} uptake and release

The uptake and release of Ca^{2+} from endoplasmic reticulum and plasma membrane vesicles was assayed by using a previously described filtration method (Pershadsingh *et al.*, 1980). Ca^{2+} uptake was initiated by the addition of membranes to assay medium in a final volume of 0.5 ml or 1.0 ml containing: 200 mM-sucrose, 20 mM-NaN_3 (which completely inhibits energy-dependent processes in the small fraction of contaminating mitochondria) (Mitchell, 1961), 100 mM-KCl, 2 mM-$MgCl_2$, 0.5 mM-EGTA and $CaCl_2$ with the Ca^{2+}/EGTA ratios adjusted to obtain the desired free Ca^{2+} concentrations, 2–3 μCi of $^{45}CaCl_2$/ml, and 50 mM-Tris/Pipes. The pH was adjusted to 7.5 for plasma membrane Ca^{2+} uptake and pH 7.0 for endoplasmic reticulum Ca^{2+} uptake. Free Ca^{2+} concentrations were determined as previously described by Pershadsingh & McDonald (1980) and confirmed by using a Ca^{2+}-specific electrode (Orion). Assays were carried out in the presence and absence of ATP (2 mM) to determine ATP-dependent Ca^{2+} uptake. When initial Ca^{2+} uptake rates were measured, 10 mM-Tris oxalate was included in the incubation medium to chelate intravesicular Ca^{2+}. The incubation was continued for various times at 30 °C and Ca^{2+} uptake was terminated by rapid filtration of an aliquot of the assay mixture on a 0.45 μm Millipore filter followed by washing the filter three times with 5 ml of iso-osmotic sucrose. The filters were dried and $^{45}Ca^{2+}$ quantified by liquid-scintillation counting. The fact that vesicles were quantitatively retained on these filters has been previously shown for S_1-ER by Bruns *et al.* (1976) and was experimentally confirmed for plasma membranes by using a Chelex column for measuring Ca^{2+} uptake (Lin, 1985). All assays were performed in triplicate. Ca^{2+}-uptake results obtained using the S_1-ER fraction are expressed in terms of nmol of Ca^{2+}/mg of endoplasmic reticulum protein. On the basis of protein concentration, the endoplasmic reticulum membranes typically represented 8–12% of the S_1-ER fraction, which is consistent with previously reported results from this laboratory (Bruns *et al.*, 1976). Under these experimental conditions, the only membrane vesicles which are capable of ATP-dependent Ca^{2+} transport are those that are orientated such that the cytoplasmic leaflet of their membrane bilayer is accessible to the assay medium (Pershadsingh *et al.*, 1980; Black *et al.*, 1980).

Ca^{2+} release in the subcellular membrane fractions was performed as follows. Ca^{2+} uptake was allowed to proceed to steady state (15 min) at which time an aliquot of the assay mixture was filtered and washed to obtain an initial value for the amount of Ca^{2+} loaded. At 16 min, additions as indicated, were made in 1/100th of the assay volume and Ca^{2+} retained by the vesicles was quantified by filtering aliquots at various subsequent times.

Assay of $(Ca^{2+}+Mg^{2+})$-ATPase

The $(Ca^{2+}+Mg^{2+})$-ATPase activity in the plasma membrane fraction was quantified by monitoring the $^{32}P_i$ released from [γ-^{32}P]ATP by the method of Seals *et al.* (1978). The standard incubation medium contained 20 mM-NaN_3, 0.5 mM-EGTA, and $CaCl_2$ with Ca^{2+}/EGTA ratios adjusted to obtain the desired free

Ca^{2+} concentration (as described above), 2 mM-ATP, and 50 mM-Tris/Pipes, pH 7.5, in a total assay volume of 500 μl. The reaction was initiated by the addition of membranes (20–30 μg) and terminated after a 10 min incubation period at 37 °C by the addition and rapid mixing of SDS to the assay mixture (final concentration 1%). All assays were performed in triplicate. Ca^{2+}-stimulated ATPase activity was calculated by subtracting the rate of ATP hydrolysis obtained in the presence of chelator alone from the rate obtained with Ca^{2+} plus chelator.

Preparation of IP_3 and IP_2

Red blood cells from 3 litres of whole blood, not over 2 weeks old, obtained from polycythaemic patients were metabolically activated as described in Shukla *et al.* (1979). Briefly, washed red cells were incubated for 2 h at 37 °C with Hepes/citrate buffer containing sodium pyruvate (50 mM), sodium phosphate (50 mM), inosine (50 mM), glucose (100 mM) and adenine (5 μM). This procedure restored the phosphoinositides to normal levels prior to both lysis (Wolf *et al.*, 1985) and calcium-stimulated phospholipase C cleavage to inositol phosphates. The inositol polyphosphates were released by incubating the red cell ghosts in 2 mM-$CaCl_2$, and IP_2 and IP_3 were separated and isolated from the supernatant using a Dowex column as described by Downes & Michell (1981). IP_3 was identified as *myo*-inositol 1,4,5-trisphosphate (Irvine *et al.*, 1984), the same isomer as the predominant product obtained by base hydrolysis of phosphatidylinositol 4,5-bisphosphate (Grado & Ballou, 1961; Tomlinson & Ballou, 1961). *myo*-Inositol 1,4-bisphosphate is the known product of phospholipase C action on red cell phosphatidylinositol 4-phosphate (Downes & Michell, 1981). The purities of IP_2 and IP_3 were established by electrophoresis (Seiffert & Agranoff, 1965) using *myo*-inositol 1,4,5-trisphosphate kindly provided by Dr. R. F. Irvine (Cambridge, U.K.) as a reference standard. The concentrations of IP_2 and IP_3 used in the experiments were determined by alkaline phosphatase treatment followed by phosphate analysis and g.l.c. analysis of *myo*-inositol, which were in agreement for the expected ratios of those substances. Based on these results, the purity of IP_3 was 99%.

RESULTS

Characterization of IP_3-induced Ca^{2+} release from S_1-ER

IP_3-induced Ca^{2+} release was first characterized in endoplasmic reticulum vesicles suspended in diluted cytosol (S_1-ER). As shown in Fig. 1, ATP-dependent accumulation of Ca^{2+} into these vesicles increased to a steady state level by 10–15 min. When the Ca^{2+} ionophore, A23187, was added in the presence of ATP, Ca^{2+} accumulation did not occur. Vanadate (1 mM) inhibited ATP-dependent Ca^{2+} uptake by 80%, consistent with our earler findings using isolated endoplasmic reticulum vesicles (Delfert & McDonald, 1985).

The effect of IP_3 on Ca^{2+} release was then investigated in the presence and absence of vanadate, which prevents re-uptake of released Ca^{2+} via the Ca^{2+} pump (Fig. 2). Addition of water alone caused little loss of Ca^{2+} from vesicles. Addition of IP_3 alone (20 mM) caused the endoplasmic reticulum vesicles to release Ca^{2+} even at the earliest measured time point (1 min). Under these

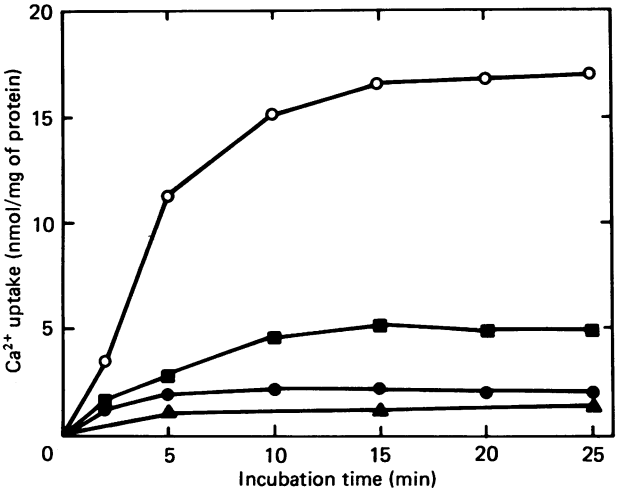

Fig. 1. Time course of Ca^{2+} uptake by S_1-ER vesicles

S_1-ER (900 μg) was added to initiate Ca^{2+} uptake. The assay medium contained: 200 mM-sucrose, 20 mM-NaN_3, 100 mM-KCl, 2 mM-$MgCl_2$, 0.5 mM-EGTA, 0.15 mM-$CaCl_2$ (0.13 μM free Ca^{2+}), approx. 2 μCi of $^{45}CaCl_2$/ml and 50 mM-Tris/Pipes buffer, pH 7.0, at 30 °C in a final volume of 1 ml. Aliquots (0.1 ml each) were removed and rapidly filtered at the times indicated, in the absence (●) or presence (○) of ATP, ATP+2 μM-A23187 (▲) or ATP+1 mM-vanadate (■). The concentration of ATP was 2 mM when present. The data were obtained from a representative experiment. The coefficient of variation at each data point was less than 10%.

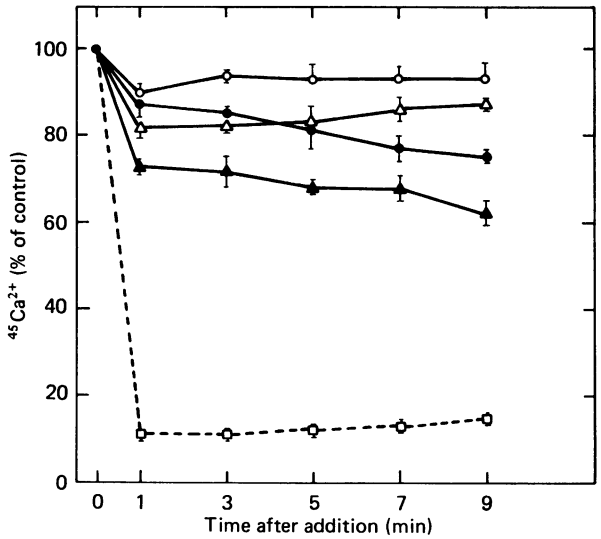

Fig. 2. Time course of IP_3-induced Ca^{2+} efflux from S_1-ER vesicles

S_1-ER vesicles were loaded with Ca^{2+} for 15 min in the presence of ATP (2 mM) as described in the legend to Fig. 1. Ca^{2+} efflux was monitored as described in the Materials and methods section. The amount of Ca^{2+} accumulated at 15 min was taken to be the control value of 100%. At 16 min ('zero' time on the abcissa) the following additions were made to yield the indicated final concentrations: water (○), 1 mM-vanadate (●), 20 μM-IP_3 (△), 1 mM-vanadate+20 μM-IP_3 (▲), or 2 μM-A23187 (□). The data were obtained from a representative experiment. Results are expressed as the mean ± S.E.M.

conditions, maximal Ca^{2+} release was 9–11% of the total exchangable Ca^{2+} pool (defined as the difference between Ca^{2+} efflux observed in the presence of the water control and efflux observed with A23187) (Fig. 2). Addition of vanadate alone (1 mM) resulted in a time-dependent loss of vesicular Ca^{2+}, which presumably resulted from inhibition of the Ca^{2+} pump (Fig. 1). When vanadate and IP_3 were added together, Ca^{2+} release was further enhanced, being 12–17% of the exchangeable Ca^{2+} pool in the presence of vanadate (defined as the difference between Ca^{2+} efflux observed in the presence of vanadate and efflux observed with A23187) (Fig. 2). The time course paralleled the curve obtained with vanadate alone, indicating that Ca^{2+} re-uptake was inhibited. IP_3 (20 μM) released $15.6 \pm 3.2\%$ ($P < 0.0001$, $n = 10$) of the exchangeable Ca^{2+} pool in the presence of vanadate at 5 min after initiation of release. Thus, the addition of vanadate permits a quantitative assessment of Ca^{2+} release independent of uptake and therefore was included in the following experiments.

The effect of IP_3 on Ca^{2+} release was concentration-dependent in the micromolar range (Fig. 3) Maximum Ca^{2+} release (approx. 20%) occurred above 20 μM-IP_3. The concentration of IP_3 that produced half-maximal release of Ca^{2+} was 7 μM. The phosphoinsotide metabolite IP_2 (10 μM) had no effect on Ca^{2+} efflux (results not shown; $n = 6$).

IP_3-induced Ca^{2+} efflux from adipocyte S_1-ER was shown to be dependent on the extravesicular Ca^{2+} concentration. Vesicles were incubated in the standard uptake medium (see the Materials and methods section) at the following free Ca^{2+} concentrations: 0.08, 0.13, 0.47, 1.2 and 12 μM free Ca^{2+}. After Ca^{2+} accumulation had reached a steady state, IP_3 was added to the assay medium and the amount of Ca^{2+} efflux was quantified. Maximal Ca^{2+} efflux occurred at approx. 0.13 μM-Ca^{2+} and declined progressively with increasing Ca^{2+} concentration (Fig. 4).

Using a microsomal system prepared from rat liver, Dawson (1985) showed that GTP in the presence of

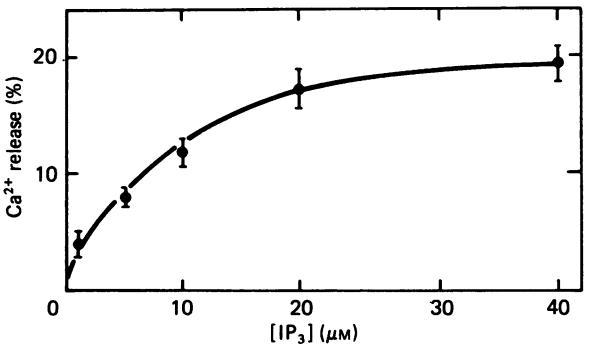

Fig. 3. Dose–response curve of IP_3-induced Ca^{2+} release by S_1-ER vesicles

S_1-ER vesicles were loaded with Ca^{2+} as described in the Materials and methods section and efflux was measured as described in Fig. 2 at 5 min after the addition of IP_3 (1–40 μM) and 1 mM-vanadate. Ca^{2+} release is expressed as a percentage of exchangeable Ca^{2+}. Exchangeable Ca^{2+} is defined as the difference in Ca^{2+} efflux observed in the presence of vanadate and efflux observed with A23187. Results are expressed as the mean ± S.E.M. of data from two experiments.

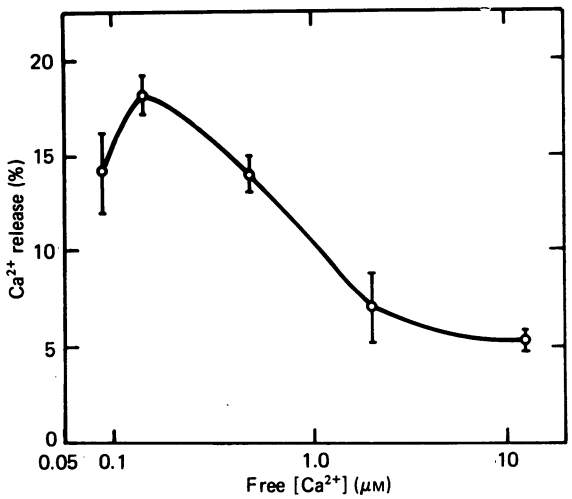

Fig. 4. Ca^{2+}-dependence of IP_3-induced Ca^{2+} release from S_1-ER vesicles

S_1-ER vesicles were incubated in the standard uptake medium (see the Materials and methods section) at the free Ca^{2+} concentrations indicated. After 15 min of Ca^{2+} accumulation (steady state), IP_3 (20 μM) and vanadate (1 mM) were added to the assay medium. After 5 min of additional incubation the amount of Ca^{2+} released was quantified. Ca^{2+} release is expressed as a percentage of exchangeable Ca^{2+}, as defined in the text. Results are expressed as the mean ± S.E.M. of data from three experiments.

poly(ethylene glycol) greatly potentiated IP_3-induced Ca^{2+} release. Under our assay conditions, which were similar to theirs, the presence of 10 μM-GTP and 3% (w/v) poly(ethylene glycol) had no effect on IP_3-induced Ca^{2+} release. Thus, the ability of these agents to enhance Ca^{2+} release by IP_3 may be dependent upon the method by which the microsomal fraction is prepared or be specific to the cell type from which it is derived.

IP_3 is thought to release Ca^{2+} from endoplasmic reticulum by increasing its permeability to Ca^{2+} and not by inhibition of the Ca^{2+}-sequestering system (Williamson *et al.*, 1985); however, there have been few data to determine if IP_3 affects the Ca^{2+}-transport system of the endoplasmic reticulum. Therefore, the effect of IP_3 on unidirectional Ca^{2+} uptake by the endoplasmic reticulum was examined. For this experiment oxalate was added to the incubation medium. The endoplasmic reticulum is permeable to oxalate which serves to trap Ca^{2+} intravesicularly and thereby eliminate the efflux component. Under these conditions, the rate of Ca^{2+} uptake was linear. We found that there was no difference between the rate of ATP-dependent Ca^{2+} uptake in the presence or absence of IP_3 (Fig. 5), thus demonstrating that IP_3 had no effect on the Ca^{2+}-sequestering mechanism of the adipocyte endoplasmic reticulum.

Effect of IP_3 on isolated endoplasmic reticulum and high and low density fractions

In the experiments described above the effects of IP_3 were studied using endoplasmic reticulum that was suspended in diluted cytosol (S_1-ER). In order to address the possibility that cytoplasmic factors may effect IP_3-dependent Ca^{2+} release, endoplasmic reticulum free

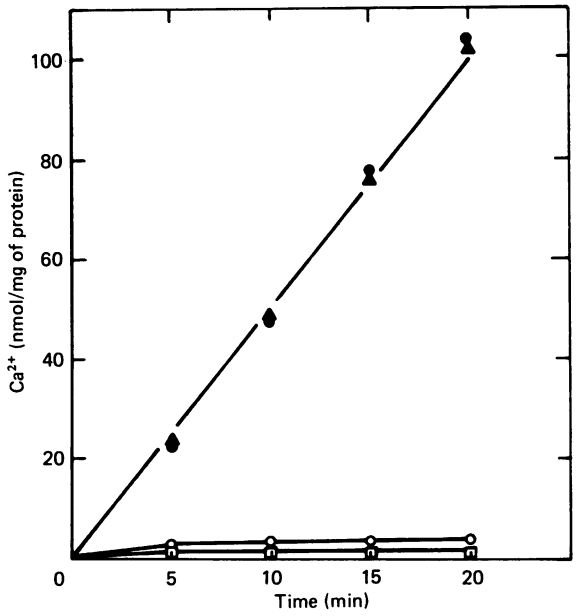

Fig. 5. Effect of IP_3 on the rate of Ca^{2+} uptake by S_1-ER vescicles

Initial rate of Ca^{2+} uptake was measured in the presence of 10 mM-Tris oxalate (as described in the Materials and methods section) in the absence of ATP (○), and in the presence of 2 mM-ATP, without (●) and with (▲) 20 μM-IP_3 and with A23187 (□). Extravesicular free Ca^{2+} concentration was 0.13 μM. These data were obtained from a representative experiment. The coefficient of variation at each data point was less than 10%.

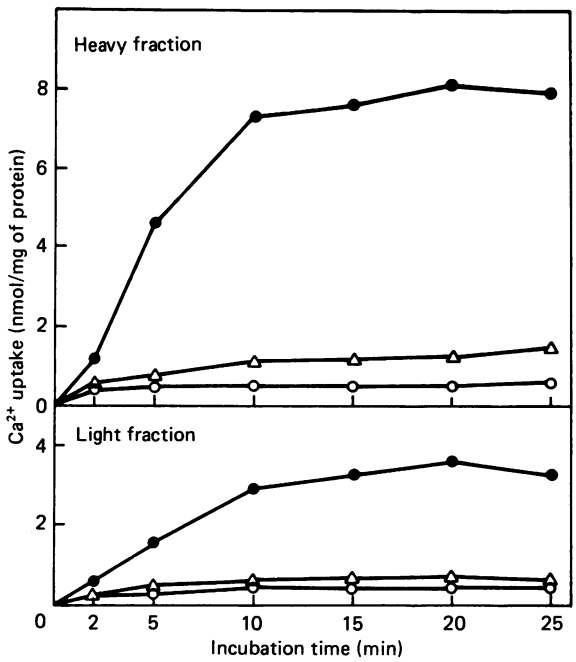

Fig. 6. Time course of Ca^{2+} uptake by heavy and light endoplasmic reticulum vesicles

Ca^{2+} uptake was assayed as described in Fig. 1 by addition of 100 μg of membrane protein in the absence of (○) and presence (● and △) of 2 mM-ATP, without (●) or with (△) 1 mM-vanadate. The data were obtained from a representative experiment. The coefficient of variation at each data point was less than 10%.

of cytosol (prepared as described in the Materials and methods section) was employed. An amount of endoplasmic reticulum membrane protein (80–100 μg/ml) was added to the assays to yield a similar number of nmol of Ca^{2+} uptake as shown in Fig. 1 for the S_1-ER. Otherwise the Ca^{2+}-efflux assays were carried out in the same manner. The profile for Ca^{2+} uptake was almost identical to that shown in Fig. 1 for S_1-ER (results not shown). In three experiments using three separate membrane preparations the average amount of IP_3-induced (20 μM) Ca^{2+} efflux was $13\pm3\%$ of the exchangable Ca^{2+} pool in the presence of vanadate. Addition of cytosol back to the efflux assay, in an amount equivalent to that present in assays performed with the S_1-ER, had no effect on IP_3-induced Ca^{2+} efflux.

Endoplasmic reticulum was further fractionated into heavy and light fractions by differential centrifugation as described in the Materials and methods section. Both the heavy and light fractions demonstrated ATP-dependent Ca^{2+} uptake (Fig. 6). As with the S_1-ER and isolated endoplasmic reticulum preparations, the Ca^{2+}-uptake activities of the heavy and light fractions were inhibited by vanadate. In three separate experiments using three different membrane preparations it was found that the heavy fraction, but not the light fraction, was sensitive to IP_3-induced Ca^{2+} efflux (Fig. 7). IP_3 (20 μM) caused the release of $15\pm3\%$ of Ca^{2+} sequestered by S_1-ER and $14\pm2\%$ of that sequestered by the heavy fraction, while no ($2\pm2\%$) vesicular Ca^{2+} was released by the light fraction. These percentages represent fractional Ca^{2+} release with IP_3 plus vanadate compared with vanadate alone. Interestingly, the amount of Ca^{2+} released by vanadate alone in the light fraction was equivalent to the amount of Ca^{2+} released from the heavy fraction in the presence of both vanadate and IP_3 (Fig. 7). These observations suggest that the light fraction, although insensitive to IP_3, is more permeable to Ca^{2+} than is the heavy fraction.

Effect of IP_3 on isolated plasma membrane

Since the plasma membrane plays a key role in maintaining a low intracellular Ca^{2+} concentration, we studied the effects of IP_3 on plasma membrane Ca^{2+} uptake, the $(Ca^{2+}+Mg^{2+})$-ATPase activity, and Ca^{2+} release from plasma membrane vesicles. IP_3 (20 μM) had no effect on these processes (Table 1). IP_2 (10 μM) also had no significant effect on plasma membrane Ca^{2+} uptake or release under the same assay conditions used to study IP_3 (results not shown).

DISCUSSION

The effect of IP_3 on the release of Ca^{2+} has been investigated using a variety of permeabilized cells or preparations of isolated endoplasmic reticulum. It is generally concluded from these studies that the source for IP_3-mediated Ca^{2+} release is the endoplasmic reticulum and not the mitochondria. However, it is possible that IP_3 could also induce increases in cytoplasmic Ca^{2+} via: (*a*) inhibition of active Ca^{2+} uptake into the endoplasmic reticulum, (*b*) inhibition of active Ca^{2+} extrusion from the cell via the plasma membrane Ca^{2+} pump, (*c*) increased

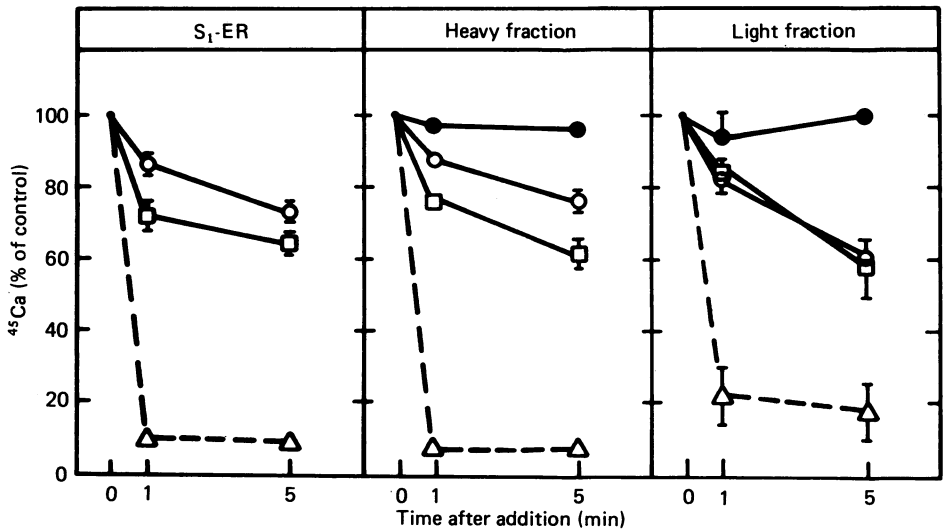

Fig. 7. Time course of IP_3-induced Ca^{2+} efflux from S_1-ER, heavy and light endoplasmic reticulum vesicles

S_1-ER (900 μg), heavy (100 μg) and light (100 μg) membrane proteins were loaded and efflux was measured as described in Figs. 1 and 2. Ca^{2+} efflux was measured after the addition of: water (●), 20 μM-IP_3 + 1 mM-vanadate (□), 1 mM-vanadate (○) and 2 μM-A23187 (△). Each point is the mean ± S.E.M. of results obtained from three experiments.

Table 1. Effect of IP_3 on plasma membrane Ca^{2+} uptake, (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} release

The Ca^{2+} uptake assay was performed as described in the Materials and methods section. The assay was started by the addition of 130 μg of plasma membrane protein/ml and incubated at 30 °C for 15 min. For the Ca^{2+}-release study, Ca^{2+} uptake in the absence of oxalate was allowed to proceed for 15 min. At 16 min, 1 mM-vanadate or 1 mM-vanadate plus 20 μM-IP_3 were added to the assay and the amount of Ca^{2+} remaining in the plasma membrane vesicles was determined after 5 min. The (Ca^{2+} + Mg^{2+})-ATPase assay was carried out under the standard conditions described in the Materials and methods section. Data are presented as means ± S.E.M. for two experiments.

Free [Ca^{2+}] (μM)	IP_3 (20 μM)	Ca^{2+} uptake (nmol/15 min per mg)	(Ca^{2+} + Mg^{2+})-ATPase (nmol/10 min per mg)	Ca^{2+} remaining (% of control)
0.059	−	2.2 ± 0.1	235.9 ± 12	89 ± 1
	+	2.5 ± 0.2	272.7 ± 14	89 ± 3
0.13	−	4.6 ± 0.1	313.1 ± 16	92 ± 2
	+	4.8 ± 0.1	333.8 ± 17	88 ± 2

plasma membrane permeability to Ca^{2+}. The present study has addressed these possibilities. IP_3 promoted Ca^{2+} release from adipocyte endoplasmic reticulum but had no significant effect on the endoplasmic reticulum Ca^{2+} pump, the plasma membrane Ca^{2+} pump and (Ca^{2+} + Mg^{2+})-ATPase, or on Ca^{2+} release from plasma membrane vesicles. The results are consistent with the concept that IP_3 has its effect exclusively on the endoplasmic reticulum.

One other study which determined the effect of IP_3 on both Ca^{2+} uptake and release by endoplasmic reticulum utilized permeabilized macrophages (Hirata *et al.*, 1985) and showed that the initial Ca^{2+} uptake rate was inhibited 12–16% by IP_3. These observations contrast with our findings, in which the Ca^{2+} uptake rate was unaffected by IP_3. Nevertheless, the amount of Ca^{2+} released in the presence of IP_3 in the peritoneal macrophage far exceeded that which could be accounted for by inhibition of the Ca^{2+} pump alone.

IP_3 alone caused rapid Ca^{2+} release from S_1-ER followed by slow Ca^{2+} re-uptake into the vesicles. In the presence of vanadate, which inhibits the endoplasmic reticulum Ca^{2+} pump, the increment of Ca^{2+} release by IP_3 was enhanced. These observations are similar to those described for other tissues. The concentration of IP_3 which results in half-maximal release of Ca^{2+} ranges from about 0.1 μM (Joseph *et al.*, 1984*a*,*b*) to 5 μM (Volpe *et al.*, 1985). The amount of accumulated Ca^{2+} maximally released was typically reported to be between 20 and 50% of exchangeable Ca^{2+}. Comparably, the adipocyte endoplasmic reticulum maximally released 20% of the exchangeable Ca^{2+}.

Whether or not isolated endoplasmic reticulum is less sensitive than the permeabilized cell systems to IP_3 has not been fully addressed. However, in studies using endoplasmic reticulum obtained from rat insulinoma cells the concentration of IP_3 required for half-maximal Ca^{2+} release was 3 μM (Prentki *et al.*, 1984) but only 0.5 μM for permeabilized insulinoma cells (Joseph *et al.*, 1984*b*). Therefore, it is possible that the sensitivity of the endoplasmic reticulum to IP_3 in intact cells may be significantly greater than that seen with isolated endoplasmic reticulum. Importantly, we determined that IP_3-induced Ca^{2+} release was similar in two different

preparations of endoplasmic reticulum vesicles; one in which vesicles were suspended in dilute cytosol (S_1-ER) and the other in which vesicles were separated from cytosol. Thus, the cytosol and its constituents appeared to have no effect on the ability of IP_3 to release Ca^{2+}.

The extent of IP_3-induced Ca^{2+} release was dependent on the concentration of free Ca^{2+} in the assay medium. The highest fraction of loaded Ca^{2+} was released when the free Ca^{2+} concentration was 0.13 μM, close to estimated free Ca^{2+} concentration in the cytoplasm of unstimulated cells (Borle & Snowdowne, 1982). The ability of IP_3 to promote Ca^{2+} release was progressively diminished at higher free Ca^{2+} concentrations. A similar Ca^{2+}-dependence of IP_3-induced Ca^{2+} efflux has been observed in other systems. Wolf *et al.* (1985), using permeabilized islets, found that the ability of IP_3 to induce Ca^{2+} release occurred only in the submicromolar range. Suematsu *et al.* (1984), using permeabilized cells from porcine coronary artery, and Hirata *et al.* (1984), using permeabilized peritoneal macrophages, found that maximal Ca^{2+} release from endoplasmic reticulum occurred at Ca^{2+} concentrations below 1 μM, becoming significantly reduced at Ca^{2+} concentrations above 1 μM. On the other hand, Burgess *et al.*, (1984), using permeabilized guinea pig hepatocytes, showed that Ca^{2+} release was unaffected at concentrations of Ca^{2+} up to 3 μM. Although the mechanism by which IP_3 promotes Ca^{2+} release is unknown, it is conceivable that the Ca^{2+}-release system could be regulated by cytoplasmic Ca^{2+} alone. For instance, one may speculate that the cellular Ca^{2+} concentration increases only to a level required to promote metabolic changes, following which the release system is shut down thereby preventing further increases in cytosolic Ca^{2+}.

We further examined the effects of IP_3 on two subfractions of isolated endoplasmic reticulum, the heavy and light fractions. Although both membrane fractions accumulated Ca^{2+} in an ATP-dependent fashion, only the heavy fraction exhibited significant release of accumulated Ca^{2+} in response to IP_3. If the heavy fraction contained a population of endoplasmic reticulum vesicles enriched in IP_3-sensitive vesicles, an enhanced Ca^{2+} release would have been expected compared with that obtained with the isolated endoplasmic reticulum fraction (160000 ***g*** pellet). However, no such difference was observed. Further investigation will be required to explain this dilemma.

The possibility that IP_3 may play a key role in Ca^{2+}-dependent regulatory mechanisms in the adipocyte derive from the recent study of Cheng & Larner (1985), who demonstrated that pyruvate dehydrogenase and glycogen phosphorylase, both of which are activated by Ca^{2+}-dependent mechanisms, and glycogen synthase, which is inactivated by a Ca^{2+}-dependent mechanism, (McDonald *et al.*, 1984, for review) are regulated by α_1-agonists via a mechanism that is apparently dependent on intracellular Ca^{2+}. Therefore, analogous to the hypothesized mechanism of action of α_1-agonists in other cells (for review see Exton, 1985), the ability of IP_3 to promote Ca^{2+} release from endoplasmic reticulum may be responsible for the regulation of these metabolic events in adipocytes. IP_3 may also play a role in insulin action since: (*a*) substantial indirect evidence suggests that Ca^{2+} plays a role in the mechanism of insulin action (Pershadsingh & McDonald, 1984), (*b*) insulin activates phospholipase C in fat cells (Koepfer-Hobelsberger & Wieland, 1984), and (*c*) the insulin receptor kinase from human placenta can phosphorylate phosphatidylinositol (Machicao & Wieland, 1984). Although our study establishes that IP_3 can promote Ca^{2+} release from adipocyte endoplasmic reticulum, further study is required to establish firmly a link between intracellular Ca^{2+} release from endoplasmic reticulum, IP_3 and hormone action in the adipocyte.

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