

***myo*-Inositol 1,4,5-trisphosphate mobilizes Ca²⁺ from isolated adipocyte endoplasmic reticulum but not from plasma membranes**

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The effects of *myo*-inositol 1,4,5-trisphosphate (IP₃) on Ca²⁺ uptake and release from isolated adipocyte endoplasmic reticulum and plasma membrane vesicles were investigated. Effects of IP₃ were initially characterized using an endoplasmic reticulum preparation with cytosol present (S₁-ER). Maximal and half-maximal effects of IP₃ on Ca²⁺ release from S₁-ER vesicles occurred at 20 μM- and 7 μM-IP₃, respectively, in the presence of vanadate which prevents the re-uptake of released Ca²⁺ via the endoplasmic reticulum Ca²⁺ pump. At saturating IP₃ concentrations, Ca²⁺ release in the presence of vanadate was 20% of the exchangeable Ca²⁺ pool. IP₃-induced release of Ca²⁺ from S₁-ER was dependent on extravesicular free Ca²⁺ concentration with maximal release occurring at 0.13 μM free Ca²⁺. At 20 μM-IP₃ there was no effect on the initial rate of Ca²⁺ uptake by S₁-ER. IP₃ promoted Ca²⁺ release from isolated endoplasmic reticulum vesicles (cytosol not present) to a similar level as compared with S₁-ER. Addition of cytosol to isolated endoplasmic reticulum vesicles did not affect IP₃-induced Ca²⁺ 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 Ca²⁺ when challenged with IP₃. IP₃ (20 μM) did not promote Ca²⁺ release from plasma membrane vesicles and had no effect on the (Ca²⁺ + Mg²⁺)-ATPase activity or on the initial rate of ATP-dependent Ca²⁺ uptake by these vesicles. These results support the concept that IP₃ acts exclusively at the endoplasmic reticulum to promote Ca²⁺ release.

INTRODUCTION

It has been proposed that the wide variety of hormones and neurotransmitters which exert their effects via Ca²⁺-dependent mechanisms do so by triggering the mobilization of intracellular Ca²⁺. One proposed mechanism for intracellular Ca²⁺ mobilization involves the production of IP₃ by rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate following agonist-induced activation of phospholipase C at the plasma membrane. IP₃ then interacts with the endoplasmic reticulum to initiate the release of Ca²⁺ stored in this organelle (Creba *et al.*, 1983). In support of this hypothesis, IP₃ has been shown to induce Ca²⁺ efflux from non-mitochondrial stores (presumably endoplasmic reticulum) of a variety of different permeabilized cells types (Streb *et al.*, 1983; Joseph *et al.*, 1984a,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 IP₃ also causes Ca²⁺ 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 IP₃-induced Ca²⁺ release from endoplasmic reticulum is unknown.

Although phosphoinositide regulation of Ca²⁺ homeostasis in the adipocyte has not yet been reported, there is reason to believe that IP₃ may promote Ca²⁺ release from their endoplasmic reticulum in response to hormonal stimulation. Activation of α₁-adrenergic receptors is one mechanism by which the breakdown of phosphatidylinositol 4,5-bisphosphate occurs in the plasma membrane to produce IP₃ (Exton, 1985). Recently, Cheng & Larner (1985) demonstrated, using adipocytes, that α₁-agonists regulate the activity of enzymes (glycogen synthase, glycogen phosphorylase, pyruvate dehydrogenase) that require Ca²⁺ for either activation or inactivation and that this regulation was dependent on intracellular and not extracellular Ca²⁺. The adipocyte is also a target cell for insulin and several lines of indirect evidence suggest that intracellular Ca²⁺ may play a part in the mechanism of insulin action (for review see Pershadsingh & McDonald, 1984). A possible role for IP₃ 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 activity associated with the insulin receptor from human placenta can act as a phosphatidylinositol kinase (Machicao & Wieland, 1984).

In order to determine if IP₃ may play a role in mobilizing Ca²⁺ from intracellular stores in the adipocyte we investigated the effects of IP₃ on Ca²⁺ uptake and

Abbreviations used: (Ca²⁺ + Mg²⁺)-ATPase, calcium-stimulated, magnesium-dependent adenosine triphosphatase; IP₃, *myo*-inositol 1,4,5-trisphosphate; IP₂, *myo*-inositol 1,4-bisphosphate; S₁-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. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from Amersham, Arlington Heights, IL, U.S.A. and $^{45}\text{CaCl}_2$ 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 $^{45}\text{CaCl}_2/\text{ml}$, 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 $^{45}\text{Ca}^{2+}$ 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 $^{32}\text{P}_i$ released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 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

Ca²⁺ concentration (as described above), 2 mM-ATP, and 50 mM-Tris/Pipes, pH 7.5, in a total assay volume of 500 μ l. The reaction was initiated by the addition of membranes (20–30 μ g) 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. Ca²⁺-stimulated ATPase activity was calculated by subtracting the rate of ATP hydrolysis obtained in the presence of chelator alone from the rate obtained with Ca²⁺ plus chelator.

Preparation of IP₃ and IP₂

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 μ M). 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-CaCl₂, and IP₂ and IP₃ were separated and isolated from the supernatant using a Dowex column as described by Downes & Michell (1981). IP₃ 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 IP₂ and IP₃ 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 IP₂ and IP₃ 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 IP₃ was 99%.

RESULTS

Characterization of IP₃-induced Ca²⁺ release from S₁-ER

IP₃-induced Ca²⁺ release was first characterized in endoplasmic reticulum vesicles suspended in diluted cytosol (S₁-ER). As shown in Fig. 1, ATP-dependent accumulation of Ca²⁺ into these vesicles increased to a steady state level by 10–15 min. When the Ca²⁺ ionophore, A23187, was added in the presence of ATP, Ca²⁺ accumulation did not occur. Vanadate (1 mM) inhibited ATP-dependent Ca²⁺ uptake by 80%, consistent with our earlier findings using isolated endoplasmic reticulum vesicles (Delfert & McDonald, 1985).

The effect of IP₃ on Ca²⁺ release was then investigated in the presence and absence of vanadate, which prevents re-uptake of released Ca²⁺ via the Ca²⁺ pump (Fig. 2). Addition of water alone caused little loss of Ca²⁺ from vesicles. Addition of IP₃ alone (20 mM) caused the endoplasmic reticulum vesicles to release Ca²⁺ even at the earliest measured time point (1 min). Under these

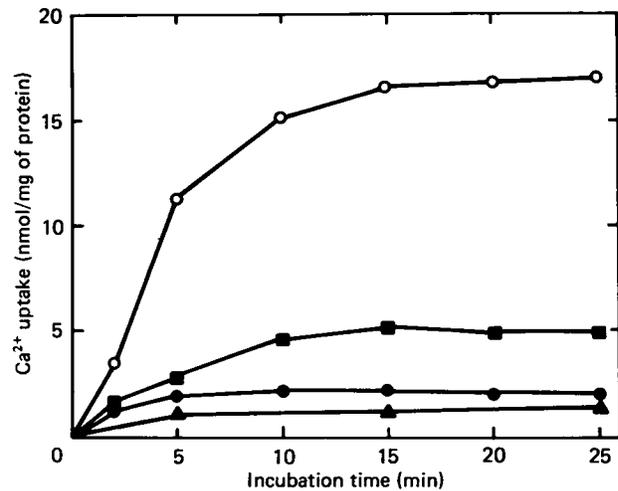


Fig. 1. Time course of Ca²⁺ uptake by S₁-ER vesicles

S₁-ER (900 μ g) was added to initiate Ca²⁺ uptake. The assay medium contained: 200 mM-sucrose, 20 mM-NaN₃, 100 mM-KCl, 2 mM-MgCl₂, 0.5 mM-EGTA, 0.15 mM-CaCl₂ (0.13 μ M free Ca²⁺), approx. 2 μ Ci of ⁴⁵CaCl₂/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 μ M-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%.

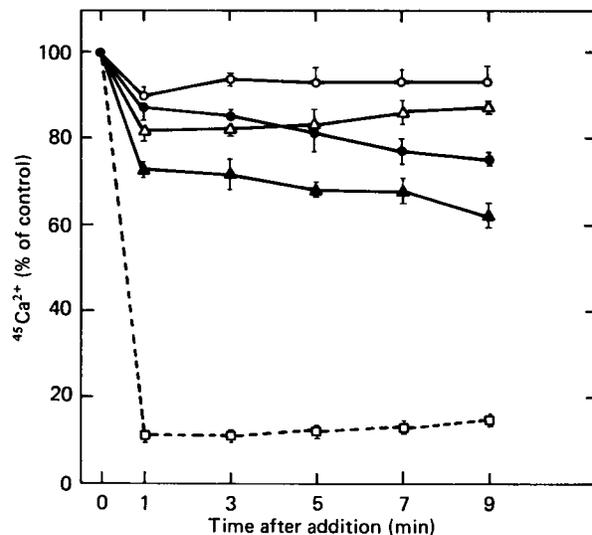


Fig. 2. Time course of IP₃-induced Ca²⁺ efflux from S₁-ER vesicles

S₁-ER vesicles were loaded with Ca²⁺ for 15 min in the presence of ATP (2 mM) as described in the legend to Fig. 1. Ca²⁺ efflux was monitored as described in the Materials and methods section. The amount of Ca²⁺ accumulated at 15 min was taken to be the control value of 100%. At 16 min ('zero' time on the abscissa) the following additions were made to yield the indicated final concentrations: water (○), 1 mM-vanadate (●), 20 μ M-IP₃ (△), 1 mM-vanadate + 20 μ M-IP₃ (▲), or 2 μ M-A23187 (□). The data were obtained from a representative experiment. Results are expressed as the mean \pm S.E.M.

conditions, maximal Ca^{2+} release was 9–11% of the total exchangeable 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 phosphoinositide 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

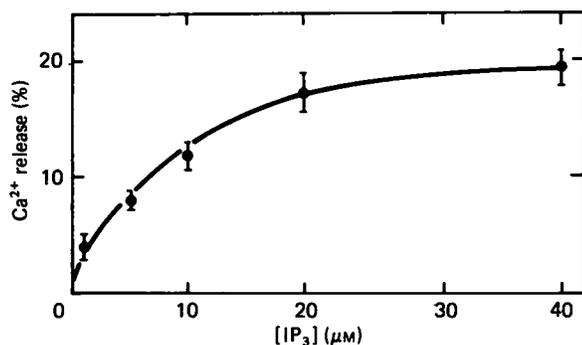


Fig. 3. Dose-response curve of IP_3 -induced Ca^{2+} release by S_1 -ER vesicles

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 \pm S.E.M. of data from two experiments.

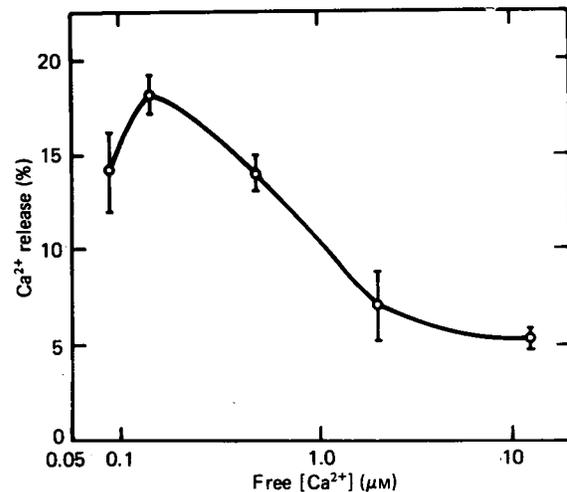


Fig. 4. Ca^{2+} -dependence of IP_3 -induced Ca^{2+} release from S_1 -ER vesicles

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 \pm 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

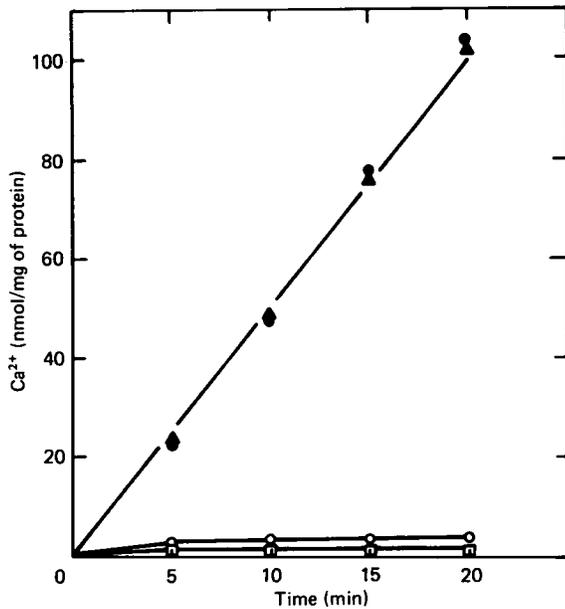


Fig. 5. Effect of IP₃ on the rate of Ca²⁺ uptake by S₁-ER vesicles

Initial rate of Ca²⁺ 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-IP₃ and with A23187 (□). Extravesicular free Ca²⁺ 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%.

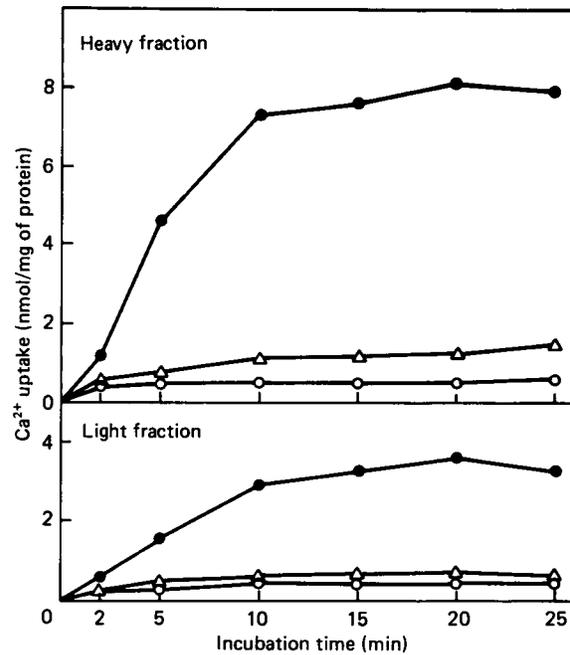


Fig. 6. Time course of Ca²⁺ uptake by heavy and light endoplasmic reticulum vesicles

Ca²⁺ 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 Ca²⁺ uptake as shown in Fig. 1 for the S₁-ER. Otherwise the Ca²⁺-efflux assays were carried out in the same manner. The profile for Ca²⁺ uptake was almost identical to that shown in Fig. 1 for S₁-ER (results not shown). In three experiments using three separate membrane preparations the average amount of IP₃-induced (20 μM) Ca²⁺ efflux was 13 ± 3% of the exchangeable Ca²⁺ 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 S₁-ER, had no effect on IP₃-induced Ca²⁺ 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 Ca²⁺ uptake (Fig. 6). As with the S₁-ER and isolated endoplasmic reticulum preparations, the Ca²⁺-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 IP₃-induced Ca²⁺ efflux (Fig. 7). IP₃ (20 μM) caused the release of 15 ± 3% of Ca²⁺ sequestered by S₁-ER and 14 ± 2% of that sequestered by the heavy fraction, while no (2 ± 2%) vesicular Ca²⁺ was released by the light fraction. These percentages represent fractional Ca²⁺ release with IP₃ plus vanadate compared with vanadate

alone. Interestingly, the amount of Ca²⁺ released by vanadate alone in the light fraction was equivalent to the amount of Ca²⁺ released from the heavy fraction in the presence of both vanadate and IP₃ (Fig. 7). These observations suggest that the light fraction, although insensitive to IP₃, is more permeable to Ca²⁺ than is the heavy fraction.

Effect of IP₃ on isolated plasma membrane

Since the plasma membrane plays a key role in maintaining a low intracellular Ca²⁺ concentration, we studied the effects of IP₃ on plasma membrane Ca²⁺ uptake, the (Ca²⁺ + Mg²⁺)-ATPase activity, and Ca²⁺ release from plasma membrane vesicles. IP₃ (20 μM) had no effect on these processes (Table 1). IP₂ (10 μM) also had no significant effect on plasma membrane Ca²⁺ uptake or release under the same assay conditions used to study IP₃ (results not shown).

DISCUSSION

The effect of IP₃ on the release of Ca²⁺ 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 IP₃-mediated Ca²⁺ release is the endoplasmic reticulum and not the mitochondria. However, it is possible that IP₃ could also induce increases in cytoplasmic Ca²⁺ via: (a) inhibition of active Ca²⁺ uptake into the endoplasmic reticulum, (b) inhibition of active Ca²⁺ extrusion from the cell via the plasma membrane Ca²⁺ pump, (c) increased

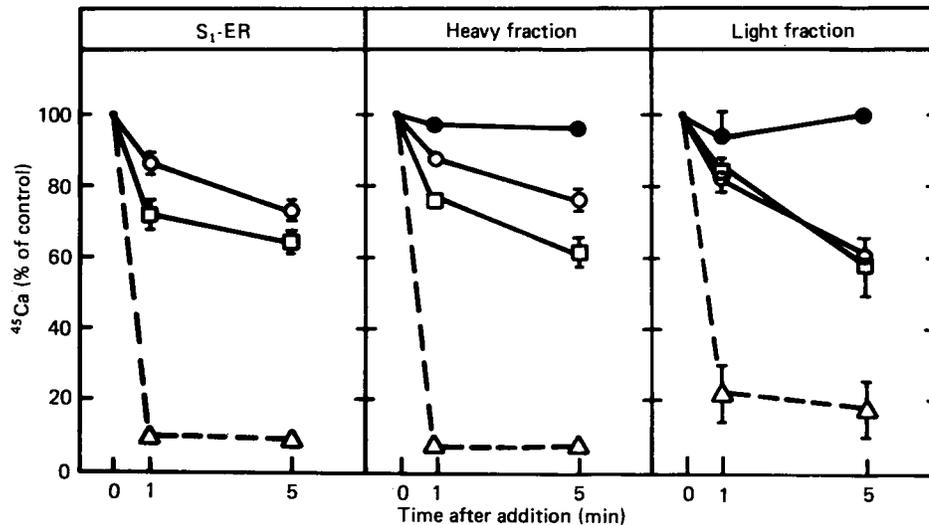


Fig. 7. Time course of IP_3 -induced Ca^{2+} efflux from S_1 -ER, heavy and light endoplasmic reticulum vesicles

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 (\bullet), 20 μM - IP_3 + 1 mM-vanadate (\square), 1 mM-vanadate (\circ) and 2 μM -A23187 (\triangle). Each point is the mean \pm S.E.M. of results obtained from three experiments.

Table 1. Effect of IP_3 on plasma membrane Ca^{2+} uptake, $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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 $^\circ\text{C}$ 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 $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase assay was carried out under the standard conditions described in the Materials and methods section. Data are presented as means \pm S.E.M. for two experiments.

Free $[\text{Ca}^{2+}]$ (μM)	IP_3 (20 μM)	Ca^{2+} uptake (nmol/15 min per mg)	$(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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 $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -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.*, 1984a,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.*, 1984b). 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 (S₁-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 IP₃ to release Ca²⁺.

The extent of IP₃-induced Ca²⁺ release was dependent on the concentration of free Ca²⁺ in the assay medium. The highest fraction of loaded Ca²⁺ was released when the free Ca²⁺ concentration was 0.13 μM, close to estimated free Ca²⁺ concentration in the cytoplasm of unstimulated cells (Borle & Snowdowne, 1982). The ability of IP₃ to promote Ca²⁺ release was progressively diminished at higher free Ca²⁺ concentrations. A similar Ca²⁺-dependence of IP₃-induced Ca²⁺ efflux has been observed in other systems. Wolf *et al.* (1985), using permeabilized islets, found that the ability of IP₃ to induce Ca²⁺ 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 Ca²⁺ release from endoplasmic reticulum occurred at Ca²⁺ concentrations below 1 μM, becoming significantly reduced at Ca²⁺ concentrations above 1 μM. On the other hand, Burgess *et al.*, (1984), using permeabilized guinea pig hepatocytes, showed that Ca²⁺ release was unaffected at concentrations of Ca²⁺ up to 3 μM. Although the mechanism by which IP₃ promotes Ca²⁺ release is unknown, it is conceivable that the Ca²⁺-release system could be regulated by cytoplasmic Ca²⁺ alone. For instance, one may speculate that the cellular Ca²⁺ 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 Ca²⁺.

We further examined the effects of IP₃ on two subfractions of isolated endoplasmic reticulum, the heavy and light fractions. Although both membrane fractions accumulated Ca²⁺ in an ATP-dependent fashion, only the heavy fraction exhibited significant release of accumulated Ca²⁺ in response to IP₃. If the heavy fraction contained a population of endoplasmic reticulum vesicles enriched in IP₃-sensitive vesicles, an enhanced Ca²⁺ 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 IP₃ may play a key role in Ca²⁺-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 Ca²⁺-dependent mechanisms, and glycogen synthase, which is inactivated by a Ca²⁺-dependent mechanism, (McDonald *et al.*, 1984, for review) are regulated by α₁-agonists via a mechanism that is apparently dependent on intracellular Ca²⁺. Therefore, analogous to the hypothesized mechanism of action of α₁-agonists in other cells (for review see Exton, 1985), the ability of IP₃ to promote Ca²⁺ release from endoplasmic reticulum may be responsible for the regulation of these metabolic events in adipocytes. IP₃ may also play a role in insulin action since: (a) substantial indirect evidence suggests that Ca²⁺ 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 IP₃ can promote Ca²⁺ release from adipocyte endoplasmic reticulum, further study is required to establish firmly a link between intracellular Ca²⁺ release from endoplasmic reticulum, IP₃ and hormone action in the adipocyte.

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