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Relationship between the calcium-mobilizing action of inositol 1,4,5-trisphosphate in permeable AR4-2J cells and the estimated levels of inositol 1,4,5-trisphosphate in intact AR4-2J cells

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Various experimental strategies were employed in an effort to explain the previously reported [Horstman, Takemura & Putney (1988) J. Biol. Chem. **263**, 15297–15303] paradoxically high levels of inositol 1,4,5-trisphosphate [(1,4,5)IP_3] in resting and substance-P-stimulated AR4-2J cells. The concentration–effect curves for substance-P-induced [^{3}H](1,4,5)IP_3 formation in [^{3}H]inositol-labelled cells and substance-P-induced increase in intracellular [Ca^{2+}] were essentially superimposable, suggesting that formation of (1,4,5)IP_3 is limiting for cellular Ca^{2+} mobilization. In electrically permeabilized AR4-2J cells, (1,4,5)IP_3 and other inositol polyphosphates stimulated Ca^{2+} release with potencies similar to those reported for other cell types, including the parent pancreatic acinar cell. Compartmentalization of basal (1,4,5)IP_3 was suggested by the fact that this material was stable in the presence of antimycin A, although this toxin completely blocked agonist stimulation of phospholipase C. However, subcellular fractionation as well as permeabilization of the cells with *Staphylococcus aureus* α-toxin failed to provide evidence for binding or sequestration of [^{3}H](1,4,5)IP_3 in AR4-2J cells. The density of (1,4,5)IP_3 receptors in AR4-2J cells was not sufficiently large to impose non-linearity in the relationship between (1,4,5)IP_3 concentration and (1,4,5)IP_3-induced Ca^{2+} release. Thus the apparent high concentrations of (1,4,5)IP_3 in resting and stimulated AR4-2J cells are not indicative of atypically low sensitivity or high concentration of (1,4,5)IP_3 receptors, nor is there evidence for compartmentalization of (1,4,5)IP_3 outside of the cytoplasm in these cells. It is possible that soluble factors in the cytoplasm of AR4-2J cells regulate the free concentration of (1,4,5)IP_3 or the sensitivity of receptors to (1,4,5)IP_3.

INTRODUCTION

In a wide variety of cellular systems, activation of cellular Ca^{2+} mobilization by agonists for surface membrane receptors is associated with stimulation of a polyphosphoinositide-specific phospholipase C and generation of inositol polyphosphates (Shears, 1989). In these instances, cellular Ca^{2+} mobilization generally consists of an initial release of intracellular sequestered Ca^{2+} to the cytosol, followed or accompanied by an increased rate of Ca^{2+} entry across the plasma membrane from the extracellular space (Putney, 1987). There is considerable evidence to suggest that the initial intracellular release of Ca^{2+} is due to the primary breakdown product of phosphatidylinositol 4,5-bisphosphate, inositol 1,4,5-trisphosphate [(1,4,5)IP_3], which is believed to bind to a specific receptor, thereby opening a Ca^{2+} channel in the limiting membrane of a specific intracellular Ca^{2+}-sequestering organelle (Berridge & Irvine, 1989). The primary evidence for this idea is: (1) activation of phospholipase C-linked receptors causes a rapid increase in [^{3}H](1,4,5)IP_3 in cells previously incubated in medium containing [^{3}H]inositol to achieve radiolabelling of the precursor polyphosphoinositides (Berridge, 1983), (2) the addition of (1,4,5)IP_3 to permeable cells or microsomal preparations causes a rapid release of sequestered Ca^{2+} (Streb *et al.*, 1983), and (3) in permeable cells which retain the ability to respond to agonists and to (1,4,5)IP_3, the release of sequestered Ca^{2+} by agonist and (1,4,5)IP_3 was not additive (Streb *et al.*, 1985).

One piece of important information which has been only recently forthcoming is the demonstration that activated cells produce sufficient quantities of (1,4,5)IP_3 to account fully for the observed release of cellular Ca^{2+}. This is due to the difficulties in measuring the mass of inositol polyphosphates in cells. The cellular levels of (1,4,5)IP_3 have been recently estimated by a variety of methods, including enzymic phosphorylation to (1,3,4,5)IP_4 with [γ-^{32}P]ATP (Tarver *et al.*, 1987), competitive receptor-binding assay (Bradford & Rubin, 1986; Challiss *et al.*, 1988; Palmer *et al.*, 1988; Bredt *et al.*, 1989), microspectrophotometry (Shayman & Kirkwood, 1987; Underwood *et al.*, 1988), and extrapolation of the mass of ^{3}H-labelled h.p.l.c.-resolved compounds from the measured specific radioactivity of the precursor lipid under conditions of isotopic equilibrium (Horstman *et al.*, 1988). Among reports in which intracellular concentrations were estimated, resting levels of (1,4,5)IP_3 varied widely, from 0.1 to 3 μM, and increased on stimulation to levels ranging from 1 to 20 μM. Thus, in general it can be concluded that the cellular levels of inositol polyphosphates appear sufficient to activate Ca^{2+} mobilization, since the observed EC_{50} values (concn. giving 50% of maximum effect) for (1,4,5)IP_3-induced Ca^{2+} release in permeable cells and membranes are in the 0.1 μM range (Berridge, 1986).

At the high end of these ranges are the levels of (1,4,5)IP_3 in resting and substance-P-stimulated AR4-2J cells (Horstman *et al.*, 1988). Extrapolating from the specific radioactivity of [^{3}H]phosphatidylinositol 4,5-bisphosphate after achievement of

Abbreviations used: the inositol phosphates are abbreviated according to the 'Chilton Convention' (Agranoff *et al.*, 1985) as, for example, (1,4,5)IP_3 for D-*myo*-inositol 1,4,5-trisphosphate; EC_{50}, concn. giving 50% of maximum effect; DME, Dulbecco's modified Eagle medium; $[Ca^{2+}]_i$, free intracellular calcium concentration; AIB, α-aminoisobutyric acid; $GPIP_2$, glycerophosphoinositol 4,5-bisphosphate.

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demonstrated isotopic equilibrium (i.e. when the measured specific radioactivities of [^{3}H]phosphatidylinositol 4,5-bisphosphate and [^{3}H]inositol were equal), the resting concentration was estimated to be about 2 μM, rising transiently to about 20 μM on stimulation with substance P. Studies of the metabolism of the ^{3}H-labelled peaks attributed to (1,4,5)IP_3 confirmed that the great majority, if not all, of this material is indeed authentic (1,4,5)IP_3 (Nogimori *et al.*, 1990). Therefore, the major purpose of the studies reported here was to characterize the (1,4,5)IP_3-induced intracellular Ca^{2+} release in permeabilized AR4-2J cells, especially with regard to comparing the sensitivity of this mechanism to (1,4,5)IP_3 with the estimated levels of this inositol polyphosphate in basal and stimulated cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DME), fetal-bovine serum, glutamine and Puck's saline A were purchased from Gibco. $^{45}Ca^{2+}$ and [^{3}H]mannose were purchased from DuPont, and inositol phosphates were purchased from Boehringer or Calbiochem.

AR4-2J cell culture

Stock cultures of the AR4-2J cell line were grown in DME containing high glucose (4.5 g/l) in 650 ml tissue-culture flasks as previously described (Horstman *et al.*, 1988). The medium was supplemented with 10% (v/v) fetal-bovine serum and 2 mM-glutamine.

Preparation of permeabilized cells for $^{45}Ca^{2+}$-uptake studies

Cells were centrifuged and resuspended in a permeabilizing solution containing 300 mM-mannitol, 5 mM-Hepes (pH 7.2) and 0.1 mM-EGTA. By using an Electroporator 6000 (BTX Inc.), the suspension of cells was exposed repeatedly (10 ×) to an intense electric field (2.5 kV/cm) each of 50 μs duration. These conditions resulted in cells that were greater than 99% permeable to Trypan Blue.

The cells were then washed and suspended in a medium resembling the intracellular milieu, which had the following composition (mM): NaCl, 20.0; KCl, 100; $MgSO_4$, 2.0; Hepes, 20.0 (pH 7.2); EGTA, 1.0. Total Ca^{2+} was added so that the free Ca^{2+} was set at 150 nM, calculated as described by Fabiato (1988). The medium also contained the mitochondrial inhibitors oligomycin (10 μg/ml) and antimycin (10 μM) and an ATP-regenerating system (phosphocreatine 10 mM; creatine kinase 10 units/ml). MgATP (3 mM) was added to initiate Ca^{2+} uptake.

$^{45}Ca^{2+}$ uptake

The uptake and release of $^{45}Ca^{2+}$ by permeabilized AR4-2J cells were measured essentially as described previously for guinea-pig hepatocytes (Burgess *et al.*, 1983). Permeabilized cells were incubated with 1 μCi of $^{45}Ca^{2+}$/ml at a density of 2 mg of protein/ml, and uptake of $^{45}Ca^{2+}$ was initiated by addition of 3 mM-MgATP. Tissue content of $^{45}Ca^{2+}$ was determined by rapidly diluting 200 μl samples of the cell suspension in 5 ml of ice-cold iso-osmotic sucrose (310 mM) containing EGTA (4 mM) and 0.5 μCi of [^{3}H]mannose/ml for determination of trapped volume. The samples were subsequently filtered rapidly through GF/C (Whatman) glass-fibre filters and washed with 5 ml of ice-cold iso-osmotic sucrose. The radioactive contents of the filters were then determined by liquid-scintillation spectroscopy. In a few experiments (results not shown), effects of (1,4,5)IP_3 were also determined in cells permeabilized with saponin as described by Burgess *et al.* (1984), with essentially equivalent results.

Determination of intracellular Ca^{2+} in intact cells

Intracellular Ca^{2+} in AR4-2J cells was measured essentially as described previously (Horstman *et al.*, 1988). The cells were grown in 150 mm-diam. dishes for 3 days in DME, at which time the medium was replaced by 15 ml of DME containing 2 μM fura-2 acetoxymethyl ester (fura-2 AM), and the cells were incubated in this medium for 45 min. The medium was removed and the cells were detached from the flask with 5 mM-EDTA in Puck's saline A. The cells were resuspended in Krebs–Ringer solution, containing 120 mM-NaCl, 5.4 mM-KCl, 0.8 mM-$MgSO_4$, 1.0 mM-$CaCl_2$, 20 mM-Hepes (pH 7.4) and 11.1 mM-glucose. The cell concentration was approx. $(1–5) \times 10^6$ cells/ml. The cell were > 90% viable, as determined by their ability to exclude Trypan Blue. The final cytosolic concentration of fura-2 was estimated to be 100 μM. A 3 ml portion of cell suspension was pipetted into a quartz cuvette and placed in a thermostatically controlled (37 °C) chamber of a Delta-Scan 1 spectrofluorimeter (Photon Technology, Princeton, NJ, U.S.A.). Free calcium concentration ($[Ca^{2+}]_i$) was calculated from the measurement of the ratio of fluorescence intensities as described by Grynkiewicz *et al.* (1986). The excitation monochromators were set at 340 nm and 380 nm and the emission monochromator at 500 nm. The maximum and minimum fluorescence ratios were determined with 0.09% Triton X-100 in the presence of 1 mM-Ca^{2+} and with Tris base (pH > 8.3) in the presence of 5 mM-EGTA, respectively. Cell autofluorescence was subtracted before $[Ca^{2+}]_i$ was estimated.

Measurement of ^{3}H-labelled inositol phosphates in intact cells

The methods for measurement of [^{3}H]inositol phosphates in AR4-2J cells were similar to those reported previously (Horstman *et al.*, 1988; Menniti *et al.*, 1990). Cells were grown for 3 days in DME containing [^{3}H]inositol. The cells were incubated for 20 min with serum-free non-radioactive DME.

For measurements of [^{3}H](1,4,5)IP_3 in response to various concentrations of substance P, appropriate concentrations of the peptide were added to dishes of cells, and 5 s later reactions were stopped by rapid vacuum aspiration of the substance-P-containing medium and addition to the culture dish of 5 ml of ice-cold 7.5% (w/v) trichloroacetic acid containing 250 μg of phytic acid (IP_6). The culture dishes were placed on ice for 20 min. The supernatant containing the inositol phosphates was pipetted from the dish, and the acid was removed from the sample by extraction with 10 vol. of water-saturated ether (10 extractions, 5 ml each). The pH of the samples was adjusted to 8–9 with 1 M-NH_3. [^{3}H](1,4,5)IP_3 was separated from other inositol phosphates by anion-exchange chromatography as previously described (Berridge *et al.*, 1983; Sugiya *et al.*, 1987). In substance-P-stimulated AR4-2J cells, the IP_3 fraction from the 0.8 M-ammonium phosphate fractions is > 95% (1,4,5)IP_3 (Horstman *et al.*, 1988).

Measurement of ATP in AR4-2J cells

AR4-2J cells in tissue culture dishes were treated with 10 μM-antimycin A or vehicle for various times, and quenched by addition of 6% (w/v) $HClO_4$. After incubation for 20 min at 4 °C, the $HClO_4$-precipitated material was removed by centrifugation, and the $HClO_4$ was then precipitated with KOH and removed by centrifugation. The ATP content of the supernatants was determined by the luciferin/luciferinase method as described previously (Sugiya & Putney, 1988).

Permeabilization and subcellular fractionation of AR4-2J cells

Cells were labelled with [^{3}H]inositol as described above, while in parallel dishes cultures of cells at confluence were washed with

Hanks balanced salt solution (HBSS) and then loaded for 1 h with [^{3}H]α-aminoisobutyric acid ([^{3}H]AIB; 1 μCi/ml) in HBSS, just before subcellular fractionation or permeabilization. For permeabilization studies, the cells were washed for 30 min with iso-osmotic sucrose (contents, in mM: sucrose, 325; $CaCl_2$, 0.1; $MgCl_2$, 0.1; Hepes, 15) and then permeabilized with iso-osmotic sucrose containing *Staphylococcus aureus* α-toxin (25 units/ml). After various times of incubation with α-toxin, the supernatant was removed and the cells were washed once with α-toxin-free sucrose. For the [^{3}H]AIB-labelled cells, the remaining cell-associated [^{3}H]AIB was released by addition of 2% Triton X-100. The radioactivity in the three samples (α-toxin, wash and Triton) was determined by liquid-scintillation spectroscopy. For the [^{3}H]inositol-labelled cells, after permeabilization, the cells were washed once with α-toxin-free sucrose, and both solutions (α-toxin and wash) were added to a trichloroacetic acid/IP_6 stop solution (as above). The remaining cell-associated inositol phosphates were recovered by addition of 0.5 ml of trichloroacetic acid/IP_6 stop solution. These samples were analysed by h.p.l.c. techniques previously described (Horstman *et al.*, 1988; Menniti *et al.*, 1990).

For subcellular fractionation, [^{3}H]AIB or *myo*-[^{3}H]inositol-labelled AR4-2J cells were disrupted by nitrogen cavitation (Shears *et al.*, 1987). After removal of intact cells and nuclei by centrifugation at 200 *g* for 5 min, the soluble fraction was separated from the particulate fraction by centrifugation at 100000 *g* for 1 h. The content of [^{3}H]AIB in the fractions was determined by liquid-scintillation spectroscopy, and the inositol phosphates were determined by h.p.l.c.

Binding of [^{3}H](1,4,5)IP_3 to AR4-2J cell membranes

A crude AR4-2J membrane fraction was prepared by nitrogen cavitation and differential centrifugation as described above. The membranes (1 mg of protein) were incubated in 1.0 ml of an intracellular-like solution (details above) with 3 nM-[^{3}H](1,4,5)IP_3 (20 Ci/mmol) and various concentrations of unlabelled (1,4,5)IP_3. The mixture was incubated for 20 min at 4 °C, and the incubations were terminated by rapid vacuum filtration through presoaked glass-fibre filters (Whatman GF/B) followed by two 5 ml washes with ice-cold intracellular solution. Filtration and washing of the filters required about 5 s, an interval considerably shorter than the half-time for binding of (1,4,5)IP_3 at 0 °C (approx. 30 s; results not shown). The radioactivity retained on the filters was determined by liquid-scintillation spectroscopy. Specific receptor-bound radioactivity at each total (1,4,5)IP_3 concentration was taken as the difference in radioactivities in the presence and absence of 100 μM-(1,4,5)IP_3. Total binding of [^{3}H](1,4,5)IP_3 ranged from 400 to 750 d.p.m./tube, and non-specific binding, with no added unlabelled ligand, was about 10% of total. The density and dissociation constant of specific (1,4,5)IP_3-binding sites were determined by Scatchard analysis.

RESULTS

Previous estimates of the level of (1,4,5)IP_3 in AR4-2J cells suggested that (1,4,5)IP_3 increases from a basal value around 2 μM to around 20 μM after stimulation with substance P (Horstman *et al.*, 1988). Since these values are substantially greater than those necessary for Ca^{2+} release in most experimental systems, we first considered the possibility that the (1,4,5)IP_3-forming capacity of AR4-2J cells greatly exceeded that necessary for maximal Ca^{2+} release. A resulting prediction is that the dose–response curve for increasing $[Ca^{2+}]_i$ due to substance P would lie to the left of that for generation of [^{3}H](1,4,5)IP_3. However, as shown in Fig. 1, the dose–response curves for substance-P-induced increases in $[Ca^{2+}]_i$ and [^{3}H](1,4,5)IP_3 were

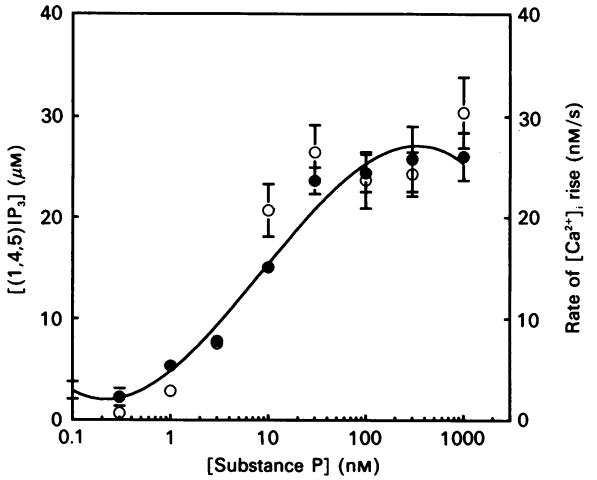

Fig. 1. Concentration–effect curves for substance-P-induced (1,4,5)IP_3 formation and Ca^{2+} mobilization

For (1,4,5)IP_3 measurements (●), the polyphosphoinositides of AR4-2J cells were labelled by incubating in the presence of [^{3}H]inositol for 3 days as described in the Materials and methods section. The indicated concentrations of substance P were added to cells on culture dishes, and ^{3}H-labelled (1,4,5)IP_3 was determined as described in the Materials and methods section. For $[Ca^{2+}]_i$ measurements (○), the indicated concentrations of substance P were added to AR4-2J cells previously loaded with the Ca^{2+} indicator fura-2 in a cuvette for fluorescence measurements of $[Ca^{2+}]_i$ as described in the Materials and methods section. Results are means ± S.E.M. of 3–5 independent determinations.

essentially superimposable, suggesting that the levels of (1,4,5)IP_3 formed in stimulated AR4-2J cells are indeed limiting for Ca^{2+} signalling. Thus we next considered the possibility that the intracellular-Ca^{2+} release mechanism in AR4-2J cells might be unusually insensitive to (1,4,5)IP_3, and examined the effects of (1,4,5)IP_3 and other inositol polyphosphates on release of $^{45}Ca^{2+}$ in electrically permeabilized AR4-2J cells to examine this prospect.

When incubated in medium with $[Ca^{2+}]$ buffered to 150 nM, and in the absence of ATP, AR4-2J cells bound $^{45}Ca^{2+}$ to about 0.5 nmol/mg of protein. Addition of 3.0 mM-ATP to the cells

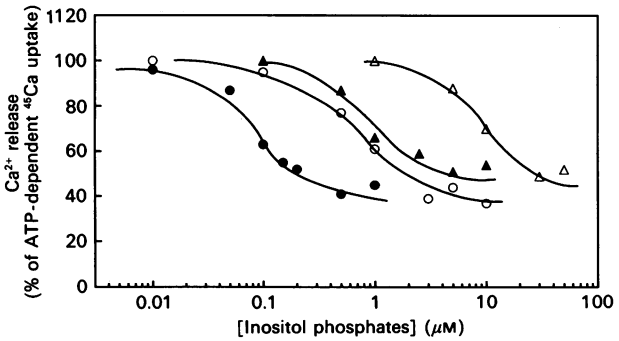

Fig. 2. Concentration–effect curves for $^{45}Ca^{2+}$ release by inositol polyphosphates from permeable AR4-2J cells

Release of $^{45}Ca^{2+}$ from permeable AR4-2J cells was determined as described in the Materials and methods section. The data are expressed as a percentage of the ATP-dependent $^{45}Ca^{2+}$ uptake. The inositol polyphosphates were: (1,4,5)IP_3 (●), (2,4,5)IP_3 (○), $GPIP_2$ (▲), (4,5)IP_2 (△). Representative experiments are shown; the experiments with (1,4,5)IP_3 were repeated 11 times, and those with other inositol polyphosphates 3 times.

caused a further accumulation to 1.5 nmol/mg of protein (results not shown). After $^{45}Ca^{2+}$ accumulation attained an apparent steady state, approx. 60% of the accumulated Ca^{2+} was released in response to addition of 1 μM-(1,4,5)IP_3 (Fig. 2). Preliminary experiments indicated that, at the protein concentration used in these experiments, the addition of maximal or sub-maximal concentrations of (1,4,5)IP_3 caused a rapid fall in cellular $^{45}Ca^{2+}$, and the achievement of a new steady state that was stable for 1–2 min after the addition (results not shown). This indicates that, under these experimental conditions, metabolism of the added (1,4,5)IP_3 did not significantly affect the apparent extent of $^{45}Ca^{2+}$ release, and thus would not affect the estimated EC_{50} values for (1,4,5)IP_3-induced $^{45}Ca^{2+}$ release.

Release of $^{45}Ca^{2+}$ was measured as a function of various concentrations of (1,4,5)IP_3 and also as a function of concentration of other inositol polyphosphates. These results from a typical determination are summarized in Fig. 2. From 11 such determinations for (1,4,5)IP_3, the EC_{50} for $^{45}Ca^{2+}$ release averaged 104±9 nM. The experiments with inositol phosphates other than (1,4,5)IP_3 were repeated with three different preparations, with similar results. The rank order of potency for releasing $^{45}Ca^{2+}$ was: (1,4,5)IP_3 > (2,4,5)IP_3 ≈ $GPIP_2$ > (4,5)IP_2. The maximal extent of Ca^{2+} release was not significantly different among these inositol polyphosphates. Inositol polyphosphates tested and found to be without effect were: (1)IP, (4)IP, (1,4)IP_2, (1,3,4,5)IP_4 (at 100 μM), (1,3,4)IP_3 (at 50 μM) and (1,3,4,5,6)IP_5 (0.5 mM). Note that some lots of (1,3,4,5)IP_4 and (1,3,4,5,6)IP_5 from some suppliers had Ca^{2+}-releasing activity which was not additive with that of (1,4,5)IP_3, and which was removed upon re-purification of the compounds by ion-exchange chromatography. We attribute this activity to contaminating (1,4,5)IP_3. Purified (1,3,4,5)IP_4 did not release $^{45}Ca^{2+}$ on its own, and did not affect the ability of (1,4,5)IP_3 to do so (results not shown).

The purpose of these experiments with permeabilized cells was to determine whether previous estimates of high cellular levels of (1,4,5)IP_3 were indicative of an unusually low sensitivity of the (1,4,5)IP_3-induced Ca^{2+} release mechanism in these cells. However, the data indicate that the sensitivity, as well as the specificity, of the (1,4,5)IP_3-induced Ca^{2+} release mechanism in permeabilized AR4-2J cells is similar to that in most eukaryotic cells (for examples, see Berridge, 1986). Thus we next focused on the apparent high basal levels of (1,4,5)IP_3 in the AR4-2J cells, and endeavoured to determine whether this material was in some manner bound or compartmentalized in the cells.

Treatment of AR4-2J cells with antimycin A caused a time-dependent loss of cellular ATP and a parallel loss in the ability of substance P to increase [^{3}H](1,4,5)IP_3 (Fig. 3). However, there was no decrease in basal [^{3}H](1,4,5)IP_3 due to antimycin treatment, indicating an extremely slow rate of turnover of basal (1,4,5)IP_3 as compared with the (1,4,5)IP_3 formed owing to receptor activation.

Since this result suggested binding or compartmentalization of basal (1,4,5)IP_3, we next examined the distribution of [^{3}H](1,4,5)IP_3 after simple fractionation of cells into soluble and particulate fractions, as well as the release of [^{3}H](1,4,5)IP_3 after cell permeabilization. For both strategies, [^{3}H]AIB was used as a marker for the movements and distribution of cytoplasmic material. Because the level of [^{3}H](1,4,5)IP_3 in substance-P-stimulated AR4-2J cells rises and declines in a shorter time than the procedures for fractionation or permeabilization take, these experiments were carried out only on unstimulated cells.

After homogenization and fractionation of [^{3}H]inositol-labelled or [^{3}H]AIB-labelled AR4-2J cells, in two of three separate experiments no ^{3}H-labelled materials were detected in the particulate fraction, and in the third only 12% of the [^{3}H]AIB and 16% of the [^{3}H](1,4,5)IP_3 were detected in the pellet. Thus, with

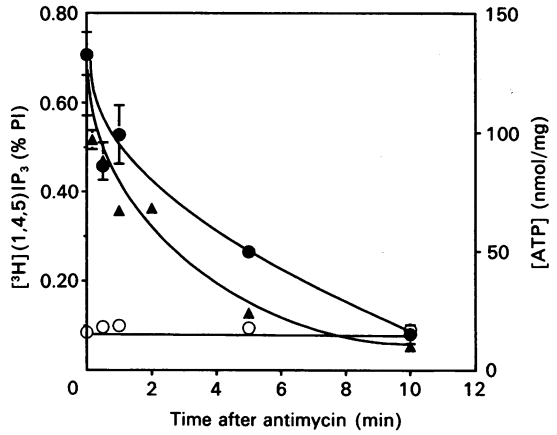

Fig. 3. Effect of antimycin on ATP levels and on basal and substance-P-stimulated [^{3}H](1,4,5)IP_3 in AR4-2J cells

Cells were labelled with [^{3}H]inositol, as described in the Materials and methods section for [^{3}H](1,4,5)IP_3 measurements. Antimycin A (10 μM) was added to the dishes, and the level of ATP (▲), the basal level of [^{3}H](1,4,5)IP_3 (○) or the level of [^{3}H](1,4,5)IP_3 produced by a 10 s stimulation with 1 μM-substance P (●) determined at the indicated times. The levels in the absence of antimycin A were also determined and are shown as values at $t = 0$. [^{3}H](1,4,5)IP_3 levels are expressed as a percentage of ^{3}H-labelled inositol lipids (%PI) and ATP levels as nmol/mg of protein (nmol/mg). Results are means± S.E.M. from 3 separate experiments.

this protocol, the behaviour of [^{3}H](1,4,5)IP_3 is similar to that of the cytosolic marker [^{3}H]AIB. However, it is possible that during the homogenization and fractionation either a (1,4,5)IP_3-containing organelle was disrupted, or the (1,4,5)IP_3 diffused from it. Therefore, we next determined the rate of release of [^{3}H]AIB and [^{3}H](1,4,5)IP_3 after permeabilization of AR4-2J cells with *Staph. aureus* α-toxin. This mode of permeabilization was chosen because α-toxin creates holes in the plasma membrane of cells with a pore diameter of approx. 2–3 nm. The α-toxin cannot pass through the pores and gain access to intracellular membranes, because its size (34 kDa) exceeds this small diameter.

Labelled AR4-2J cells ([^{3}H]AIB or [^{3}H]inositol) were treated with iso-osmotic sucrose containing α-toxin (25 units/ml) for various times. This permeabilizing solution was removed and the cells were washed once with α-toxin-free sucrose. The remaining

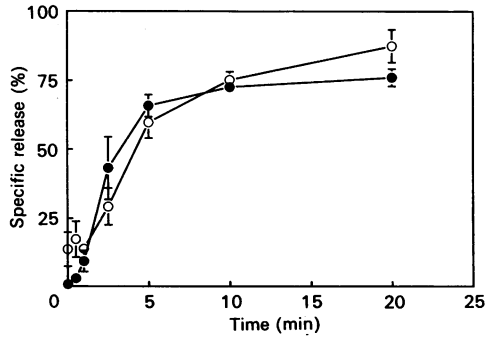

Fig. 4. Release of [^{3}H]AIB or [^{3}H](1,4,5)IP_3 from AR4-2J cells after permeabilization with *Staph. aureus* α-toxin

AR4-2J cells previously labelled with [^{3}H]AIB (●) or [^{3}H]inositol (○) were treated with the α-toxin and the incubation media removed from the dishes at the indicated times to determine the percentage of intracellular ^{3}H-labelled materials released. Results are means± S.E.M. from 4 separate experiments.

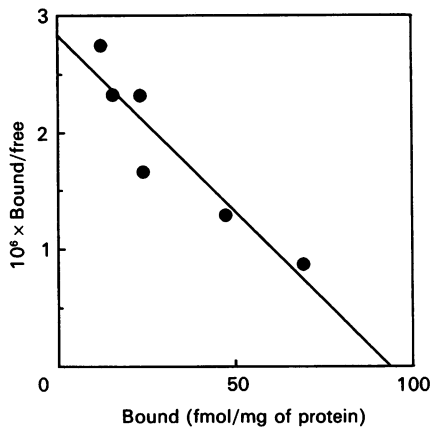

Fig. 5. Scatchard analysis of [^{3}H](1,4,5)IP$_3$ binding to AR4-2J membranes

One experiment is shown; two others gave similar results.

cell-associated label was released as described above. Non-specific release was determined by a parallel incubation of cells in the absence of toxin and was subtracted from the release observed in the presence of toxin. Fig. 4 shows that the kinetics of release of [^{3}H]AIB and [^{3}H](1,4,5)IP$_3$ from α-toxin-treated AR4-2J cells are similar. [Apparent differences in the early time points are due to high values for (1,4,5)IP$_3$ in two of the experiments, but do not reflect significant differences.] Therefore, consistent with the results from subcellular fractionation, these findings suggest that most of the basal (1,4,5)IP$_3$ contained in AR4-2J cells appears to be freely diffusible within the cytoplasm.

One possible explanation for the disagreement between measured cellular levels of (1,4,5)IP$_3$ in basal and stimulated cells is that the concentration of (1,4,5)IP$_3$ receptor might be high relative to its K_D, such that the receptor represents a substantial depot for cytoplasmic (1,4,5)IP$_3$. Therefore, the affinity and concentration of (1,4,5)IP$_3$ receptors present in a crude membrane preparation from AR4-2J cells were determined. As shown in Fig. 5, AR4-2J cell membranes appear to contain high-affinity (1,4,5)IP$_3$-binding sites; from three such experiments, the average K_D was 41 ± 4 nM, and the receptor density was 107 ± 26 fmol/mg of protein, which corresponds to a cellular concentration of 18 ± 4 nM (calculated as in Horstman *et al.*, 1988). Since the apparent binding constant determined in such assays generally underestimates the physiological binding constant (Nunn & Taylor, 1990), it is unlikely that the concentration of intracellular (1,4,5)IP$_3$ receptors is sufficiently high to act as a significant depot for basal or stimulated (1,4,5)IP$_3$ in the AR4-2J cells.

DISCUSSION

In a previous report from this laboratory, intracellular concentrations of (1,4,5)IP$_3$ were estimated by extrapolations from measured specific radioactivity of its ^{3}H-labelled precursor, phosphatidylinositol 4,5-bisphosphate (Horstman *et al.*, 1988). Assuming uniform distribution of the ^{3}H-labelled (1,4,5)IP$_3$, the basal concentration was calculated to be about 2 μM, increasing to around 20 μM after stimulation with substance P. Whereas for the initial estimates [^{3}H](1,4,5)IP$_3$ was identified solely on the basis of its h.p.l.c. mobility, in a subsequent report it was shown, by use of specific (1,4,5)IP$_3$-metabolizing enzymes, that the material from AR4-2J cells running as (1,4,5)IP$_3$ on the SAX h.p.l.c. column used for these studies was in fact essentially all D-*myo*-inositol 1,4,5-trisphosphate (Nogimori *et al.*, 1990).

These estimated values appear high, given the numerous reports of sub-micromolar concentrations of (1,4,5)IP$_3$ inducing Ca^{2+} mobilization from various preparations *in vitro* (Berridge, 1986). We therefore considered the following possible explanations for this apparent paradox: (*a*) when stimulated with supramaximal concentrations of substance P, AR4-2J cells make considerably greater quantities of (1,4,5)IP$_3$ than is necessary to saturate the available (1,4,5)IP$_3$ receptors [however, this would not provide an explanation for the high basal levels of (1,4,5)IP$_3$]; (*b*) (1,4,5)IP$_3$ receptors in AR4-2J cells are atypically insensitive to (1,4,5)IP$_3$; (*c*) basal and/or stimulated (1,4,5)IP$_3$ is compartmentalized or bound to macromolecules in AR4-2J cells.

Possibility (*a*) is similar to the proposal of 'spare receptors', but suggests that the 'reserve' occurs between (1,4,5)IP$_3$ production and Ca^{2+} mobilization. A prediction from this proposal is that the concentration–response curve for (1,4,5)IP$_3$ production will lie considerably to the right of the curve for Ca^{2+} mobilization. However, as shown by the data in Fig. 1, this was clearly not the case for substance-P stimulation of AR4-2J cells. In fact, maximal concentrations of substance P do not produce increases in $[Ca^{2+}]_i$ or in (1,4,5)IP$_3$ as great as those with bombesin (Nogimori *et al.*, 1990), making it extremely unlikely that substance-P activation leads to the production of excess (1,4,5)IP$_3$. Therefore, the conclusion is that all of the apparent 20 μM-(1,4,5)IP$_3$ in substance-P-activated AR4-2J cells is needed for generation of the observed Ca^{2+} signal.

Possibility (*b*) was investigated by examining the ability of (1,4,5)IP$_3$, as well as other inositol polyphosphates, to release $^{45}Ca^{2+}$ from electrically permeabilized AR4-2J cells. The use of $^{45}Ca^{2+}$ and Ca^{2+}–EGTA buffers for Ca^{2+}-transport studies allows precise control of $[Ca^{2+}]$, and permits use of dilute suspensions of cells, such that the rate of metabolism of (1,4,5)IP$_3$ does not complicate the interpretation of the concentration–effect relationships. As shown in Fig. 2, (1,4,5)IP$_3$ release Ca^{2+} from the permeabilized AR4-2J cells in the concentration range 0.01–1.0 μM, which is similar to the range for a variety of other preparations (Burgess *et al.*, 1984), including the pancreatic acinar cell (Streb *et al.*, 1983) from which this line is presumably derived (Womack *et al.*, 1985). In addition, the (1,4,5)IP$_3$ receptor was less potently activated by other inositol polyphosphates containing vicinal 4,5-phosphate groups, as shown for a variety of other systems (Nahorski & Potter, 1989). Thus, at least under these assay conditions, the (1,4,5)IP$_3$-sensitivity of the Ca^{2+}-releasing organelle in AR4-2J cells appears similar to that in other systems, and does not readily provide an explanation for the apparent high level of this signalling molecule in resting and substance-P-stimulated cells.

A number of strategies were employed to investigate possibility (*c*), that is, that binding or compartmentation of (1,4,5)IP$_3$ might lower its biologically effective concentration in the cell. Indeed, the results with antimycin A strongly indicate some mechanism of compartmentation of basal (1,4,5)IP$_3$. This conclusion comes from the observed stability of basal (1,4,5)IP$_3$ in the presence of antimycin A, despite an inhibition of the substance-P-mediated increase in (1,4,5)IP$_3$. A primary effect of lowering cellular ATP in this manner is to deplete cells of the polyphosphoinositide substrates for phospholipase C (Irvine *et al.*, 1984; Putney *et al.*, 1978). Soluble (1,4,5)IP$_3$ in stimulated cells is rapidly degraded when receptor activation is halted (Horstman *et al.*, 1988; Hughes *et al.*, 1988). Thus it would be expected that basal (1,4,5)IP$_3$, if attributable to a slow release of material to the cytosol from basal activity of phospholipase C, would similarly be rapidly degraded after antimycin A poisoning. However, as shown in Fig. 3, this was clearly not the case. This would suggest that this material, which has been previously shown to be essentially pure (1,4,5)IP$_3$, is protected in some manner from the cytosolic enzymes that metabolize the (1,4,5)IP$_3$ formed upon receptor

activation. A similar suggestion has been put forth by Lambert & Nahorski (1990) to explain the apparent high basal concentrations of (1,4,5)IP_3 in SH-SY5Y human neuroblastoma cells.

However, no evidence in support of this interpretation was obtained from subcellular-fractionation and cell-permeabilization studies. After disruption of AR4-2J cells by nitrogen cavitation, all of the [^{3}H](1,4,5)IP_3 in unstimulated cells was recovered in the 100000 *g* supernatant. In addition, when cells were permeabilized with *Staph. aureus* α-toxin, which is purported to cause the formation of 2–3 nm pores in the cell membrane (Kitazawa *et al.*, 1989), [^{3}H](1,4,5)IP_3 was released from the cells with a similar time course to the cytosolic marker, [^{3}H]AIB (Fig. 4). Substantial binding of [^{3}H](1,4,5)IP_3 to an AR4-2J-cell membrane fraction was detected. This presumably reflects binding of (1,4,5)IP_3 to its physiological receptor (Nahorski & Potter, 1989; Spat *et al.*, 1986). As argued previously (Spat *et al.*, 1986), if the concentration of these receptors in the cell were substantially greater than the K_D of the receptor for its ligand, then considerably non-linearity in the responsiveness of the system would result, and the apparent K_D in the intact cell would be increased (Chang *et al.*, 1975). However, the (1,4,5)IP_3-receptor concentration in AR4-2J cells was estimated to be less than 20 nM, which clearly does not exceed the estimated affinity for (1,4,5)IP_3-induced Ca^{2+} release.

Therefore, the various strategies employed in this study have not succeeded in resolving the paradox of apparent high levels of (1,4,5)IP_3 in AR4-2J cells, especially under conditions of receptor activation. Each of these experimental tactics has potential difficulties that could lead to a falsely negative conclusion. For example, the sensitivity of the (1,4,5)IP_3 receptor to its ligand could be regulated, i.e. diminished, by factors whose effects are rapidly lost on cell permeabilization. Likewise, if (1,4,5)IP_3 were reversibly bound or complexed with macromolecules or even low-molecular-mass substances, then this binding might be rapidly reversed after dilution by permeabilization or cellular disruption. Experiments are needed to determine if soluble factors exist in AR4-2J cells which might affect the ability of (1,4,5)IP_3 to bind to its receptor and activate Ca^{2+} intracellular release.

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