The intracellular distribution of inositol polyphosphates in HL60 promyeloid cells

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1. HL60 promyeloid cells contain high intracellular concentrations of inositol polyphosphates, notably inositol 1,3,4,5,6-pentakisphosphate (InsP_5) and inositol hexakisphosphate (InsP_6). To determine their intracellular location(s), we studied the release of inositol (poly)phosphates, of ATP, and of cytosolic and granule-enclosed enzymes from cells permeabilized by four different methods. 2. When cells were treated with digitonin, all of the inositol phosphates were released in parallel with the cytosolic constituents. Most of the InsP_5 and InsP_6 was released before significant permeabilization of azurophil granules. 3. Similar results were obtained from cells preloaded with ethylene glycol and permeabilized by osmotic lysis. 4. Electroporation at ~ 500 V/cm caused rapid release of free inositol. Higher field strengths provoked release of most of the ATP, InsP_5 and InsP_6 , but only slight release of the intracellular enzymes. Multiple discharges released ~ 80 – 90% of total InsP_5 and InsP_6 . In the

absence of bivalent-cation chelators, InsP_5 and InsP_6 were released less readily than ATP. 5. Treatment of cells with *Staphylococcus aureus* α -toxin caused quantitative release of inositol and ATP, without release of intracellular enzymes. However, inositol phosphates were released much less readily than inositol or ATP. Even after prolonged incubation with a high concentration of α -toxin, only ~ 50 – 70% of InsP_2 , InsP_3 and InsP_4 and $\leq 20\%$ of InsP_5 and InsP_6 were released, indicating that the high charge or large hydrated radius of InsP_5 and InsP_6 might limit their release through small toxin-induced pores. 6. These results indicate that most intracellular inositol metabolites are either in, or in rapid exchange with, the cytosolic compartment of HL60 cells. However, they leave open the possibility that a small proportion of cellular InsP_5 and InsP_6 (≤ 10 – 20%) might be in some intracellular bound form.

INTRODUCTION

Many inositol phosphates have been identified in eukaryote cells. Of these, the only compound with an undisputed function is $\text{Ins}(1,4,5)\text{P}_3$, a second messenger whose formation links receptor activation to intracellular Ca^{2+} mobilization (Berridge, 1993). Interest has recently focused on possible roles for inositol 1,3,4,5,6-pentakisphosphate (InsP_5) and inositol hexakisphosphate (InsP_6), two very polar compounds that are major inositol phosphates of mammalian and other eukaryote cells (reviewed by Shears, 1992; Hughes and Michell, 1993; Menitti et al., 1993a). It is not known why cells synthesize, maintain and regulate fairly high intracellular concentrations of these compounds (e.g. ~ 60 μM InsP_6 in HL60 cells: French et al., 1991), and an understanding of their cellular function(s) will require, among other things, knowledge of their location(s) in the cell. Studies of changes in their levels during functional changes in intact cells suggest that they may be implicated in cell regulatory processes. For example, intracellular [InsP_5] increases when HL60 promyeloid cells become committed to neutrophilic differentiation (French et al., 1991; Mountford et al., 1994), and intracellular concentrations of InsP_5 and InsP_6 change substantially during cell-cycle progression in thymocytes (Guse et al., 1993).

Several possible biological functions for InsP_5 and InsP_6 have been suggested, not all of which could be achieved by inositol polyphosphates located in any single intracellular compartment. The one well-established cytosolic function of InsP_5 is modulation of the oxygen affinity of haemoglobin in avian erythrocytes

(Isaacs and Harkness, 1980). Some reports have suggested that InsP_6 may have an extracellular role in controlling neuronal excitability, heart rate and blood pressure (Vallejo et al., 1987; Barraco et al., 1989), and InsP_6 has been proposed to regulate pituitary function by stimulating Ca^{2+} uptake (Sortino et al., 1990). For these proposals to be correct, InsP_6 would need to be intracellularly compartmentalized, so as to allow its controlled release to the extracellular medium. Other suggested roles for InsP_5 and/or InsP_6 , for example as antioxidants (Graf et al., 1987) or anti-neoplastic agents (Shamsuddin et al., 1992), would be best served if these compounds were located in the cytosolic compartment of cells.

Other clues as to possible roles for InsP_5 and InsP_6 have come from studies of certain enzymes whose activities they influence, and from studies of intracellular sites to which they bind with high affinity and selectivity. InsP_5 and InsP_6 potentially inhibit $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase from rat parotid glands or bovine testis (Hughes and Shears, 1990; Hughes et al., 1994). $\text{Ins}(1,3,4,5,6)\text{P}_5$ also binds Aldolase A with high affinity (Koppitz et al., 1986). High-affinity binding sites that can be regarded as putative receptors for InsP_6 have been found in several cell types (Nicoletti et al., 1990; Theibert et al., 1991; Regunathan et al., 1992), and one protein which binds InsP_6 with sub-micromolar affinity is AP-2, a clathrin assembly protein of coated vesicles which has an associated K^+ -channel activity and may be important in receptor-mediated endocytosis (Voglmaier et al., 1992; Timerman et al., 1992). InsP_6 also binds to arrestin, a protein that prevents G-protein-mediated closure of $\text{Na}^+/\text{Ca}^{2+}$ channels

Abbreviations used: 6-PGDH, 6-phosphogluconate dehydrogenase; FCS, foetal-calf serum; BAPTA, 1,2-bis-(2-aminophenoxy)ethanetetra-acetic acid.

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by metarhodopsin ($K_d \sim 5 \mu\text{M}$; Palczewski et al., 1991). Higher concentrations of InsP_6 are needed to inhibit specific binding of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ to rat cerebellar membranes ($\text{IC}_{50} \sim 36 \mu\text{M}$ and $\sim 3.6 \mu\text{M}$ respectively; Challiss et al., 1991) and $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release [$\text{IC}_{50} \sim 260 \mu\text{M}$ in brain microsomes (Palade et al., 1989) and $\sim 60 \mu\text{M}$ in cerebellar microsomes (G. R. Brown and F. Michelangeli, unpublished work)], but even these processes could be influenced by known intracellular concentrations of InsP_6 (e.g. $\sim 60 \mu\text{M}$ in HL60 cells).

An additional and quite different role for InsP_5 and InsP_6 is suggested by the recent discovery that at least some eukaryotic cells [AR4-2J pancreatoma cells (Menniti et al., 1993b) and *Dictyostelium discoideum* (Stephens et al., 1993)] synthesize pyrophosphorylated derivatives of these compounds which have a high free energy of hydrolysis. These novel compounds appear to be freely soluble ionic species in the cytosol of *Dictyostelium* amoebae, are present at high concentrations (0.05–0.25 mM) and are rapidly turned over through a phosphorylation/dephosphorylation cycle in which cells must make a major energetic investment (Menniti et al., 1993b; Stephens et al., 1993). Their role is unknown, but one possibility is that they might serve as a rapidly recruited intracellular energy store.

Evidence that InsP_5 and InsP_6 might be implicated in important cell functions is therefore accumulating, but it is not possible to interpret these reports fully, since we do not know whether InsP_5 and InsP_6 are located in the required intracellular compartment(s). In this paper, we report the use of four techniques employing different physical principles to investigate the intracellular distribution of InsP_5 and InsP_6 in HL60 human promyeloid cells (French et al., 1991; Bunce et al., 1992). Our main aim was to permeabilize the plasma membrane selectively, without disruption of the intracellular organelles, in order to determine what proportions of total cellular InsP_5 and InsP_6 (and of other inositol metabolites) are either located in or in rapid exchange with the cytosol compartment. Some of this work has been reported briefly elsewhere (Michell et al., 1992, 1993; Stuart et al., 1993).

MATERIALS AND METHODS

Materials

Reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.), Fisons (Loughborough, Leicestershire, U.K.) or Boehringer Mannheim UK (Lewes, East Sussex, U.K.), unless otherwise specified. *Staphylococcus aureus* α -toxin was generously given by Professor C. A. Pasternak (Department of Cellular and Molecular Sciences, St George's Hospital Medical School, London S.W. 17, U.K.).

Cell culture

For the studies with digitonin, HL60 cells were grown in inositol-free RPMI-1640 medium (Gibco, Paisley, U.K.) supplemented with 1 mg/l inositol exactly as described by French et al. (1991). For the other studies, the cells were cultured in RPMI-1640 supplemented with 3% (v/v) foetal-calf serum (FCS; Techgen International, London, U.K.). Equilibrium labelling of cells was achieved by culturing for 6 days in media containing 1% FCS and $2 \mu\text{Ci/ml}$ [^3H]myo-inositol (Amersham International, Amersham, Bucks., U.K.). Labelled cells were recovered from their culture medium, washed in fresh unlabelled medium, and counted in a haemocytometer.

Methods of membrane permeabilization

Permeabilization of digitonin

HL60 cells ($10^7/\text{ml}$) were incubated in a sucrose-based permeabilization medium (0.25 M sucrose, 5 mM EGTA, 10 mM Hepes, pH 7.5) at 4°C for 3 min in the presence of the indicated concentrations of digitonin. The cell suspensions were then loaded on to a layer of n-bromododecane (0.5 ml) overlying 5% (w/v) trichloroacetic acid [0.5 ml in 0.3 M sucrose, containing phytate hydrolysate ($2.5 \mu\text{g/ml}$ total phosphate; Wreggett et al., 1987)] in a 1.5 ml Microfuge tube, and the cells were immediately centrifuged (13000 rev./min for 3 min in a Microfuge) through the n-bromododecane layer into the acid. A small sample of the upper layer, containing released material, was retained for ATP and enzyme analysis; the remainder was mixed with an equal volume of 10% trichloroacetic acid. The acid-treated samples were then kept on ice for 10 min, the protein precipitates were removed by centrifugation, and the trichloroacetic acid was removed by six washes with diethyl ether (Tilly et al., 1987). The samples were adjusted to neutrality with Na_4EDTA (0.1 ml, 0.1 M, pH 7.5) and stored at -20°C .

Permeabilization by electroporation

HL60 cells were suspended at $5 \times 10^6/\text{ml}$ in an 'extracellular' buffer consisting of 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM glucose and 20 mM Hepes, pH 7.5 (Chu et al., 1987). A 1 ml portion of cell suspension was placed in a plastic cuvette (10 mm $\times$ 4 mm cross-section) between platinum electrodes 4 mm apart: platinum electrodes were used to prevent the artefacts that can be caused by aluminium (Loomis-Husselbee et al., 1991). A capacitor ($2 \mu\text{F}$) was charged to the required voltage by using a Consort electrophoresis power supply (Warren Scientific, Lichfield, U.K.) and discharged through the cell suspension. The electric field decayed exponentially with a time-constant of ~ 4 ms, given by the product of the capacitance and resistance of the cell suspension (Knight and Baker, 1982). The interval between multiple discharges was usually 8–10 s. Electroporated samples were incubated at room temperature ($\sim 23^\circ\text{C}$) for 30 min before sedimentation of cells by centrifugation. The supernatants were treated with HClO_4 (3%, w/v), kept on ice for 20 min, neutralized with 1.2 M KOH containing 75 mM Hepes and 60 mM EDTA, and insoluble material was removed by centrifugation (Kirk et al., 1990).

Permeabilization by hypo-osmotic lysis of glycol-loaded cells

The method was based on the erythrocyte-lysis technique of Billah et al. (1976, 1977). A pellet of HL60 cells (5×10^6) was dispersed in $50 \mu\text{l}$ of ethylene glycol solution [between 1 M and 9 M, in sucrose buffer (0.25 M sucrose, 5 mM EGTA, 2 mM Hepes, pH 7.0)] and left on ice for 3 min. The cell suspension was then quickly diluted with 1 ml of ice-cold sucrose buffer, kept on ice for a further 3 min, and cells were separated from medium by centrifugation through an n-bromododecane barrier (see 'Permeabilization by digitonin', above).

Permeabilization by *Staphylococcus aureus* α -toxin

Samples of HL60 cells (10^7 cells/ml in 0.25 M sucrose containing 5 mM EGTA and 10 mM Hepes, pH 7.5) were incubated with *Staphylococcus aureus* α -toxin (1–10 $\mu\text{g/ml}$) at 37°C for up to 2 h. Cells were separated from the suspending medium by centrifugation through a barrier of n-bromododecane into an underlying layer of acid (see 'Permeabilization by digitonin', above).

Analysis of released cell components

6-Phosphogluconate dehydrogenase (6-PGDH)

The activity of this cytosolic enzyme was measured by using the assay conditions described by Glock and McLean (1953). Total cellular activity was assessed by measuring the activity of the soluble fraction obtained from a cell pellet (5×10^6 cells) that had been permeabilized by 0.5 ml of 0.25 M sucrose containing 10 mM Hepes, 5 mM EGTA and 0.5% (w/v) Triton X-100.

β -Galactosidase

This was measured by the fluorimetric stopped-assay method of Heyworth et al. (1981). Total cell activity was assayed in a Triton X-100 cell extract as described above.

ATP

ATP released by cell permeabilization was determined by the method of Cotton and Jackson (1984), which uses luciferin/luciferase assay reagent. A portion (15–40 μ l) of the upper layer, containing released material (prepared as described above), from one of the permeabilization protocols was mixed with 25 μ l of luciferase reagent and 1 ml of buffer (10 mM Tris/glycine, 1 mM MgSO_4 , pH 7.75) and the light emission was measured immediately in a laboratory-constructed luminometer. Total cellular ATP levels were determined by analysing an HClO_4 (5%, in 3 mM EDTA) extract of a suspension of whole cells. After 20 min on ice, the acid extract was centrifuged (5 min, 1000 g), neutralized with 1 M KOH containing 1 M KHCO_3 , centrifuged again, and ATP in the supernatant was assayed as above. Some experiments employed an alternative method for determining total cellular ATP levels, which involved sonication of cell suspensions, followed by freezing at -80°C and rapid thawing. The two methods gave similar results.

Inositol and its phosphorylated metabolites

Inositol metabolites were usually separated by using gravity-fed Dowex AG1-X8 anion-exchange mini-columns as described by Kirk et al. (1990). Portions (5 ml) of each eluate were mixed with up to 15 ml of UltimaFlo AF biodegradable scintillant (Canberra Packard) and their ^3H content was determined by liquid-scintillation spectrometry. The amount of ^3H in the supernatant from untreated cells was subtracted, and the amount of each inositol phosphate species expressed as a percentage of its amount in the total cell extract. In a limited number of experiments, detailed comparisons of the released and retained inositol phosphates were made by analytical h.p.l.c. on Partisphere 5-SAX columns, as described previously (French et al., 1991; Bunce et al., 1993).

Transmission electron microscopy

Untreated and permeabilized cell samples were concentrated by gentle centrifugation, and the pellets were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. This was followed by further fixation in 1% (w/v) osmium tetroxide in cacodylate and dehydration in ethanol. Samples were embedded in Epon resin and sectioned. The sections were stained with uranyl acetate and lead citrate and examined in a Jeol 1200 electron microscope.

RESULTS

The experiments described below, which used several techniques based on different physical principles, all had the same aim, to permeabilize the HL60-cell plasma membrane selectively, without disrupting any intracellular organelles within which InsP_5 and InsP_6 might be located. Plasma-membrane permeabilization was monitored by measuring the release of two marker components, the enzyme 6-PGDH and the nucleotide ATP: 6-PGDH has long been recognized as an exclusively cytosolic enzyme, and most of the cell ATP is in the cytosol compartment. ATP is a phosphorylated polyanion of roughly similar size and charge to the inositol polyphosphates, so its behaviour in these experiments was of particular interest. By contrast, 6-PGDH is an enzyme whose release might be expected to need the presence of larger 'pores' than would be necessary to permit the passage of inositol polyphosphates and ATP. Acid β -galactosidase is an enzyme characteristic of the azurophil granules (lysosomes) of myeloid cells. β -Galactosidase release was used to detect the permeabilization of these relatively labile intracellular organelles.

Studies of cells treated with digitonin

The plant glycoside digitonin binds specifically to unconjugated 3β -hydroxysterols, so it most readily permeabilizes those membranes that have a high cholesterol content (Mackall et al., 1979). Digitonin therefore permeabilizes the cholesterol-rich plasma membrane quickly, before more slowly gaining access to intracellular organelles whose membranes contain less cholesterol. The β -galactosidase-containing azurophil granules are among the most cholesterol-rich of the intracellular structures, so they are probably among the most digitonin-sensitive of the intracellular structures.

Permeabilization

Incubation of HL60 cells for 3 min with low concentrations of digitonin (5–100 $\mu\text{g/ml}$) caused rapid permeabilization of the plasma membrane (maximum within 5 min), releasing most of the cellular 6-PGDH and ATP, but only a small proportion of the β -galactosidase (Figure 1). Treatment with low concentrations of digitonin thus achieved permeabilization of the plasma membrane without substantial permeabilization of intracellular structures. That the integrity of the intracellular granules was maintained is supported by the maintenance of their refractility under phase-contrast microscopy (results not shown). Higher concentrations of digitonin permeabilized a greater proportion of the β -galactosidase-containing granules, but complete release of this enzyme was not achieved even by very high digitonin concentrations (Figure 1).

Release of InsP_5 , InsP_6 and other inositol metabolites

This closely paralleled the release of the two cytosolic markers, 6-PGDH and ATP. The released and cell-retained samples contained all of the InsP_5 and InsP_6 of the original cells, with 70–80% of this being released by digitonin concentrations that released only a small proportion of the lysosomal marker (Figure 1). This suggests that InsP_5 and InsP_6 were present predominantly in the cytosolic compartment before permeabilization.

Anion-exchange analysis of the released inositol phosphates and of the cell-retained material, both on Dowex mini-columns and by h.p.l.c., showed that the patterns of inositol-labelled components present, from the least polar glycerophosphoinositol and InsP isomers through to the highly charged InsP_5 and InsP_6 , were similar to those reported previously (French et al., 1991).

Figure 1 Permeabilization of HL60 cells with digitonin

This shows the percentages of each marker and inositol metabolite fraction that were released at each digitonin concentration: the main Figure compares the release of markers with the release of InsP_{5+6} , and the inset compares the release of the different inositol metabolites. The data shown are means $\pm$ S.E.M. of the results from three experiments. The amounts of radioactivity (thousands of d.p.m. per 10^7 cells) in each fraction were typically: $\text{Ins} \sim 350$; $\text{InsP} \sim 200$; $\text{InsP}_2 \sim 20$; $\text{InsP}_3 \sim 6$; $\text{InsP}_4 \sim 10$; $\text{InsP}_{5+6} \sim 250$. Similar amounts of radioactivity were recovered from each permeabilization method.

and that these patterns were also similar in the released and cell-retained material (results not shown). Thus there was no evidence for selective release or retention of any particular inositol phosphate species by the digitonin-treated cells. Moreover, h.p.l.c. revealed no differences in the degrees of efflux from cells of InsP_5 and of InsP_6 during any of the permeabilization protocols.

To investigate whether the 'unreleasable' 10–20% of InsP_5 and InsP_6 might simply be associated with membranes, we incubated digitonin-permeabilized cells with high-specific-radioactivity InsP_6 . Less than 1% was bound by the permeabilized cells, indicating that the much larger proportion of InsP_5 and InsP_6 that was retained by cell pellets was not unspecifically bound to membranes.

Studies of cells subjected to glycol-induced osmotic lysis

Characteristics of the lysis process

This procedure for the selective permeabilization of the HL60-cell plasma membrane was adapted from a well-characterized method for the controlled lysis of human erythrocytes and isolation of sealed haemoglobin-free erythrocyte 'ghosts' (Billah et al., 1976, 1977). HL60 cells were incubated for 3–4 min in a sucrose-based medium supplemented with a high concentration of ethylene glycol (up to 9 M), to allow the extracellular polyol to equilibrate with the intracellular environment. The glycol-

loaded cells were then exposed to a large volume of glycol-free medium, which caused cell lysis as a result of the rapid osmotically driven influx of water across the plasma membrane. The most effective conditions for selective disruption of the plasma membrane involved a glycol loading period of 3 min and a similar lysis period. Increasing the glycol loading period beyond 5 min caused a much greater disruption of the β -galactosidase-containing granules, possibly because the longer loading period allowed more complete pre-loading of glycol into the granule compartment and hence greater granule lysis (results not shown). Lysis of the plasma membrane occurred within 1–2 min after dilution into glycol-free medium: prolonging the lysis period had little effect on the degree of permeabilization.

Transmission electron microscopy of the cells lysed at the higher glycol concentrations (> 7 M) showed swollen but intact nuclei and extensive damage to the plasma membrane and cytoplasmic loss (compare Figures 2a and 2b). The space within the nuclear envelope was also distended, but this distension was equally apparent in cells that were loaded with 9 M glycol but not diluted, indicating that it was caused by exposing cells to high concentrations of glycol rather than the stresses of osmotic lysis (Figure 2c). Transmission electron microscopy and phase-contrast microscopy indicated that the integrity of other intracellular organelles was largely maintained during lysis. Electron microscopy of cells lysed after loading with 5 M ethylene glycol showed them to be relatively intact (results not shown): phase-contrast examination indicated that these cells 'blebbed' their cytoplasm through relatively small holes in the plasma membrane.

Release of InsP_5 and InsP_6

Figure 3 shows the release of InsP_{5+6} (a mixed InsP_5 plus InsP_6 fraction eluted from a Dowex-1 column) and cellular markers from cells lysed after pre-loading with a range of ethylene glycol concentrations. At the lower glycol concentrations, HL60 cells released InsP_{5+6} and the cytosolic enzyme 6-PGDH approximately in parallel, with much of this release occurring at glycol concentrations that caused little release of the granule enzyme β -galactosidase. ATP and other inositol phosphates were released in parallel with InsP_5 and InsP_6 . Approximately 40% of the total InsP_{5+6} and of cytosolic markers were released from cells lysed after pre-loading with 5 M ethylene glycol, a concentration which caused lysis of only about half of the cells (results not shown) and caused only slight disruption of granules (Figure 3). Approximately 70% of the total cellular InsP_{5+6} was released at the highest glycol concentration, but at this concentration there was appreciable disruption of intracellular structures, as indicated by significant release of granule contents.

Studies with electroporated cells

Characteristics of pore formation by electroporation

If a cell (or a membrane-enclosed organelle) is exposed to an electric field, a voltage gradient is set up across its bounding membrane, and when the voltage becomes sufficient to cause membrane breakdown, two pores are formed in the plasma membrane at opposite poles of the cell. Selective breakdown only of the plasma membrane can readily be achieved, because much greater applied voltages are needed to form pores in the smaller intracellular organelles. Cells re-orient during intervals between multiple discharges, so each extra discharge usually adds an extra pair of permeable pores to the plasma membranes of the majority of cells in a suspension (Knight and Baker, 1982; Knight and

Figure 2 Electron micrographs of stained sections of HL60 cells

(a) Untreated cells. (b) Cells lysed after preloading with 9 M ethylene glycol. (c) Distension of intact nuclear envelope in cells loaded with 9 M ethylene glycol but not lysed. (d) Electroporated cells (1 kV/cm, one pulse).

Scrutton, 1986; Tsong, 1991). In contrast with treatment of cells with digitonin, which forms many pores that are freely permeable to macromolecules, and with osmotic lysis, which produces larger lesions, electroporation thus creates a few transmembrane channels that pass small molecules, but not macromolecules.

We exposed cells to pulses at a range of field strengths and then monitored their structural integrity and the release of intracellular components. Cells electroporated by a single 500 V/cm discharge were not distinguishable by electron microscopy from normal untreated cells (results not shown). Cells electroporated by a single 1 kV/cm pulse varied considerably in their staining patterns, particularly in the degree of mitochondrial condensation, but all of their membranes still appeared intact (Figure 2d). Examination of cells incubated with Trypan Blue, added at intervals after electroporation, indicated that the pores formed in cells treated with a 500 V/cm discharge re-sealed, but that those formed in cells exposed to 1 kV/cm were still open after 30 min (results not shown). The re-sealing of cells exposed to the smaller discharges occurred more rapidly at 37 °C than at lower temperatures, as previously observed by Kinoshita and Tsong (1977), but was not modified by the presence or absence of EGTA (as previously observed in adrenal medullary cells: Knight and Baker, 1982).

Release of marker molecules and of InsP_5 and InsP_6

The effect of varying the field strength on the release of markers,

inositol and inositol phosphates in the presence of 5 mM EGTA (included in the electroporation buffer to prevent a Ca^{2+} -dependent and electroporation-induced activation of phosphoinositidase C: J. A. Stuart and K. L. Anderson, unpublished work) is shown in Figure 4. Inositol, a small uncharged molecule (180 Da), was released after a single discharge at voltages of 375–500 V/cm, and more readily than the charged nucleotide ATP [molecular mass (free acid) 507 Da]. The highly charged species InsP_5 and InsP_6 were released by discharges of a similar intensity to that needed to release ATP (Figure 4, and see below for more detailed analysis). Over the voltage range that released most of the cellular ATP and inositol polyphosphates, there was very little release either of cytosolic 6-PGDH or of the granule enzyme β -galactosidase.

After a single 1 kV/cm pulse in the presence of 5 mM EGTA, ~70% of the free inositol and ~55–60% of total cellular InsP_{5+6} were rapidly released (Figure 4): the process was largely complete within 5 min (results not shown). After a 500 V/cm discharge, the process was slightly slower, and efflux of 60% of the free inositol was accompanied by release of only ~20% of InsP_5 and InsP_6 . The other, less polar, inositol phosphates were released from electroporated HL60 cells in a manner intermediate between that for Ins and InsP_{5+6} (Fig. 4, inset). The slower release of the larger, more highly charged, species suggests that the lower voltages created smaller pores that act, at least to a limited degree, as a 'molecular sieve'. Inositol phosphates were released at the same rates at temperatures from 4 °C to 37 °C,

Figure 3 Release of markers and inositol metabolites from HL60 cells that were osmotically lysed after preloading with the indicated concentrations of ethylene glycol

The main Figure shows the extent of release of the various markers (ATP; 6-PGDH; β -galactosidase) and of InsP_{5+6} , and the inset compares the release of InsP_{5+6} with the release of other inositol phosphates and of free inositol. The data shown are means $\pm$ S.E.M. of the results from three experiments.

making it unlikely that metabolic processes modulated this release or that factors such as [ATP] are involved (results not shown).

The medium used for these experiments was the 'extracellular' buffer described by Chu et al. (1987) for introducing DNA into cells. Similar results were obtained when this buffer was replaced by an 'intracellular' buffer containing high $[\text{K}^+]$ and low $[\text{Na}^+]$, and when the cell density in the electroporation cuvette was varied over the range 10^6 – 10^7 cells/ml (results not shown).

Figure 4 Release of markers and inositol metabolites from electroporated HL60 cells

Cells were exposed to a single discharge of the indicated voltage. The main Figure shows the extent of release of the various markers (ATP; 6-PGDH; β -galactosidase) and of InsP_{5+6} , and the inset compares the release of InsP_{5+6} with the release of other inositol phosphates and of free inositol. The data shown are means $\pm$ S.E.M. of the results from three experiments.

Effects of chelation of multivalent cations

Since InsP_5 and InsP_6 interact strongly with multivalent cations, and such ions potentiate their association with membranes (Poyner et al., 1993), we examined the effects of cation chelators on the release of InsP_{5+6} from electroporated HL60 cells. Omission of EGTA from the electroporation buffer had no effect on the release of inositol or of ATP, but decreased the release of InsP_{5+6} by one third, from $\sim 60\%$ to $\sim 40\%$ (Table 1). EDTA

Table 1 Effects of multiple electric discharges and of the presence of various cation chelators on the release of inositol polyphosphates from HL60 cells permeabilized by electroporation

Cells were electroporated as described in the Materials and methods section, with various numbers of 1 kV/cm discharges at various intervals, and the release of InsP_{5+6} was assessed. The results presented are means $\pm$ S.E.M. ($n \geq 3$); n.a. designates conditions that were not assayed.

		Percentage of total InsP_{5+6} released by electroporation			
Chelator present	No. of discharges ... Interval between discharges ...	1	2	2	3
			8 s	15 min	8 s
None		39.2 $\pm$ 2.1	36.7 $\pm$ 5.5	65.2 $\pm$ 1.5	42.4 $\pm$ 2.5
1 mM BAPTA		56.8 $\pm$ 1.2	n.a.	n.a.	81.2 $\pm$ 12.7
5 mM EGTA		56.7 $\pm$ 2.8	73.9 $\pm$ 9.4	70.6 $\pm$ 0.6	88.7 $\pm$ 7.6
5 mM EDTA		56.8 $\pm$ 0.8	66.8 $\pm$ 0.7	n.a.	81.1 $\pm$ 2.8
1 mM desferrioxamine		44.1 $\pm$ 3.7	59.3 $\pm$ 2.3	n.a.	73.4 $\pm$ 3.9
1 mM desferrioxamine plus 5 mM EGTA		57.0 $\pm$ 1.5	n.a.	n.a.	n.a.

Figure 5 Release of markers and of inositol metabolites from HL60 cells permeabilized by exposure to staphylococcal α -toxin

(a) Release of ATP and intracellular enzymes in cells treated with 1 μ g/ml toxin. (b) Release of inositol metabolites during prolonged exposure to 10 μ g/ml toxin.

and 1,2-bis-(2-aminophenoxy)ethanetetra-acetic acid (BAPTA, a chelator with a high selectivity for Ca^{2+} over Mg^{2+} ; Tsien, 1980) had the same effect on the release of InsP_5 and InsP_6 as did EGTA

(Table 1). Desferrioxamine, an agent from *Streptomyces pylosus* which chelates both Fe^{3+} and Al^{3+} , slightly enhanced InsP_{5+6} release, but was less effective than EDTA, EGTA or BAPTA in maximizing the release of InsP_5 and InsP_6 .

Effects of multiple discharges

Larger releases of InsP_5 and InsP_6 were achieved when cells were subjected to more than one discharge (see Table 1). Three 1 kV/cm discharges, ~ 8 s apart, released 80–90% of the total InsP_{5+6} in the presence of BAPTA, EGTA or EDTA, and about 75% in the presence of desferrioxamine, but only about 40% in the absence of any chelator. However, two 1 kV/cm pulses 15 min apart provoked 65% release of InsP_{5+6} even in the absence of chelator. Two discharges of 500 V/cm, 15 min apart, were also more effective than a single discharge in causing the release of inositol metabolites. Release of inositol was 20% greater than with a single pulse at the same voltage, that of InsP was 40% higher, and the release of InsP_2 , InsP_3 , InsP_4 and InsP_{5+6} was approximately doubled. One possible interpretation of these observations is that these molecules, particularly the most highly phosphorylated species, have only restricted mobility inside cells, and that this delays their access to the small number of pores formed during electroporation.

Treatment with *Staphylococcus aureus* α -toxin

The α -toxin of *Staphylococcus aureus* is a secreted protein (~ 33 kDa) that intercalates into, and forms pores in, many membrane structures that include a fluid lipid bilayer (Tomita et al., 1992). These pores have a molecular-mass cut-off of ~ 1 kDa (Füssle et al., 1981).

Treatment of HL60 cells with 1 μ g/ml α -toxin caused release of $\sim 90\%$ of the cellular ATP within 3–10 min, after which the released ATP was rapidly degraded. Essentially no 6-PGDH or β -galactosidase was released by this treatment (Figure 5a). Over the same period, there was release of $\sim 70\%$ of free inositol. Inositol phosphates were released more slowly than inositol or ATP, with the less heavily phosphorylated species released more quickly than InsP_{5+6} (results not shown). We therefore undertook studies with a higher α -toxin concentration (10 μ g/ml) that produced maximal ATP release in 2–3 min, and these incubations were extended for longer periods. Under these conditions, up to 90% of free inositol and 50–70% of InsP_4 , InsP_3 and InsP_2 were released within 2 h (Figure 5b), but there was still less than 20% release of InsP_{5+6} even after 5 h (results not shown).

DISCUSSION

In this study of the intracellular localization of inositol polyphosphates, we elected to compare the release of inositol phosphates from cells rendered permeable by several fundamentally different methods of membrane permeabilization, so as to overcome difficulties with interpretation that might arise from results obtained by a single method. Although the four methods are all directed at permeabilization of the plasma membrane, each achieves its selectivity by a different mechanism. Digitonin particularly targets the plasma membrane because of its high cholesterol content, whereas electroporation and osmotic lysis are similarly selective because these techniques need much smaller imposed forces (electric field and osmotic gradient, respectively) to permeabilize the large-radius plasma membrane than to permeabilize small membrane-enclosed structures such as intracellular organelles. The pores formed by *Staphylococcus aureus*

α -toxin, which readily permeabilizes membranes with fluid lipid phases (Tomita et al., 1992), are so small (molecular-mass cut-off ~ 1 kDa: Füssle et al., 1981) that the toxin monomers (~ 33 kDa) cannot gain access to intracellular membranes.

Evidence that InsP_5 and InsP_6 are primarily present in the cytosol compartment

Each of these methods gave results which indicate that most of the inositol phosphates in HL60 promyeloid cells, including the relatively abundant polyphosphates InsP_5 and InsP_6 , are released by methods that permeabilize the plasma membrane but leave the intracellular lysosomes (azurophil granules) largely intact. We chose to monitor the integrity of the latter organelles, whose membranes are relatively cholesterol-rich by intracellular standards, because these lysosomal vesicles are likely to be among the most labile of the intracellular structures. Confirmation that this was a reasonable experimental design came from the complementary evidence for maintenance of the integrity of other organelles provided by phase-contrast and electron microscopy.

The results obtained by the different permeabilization methods were, however, not identical. Digitonin, electroporation by multiple discharges and α -toxin treatment all caused quantitative release of free inositol and of ATP, and the first two methods were capable of releasing up to 85–90% of total InsP_{5+6} . However, even a prolonged treatment with a high concentration of staphylococcal α -toxin only caused release of $\sim 20\%$ of the InsP_{5+6} . The most straightforward interpretation of this result is that the pores formed by the toxin act as a molecular sieve that allows the graded release of small, less highly charged, molecules ahead of larger and more highly charged species. Ali et al. (1993) have reported that InsP_6 released from cells displays an effective molecular mass of ~ 9 kDa, a size that would not readily pass through the pores formed by α -toxin. A previous study (Bird et al., 1991), which used this toxin to investigate whether $\text{Ins}(1,4,5)\text{P}_3$ might be compartmentalized in AR4-2J cells, observed a higher degree of release of InsP_3 than in our study, but found no evidence for either size selectivity of pores or sequestration of InsP_3 .

Even though the electroporation studies gave the clearest evidence for approximately parallel release of inositol polyphosphates, free inositol and ATP, this only occurred when bivalent-cation chelators were included in the electroporation medium: much less InsP_5 and InsP_6 was released by the permeabilized cells in their absence (Table 1). EDTA, EGTA and BAPTA facilitated this release equally effectively, and much better than desferrioxamine, so it is likely that Ca^{2+} was a major ion responsible for this retention of a proportion of the inositol polyphosphates after electroporation. In principle, this retention might have been caused either by a pre-existing Ca^{2+} -facilitated binding of InsP_5 and/or InsP_6 in the cells or by traces of Ca^{2+} (and other ions?) which leaked into the cells during electroporation. However, Chelex treatment of the electroporation buffer before use did not facilitate the release of InsP_{5+6} in the absence of chelators, so it seems unlikely that these effects were caused primarily by ions contaminating the experimental media (results not shown). Luttrell (1993) has shown that InsP_6 binds Ca^{2+} at normal intracellular concentrations, and Poyner et al. (1993) found that micromolar quantities of multivalent cations cause significant amounts of InsP_6 to associate with membranes: such interactions within the cells could have been responsible for the cation modulation of InsP_5 and InsP_6 release from electroporated HL60 cells.

The physical state of inositol polyphosphates in the cytosol compartment

A number of proteins exposed to the cytosol compartment bind InsP_5 and/or InsP_6 with high affinity [e.g. the adaptin AP-2, haemoglobin in avian erythrocytes, arrestin and $\text{Ins}(1,3,4)\text{P}_3$ 5/6-kinase], as do a number of other less well defined, but fairly selective, intracellular binding sites, particularly on membranes (see the Introduction for details). Inositol polyphosphate 3-phosphatase also binds InsP_5 and InsP_6 tightly, and a recent report appears to have localized hepatic $\text{Ins}(1,3,4,5)\text{P}_4/\text{InsP}_5/\text{InsP}_6$ phosphatase exclusively within the cisternae of the endoplasmic reticulum (Ali et al., 1993), where it would appear to be incapable of metabolizing cytosolic inositol polyphosphates. However, another study places an ' $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase' activity at the inner surface of the erythrocyte plasma membrane (Estrada-Garcia et al., 1991), so offering a clear suggestion that cytosolic InsP_5 and InsP_6 are likely to be accessible to at least one form of inositol polyphosphate 3-phosphatase.

It seems unlikely that any of these potential inositol polyphosphate-binding sites are present in cells in quantities sufficient to form complexes with, and thus keep out of solution, a large part of the cellular complement of inositol polyphosphates, whose concentrations range from 1 mM in *Dictyostelium discoideum*, through 50–100 μM in HL60 cells, to considerably less in some other cells. Moreover, changes in cell state such as differentiation or transit through the cell cycle are sometimes associated with substantial changes in inositol polyphosphate levels (see the Introduction), and there is no evidence for parallel changes in the amounts of the proteins that bind inositol polyphosphates.

These are a number of possible ways, such as chelation to cations, by which a cell might limit the free ionized concentrations of InsP_5 and InsP_6 in the cytosol. For example, the InsP_6 which is used as a phosphorus store in plant seeds is deposited as a mixed salt of several cations, including K^+ , Ca^{2+} and Mg^{2+} . The resulting 'phytins' are typically deposited in membrane-bound storage vesicles known as protein bodies. It has been postulated that the synthesis of this InsP_6 occurs in the cytoplasmic compartment, perhaps at the endoplasmic reticulum, and that phytin is then transported into the protein bodies (Raboy, 1990). Given the high charge of InsP_5 and InsP_6 , interactions with other ions are also possible. Interactions of InsP_5 and InsP_6 with Ca^{2+} and Zn^{2+} were investigated by Xu et al. (1992) in an attempt to clarify the well-recognized adverse effect of a combined high dietary level of calcium and InsP_6 on dietary zinc availability: at pH values typical of the gastrointestinal tract, Ca^{2+} potentiated the formation of insoluble $\text{Ca-InsP}_6\text{-Zn}$ complexes. When the $\text{InsP}_6/\text{Zn}^{2+}$ ratio was 1, Zn solubility increased with increasing $\text{Ca}^{2+}/\text{Zn}^{2+}$ ratios, suggesting that Ca^{2+} competed with Zn^{2+} for binding sites on the InsP_6 , as proposed by Graf and Eaton (1993).

Functional implications

An obvious implication of the localization of InsP_5 and InsP_6 largely or solely in the cytosol compartment is that these inositol polyphosphates are unlikely to be secreted as intercellular messengers in the manner envisaged, for example, by Vallejo et al. (1987), Barraco et al. (1989) and Sortino et al. (1990). However, it remains possible that a small proportion of the total cellular inositol polyphosphate complement could be packaged in vesicles for this purpose, since our experiments with HL60 cells were not capable of assigning 100% of cellular InsP_5 and InsP_6 to the cytosol compartment, and it is also conceivable that cells that are more specialized for the secretion of signalling

agents might commit more of their InsP_5 and InsP_6 to such compartments.

The results suggesting a cytosolic location for InsP_5 and InsP_6 naturally lead to an expectation that under intracellular conditions a substantial proportion of the cytosolic InsP_5 and InsP_6 will probably exist as insoluble complexes with bivalent cations, most notably Mg^{2+} at the concentrations which prevail in the cytosol. This poses the further question of how cells effectively regulate the concentrations of multivalent cations, particularly the essential regulatory Ca^{2+} , in this compartment in the presence of relatively large quantities of such potent intracellular chelators.

What these compounds do in the cytosol compartment of a wide variety of eukaryote cells, and why their levels should vary in a manner linked to changes in cell state, remain largely a mystery. Their interconversion with their pyrophosphorylated derivatives, which may serve as high-energy phosphate donors, might be one of their important functions (see the Introduction). However, the fact that relatively little of the cellular InsP_5 and InsP_6 appears to be modified in this way, at any particular moment, makes it unlikely that this is the sole rationale for the presence in cells of relatively high concentrations of InsP_5 and InsP_6 . Similarly, these compounds seem to be present in great excess over the amounts needed for most of their reported interactions with relatively rare cell proteins.

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