Biochem. J. (1988) 255, 131–137 (Printed in Great Britain)

1D-*myo*-Inositol 1,4,5-trisphosphate dephosphorylation by rat enterocytes involves an intracellular 5-phosphatase and non-specific phosphatase activity at the cell surface

Carlos RUBIERA,* Gloria VELASCO,* Robert H. MICHELL,† Pedro S. LAZO* and Stephen B. SHEARS†‡
*Departamento de Biologia Funcional, Universidad de Oviedo, 33071 Oviedo, Spain, and †Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

We studied the dephosphorylation of Ins(1,4,5)P_3 (inositol 1,4,5-trisphosphate) by permeabilized rat intestinal epithelial cells incubated in a medium resembling intracellular ionic strength and pH. Saponin-permeabilized cells rapidly dephosphorylated Ins(1,4,5)P_3 to a mixture of three InsP_2 (inositol bisphosphate) isomers, namely Ins(1,4)P_2, Ins(1,5)P_2 and Ins(4,5)P_2. These products were identified by h.p.l.c. analysis after dephosphorylation of both ^{3}H- and ^{32}P-labelled Ins(1,4,5)P_3. Ins(1,4)P_2 accumulated to about half of the concentration attained by Ins(1,5)P_2 and Ins(4,5)P_2. Ins(1,4,5)P_3 dephosphorylation was inhibited, by up to 75%, by 10 mM-glucose 6-phosphate. In these conditions Ins(1,4)P_2 became the predominant product, indicating that glucose 6-phosphate inhibited non-specific dephosphorylation of Ins(1,4,5)P_3, at least at the 1- and 4-phosphate groups. Ins(1,4)P_2 was further dephosphorylated, and the major Ins$P$ (inositol monophosphate) product was Ins4P. Most of the glucose 6-phosphate-inhibitable Ins(1,4,5)P_3 phosphatase activity was exposed on the cell surface. The glucose 6-phosphate-insensitive Ins(1,4,5)P_3 5-phosphatase activity was not detected until the cells were permeabilized with saponin. This intracellular 5-phosphatase activity was: (i) predominantly associated with the particulate portion of the cell; (ii) strongly inhibited by 10 mM-2,3-bisphosphoglycerate; (iii) insensitive to 50 mM-Li^+. Therefore the Ins(1,4,5)P_3 5-phosphatase activity in enterocytes appears similar to the 5-phosphatase that has been characterized in a number of cell types.

INTRODUCTION

In a variety of cells, receptor-mediated hydrolysis of PtdIns(4,5)P_2 leads to the production of Ins(1,4,5)P_3 (reviewed by Berridge, 1984; Downes & Michell, 1985), which releases intracellular Ca^{2+} pools (reviewed by Berridge & Irvine, 1984). Two routes of Ins(1,4,5)P_3 metabolism have been demonstrated. It is phosphorylated to Ins(1,3,4,5)P_4 (Batty *et al.*, 1985; Hawkins *et al.*, 1986; Irvine *et al.*, 1986*a*), which may potentiate the Ca^{2+}-mobilizing effects of Ins(1,4,5)P_3 by facilitating Ca^{2+} influx across the plasma membrane (Irvine & Moor, 1986; Morris *et al.*, 1987). Alternatively, Ins(1,4,5)P_3 is dephosphorylated to Ins(1,4)P_2 in, for example, erythrocytes (Downes *et al.*, 1982), liver (Shears *et al.*, 1987*a*), pancreatic islets (Rana *et al.*, 1986), brain (Erneux *et al.*, 1986) and platelets (Connolly *et al.*, 1985). Ins(1,4)P_2 does not mobilize Ca^{2+} (for references see Berridge & Irvine, 1984). The Ins(1,4,5)P_3 5-phosphatase removes Ins(1,4,5)P_3 from the cell, and limits the formation of Ins(1,3,4,5)P_4, thereby contributing substantially to the termination of Ca^{2+} mobilization.

Several groups have shown that enterocytes contain an Ins(1,4,5)P_3-releasable Ca^{2+} pool (Velasco *et al.*, 1986*a*; Ilundain *et al.*, 1987; Van Corven *et al.*, 1987), so it was decided to study Ins(1,4,5)P_3 metabolism by these cells. Rao & Ramakrishnan (1985) are the only workers who have published data on inositol trisphosphatase activity in intestinal mucosa. The substrate for their study was a mixture of uncharacterized inositol trisphosphate isomers produced by alkaline hydrolysis of phytic acid (see Phillippy *et al.*, 1987), so the results give no insight into the pathway of Ins(1,4,5)P_3 dephosphorylation. We have studied the metabolism of genuine Ins(1,4,5)P_3. Moreover, in intestinal cells, it is important to distinguish between substrate-specific phosphatases and the array of unspecific phosphatases involved in nutrient digestion (Moore & Veum, 1983; Rao & Ramakrishnan, 1985).

MATERIALS AND METHODS

Preparation of isolated intestinal epithelial cells

Intestinal cells were isolated from the intestine of a male rat (approx 200 g) by treatment with hyaluronidase (Velasco *et al.*, 1986*b*). The separated cells were centrifuged at 1000 *g* for 3 min. The supernatant was discarded and the cells were washed twice by suspension in 50 ml of ice-cold medium containing 120 mM-KCl, 10 mM-NaCl and 10 mM-Hepes (adjusted to pH 7.0 with KOH), followed by re-centrifugation at 0–4 °C. The cells were stored on ice, and used within 20 min of preparation,

Abbreviations used: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins, Ins$P$, Ins1P, Ins4P, InsP_2, Ins(1,4)P_2, Ins(1,5)P_2, Ins(4,5)P_2 and Ins(1,4,5)P_3 are *myo*-inositol and its mono-, bis-, and tris-phosphates (locants designated where appropriate with reference to D-*myo*-inositol 1-phosphate).

‡ To whom reprint requests should be sent. Present address: Laboratory of Cellular and Molecular Pharmacology, National Institute for Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

except when they were divided into soluble and particulate fractions. In the latter case, the cells were homogenized (1000 rev./min, 20 up-and-down strokes) in 11 ml of ice-cold medium containing 120 mM-KCl, 10 mM-NaCl and 10 mM-Hepes (pH 7.0 with KOH), and then centrifuged at 100000 *g* for 1 h at 0–4 °C. The supernatant and particulate fractions were each diluted with homogenization buffer to the concentration of the original homogenate. The supernatants contained > 84% of total cellular lactate dehydrogenase (results not shown), indicating that homogenization almost completely released cytosolic enzymes.

Determination of protein concentration

Protein concentration was determined by Bradford's (1976) assay.

Assay of inositol phosphate metabolism

The appropriate inositol phosphate was added to medium containing 120 mM-KCl, 10 mM-NaCl, 1 mM-EGTA, 4 mM-$MgSO_4$, 0.3 mM-$CaCl_2$, 20 mM-Hepes (pH 7.0 with KOH) and, unless otherwise indicated, 0.2 mg of saponin/ml. This medium contains 4 mM free Mg^{2+} and 0.2 μM free Ca^{2+} (see Burgess *et al.*, 1983). Incubations (0.5 ml at 37 °C) were initiated with either 0.025 ml of a suspension of intestinal cells (see above), or 0.2 ml portions of packed human erythrocyte ghosts. The latter were prepared by hypo-osmotic lysis in 20 mM-Tris (pH 7.5 at 25 °C with HCl) as described by Downes *et al.* (1981). The final membrane pellets were stored at −20 °C for < 1 month before use.

After appropriate times, the incubations were quenched with 0.2 ml of 1.7 M-$HClO_4$ (plus 1 mg of phytic acid/ml; see Wreggett *et al.*, 1987). The precipitated protein was removed by centrifugation at 12000 *g* for 3 min. Samples (0.65 ml) of the acid-quenched supernatants that were to be analysed by h.p.l.c. were first neutralized with 1 ml of 1,1,2-trichlorotrifluoroethane/tri-n-octylamine (1:1, v/v) [Sharpes & McCarl (1982) as modified by Shears *et al.* (1987*a*)]. The recovery of ^{32}P-labelled inositol phosphate standards through the neutralization procedures was 81 ± 2% (n = 6). H.p.l.c. of the neutralized samples was performed with a 25 cm × 0.46 cm main column and a 5 cm × 0.46 cm guard column, both containing Whatman Partisil 10-SAX. Ins(1,4,5)P_3 metabolites were separated with a gradient of ammonium formate (pH 3.7 with H_3PO_4) (Shears *et al.*, 1987*a*). Ins(1,4)P_2 metabolites were separated by using a gradient of ammonium phosphate (pH 4.6 with H_3PO_4) (Shears *et al.*, 1987*c*).

Some acid-quenched supernatants (0.65 ml) were neutralized at 0–4 °C with approx. 0.3 ml of a mixture of 1.2 M-KOH, 0.06 M-EDTA and 0.75 M-Hepes. After 30 min the $KClO_4$ precipitate was removed by centrifugation. The resulting supernatants were loaded, in 10 ml of water, on to gravity-fed ion-exchange columns (1 ml of Bio-Rad AG 1-X8; 200–400 mesh; formate form) and the Ins$P$, InsP_2 and InsP_3 fractions were separately eluted (Shears *et al.*, 1987*a*).

Preparation of [4,5-^{32}P]Ins(1,4,5)P_3

Human blood (7–14 days after donation) was obtained from the local Blood Transfusion Centre. The erythrocytes were washed three times in 188 mM-Hepes (pH 7.2 with NaOH), and then 6 ml of packed cells was incubated at 37 °C in 10 ml of buffer containing 188 mM-Hepes (pH 7.2 with NaOH), 5 mM-sodium pyruvate, 2 mM-calcium gluconate, 1.18 mM-magnesium gluconate, 1 mM-inosine, 1 mM-adenine, 10 mM-glucose, 0.7% bovine serum albumin and 4 mCi of [^{32}P]P_i (the adenine and calcium gluconate have to be dissolved individually). This buffer is similar to that developed by King *et al.* (1987) to maximize the rate of [^{32}P]P_i incorporation into PtdIns(4,5)P_2. To ensure unequal labelling of the 4- and 5-phosphates, the erythrocytes were incubated for 4 h (see King *et al.*, 1987).

After labelling, the erythrocytes were lysed at 0–4 °C in 100 ml of 20 mM-Tris/1 mM-EDTA (pH 7.5 with HCl at 25 °C). The membranes were centrifuged (16000 *g* for 10 min) and washed several times by resuspension and re-centrifugation in the same medium. The lipids were extracted (King *et al.*, 1987), dried, and deacylated in 2 ml of a mixture of 30% methylamine (in methanol)/water/n-butanol/methanol (44:44:9:3, by vol.) (Clarke & Dawson, 1981). The resultant glycerophosphodiesters were extracted (Clarke & Dawson, 1981) and treated with 1.6 ml of 10 mM-sodium periodate at room temperature in the dark for 20 min (Brown & Stewart, 1966; Irvine *et al.*, 1986*b*). Then 20 μl of ethylene glycol was added, followed by 200 μl of aq. 1% 1,1-dimethylhydrazine (pH 4.0 with formic acid; Brown & Stewart, 1966) under N_2 (Irvine *et al.*, 1986*b*). After 4 h at room temperature the sample was diluted to 20 ml with water and applied to a 0.5 ml cation-exchange column (Bio-Rad AG 1-X8; 200–400 mesh; formate form). A red contaminant (see Brown & Stewart, 1966) remained in the eluate. P_i and InsP_2 were eluted with 10 ml of 0.4 M-ammonium formate/0.1 M-formic acid; then Ins(1,4,5)P_3 was eluted with 10 ml of 0.8 M-ammonium formate/0.1 M-formic acid. The latter eluate was diluted 3-fold and re-applied to another column, and the elution procedure was repeated. Finally, the Ins(1,4,5)P_3 was desalted by treatment with 6 ml of Bio-Rad cation-exchange resin (200–400 mesh, H^+ form), followed by freeze-drying (Downes *et al.*, 1982). Between 1 and 4 μCi of [4,5-^{32}P]Ins(1,4,5)P_3 was produced, 98% of which was eluted as a single peak during h.p.l.c.

Materials

Glucose 6-phosphate and 2,3-bisphosphoglycerate were purchased from Sigma. 1,1-Dimethylhydrazine was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. Methylamine was purchased from BDH, Poole, Dorset, U.K. All radiochemicals were purchased from Amersham International. The [^{3}H]Ins(1,4,5)P_3 was routinely analysed by h.p.l.c., and some batches, particularly those stored for over 2 months, contained up to 20% of an unknown ^{3}H-labelled compound that co-eluted with Ins(4,5)P_2. Thus 1.5 μCi samples of the [^{3}H]Ins(1,4,5)P_3 were purified by h.p.l.c. (see above), and the fractions containing genuine Ins(1,4,5)P_3 were combined, neutralized and applied to an ion-exchange column as described by Shears *et al.* (1987*c*). The Ins(1,4,5)P_3 was eluted, desalted and freeze-dried as described above.

RESULTS

Ins(1,4,5)P_3 phosphatase activity in permeabilized intestinal cells

Saponin-permeabilized intestinal cells (final concn. approx. 5 μg of protein/ml) were incubated with approx.

Table 1. Dephosphorylation of [^{3}H]- and [4,5-^{32}P]-Ins(1,4,5)P_3 by rat intestinal cells and human erythrocyte ghosts

Incubations were performed as described in the Materials and methods section for either 10 min (with 25 μl of a stock suspension of intestinal cells) or 20 min (with 200 μl of erythrocyte ghosts). At zero time each incubation ($n = 3$) contained 13245 ± 846 d.p.m. of [^{3}H]Ins(1,4,5)P_3 and 6097 ± 308 d.p.m. of [4,5-^{32}P]Ins(1,4,5)P_3 (^{32}P/^{3}H ratio = 0.45). The InsP_2 products of the reactions were assessed on gravity-fed columns as described in the Materials and methods section. Data (means ± S.E.M.) were each obtained from quadruplicate determinations on a single cell preparation. Two additional cell preparations gave similar results (n.c. = not calculated). For those incubations without glucose 6-phosphate (Glc6P), the final concentration of intestinal cells (4 μg of protein/ml) was more dilute than in those incubations with Glc6P (17 μg of protein/ml).

Cell type	Glc6P (mM)	Fraction	Inositol phosphate concn. (d.p.m./fraction) ^{3}H	Inositol phosphate concn. (d.p.m./fraction) ^{32}P	^{32}P/^{3}H ratio	[^{3}H]InsP_3 metabolized (d.p.m./10 min per μg of protein)
Erythrocyte	0	InsP_2	3088 ± 77	396 ± 14	0.13	
Intestine	0	InsP_2	1520 ± 99	527 ± 42	0.35	
Erythrocyte	0	InsP_3	3428 ± 113	1537 ± 308	0.45	n.c.
Intestine	0	InsP_3	6022 ± 418	2846 ± 201	0.47	1806
Intestine	5	InsP_2	2770 ± 135	390 ± 25	0.14	
Intestine	10	InsP_2	2836 ± 159	338 ± 26	0.12	
Intestine	5	InsP_3	4648 ± 694	2014 ± 283	0.43	506
Intestine	10	InsP_3	5541 ± 295	2562 ± 151	0.46	453

8 nM-[^{3}H]Ins(1,4,5)P_3 in a medium of approximately physiological ionic strengh and pH (see the Materials and methods section). In these incubations Ins(1,4,5)P_3 was rapidly dephosphorylated ($t_{\frac{1}{2}} \simeq 5$ min), and the rate of metabolism followed first-order kinetics (results not shown).

By studying the metabolism of a mixture of [^{3}H]-Ins(1,4,5)P_3 and [4,5-^{32}P]Ins(1,4,5)P_3, the products can be identified (see Shears *et al.*, 1987*a*,*b*), provided that the distribution of ^{32}P between the 4- and 5-phosphate groups is known. The latter information was obtained by incubating both substrates with erythrocyte ghosts (Table 1), which remove only the 5-phosphate from Ins(1,4,5)P_3 (Downes *et al.*, 1982). The ^{32}P/^{3}H ratio of the Ins(1,4)P_2 product (0.13; Table 1), was 29% of the corresponding ratio for the Ins(1,4,5)P_3 substrate (0.45; Table 1). Thus in this batch of [4,5-^{32}P]Ins(1,4,5)P_3 the 4- and 5-phosphates were estimated to be labelled in the ratio 29:71. The ^{32}P/^{3}H ratios of the other two possible InsP_2 products, Ins(1,5)P_2 and Ins(4,5)P_2, would be respectively 71% and 100% of the ^{32}P/^{3}H ratio of the Ins(1,4,5)P_3.

Permeabilized intestinal cells were incubated with the same mixture of [4,5-^{32}P]Ins(1,4,5)P_3 and [^{3}H]-Ins(1,4,5)P_3, and the metabolites were analysed on small gravity-fed columns. The InsP_2 fraction had a ^{32}P/^{3}H ratio that was 78% of the corresponding ratio for the Ins(1,4,5)P_3 (0.35; Table 1). Thus the 5-phosphate was retained by some of the InsP_2 products, which must therefore include Ins(1,5)P_2 and/or Ins(4,5)P_2, but the proportions formed could not be determined from the data in Table 1.

Intestinal cells have substantial non-specific phosphatase activities (e.g. Moore & Veum, 1983; Rao & Ramakrishnan, 1985). We attempted selectively to inhibit some of these enzyme activities, to determine if they contributed to Ins(1,4,5)P_3 dephosphorylation. Glucose 6-phosphate at 5 mM does not affect Ins(1,4,5)P_3 5-phosphatase activity in pancreatic islets (Rana *et al.*, 1986). Ins(1,4,5)P_3 5-phosphatase in erythrocyte ghosts was also unaffected by up to 10 mM-glucose 6-phosphate, although higher concentrations were inhibitory, almost completely so at 100 mM (Fig. 1). In contrast, 10 mM-glucose 6-phosphate inhibited Ins(1,4,5)P_3 dephosphorylation in intestinal cells by 75% (Table 1); the remaining 25% of phosphatase activity produced an InsP_2 with a ^{32}P/^{3}H ratio (0.12; Table 1) that indicated that Ins(1,4)P_2 was the only product. Thus, in enterocytes incubated with 10 mM-glucose 6-phosphate, Ins(1,4,5)P_3 5-phosphatase activity may be observed, yet there is inhibition of other enzymes that dephosphorylate Ins(1,4,5)P_3 to Ins(1,5)P_2 and/or Ins(4,5)P_2. When the enterocyte incubations contained 5 mM-glucose 6-phosphate instead of 10 mM, the ^{32}P/^{3}H ratio of the InsP_2 fractions was increased by 19 ± 5% ($n = 4$),

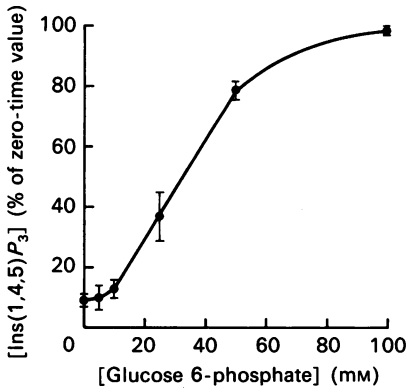

Fig. 1. Effect of glucose 6-phosphate on Ins(1,4,5)P_3 5-phosphatase activity in human erythrocyte ghosts

Erythrocyte ghosts were incubated as described in the Materials and methods section for 15 min with approx. 10000 d.p.m. of [^{3}H]Ins(1,4,5)P_3 and the indicated concentration of glucose 6-phosphate. The amount of substrate metabolized was then determined (see the Materials and methods section). Data are means ± S.E.M. obtained from three preparations of ghosts.

showing that Ins(1,4,5)P_3 1- and 4-phosphatase activities were then less effectively inhibited.

Products of Ins(1,4,5)P_3 dephosphorylation and the effect of glucose 6-phosphate

[4,5-^{32}P]Ins(1,4,5)P_3 and [^{3}H]Ins(1,4,5)P_3 were incubated with intestinal cells, and the products were analysed by h.p.l.c. In the absence of glucose 6-phosphate, three InsP_2 products were detected. The latest eluted of these (18 min; Fig. 2*a*) had a ^{32}P/^{3}H ratio that was 108±4.5% ($n = 8$) that of the Ins(1,4,5)P_3. Thus this InsP_2 had retained the 4- and 5-phosphates, and is identified as Ins(4,5)P_2. The ^{32}P/^{3}H ratios of the maxima of the overlapping peaks eluted at 16 and 16.4 min (Fig. 2*a*) were respectively 25% and 82% of the ^{32}P/^{3}H ratio for the InsP_3. In this preparation of [4,5-^{32}P]Ins(1,4,5)P_3, the 4- and 5-phosphates were ^{32}P-labelled in the ratio 23±2.4/77±2.4 ($n = 3$). Thus the peaks eluted at 16 min and 16.4 min were Ins(1,4)P_2 and Ins(1,5)P_2 respectively. In most additional experiments, these two peaks were not resolved sufficiently to determine their individual ^{32}P/^{3}H ratios. Nevertheless, the net ^{32}P/^{3}H ratio of the two combined peaks could be used to estimate the relative proportions of Ins(1,4)P_2 and Ins(1,5)P_2 therein. The Ins$P$ isomer(s) (Fig. 2*a*) were not resolved by this h.p.l.c. system.

In incubations containing glucose 6-phosphate, the cell concentration was increased 5-fold to facilitate the accumulation of substantial amounts of products, despite the resultant inhibition of Ins(1,4,5)P_3 metabolism (see Table 1). A single InsP_2 peak was eluted at 16 min (Fig. 2*b*), and its ^{32}P/^{3}H ratio [29±0.02% that of the

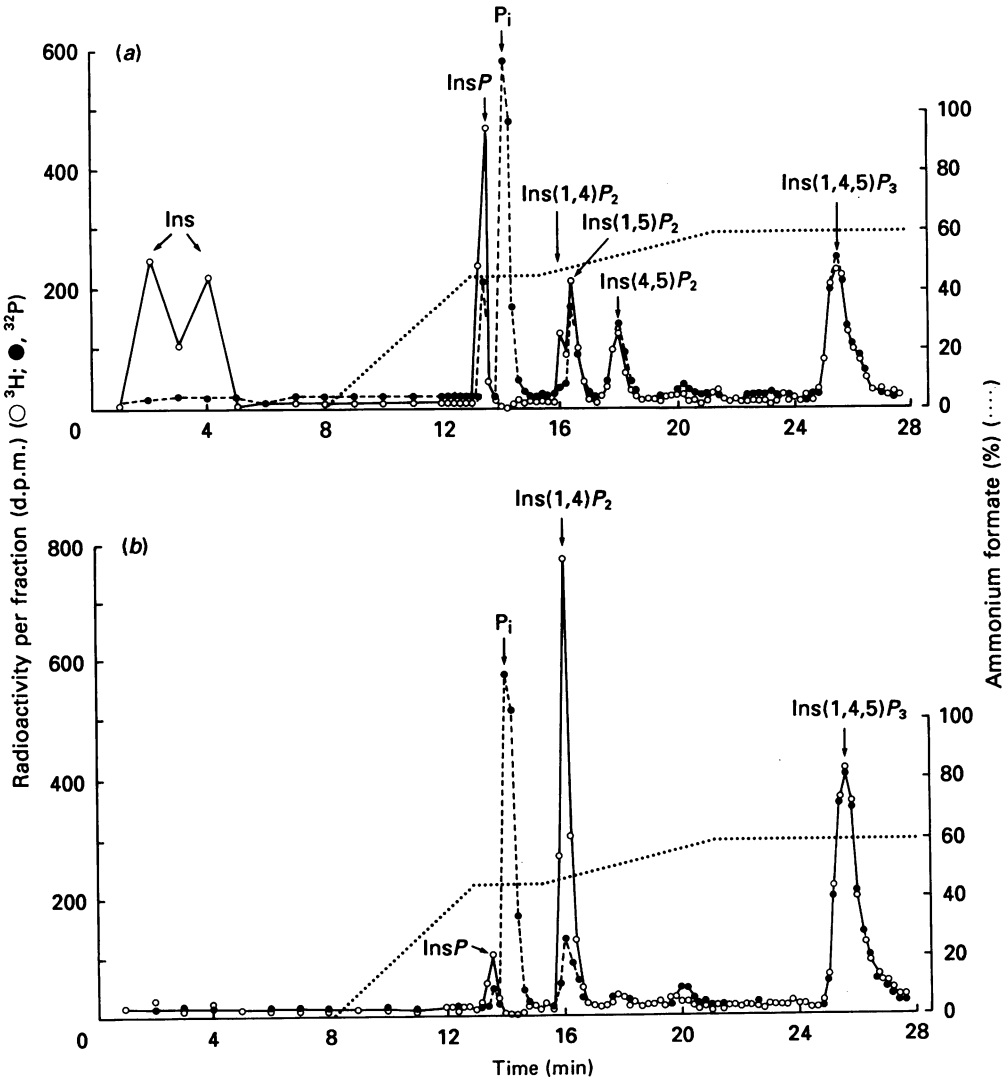

Fig. 2. H.p.l.c. analysis of the metabolism of [4,5-^{32}P]Ins(1,4,5)P_3 and [^{3}H]Ins(1,4,5)P_3, by rat intestinal cells incubated in the presence and absence of glucose 6-phosphate

Intestinal cells were incubated as described in the Materials and methods section for 10 min with approx. 4000 d.p.m. of [4,5-^{32}P]Ins(1,4,5)P_3 and approx. 4000 d.p.m. of [^{3}H]Ins(1,4,5)P_3. The final concentration of cells was either 7 μg of protein/ml (*a*; glucose 6-phosphate concn. = 0) or 34 μg of protein/ml (*b*; glucose 6-phosphate concn. = 10 mM). The incubations were quenched, and the h.p.l.c. analyses were performed, as described in the Materials and methods section, by using a gradient of ammonium formate (pH 3.7 with H_3PO_4): ●, ^{32}P; ○ ^{3}H. Data are from single experiments. Similar results were obtained in three further experiments, except that there was less resolution of the peaks in Fig. 2(*a*) that were eluted at 16 and 16.4 min (see the text). The two inositol peaks arise from the sample being injected on to the h.p.l.c. as two batches.

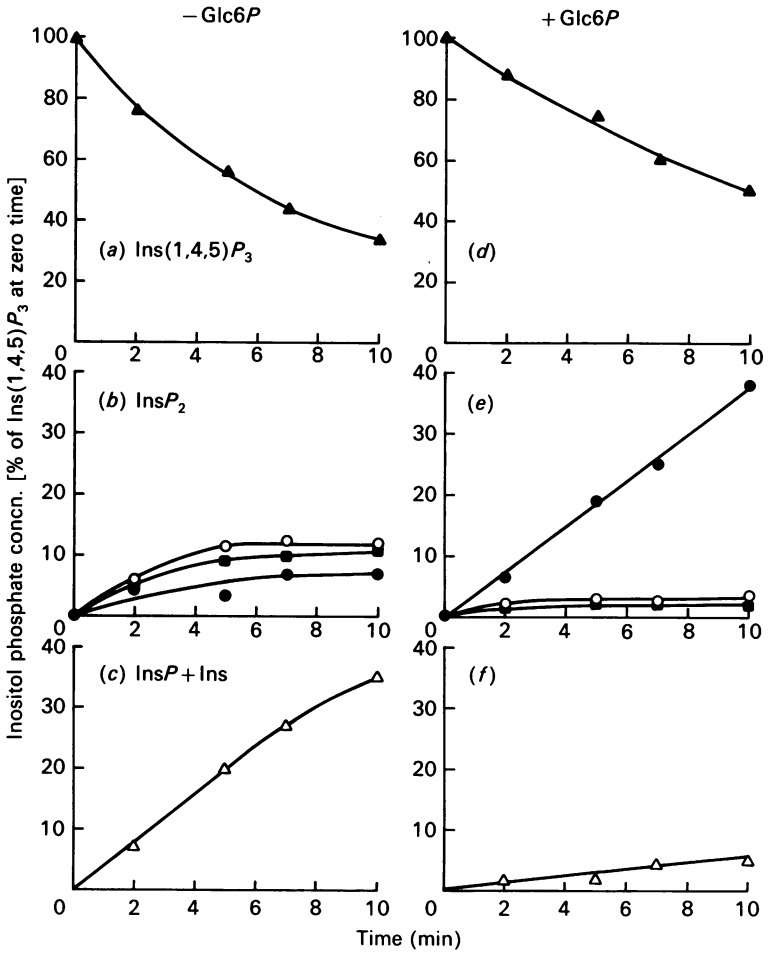

Fig. 3. Products of Ins(1,4,5)P_3 metabolism by intestinal cells incubated for various times in the presence and the absence of glucose 6-phosphate (Glc6*P*)

Intestinal cells were incubated with [4,5-^{32}P]Ins(1,4,5)P_3 (approx. 5000 d.p.m.) and [^{3}H]Ins(1,4,5)P_3 (approx. 12000 d.p.m.) for 0–10 min as described in the legend to Fig. 2. The final protein concentration was either 5 μg/ml (−Glc6*P*; *a*, *b*, *c*) or 25 μg/ml (+10 mM-Glc6*P*; *d*, *e*, *f*). The relative proportions of the various products were assessed by h.p.l.c. (see Fig. 2). Ins(1,4)P_2 and Ins(1,5)P_2 were not resolved into separate peaks, but the total ^{32}P/^{3}H ratio of the combined peaks was used to estimate the relative proportions of the two isomers (see the text). ▲, Ins(1,4,5)P_3; ○, Ins(1,5)P_2; ■, Ins(4,5)P_2; ●, Ins(1,4)P_2; △, Ins+Ins*P*. Data are averages of experiments performed on two cell preparations, each in duplicate.

Ins(1,4,5)P_3; $n = 19$] identified it as predominantly consisting of [^{3}H]- and [4-^{32}P]-Ins(1,4)P_2. This was itself dephosphorylated to an Ins*P* with a similar ^{32}P/^{3}H ratio [24±0.02% of the ratio for Ins(1,4,5)P_3; $n = 19$], so the ^{32}P label was retained by the monophosphate, identifying it as Ins4*P*.

Permeabilized intestinal cells were also incubated for various times with [^{3}H]- and [4,5-^{32}P]-Ins(1,4,5)P_3. In the absence of glucose 6-phosphate Ins(1,4)P_2 was the least of the three InsP_2 products. Substantial amounts of the InsP_2 isomers were further dephosphorylated to Ins and Ins*P* (Fig. 3). When glucose 6-phosphate was added to incubations containing a 5-fold greater cell concentration (see above), Ins(1,5)P_2 and Ins(4,5)P_2 were now the minor products [<3.5% of the initial concentration of Ins(1,4,5)P_3; Fig. 3]. Much larger quantities of Ins(1,4)P_2 accumulated at a near-linear rate, confirming the predominance of Ins(1,4,5)P_3 5-phosphatase in these conditions. When glucose 6-phosphate was present, around 90% of the products of Ins(1,4,5)P_3 dephosphorylation were InsP_2 isomers, suggesting that their further dephosphorylation was inhibited. This was confirmed in further experiments analysed either by h.p.l.c. or on gravity-fed columns (results not shown), in which 10 mM-glucose 6-phosphate inhibited, by 95%, the first-order rate constant for [^{3}H]Ins(1,4)P_2 metabolism.

The products of Ins(1,4)P_2 metabolism were analysed by h.p.l.c. as described in the Materials and methods section. Ins4*P* was the predominant isomer (80 and 95% of total Ins*P* in the absence and presence of glucose 6-phosphate respectively; results not shown). These data independently confirm the dephosphorylation pathway for Ins(1,4)P_2 determined above.

Intra- and extra-cellular distribution of intestinal phosphatase activities towards Ins(1,4,5)P_3

From the information given above, it is clear that 10 mM-glucose 6-phosphate strongly inhibits non-specific phosphatase activity towards Ins(1,4,5)P_3, whereas specific 5-phosphatase activity is not substantially affected. We have used this characteristic, in intact and saponin-

Table 2. Effect of saponin on Ins(1,4,5)P_3 dephosphorylation by rat intestinal cells in the presence and the absence of glucose 6-phosphate

Around 10000 d.p.m. of [^{3}H]Ins(1,4,5)P_3 was incubated for 10 min with intestinal cells [approx. 4 and 20 μg of protein/ml in the presence and absence of glucose 6-phosphate (Glc6P) respectively] as described in the Materials and methods section, in the presence or absence of saponin. Data (means ± S.E.M.) were obtained from seven preparations of intestinal cells.

[Glc6P] (mM)	Rate constant for Ins(1,4,5)P_3 metabolism (s^{-1}/mg of protein)		
	−saponin	+saponin	(+saponin)−(−saponin)
0	0.16±0.016	0.26±0.04	0.1
10	0.025±0.007	0.1±0.02	0.075
Inhibition by Glc6P (%)...	84	62	25

Table 3. Effects of 2,3-bisphosphoglycerate on Ins(1,4,5)P_3 dephosphorylation by rat enterocytes in the presence and absence of glucose 6-phosphate

Around 10000 d.p.m. of [^{3}H]Ins(1,4,5)P_3 was incubated with intestinal cells in the presence of saponin for 10 min as described in the legend to Table 2. Glucose 6-phosphate (Glc6P) and 2,3-bisphosphoglycerate (2,3-DPG) were added where indicated. Data (means ± S.E.M.) are from three experiments.

[Glc6P] (mM)	[2,3-DPG] (mM)	Ins(1,4,5)P_3 metabolism (% per 10 min)	Inhibition (%)
0	0	59±4	-
10	0	30±7	49
0	10	17±6	71
10	10	6±0.7	90

permeabilized cells, to determine what proportions of these phosphatase activities are intracellular.

In all the experiments described above, intestinal cells were permeabilized by saponin. In such incubations, the rate of Ins(1,4,5)P_3 metabolism was about 1.6-fold faster than when saponin was omitted (Table 2). Cells incubated in the absence of saponin are relatively intact (Velasco *et al.*, 1986*b*), so about two-thirds of total cellular Ins(1,4,5)P_3 phosphatase activity must be expressed on the outer cell surface. This extracellular activity was strongly inhibited by glucose 6-phosphate (Table 2), and is therefore the relatively non-specific phosphatase(s). The glucose 6-phosphate-insensitive, Ins(1,4,5)P_3 5-phosphatase activity was increased 4-fold by saponin, indicating that this enzyme is predominantly intracellular.

The distribution of Ins(1,4,5)P_3 5-phosphatase activity between the particulate and soluble fractions of the cell was studied in incubations containing 10 mM-glucose 6-phosphate. In such experiments, the particulate fractions contained 83±2% ($n = 3$) of total activity, and the remainder was located in the soluble fractions.

Effect of 2,3-bisphosphoglycerate on Ins(1,4,5)P_3 phosphatase activity

A characteristic of Ins(1,4,5)P_3 5-phosphatase activity in several cell types is its inhibition by 2,3-bisphosphoglycerate (Downes *et al.*, 1982; Connolly *et al.*, 1985; Rana *et al.*, 1986). In three further preparations of enterocytes, about half of the total Ins(1,4,5)P_3 phosphatase activity was sensitive to glucose 6-phosphate (Table 3), and therefore due to non-specific dephosphorylation (see above). Ins(1,4,5)P_3 metabolism was more potently inhibited by 10 mM-2,3-bisphosphoglycerate (71%; Table 3), which must therefore have acted on both non-specific and 5-phosphate-specific phosphatases. More importantly, 10 mM-2,3-bisphosphoglycerate strongly inhibited the Ins(1,4,5)P_3 dephosphorylation that persisted in the presence of 10 mM-glucose 6-phosphate, which Fig. 2 demonstrates is almost entirely due to removal of the 5-phosphate.

DISCUSSION

Several agonists (carbachol, 5-hydroxytryptamine and substance P) stimulate intestinal-fluid secretion in a Ca^{2+}-dependent manner (reviewed by Donowitz & Welsh, 1986). It seems likely that these responses are mediated through receptor-activated inositol lipid turnover, as are responses to these stimuli in other cell types. Although we are unaware of any studies confirming this prediction, recent work has shown that intestinal cells possess several key components of the inositol-lipid signalling system, namely protein kinase C (Velasco *et al.*, 1986*c*), an Ins(1,4,5)P_3-releasable Ca^{2+} pool (Velasco *et al.*, 1986*a*; Ilundain *et al.*, 1987; Van Corven *et al.*, 1987), and Ins(1,4,5)P_3 kinase (Shears *et al.*, 1987*d*). In the present paper, we add to this list a specific intracellular Ins(1,4,5)P_3 5-phosphatase, which bears a number of similarities to that in other cells, i.e.: (i) its intracellular location (largely membrane-bound; see also Shears *et al.*, 1987*b*; Erneux *et al.*, 1986; Rana *et al.*, 1986); (ii) its inhibition by 2,3-bisphosphoglycerate (compare Table 3 with Downes *et al.*, 1982; Connolly *et al.*, 1985; Rana *et al.*, 1986); (iii) its insensitivity to 50 mM-Li^+ (results not shown; see also Shears *et al.*, 1987*a*; Erneux *et al.*, 1986).

The demonstration of Ins(1,4,5)P_3 5-phosphatase in enterocytes has not been straightforward, because these cells contain other phosphatase(s) that attack the 1- and 4-phosphate groups. Fortunately the latter enzyme activities were strongly inhibited by 10 mM-glucose 6-phosphate. In contrast, Ins(1,4,5)P_3 5-phosphatase was not substantially inhibited by up to 10 mM-glucose 6-phosphate in pancreatic islets (Rana *et al.*, 1986), enterocytes and human erythrocytes (see the Results section). The intracellular concentration of glucose 6-phosphate in enterocytes (50 μM; Kellett *et al.*, 1984) is far too low to affect Ins(1,4,5)P_3 5-phosphatase; the same conclusion was reached in studies on pancreatic islets (Rana *et al.*, 1986).

The non-specific and 5-phosphate-specific phosphatase activities towards Ins(1,4,5)P_3 were also distinguished by their cellular location. The non-specific activity was expressed on the surface of intact cells, whereas the 5-phosphatase activity was intracellular, as indicated by its 4-fold activation when the cells were permeabilized with saponin (Table 2). In fact the proportion of 5-phosphatase

that was intracellular may have been underestimated if some of the cells were damaged and therefore leaky to Ins(1,4,5)P_3 even in the absence of saponin.

We have not tried to identify those enzymes that non-specifically dephosphorylate Ins(1,4,5)P_3, but candidates include alkaline phosphatase, phytase and a relatively non-specific inositol polyphosphatase (Moore & Veum, 1983; Rao & Ramakrishnan, 1985). One function of these enzymes *in vivo* may be the digestion of dietary inositol polyphosphates at the mucosal surface of the cell, before inositol absorption. Additionally, recent evidence indicates that certain inositol polyphosphates may act extracellularly (Vallejo *et al.*, 1987). This raises the expectation that these putative signalling molecules would be deactivated by extracellular inositol polyphosphatases. If an enzyme had this function in enterocytes, it would be expected to act at the exterior of the serosal cell surface. Whatever the normal roles of the extracellular phosphatases, they attack Ins(1,4,5)P_3, at least at the 1- and 4-positions. Since these reactions occur predominantly at the cell surface, Ins(1,5)P_2 and Ins(4,5)P_2 may not be normal metabolites of any Ins(1,4,5)P_3 formed within enterocytes.

There have been only a few attempts (e.g. Irvine *et al.*, 1987; Hughes & Drummond, 1987) to identify the InsP_2 isomers in intact cells. Recently a minor InsP_2 in GH3 cells, the concentration of which trebled on stimulation with thyrotropin-releasing hormone, was tentatively identified as Ins(4,5)P_2, largely by its co-elution with Ins(4,5)P_2 standards during h.p.l.c. (Dean & Moyer, 1987; Hughes & Drummond, 1987). A small amount of Ins(4,5)P_2 was formed when Ins(1,4,5)P_3 was added to GH3-cell homogenates (Hughes & Drummond, 1987). However, this does not prove that, in intact cells, Ins(4,5)P_2 is formed exclusively from Ins(1,4,5)P_3 and not from some other inositol polyphosphate(s). If any Ins(1,5)P_2 is produced in cells, it might have escaped detection in many studies because of its close elution with Ins(1,4)P_2, at least with the popular h.p.l.c. protocol used in our studies. However, the structures of the InsP_2 isomers from GH4 cells have been analysed more rigourously, and there was no evidence of any Ins(1,5)P_2 or Ins(4,5)P_2 (Irvine *et al.*, 1987).

This work was supported by the Medical Research Council, U.K., C.I.C.Y.T., Spain (grant no. PP85/0399) and F.I.S., Spain (grant no. 88/1459). C.R. and G.V. are both in receipt of fellowships from F.I.S. We also thank the British Council and the M.E.C. (Spain) for granting an Accion Integrada.

REFERENCES

Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) Biochem. J. **232**, 211–215

Berridge, M. J. (1984) Biochem. J. **220**, 345–360

Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Brown, D. M. & Stewart, J. C. (1966) Biochim. Biophys. Acta **215**, 413–421

Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. R. (1983) J. Biol. Chem. **258**, 15336–15345

Clarke, N. G. & Dawson, R. M. C. (1981) Biochem. J. **195**, 301–309

Connolly, T. M., Bross, T. E. & Majerus, P. W. (1985) J. Biol. Chem. **260**, 7868–7874

Dean, N. M. & Moyer, J. D. (1987) Biochem. J. **242**, 361–366

Donowitz, M. & Welsh, M. J. (1986) Annu. Rev. Physiol. **48**, 135–150

Downes, C. P. & Michell, R. H. (1985) in Molecular Mechanisms in Transmembrane Signalling (Cohen, P. & Houslay, M. D.), pp. 3–56, Elsevier, Amsterdam.

Downes, C. P., Simmonds, S. H. & Michell, R. H. (1981) Cell Calcium **2**, 473–482

Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) Biochem. J. **203**, 169–177

Erneux, C., Delvaux, A., Moreau, C. & Dumont, J. E. (1986) Biochem. Biophys. Res. Commun. **134**, 351–358

Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) Biochem. J. **238**, 507–516

Hughes, P. J. & Drummond, A. H. (1987) Biochem. J. **248**, 463–470

Ilundain, A., O'Brien, J. A., Burton K. A. & Sepulveda, F. V. (1987) Biochim. Biophys. Acta **896**, 113–116

Irvine, R. F. & Moor, R. M. (1986) Biochem. J. **240**, 917–920

Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986*a*) Nature (London) **320**, 631–634

Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986*b*) Biochem. J. **240**, 301–304

Irvine, R. F., Letcher, A. J., Lander, D. J., Heslop, J. P. & Berridge, M. J. (1987) Biochem. Biophys. Res. Commun. **143**, 353–359

Kellett, G. L., Jamal, A., Robertson, J. P. & Wollen, N. (1984) Biochem. J. **219**, 1027–1035

King, C. E., Stephens, L. R., Hawkins, P. T., Guy, G. R. & Michell, R. H. (1987) Biochem. J. **244**, 209–217

Moore, R. J. & Veum, T. L. (1983) Br. J. Nutr. **49**, 145–152

Morris, A. P., Gallacher, D. V., Irvine, R. F. & Peterson, O. H. (1987) Nature (London) **330**, 653–655

Rana, R. S., Sekar, M. C., Hokin, L. E. & MacDonald, M. J. (1986) J. Biol. Chem. **261**, 5237–5240

Rao, R. K. & Ramakrishnan, C. V. (1985) Enzyme **33**, 205–215

Phillippy, B. P., White, K. D., Johnston, M. R., Tao, S.-H. & Fox, R. S. (1987) Anal. Biochem. **162**, 115–121

Sharpes, E. S. & McCarl, R. L. (1982) Anal. Biochem. **124**, 421–424

Shears, S. B., Storey, D. J., Morris, A. J., Cubitt, A. B., Parry, J. B., Michell, R. H. & Kirk, C. J. (1987*a*) Biochem. J. **242**, 393–402

Shears, S. B., Parry, J. B., Tang, E. K. Y., Irvine, R. F., Michell, R. H. & Kirk, C. J. (1987*b*) Biochem. J. **246**, 139–147

Shears, S. B., Kirk, C. J. & Michell, R. H. (1987*c*) Biochem. J. **248**, 977–980

Shears, S. B., Parry, J. B., Tang, E. K. Y., Velasco, G., Lazo, P. S., Kirk, C. J. & Michell, R. H. (1987*d*) in Hormones and Cell Regulation (Dumont, J. E. & Nunez, J., eds.), vol. 153, pp. 27–40, INSERM/John Libbey Eurotext, London

Vallejo, M., Jackson, T., Lightman, S. & Hanley, M. R. (1987) Nature (London) **330**, 656–658

Van Corven, E. J. J. M., Verbost, P. M., de Jong, M. D. & Van Os, C. H. (1987) Cell Calcium **8**, 197–206

Velasco, G., Shears, S. B., Michell, R. H. & Lazo, P. S. (1986*a*) Biochem. Biophys. Res. Commun. **139**, 612–618

Velasco, G., Dominguez, P., Shears, S. B. & Lazo, P. S. (1986*b*) Biochim. Biophys. Acta **889**, 361–365

Velasco, G., Iglesias, C. F., Dominguez, P., Barros, F., Gascon, S. & Lazo, P. S. (1986*c*) Biochem. Biophys. Res. Commun. **139**, 875–882

Wreggett, K. A., Howe, L. R., Moore, R. F. & Irvine, R. F. (1987) Biochem. J. **245**, 933–934

Received 11 April 1988/16 May 1988; accepted 16 May 1988