Kinetic and inhibitor profiles of soluble and particulate inositol 1,4,5-trisphosphate 5-phosphatase from GH₃ and IMR-32 cells

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A simple procedure for assay of Ins(1,4,5) P_3 5-phosphatase is described. The reaction products ([3H]Ins(1,4) P_2 , [3H]InsP and *myo*-[3H]inositol) are completely separated from one another, with quantitative yield, on Amprep SAX (100 mg) minicolumns. [3H]Ins(1,4,5) P_3 and [3H]Ins(1,3,4,5) P_4 are adsorbed to the columns but not released to any appreciable extent by the elution conditions used. In GH₃ cells, the stepwise dephosphorylation of [3H]Ins(1,4,5) P_3 to *myo*-[3H]inositol was demonstrated, and was inhibited by 2,3-bisphosphoglycerate. The K_m of the soluble form of the enzyme was lower in GH₃ cells (8–13 μM) than in IMR-32 cells (26–32 μM) or in rat cerebral-cortical samples (22 μM). The K_m of the particulate form of the enzyme was similar in all three preparations (10–16 μM). The pH profiles of the two soluble 5-phosphatases differed, with a wider pH optimum for the GH₃-cell activity than for the IMR-32-cell activity. The soluble and particulate GH₃ enzymes were more sensitive than the corresponding IMR-32 enzymes to inhibition by *p*-hydroxymercuribenzoate, whereas there were no differences in their sensitivities to glucose 6-phosphate, 2,3-bisphosphoglycerate, fructose 1,6- and 2,6-bisphosphate and non-radioactive Ins(1,3,4,5) P_4 . Dialysis of the soluble fractions and washing of the particulate fractions did not affect the inhibitor sensitivities, except for the soluble IMR-32 fraction and *p*-hydroxymercuribenzoate. The K_m value of the soluble GH₃ 5-phosphatase activity was lower, and the inhibition by Ins(1,3,4,5) P_4 greater, after adsorption to and elution from phosphocellulose. It is concluded that there are qualitative differences in the properties of the soluble 5-phosphatase activity from GH₃ and IMR-32 cells.

INTRODUCTION

Ins(1,4,5) P_3 is an intracellular messenger generated by the receptor-coupled phosphodiesteratic cleavage of PtdIns(4,5) P_2 . Ins(1,4,5) P_3 acts synergistically with Ins(1,3,4,5) P_4 to mobilize Ca²⁺ from intracellular stores (for review, see Berridge & Irvine, 1989). Ins(1,4,5) P_3 is rapidly metabolized either by a 5-phosphatase [to form Ins(1,4) P_2] or, in the presence of ATP, by a 3-kinase to form Ins(1,3,4,5) P_4 , which in turn is a substrate for the 5-phosphatase (Downes *et al.*, 1982; Irvine *et al.*, 1986; for review, see Shears, 1989).

Ins(1,4,5) P_3 5-phosphatase is found in the cytosol and in a membrane-bound form (see Shears, 1989). Recent studies have suggested that the soluble 5-phosphatase enzyme is heterogeneous in nature. Thus two enzyme activities, termed Type I and Type II, have been purified from soluble rat brain, ox brain and human platelet fractions (Hansen *et al.*, 1987; Mitchell *et al.*, 1989; Erneux *et al.*, 1989). The Type I enzyme has a lower K_m value for Ins(1,4,5) P_3 than the Type II enzyme, is able to metabolize Ins(1,3,4,5) P_4 (Type II activity has only a low affinity for this substrate), and is phosphorylated by protein kinase C, whereas Type II is not (Hansen *et al.*, 1987; Mitchell *et al.*, 1989; Erneux *et al.*, 1989).

In the present study, the kinetic parameters and inhibitor sensitivities of membrane-bound and soluble Ins(1,4,5) P_3 5-phosphatase have been determined in GH₃ rat pituitary cells, IMR-32 human neuroblastoma cells and, as a comparison, in rat cerebral cortex by use of a simple assay method whereby the reaction products are separated from [3H]Ins(1,4,5) P_3 on Amprep SAX columns. The results indicate that there are qualitative differences in the soluble 5-phosphatase properties in the IMR-32 and GH₃ cells.

MATERIALS AND METHODS

Materials

Rat GH₃ pituitary cells (ATCC no. CCL 82.1) and human

IMR-32 neuroblastoma cells (ATCC no. CCL 127) were obtained from Flow Laboratories, Irvine, Scotland, U.K., and were used at passages 37–45 and 67–75 respectively. *D*-*myo*-[2- 3H]inositol 1,4,5-trisphosphate {[3H]Ins(1,4,5) P_3 , sp. radioactivity 1 Ci/mmol}, *D*-*myo*-[2- 3H]inositol 1,3,4,5-tetrakisphosphate {[3H]Ins-(1,3,4,5) P_4 , sp. radioactivity 1 Ci/mmol}, *D*-*myo*-[2- 3H]inositol 1,4-bisphosphate {[3H]Ins(1,4) P_2 , sp. radioactivity 1 Ci/mmol}, *D*-*myo*-[2- 3H]inositol 1-phosphate {[3H]InsP, sp. radioactivity 1 Ci/mmol}, *myo*-[2- 3H]inositol ([3H]Ins, sp. radioactivity 21.6 Ci/mmol, with added PT6-271 to adsorb breakdown products), non-radioactive Ins-(1,4,5) P_3 and Amprep SAX (100 mg) minicolumns were obtained from Amersham International, Amersham, Bucks., U.K. The molecular mass of the non-radioactive Ins(1,4,5) P_3 was taken to be that of the free acid (420 Da). Ins(1,3,4,5) P_4 heptammonium salt was obtained from Boehringer Mannheim G.m.b.H., Mannheim, Germany. Glucose 6-phosphate (monosodium salt), 2,3-bisphospho-D-glycerate (pentacyclohexylammonium salt), D-fructose 1,6- and 2,6-bisphosphates (sodium salts) and *p*-hydroxymercuribenzoate (sodium salt) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. (–)-*p*-Bromotetramisole oxalate was obtained from Research Biochemicals, Natick, MA, U.S.A. Minimal essential medium Eagle (modified) with Hanks salts (HMEM) and Ham's F-10 medium were obtained from Flow Laboratories. HMEM was supplemented with 1% (w/v) non-essential amino acids (Gibco, Paisley, Scotland, U.K.), and both media were supplemented with 10 mM-Hepes, 2 mM-glutamine, 100 μ g streptomycin sulphate (Sigma)/ml and 70 μ g of benzylpenicillin K (Astra)/ml. Foetal-calf serum was obtained from Gibco.

Preparation of homogenates

Homogenates were prepared by a modification of the method described by Dean & Moyer (1988). The GH₃ cells were cultured in 175 cm² flasks in supplemented Ham's F10 medium + 10% foetal-calf serum, and harvested when 50% confluency was reached. Upon harvesting, the cells were washed with ice-

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cold sodium phosphate-buffered saline (PBS; 137 mM-NaCl/2.68 mM-KCl/8.38 mM- Na_2HPO_4 /1.47 mM- KH_2PO_4) and allowed to detach by addition of PBS+0.02% EDTA. The cells were sedimented by centrifugation at 1000 *g* for 4 min and resuspended in 20 mM-Tris/HCl with 0.2 mM-EDTA, pH 7.6. The presence of EDTA during these preparative stages optimized the yield of cells, but did not affect the observed specific activity of Ins(1,4,5) P_3 5-phosphatase in GH_3 -cell homogenates. The suspensions, in a volume of 300–350 μl /flask, were left on ice for 10 min and then homogenized with a Dounce homogenizer (20 strokes). In the initial experiments (Fig. 2), portions of the homogenate were frozen at -20°C , followed by -70°C , and stored until assay. In the remaining experiments, the homogenate was centrifuged at 100000 *g* for 60 min to yield a supernatant ('soluble fraction') and a pellet ('particulate fraction'). The pellet was resuspended in 20 mM-Tris/HCl+0.2 mM-EDTA, pH 7.6, to the same volume as the supernatant, and portions of both were frozen until used for assay.

Human neuroblastoma IMR-32 cells were cultured in supplemented HMEM+10% heat-inactivated foetal-calf serum in 175 cm^2 flasks. Harvesting and homogenate preparation was essentially as described as for GH_3 cells, but without the PBS washing phase. In a separate experiment, both GH_3 and IMR-32 pellet fractions were washed, by resuspension in homogenization buffer followed by re-centrifugation at 100000 *g* for 60 min, and then resuspended before freezing. For the batch used in Table 2, a total of approx. 6×10^8 GH_3 cells harvested from 36 175 cm^2 flasks yielded ~80 and ~160 mg of protein in the soluble and particulate fractions respectively. For the IMR-32 cells (see below), the corresponding yields were ~70 and ~120 mg of protein respectively.

Cerebral cortices from male Sprague-Dawley rats (Alab Laboratorietjänst, Sollentuna, Sweden; body weight 250–300 g) were homogenized 1:10 (w/v) by Ultra-Turrax (3 $\times$ 3 s) followed by a Dounce homogenizer (10 strokes), and were centrifuged at 1000 *g* for 6.5 min. The supernatants were centrifuged at 100000 *g* for 60 min as described above for the GH_3 cells. All particulate fractions (and, for the initial experiments on GH_3 cells, the homogenates) from all three sources were sonicated before assay.

Phosphocellulose treatment of soluble fractions

The phosphocellulose treatment was based on that described by Hansen *et al.* (1987). A 1 g portion of cellulose phosphate P11 fibrose cation exchanger was precycled and equilibrated with 10 mM-Tris/HCl/10 mM-KCl/1 mM-dithiothreitol, pH 8.1, as described by the manufacturer (Whatman Biosystems, Maidstone, Kent, U.K.). Then 250 mg of the phosphocellulose was added to 4 ml of the soluble fractions from the GH_3 and IMR-32 cells (corresponding to ~30 mg of protein). The mixtures were stirred for 2.5 h on ice and then poured into centrifuge tubes. The phosphocellulose was centrifuged down (2300 *g* for 10 min) and the supernatant collected. The phosphocellulose was washed (by resuspension followed by re-centrifugation) with 4 ml of (1) distilled water, (2) 50 mM-potassium phosphate+1 mM-dithiothreitol, (3) 300 mM-potassium phosphate+1 mM-dithiothreitol and (4) 600 mM-potassium phosphate+1 mM-dithiothreitol (pH 7.6). All fractions, together with samples (1.5 ml) of the untreated soluble preparations, were dialysed overnight against homogenization buffer (at pH 8.15), with one change, by using Spectra/Por dialysis tubing with a 1000 Da molecular-mass cut-off (Spectrum Laboratory Products, Los Angeles, CA, U.S.A.). The samples were then frozen until used for assay.

Assay of Ins(1,4,5) P_3 5-phosphatase activity

The assay medium was essentially the same as described by Dean & Moyer (1988), but the sample work-up is different. In

2 ml Sarstedt tubes, samples [20 μl (40 μl in Table 2), corresponding to 20–200 μg of protein (except for the phosphocellulose extracts, where ~2–10 μg of protein was used), as indicated in the Table and Figure legends] were added to 200 μl (190 μl in Table 2) of assay buffer [10 mM-Hepes, 10 mM-KCl, 4 mM- MgCl_2 , 1 mM-EGTA, pH 7.2, containing the test compounds, when present (Fig. 5, Tables 1 and 2)] and incubated for 10 min at 37°C . This concentration of EGTA was found in preliminary experiments not to affect the 5-phosphatase activity. The tubes were then placed on ice and 30 μl (20 μl in Table 2) of [^3H]Ins(1,4,5) P_3 (to give a final concentration of 10 μM , unless otherwise stated) was added. For the experiments with Ins(1,3,4,5) P_4 (Table 2), a final volume of 200 μl was used, and 50 μl of assay buffer was added after the reactions were stopped.

The samples were incubated at 37°C at the times indicated in the Tables and Figures, after which reactions were stopped and the [^3H]inositol phosphates were extracted from the cell/tissue fractions essentially as described by Berridge *et al.* (1982). Briefly, 600 μl of ice-cold chloroform/methanol (1:2, v/v), was added, and the samples were vortex-mixed and placed on ice. When all samples had been treated in this manner, they were vortex-mixed for 10 min with a SMI model 2601 multi-tube vortexer (Scientific Manufacturing Industries, Emeryville, CA, U.S.A.). Then 750 μl of water was added, and the samples were vortex-mixed for 60 s, followed by centrifugation for 4 min at 12000 rev./min in an Eppendorf centrifuge to separate the phases.

The ^3H -labelled inositol phosphates were separated by a modification of the method of Minchin & Wood (1986), but with different SAX columns. Amprep SAX (100 mg) mini-columns were placed in an Amersham Manifold-10 vacuum manifold and conditioned with 1 ml of methanol followed by 1 ml of distilled water. Samples (1 ml) of the aqueous phase were added and the following elution scheme was employed: stage I, run through of the aqueous phase followed by 1 ml of water; stage II, 1 ml of 10 mM-NaCl; stage III, 2 $\times$ 1 ml of 80 mM-NaCl; stage IV, 1 ml of 300 mM-NaCl followed by 2 $\times$ 1 ml of 400 mM-NaCl; and, in the initial experiments, stage V, 1 ml of 1 M-NaCl+0.1 M-HCl. The radioactivity (d.p.m.) in the fractions was determined by liquid-scintillation spectroscopy with quench correction, by using Ultima Gold (Packard Instruments, Downers Grove, IL, U.S.A.) as scintillant.

Protein determinations

Protein contents of the samples were determined by the method of Markwell *et al.* (1978), with BSA as standard.

RESULTS

Assay of Ins(1,4,5) P_3 5-phosphatase

The ^3H -labelled inositol phosphates were separated by ion-exchange chromatography on Amprep SAX (100 mg) mini-columns. Almost quantitative yields of the [^3H]Ins, [^3H]Ins P and [^3H]Ins(1,4) P_2 standards were obtained, with elution occurring almost entirely at stages I, III and IV respectively (Fig. 1). [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4,5) P_4 bound very tightly to the columns and were poorly eluted (Fig. 1). In these initial experiments, the recovery of [^3H]Ins(1,4,5) P_3 from the columns was about 20%, mainly in stage IV (Fig. 1), but in subsequent batches the recovery was much lower (see, e.g., blank values in Fig. 2d): over a range of 2–20 μM -[^3H]Ins(1,4,5) P_3 , recovery from the columns was found to be consistently about 3%. This recovery (range 2–7%) has been found in all subsequent experiments performed on eight separate batches of [^3H]Ins(1,4,5) P_3 during the period June 1989–June 1990. In the remaining experiments presented here, the radioactivity eluted in stages I, III and IV has been termed [^3H]Ins, [^3H]Ins P and [^3H]Ins(1,4) P_2 respectively.

Fig. 1. Elution profiles of [^3H]inositol phosphates from Amprep SAX (100 mg) mini-columns

To GH₃-cell homogenates were added (in the absence of incubation) 50 nCi of *myo*-[^3H]inositol (Ins), [^3H]Ins-1-*P* (InsP), [^3H]Ins(1,4) P_2 (InsP₂), [^3H]Ins(1,4,5) P_3 (InsP₃) + 10 μM -Ins(1,4,5) P_3 ('cold') or [^3H]Ins(1,3,4,5) P_4 (InsP₄) + 10 μM -Ins(1,4,5) P_3 . Samples (1 ml) of the aqueous-phase extracts were applied to conditioned Amprep SAX mini-columns and eluted as described in the Materials and methods section. Data are means $\pm$ S.D. ($n = 4$); the radioactivity (d.p.m.) recovered from the columns is shown as a percentage of the d.p.m. applied to the columns.

Time courses of [^3H]Ins(1,4) P_2 , [^3H]InsP and [^3H]Ins production in GH₃-cell homogenates are shown in Fig. 2. In all cases, the extraction of the ^3H from the homogenate was virtually complete. Thus, for example, yields in the aqueous phase of 92 ± 9 , 98 ± 3 , 99 ± 2 and 99 ± 1 [means $\pm$ S.D., $n = 3$; values as % of added [^3H]Ins(1,4,5) P_3 d.p.m.] were found after incubation for 0, 5, 10 and 20 min with GH₃-cell homogenates (200 μg of protein/assay tube). High extraction rates (with few exceptions $> 80\%$, and usually $> 90\%$) were found regardless of the enzyme source, [^3H]Ins(1,4,5) P_3 concentration (in the range

2–20 μM), or protein concentration. The presence of Ins(1,3,4,5) P_4 , however, decreased the ^3H yield in a manner dependent on both its own concentration and the protein concentration present. Thus, for example, in the absence of protein, 0 and 15 μM -Ins(1,3,4,5) P_4 gave extraction rates of 98 and 99 %, whereas in the presence of GH₃-cell soluble fraction (24 μg of protein/assay), rates of 84 and 67 % were found. Addition of Ins(1,3,4,5) P_4 (5–50 μM) after the incubation with [^3H]Ins(1,4,5) P_3 had minor effects on both extraction efficiency and the pattern of elution of the [^3H]Ins(1,4,5) P_3 breakdown products (results not shown). Acidification of the chloroform/methanol with 25 μl of 0.1 M-HCl greatly decreased the extraction from the GH₃ homogenates of [^3H]Ins(1,4,5) P_3 , without effect on the other inositol phosphates (results not shown). In the remaining experiments, unless otherwise stated, the neutral extraction procedure was used, and the [^3H]Ins(1,4) P_2 , [^3H]InsP and [^3H]Ins d.p.m. eluted from the Amprep columns (after compensation for the fact that only part of the aqueous phase was used) was expressed as a percentage of the total starting [^3H]Ins(1,4,5) P_3 added to the homogenates. Preliminary experiments indicated that the columns could not be re-used after reconditioning with distilled water followed by methanol and distilled water, since the ^3H eluted from the columns was affected by this procedure. Thus, for example, the ^3H extracted as [^3H]Ins(1,4) P_2 after incubation of [^3H]Ins(1,4,5) P_3 with a particulate fraction from GH₃ cells was decreased from 46 % to 32 % of the total ^3H added after this column-regeneration protocol.

From the time course of the combined [^3H]Ins(1,4) P_2 , [^3H]InsP and [^3H]Ins d.p.m. shown in Fig. 2(d), it is clear that the reaction is linear until remarkably high levels of substrate utilization are reached. Indeed, until 50–60 % of the substrate is utilized, linearity with respect to both incubation time and enzyme concentration is found. At prolonged incubation times, the metabolism of the added [^3H]Ins(1,4,5) P_3 is virtually complete (Fig. 2d). No time-dependent utilization of substrate was found in the absence of homogenate (Fig. 2d) or when boiled homogenate was used (results not shown). In the remaining experiments, blank values were determined with the same incubation time in the absence of homogenate.

At a protein concentration of 20 μg /assay (Fig. 2a), [^3H]Ins(1,4) P_2 production proceeds linearly, whereas [^3H]InsP production starts after a lag phase. The pattern of product formation is similar after 20 min to that found between 5 and 10 min at a protein concentration of 60 μg of protein/assay (Fig. 2b). After 20 min at this protein concentration, the pattern is similar to that found after 5 min at 200 μg of protein/assay (Fig. 2c), and the rate of [^3H]Ins(1,4) P_2 metabolism to [^3H]InsP becomes greater than its production from [^3H]Ins(1,4,5) P_3 , so that the levels approach blank values at long incubation times. At the longest incubation time used in this experiment (20 min), formation of *myo*-[^3H]inositol was minor, but at longer incubation times there was considerable formation (results not shown).

The Ins(1,4,5) P_3 5-phosphatase inhibitor 2,3-bisphosphoglycerate was found concentration-dependently to increase blank values, owing to ^3H elution in stage IV. Thus, for example, the percentage of ^3H present in the aqueous phase of the homogenate-free blanks eluted in stage IV by 0, 0.5 mM, 1 mM, 2 mM, 5 mM and 20 mM-2,3-bisphosphoglycerate in a preliminary experiment was 3, 6, 6, 13, 20 and 32 respectively. In consequence, in subsequent experiments, activities are related to blank values containing the same concentration of the test compound. Under these conditions, 2,3-bisphosphoglycerate (0.2, 0.5, 1 and 2 mM) inhibited the production of the combined [^3H]Ins(1,4) P_2 , [^3H]InsP and [^3H]Ins in a GH₃-cell homogenate preparation after incubation for 10 min with 10 μM -[^3H]Ins(1,4,5) P_3 by 17, 40, 54 and 65 % respectively.

Fig. 2. Time course of the dephosphorylation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ ($10\ \mu\text{M}$) by a GH_3 -cell homogenate

In (a)–(c), the concentrations of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ (∇), $[^3\text{H}]\text{InsP}$ ($\blacktriangle$) and *myo*- $[^3\text{H}]\text{inositol}$ ($\circ$) are shown respectively. The protein concentrations used were 20 (a), 60 (b) and 200 (c) $\mu\text{g}/\text{assay}$. In (d), the combined $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ + $[^3\text{H}]\text{InsP}$ + *myo*- $[^3\text{H}]\text{inositol}$ production at 0 ($\times$), 20 ($\triangle$), 60 ($\bullet$) and 200 ($\square$) μg of protein/assay is shown. Data are means $\pm$ S.D. for separate determinations of activity on three samples derived from the same homogenate.

Table 1. Kinetic and pharmacological characteristics of soluble and particulate $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase

'% Activity in fraction' represents the $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ metabolized/min in the whole fraction, with the total soluble + particulate activities defined as 100%. The kinetic parameters were determined with 2 μM , 4 μM , 8 μM , 12 μM , 16 μM and 20 μM $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, and the following protein concentrations/assay and incubation times respectively: rat cerebral cortex, soluble $60 \pm 4\ \mu\text{g}$ and 5 min, particulate $54 \pm 6\ \mu\text{g}$ and 5 min; IMR-32, soluble 82 μg and 10 min, particulate 130 μg and 5 min; GH_3 , soluble 32 μg and 10 min, particulate 102 μg and 5 min. The inhibitor-sensitivities were determined after a 10 min preincubation of the enzyme preparations with the test compounds. The incubation time with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ ($10\ \mu\text{M}$) was 10 min in all cases and the protein concentration was as above except for IMR-32-cell particulate fraction (194 $\mu\text{g}/\text{assay}$) and rat brain particulate fraction ($82 \pm 9\ \mu\text{g}/\text{assay}$). Data are means $\pm$ S.D. of three experiments [except for 25 μM -glucose-6-phosphate ($n = 5$) and CdCl_2 for rat and GH_3 cells, where the individual values are shown] undertaken separately on different samples either from the same (GH_3 , IMR-32) or different (rat cerebral cortex) preparations. Abbreviations: Sol, soluble; Part, particulate.

		Rat cerebral cortex		IMR-32 cells		GH_3 cells	
		Sol	Part	Sol	Part	Sol	Part
% Activity in fraction		33 ± 4	67 ± 4	19 ± 6	81 ± 6	28 ± 4	72 ± 4
K_m (μM)		22 ± 3	16 ± 0.5	26 ± 10	11 ± 1	8 ± 1	10 ± 1
V_{max} (nmol/min per mg of protein)		5.5 ± 0.6	8.4 ± 1.7	0.9 ± 0.2	1.7 ± 0.3	2.6 ± 0.2	2.2 ± 0.4
Activity remaining (%) in presence of (mM):							
D-Glucose 6-phosphate	5	81 ± 9	95 ± 9	92 ± 8	83 ± 13	75 ± 13	81 ± 18
	25	25 ± 8	40 ± 19	50 ± 22	40 ± 8	15 ± 9	30 ± 3
	100	2 ± 1	3 ± 2	7 ± 10	2 ± 3	2 ± 2	1, 9
2,3-Bisphospho-D-glycerate	0.2	69 ± 8	81 ± 2	80 ± 8	71 ± 8	69 ± 16	72 ± 4
	0.5	48 ± 13	62 ± 6	52 ± 3	49 ± 10	56 ± 8	50 ± 8
	1	29 ± 5	42 ± 1		31 ± 9	29 ± 9	26 ± 6
	2	18 ± 1	29 ± 3		20 ± 6		15 ± 8
	5	8 ± 1	16 ± 4	10 ± 4	7 ± 5	9 ± 8	5 ± 4
Fructose 1,6-bisphosphate	5	46 ± 5	49 ± 8	64 ± 4	36 ± 3	23 ± 2	27 ± 4
Fructose 2,6-bisphosphate	5	73 ± 1	78 ± 14	96 ± 5	64 ± 3	52 ± 2	60 ± 11
(-)-p-Bromotetramisole oxalate	0.1	104 ± 7	114 ± 9	131 ± 28	101 ± 1	87 ± 8	110 ± 19
p-Hydroxymercuribenzoate	0.01	28 ± 7	34 ± 18	68 ± 12	77 ± 10	3 ± 3	27 ± 14
	0.03	17 ± 3	8 ± 2	9 ± 5	19 ± 7	2 ± 1	1 ± 1
	0.1	16 ± 9	4 ± 1	20 ± 8	1 ± 2	4 ± 1	1 ± 2
EDTA	10	2 ± 4	3 ± 3	8 ± 7	1 ± 1	4 ± 5	3 ± 2
CdCl_2	1	4, 9	3, 5	12 ± 2	1 ± 1	2, 3	1, 2

Table 2. Characteristics of soluble Ins(1,4,5) P_3 5-phosphatase from GH $_3$ and IMR-32 cells after dialysis or phosphocellulose treatment

The yields for the dialysed fractions from both cells and for the GH $_3$ dialysed phosphocellulose extract were calculated by using $V_{\max}$ values from determinations of activity with 4 μM , 8 μM , 12 μM , 16 μM and 20 μM - $[\text{^3H}]\text{Ins}(1,4,5)P_3$. The yield for the IMR-32-cell dialysed phosphocellulose extract was calculated from activity determinations at 10 μM - $[\text{^3H}]\text{Ins}(1,4,5)P_3$. The inhibitory effects of Ins(1,3,4,5) P_4 were tested by using 3.75 μM - $[\text{^3H}]\text{Ins}(1,4,5)P_3$, no preincubation and an incubation time of 20 min. The values are normalized to compensate for the effects of Ins(1,3,4,5) P_4 on the ^3H extraction (see the Results section). The inhibitory effects of glucose 6-phosphate (G-6-P), 2,3-bisphosphoglycerate (2,3bisP), fructose 1,6-bisphosphate [F(1,6) P_2] and *p*-hydroxymercuribenzoate (HgBz) were tested by using 10 μM - $[\text{^3H}]\text{Ins}(1,4,5)P_3$, 10 min preincubation and 30 min incubation. Data are means of 2–3 experiments on the same preparations.

Fraction	Yield (%)	K_m (μM)	Activity remaining (%) in presence of:						
			Ins(1,3,4,5) P_4 (μM):			G-6-P (25 mM)	2,3bisP (0.5 mM)	F(1,6) P_2 (5 mM)	HgBz (0.01 mM)
			5	15	50				
GH ₃ cells									
Initial fraction	100	13	65	45	42	19	29	25	1
Dialysed fraction	83	14	62	46	48	18	36	32	2
Phosphocellulose extract	6.5	6.0	34	11	12	32	41	21	3
IMR-32 cells									
Initial fraction	100	32	73	64	61	19	31	33	17
Dialysed fraction	93	22	69	52	51	17	33	35	8
Phosphocellulose extract	7.3	–	53	0	19	40	23	19	7

Fig. 3. Time courses of the dephosphorylation of $[\text{^3H}]\text{Ins}(1,4,5)P_3$ (10 μM) by rat brain soluble and particulate fractions

The concentrations of $[\text{^3H}]\text{Ins}(1,4)P_2$ (∇), $[\text{^3H}]\text{Ins}P$ ($\blacktriangle$), *myo*- $[\text{^3H}]\text{inositol}$ ($\circ$) and all three combined ($\bullet$) are shown. Mean protein contents of the samples were 60 and 82 $\mu\text{g}/\text{assay}$ for the soluble and particulate fractions respectively. Data are means $\pm$ S.D. for three preparations, each assayed separately: ** $P < 0.01$, * $P < 0.05$ versus corresponding 'soluble' value (two-tailed paired *t* test).

Comparison of soluble and particulate Ins(1,4,5) P_3 5-phosphatase activities in GH $_3$ and IMR-32 cells and in rat cerebral cortex

Ins(1,4,5) P_3 5-phosphatase activities were found in both soluble and particulate fractions from all three sources. Most (67, 81 and 72 % for rat cerebral cortex, IMR-32 and GH $_3$ cells respectively) of the activity was located in the particulate fraction (Table 1). For a second batch (Table 2) prepared 9 months later, the corresponding values were 76 and 65 % for IMR-32 and GH $_3$ cells respectively. Time-course experiments for the soluble and particulate rat cerebral-cortical fractions are shown in Fig. 3. The soluble fraction shows a pattern of product formation akin to that in the GH $_3$ -cell homogenates, whereas the rate of $[\text{^3H}]\text{Ins}P$ formation is considerably lower (and the corresponding observed levels of $[\text{^3H}]\text{Ins}(1,4)P_2$ are much higher) in the particulate fraction. Similar time-course patterns were found for the soluble and particulate IMR-32- and GH $_3$ -cell fractions (results not shown). For the IMR-32-cell soluble fractions used in Table 2,

18.7, 14.9, 9.0 and 5.0 % of the added $[\text{^3H}]\text{Ins}(1,4,5)P_3$ (10 μM) was metabolized after incubation of 29, 22, 15 and 7.4 μg portions for 30 min at 37 $^{\circ}\text{C}$.

Kinetic analyses of the Ins(1,4,5) P_3 5-phosphatase activities in the different preparations were undertaken by using six concentrations of $[\text{^3H}]\text{Ins}(1,4,5)P_3$ (2, 4, 8, 12, 16 and 20 μM) and measuring the total $[\text{^3H}]\text{Ins}(1,4)P_2$, $[\text{^3H}]\text{Ins}P$ and $[\text{^3H}]\text{Ins}$ produced. The fractions from the different sources were assayed concomitantly rather than sequentially, to rule out the risk that differences in K_m values could be ascribed to changes in substrate batch quality. The K_m and $V_{\max}$ values derived are given in Table 1. The K_m values for the particulate enzyme activities are rather similar in the three different preparations (Table 1). On the other hand, the K_m value for the soluble enzyme activity in GH $_3$ cells is lower than the corresponding values in the rat cerebral cortex and IMR-32 cells (Table 1, Fig. 4). This difference was also found in a separate experiment using different batches of $[\text{^3H}]\text{Ins}(1,4,5)P_3$ and of the GH $_3$ - and IMR-32-cell soluble frac-

Fig. 4. Hanes plot of the soluble Ins(1,4,5) P_3 5-phosphatase activity from GH $_3$ cells (○) and from rat cerebral cortex (●)

Incubation times and protein concentrations/assay were GH $_3$ cells 10 min and 32 μ g, and rat cerebral cortex 5 min and 60 $\pm$ 4 μ g, respectively. Data are means of three separate determinations of activity on different samples derived from the same (GH $_3$) or different (rat) preparations. Regression lines of best fit for the mean data are shown. Initial velocities (v) are expressed as nmol/min per mg of protein.

tions (Table 2), although the $V_{\max}$ values (4.2 and 2.1 nmol/min per mg of protein respectively) were higher in this experiment. Dialysis of the soluble fractions did not affect the K_m value for the GH $_3$ cells, but decreased slightly the K_m value for the IMR-32 cells (Table 2).

The effects of a number of compounds on soluble and particulate Ins(1,4,5) P_3 5-phosphatase were investigated (Tables 1 and 2). Non-radioactive Ins(1,3,4,5) P_4 decreased the activity of the soluble 5-phosphatase from GH $_3$ and IMR-32 cells to about half over the range of 5–50 μ M (Table 2), although the effects of this compound on the 3 H extraction (see above) make these values approximate in nature. D-Glucose 6-phosphate, 2,3-bisphospho-D-glycerate, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, EDTA and CdCl $_2$ inhibited the 5-phosphatase activity in all preparations, whereas the non-specific alkaline phosphatase inhibitor (–)-*p*-bromotetramisole oxalate was without effect at the concentration used (0.1 mM). *p*-Hydroxymercuribenzoate also inhibited the 5-phosphatase activities, but the sensitivity to inhibition was lower in the IMR-32 cells than in the other preparations (Table 1). However, dialysis of the IMR-32 soluble fraction appeared to increase the sensitivity to inhibition by this compound, whereas the sensitivities to inhibition by Ins(1,3,4,5) P_4 , glucose 6-phosphate, 2,3-bisphosphoglycerate and fructose 1,6-bisphosphate were not changed (Table 2). Washing of the particulate fractions from GH $_3$ and IMR-32 cells (by resuspension in homogenization buffer, followed by re-centrifugation) gave protein yields of 75 % and 97 %, and 5-phosphatase yields {assayed at 10 μ M- 3 H]Ins(1,4,5) P_3 } of 82 % and 72 % respectively. The washing procedure did not affect the sensitivities of the fractions to inhibition by 2,3-bisphosphoglycerate (0.5 mM), fructose 1,6-bisphosphate (5 mM) or *p*-hydroxymercuribenzoate (0.01 mM). Thus, for example, the percentage of activity remaining in the presence of *p*-hydroxymercuribenzoate was, for GH $_3$ cells, 43 and 36, and for IMR-32 cells, 60 and 60, for control and washed fractions respectively.

In all three preparations, glucose 6-phosphate inhibited the dephosphorylation of 3 H]Ins(1,4) P_2 to a greater extent than the dephosphorylation of 3 H]Ins(1,4,5) P_3 in both soluble and par-

Fig. 5. pH profiles of soluble Ins(1,4,5) P_3 5-phosphatase activities from GH $_3$ (○) and IMR-32 (▲) cells

pH was varied by using mixtures of Hepes free acid and sodium salt, with addition of KOH at the two highest pH values tested. Incubation time with 10 μ M- 3 H]Ins(1,4,5) P_3 was 10 min. The activity at pH 7.08 was defined as 100 %. Data are means from two separate experiments.

ticulate fractions. Thus in the rat brain soluble fractions, for example, the total utilization of 3 H]Ins(1,4,5) P_3 was decreased from a mean of 38.6 % to 11.6 % by 25 mM-glucose (for assay conditions, see Table 1). The 3 H recovered as 3 H]Ins(1,4) P_2 , however, increased from 6.4 % to 10.2 % of the starting 3 H]Ins(1,4,5) P_3 , whereas the 3 H recovered as 3 H]InsP was almost totally inhibited (32.0 % versus 1.1 % of the starting 3 H]Ins(1,4,5) P_3). Similar effects were found with fructose 1,6-bisphosphate and fructose 2,6-bisphosphate in all three preparations, but not with 2,3-bisphosphoglycerate (results not shown).

The pH-dependencies of the soluble GH $_3$ - and IMR-32-cell activities are shown in Fig. 5. The pH optimum for the GH $_3$ -cell activity was over a much wider pH range than that for the IMR-32 activity.

Phosphocellulose extraction of the soluble 5-phosphatase

Soluble fractions from GH $_3$ and IMR-32 cells were treated with phosphocellulose as described in the Materials and methods section. The 5-phosphatase activity was eluted in fraction 3 (300 mM-potassium phosphate + 1 mM-dithiothreitol), consistent with the study by Hansen *et al.* (1987). The yields of the 5-phosphatase (~ 7 %; see Table 2) were considerably lower than the 68 % recovery reported for a larger-scale experiment using rat brain supernatant (Hansen *et al.*, 1987). However, the yields of the protein (~ 1 % and 7 % for GH $_3$ and IMR-32 cells respectively) are in line with the 3.4 % yield of protein found in that study.

The 300 mM-potassium phosphate + 1 mM-dithiothreitol eluate did not contain any Ins(1,4) P_2 phosphatase activity. Thus, after a 30 min incubation of a 40 μ l sample with 10 μ M- 3 H]Ins(1,4,5) P_3 , 46.5, 0.7 and 0 % of the starting 3 H was recovered as 3 H]Ins(1,4) P_2 , 3 H]InsP and 3 H]Ins respectively. For the dialysed soluble fraction, at a concentration giving a similar rate of breakdown, the corresponding recoveries were 5.8, 36.9 and 4.7 % respectively.

The kinetic and pharmacological characterizations of the phosphocellulose extracts are shown in Table 2. The K_m of the GH $_3$ -cell fraction was lower than for the corresponding control sample, and the inhibition by Ins(1,3,4,5) P_4 was higher. The very low activity in the IMR-32-cell eluate precluded detailed kinetic investigation, and warrants caution in interpretation of the inhibitor data, but the inhibition by Ins(1,3,4,5) P_4 also appeared to be greater. Slightly less inhibition by 25 mM-glucose 6-

phosphate was found after the phosphocellulose treatment, whereas the sensitivities to 2,3-bisphosphoglycerate, fructose 1,6-bisphosphate and *p*-hydroxymercuribenzoate showed no consistent change (Table 2).

DISCUSSION

In the present study, the activity of Ins(1,4,5) P_3 5-phosphatase has been investigated in GH₃-cell preparations and compared with that found in IMR-32-cell and rat cerebral-cortical preparations. The metabolism of Ins(1,4,5) P_3 has been described in GH₃ cells by Dean & Moyer (1988). In the absence of ATP there is, as in other cells, no Ins(1,3,4,5) P_4 formation, and > 99 % of the Ins(1,4,5) P_3 is metabolized to Ins(1,4) P_2 , which in turn is metabolized almost exclusively to Ins(4) P (Dean & Moyer, 1988). Thus the stepwise metabolism in GH₃ cells of Ins(1,4,5) P_3 , without the production of inositol phosphate isomers [such as Ins(1,3,4) P_3 , Ins(1,3) P_2 and Ins(3,4) P_2] found when the Ins-(1,4,5) P_3 3-kinase activity is permitted by the presence of ATP (Dean & Moyer, 1988; for review see Berridge & Irvine, 1989), allows the use of simple ion-exchange separation techniques. Most published studies of the Ins(1,4,5) P_3 5-phosphatase have used Dowex ion exchanger (see, e.g. Downes *et al.*, 1982; Rana *et al.*, 1986; Erneux *et al.*, 1986, 1989; Milani *et al.*, 1988). In the present study, however, Amprep SAX (100 mg) mini-columns have been used, with elution conditions similar to those reported by Minchin & Wood (1986), who used different SAX columns. The advantages of the use of the Amprep SAX columns is the low rate of elution of [3 H]Ins(1,4,5) P_3 , giving rise to low blank values. The extraction of the [3 H]inositol phosphates from the homogenates (which can be problematic in some assays; see Wreggett *et al.*, 1987) and the yields of the [3 H]Ins(1,4) P_2 , [3 H]Ins P and *myo*-[3 H]inositol from the mini-columns are essentially quantitative, although the presence of Ins(1,3,4,5) P_4 affected the recovery (see the Results section).

Validity of the assay was confirmed by the following observations.

(a) The dephosphorylation observed is stepwise, [3 H]Ins(1,4) P_2 formation preceding [3 H]Ins P formation, with *myo*-[3 H]inositol formation occurring only after long incubation times (Fig. 2).

(b) The K_m values for the observed Ins(1,4,5) P_3 5-phosphatase activity (see Table 1) are in line with the literature (see Shears, 1989).

(c) The dephosphorylation of [3 H]Ins(1,4) P_2 is inhibited by 2,3-bisphosphoglycerate, fructose 1,6- and -2,6-bisphosphates, *p*-hydroxymercuribenzoate, CdCl₂ and EDTA (Table 1), in agreement with the literature (see, e.g., Downes *et al.*, 1982; Seyfred *et al.*, 1984; Rana *et al.*, 1986; Erneux *et al.*, 1986; Hansen *et al.*, 1987; Milani *et al.*, 1988). Glucose 6-phosphate inhibited the activity only at concentrations > 5 mM, a result found also in erythrocyte ghosts, but not in rat enterocytes, where there is a considerable non-specific phosphatase activity inhibited by low glucose 6-phosphate concentrations (Rubiera *et al.*, 1988). *p*-Hydroxymercuribenzoate appeared to inhibit the IMR-32-cell 5-phosphatase activity to a lesser extent than the corresponding activities in rat brain and GH₃ cells. This difference was not affected by washing of the particulate fractions, but was decreased by dialysis of the soluble fractions (Table 2). The non-specific alkaline phosphatase inhibitor (–)-*p*-bromotetramisole oxalate (Borgers & Thone, 1975) was without effect on the Ins(1,4,5) P_3 5-phosphatase activity (Table 1).

(d) The Ins(1,4,5) P_3 5-phosphatase activity is found in both soluble and particulate fractions, whereas the Ins(1,4) P_2 phosphatase activity is essentially cytosolic in nature (Fig. 3), in agreement with the literature (for review, see Shears, 1989). The 5-phosphatase activity associated with the particulate fraction is

not due to large-scale contamination with the soluble enzyme, since washing of the membranes did not affect to any great degree the specific activity (or the inhibitor sensitivity) of the particulate enzyme.

The assay procedure used has two disadvantages. First, the Amprep columns cannot be re-used. Secondly, the assay cannot be used to measure the dephosphorylation of [3 H]Ins(1,3,4,5) P_4 by the 5-phosphatase, since the primary product, [3 H]Ins(1,3,4) P_3 , is unlikely to be eluted from the Amprep columns under the conditions used. Nevertheless, it is useful as a simple and accurate measure of [3 H]Ins(1,4,5) P_3 dephosphorylation by the 5-phosphatase enzymes.

There has been considerable interest in the literature with respect to the heterogeneity of Ins(1,4,5) P_3 5-phosphatase. Different K_m values for the 5-phosphatase activity towards Ins(1,4,5) P_3 have been reported for different preparations, although this may be due in part to differences in substrate batch quality (for review, see Shears, 1989). In the present study, where this problem is circumvented by the concomitant, rather than sequential, assay of the different preparations, similar K_m values for the particulate preparations were found (Table 1). However, 3-fold differences in the K_m values for the soluble 5-phosphatase activities were seen, values of 8–13, 22 and 26–32 μ M being found for GH₃-cell, rat cerebral-cortical and IMR-32-cell preparations respectively (Tables 1 and 2, Fig. 4). The values for the GH₃- and IMR-32-cell soluble activities correspond rather well to those found for the 45 kDa (Type I) and 75 kDa (Type II) forms of the soluble enzyme purified from human platelets (7.5 and 24 μ M respectively; Mitchell *et al.*, 1989), and are in line with the values reported by Erneux *et al.* (1989) for Type I and Type II soluble 5-phosphatase activities from bovine brain (11 and 72 μ M respectively) and by Hansen *et al.* (1987) for rat brain (3 and 18 μ M respectively). The higher K_m value for the soluble IMR-32-cell fractions, however, appears to be decreased upon dialysis, suggesting that the soluble fractions contain compounds (presumably organic or inorganic phosphates) at high enough concentrations to compete with the substrate for the enzyme active site. The presence of such compounds, however, would be expected to result in non-linearity between the rate of [3 H]Ins(1,4,5) P_3 dephosphorylation and the protein concentration/assay, which is not the case. The different pH profiles found for the GH₃- and IMR-32-cell soluble activities (Fig. 5) is in line with the finding of different pH optima (7.5 and 6.5–7.0 respectively) for soluble Type I and Type II activities purified from rat brain (Hansen *et al.*, 1987).

Although the findings described above suggest at first sight that the Type I and Type II soluble activities are differentially expressed in the GH₃ and IMR-32 cells, the experiments with Ins(1,3,4,5) P_4 (Table 2) would suggest that these differences are more likely species differences. This compound has a high affinity ($K_m \sim 1 \mu$ M) for the Type I enzyme, but a low affinity for the Type II enzyme (Hansen *et al.*, 1987; Mitchell *et al.*, 1989; Erneux *et al.*, 1989). By using non-radioactive Ins(1,3,4,5) P_4 to compete with [3 H]Ins(1,4,5) P_3 , similar inhibitions were found for GH₃- and IMR-32-cell soluble fractions (Table 2). The inhibition appears to reach a plateau at about 40–60 % for both cell types, which suggests similar contributions to the metabolism of 3.75 μ M-[3 H]Ins(1,4,5) P_3 by both 5-phosphatase types. Interestingly, the phosphocellulose extract has a lower K_m for [3 H]Ins(1,4,5) P_3 and is inhibited to a greater extent by Ins(1,3,4,5) P_4 (Table 2). Although the latter finding should be interpreted with caution, given the effects of Ins(1,3,4,5) P_4 on the 3 H extraction, it is possible that there is a selective enrichment of the Type I form by the phosphocellulose treatment, analogous with the separation of Ins(1,4,5) P_3 5-phosphatase and Ins(1,4) P_2 phosphatase activities by this procedure (see the Results section).

In conclusion, a simple assay for measurement and characterization of the soluble and particulate 5-phosphatase activities in rat brain, GH₃- and IMR-32-cell preparations has been described. The 5-phosphatase activity in the soluble GH₃ fractions appears to be qualitatively different from that in the soluble IMR-32 fractions, the former having a lower K_m for [³H]Ins(1,4,5)P₃, a wider pH optimum and a higher sensitivity to *p*-hydroxymercuribenzoate than the latter. Although some of these effects can be decreased on dialysis, they may reflect species variations in the properties of the 5-phosphatase enzymes.

REFERENCES

- Berridge, M. J. & Irvine, R. F. (1989) *Nature* (London) **341**, 197–205
 Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
 Borgers, M. & Thone, E. (1975) *Histochemistry* **44**, 277–280
 Dean, N. M. & Moyer, J. D. (1988) *Biochem. J.* **250**, 493–500
 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
 Erneux, C., Lemos, M., Verjans, B., Vanderhaeghen, P., Delvaux, A. & Dumont, J. E. (1989) *Eur. J. Biochem.* **181**, 317–322
 Hansen, C. A., Johanson, R. A., Williamson, M. T. & Williamson, J. R. (1987) *J. Biol. Chem.* **262**, 17319–17326
 Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature* (London) **320**, 631–634
 Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. G. (1978) *Anal. Biochem.* **87**, 206–210
 Milani, D., Volpe, P. & Pozzan, T. (1988) *Biochem. J.* **254**, 525–529
 Minchin, M. C. W. & Wood, M. D. (1986) *Br. J. Pharmacol.* **89**, 784P
 Mitchell, C. A., Connolly, T. M. & Majerus, P. W. (1989) *J. Biol. Chem.* **264**, 8873–8877
 Rana, R. S., Sekar, M. C., Hokin, L. E. & MacDonald, M. J. (1986) *J. Biol. Chem.* **261**, 5237–5240
 Rubiera, C., Velasco, G., Michell, R. H., Lazo, P. S. & Shears, S. B. (1988) *Biochem. J.* **255**, 131–137
 Seyfred, M. A., Farrell, L. E. & Wells, W. W. (1984) *J. Biol. Chem.* **259**, 13204–13208
 Shears, S. B. (1989) *Biochem. J.* **260**, 313–324
 Wreggett, K. A., Howe, L. R., Moore, J. P. & Irvine, R. F. (1987) *Biochem. J.* **245**, 933–934

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