A simple enzymic method to separate [^3H]inositol 1,4,5- and 1,3,4-trisphosphate isomers in tissue extracts

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A novel method to separate [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4) P_3 in tissue extracts is described. It is based on the selective metabolism of Ins(1,3,4) P_3 by a crude cerebral supernatant in a Mg^{2+} -free buffer followed by separation of [^3H]inositol trisphosphates using conventional anion-exchange chromatography. Evaluation of the assay was performed using [^3H]Ins(1,3,4) P_3 standards and tissue extracts containing different proportions of [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4) P_3 . Parallel h.p.l.c. separations of extracts established the selective and complete metabolism of [^3H]Ins(1,3,4) P_3 under the above conditions and demonstrated that the enzymic method provides an accurate estimate of the trisphosphate isomers in rat cerebral cortex, parotid gland and bovine tracheal smooth muscle.

INTRODUCTION

It has now been clearly established that Ins(1,4,5) P_3 is an intracellular second messenger linking the activation of several cell-surface receptors to the release of intracellular Ca^{2+} [1,2]. However, the metabolism of the inositol headgroup is complex and can proceed either by dephosphorylation to Ins(1,4) P_2 [3] or phosphorylation by a specific 3-kinase to generate Ins(1,3,4,5) P_4 [4] which can then give rise to another trisphosphate [Ins(1,3,4) P_3] by dephosphorylation [5]. Agonist stimulation of receptors linked to phosphoinositidase C therefore often generates a complex mixture of these inositol trisphosphate isomers in cells (see [2]), and since only Ins(1,4,5) P_3 is active in releasing Ca^{2+} from intracellular stores [6], separation of these isomeric species is crucial in order to interpret the significance of most experiments. To date this has only been achieved by labelling cells with [^3H]inositol and separating [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4) P_3 by anion-exchange h.p.l.c. [7]. These techniques are expensive, laborious and quite unsuitable for the assay of multiple samples. We describe here an alternative separation method based on our recent understanding of the cerebral metabolism of Ins(1,4,5) P_3 and Ins(1,3,4) P_3 . In the absence of Mg^{2+} , a selective dephosphorylation of Ins(1,3,4) P_3 can proceed via Ins(1,3) P_2 [8,9] and this provides a convenient enzymic method to selectively degrade and therefore separate Ins(1,3,4) P_3 from Ins(1,4,5) P_3 . A simple separation of trisphosphates on Dowex formate columns following incubation of crude extracts with active or heat-inactivated cerebral supernatants is shown to provide an accurate assessment of tissue [^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4) P_3 isomers that closely agrees with that obtained in parallel h.p.l.c. analysis.

MATERIALS AND METHODS

Tissue preparation

Tissue slices from rat cerebral cortex ($350\ \mu\text{m} \times 350\ \mu\text{m}$) [10], rat parotid gland ($150\ \mu\text{m} \times 150\ \mu\text{m}$) [11] and

bovine cervical trachealis muscle ($300\ \mu\text{m} \times 300\ \mu\text{m}$) [12] were prepared as described previously and preincubated for 60 min in oxygenated Krebs–Henseleit buffer at 37°C . In the cerebral cortex studies $50\ \mu\text{l}$ aliquots of gravity-packed slices were labelled with $5\ \mu\text{Ci}$ of *myo*-[2- ^3H]inositol for 60 min in $250\ \mu\text{l}$ of buffer and stimulated with $10^{-3}\ \text{M}$ -carbachol for 30 min. Parotid gland and tracheal smooth muscle slices [13] were labelled in bulk with $100\ \mu\text{Ci}$ and $50\ \mu\text{Ci}$ of *myo*-[2- ^3H]inositol/ml of packed slices respectively, and then stimulated for 30 min with either $10^{-3}\ \text{M}$ -carbachol in the presence of $10\ \text{mM}$ -lithium (parotid) or $10^{-4}\ \text{M}$ -carbachol in the absence of lithium (tracheal smooth muscle). Reactions were terminated with ice-cold $1\ \text{M}$ -trichloroacetic acid and the supernatants were treated with $5 \times 2\ \text{vol.}$ of water-saturated diethyl ether or tri-*n*-octylamine/1,1,2-trichloro-1,2,2-trifluoroethane (50:50, v/v) as described by Hawkins *et al.* [14] and adjusted to pH 7.0 with a Hepes/EDTA buffer (final concentrations: Hepes, $25\ \text{mM}$; EDTA, $5\ \text{mM}$).

Preparation of standards

[^3H]Ins(1,3,4) P_3 was prepared by dephosphorylation of authentic [^3H]Ins(1,3,4,5) P_4 with human erythrocyte membranes according to the method of Downes *et al.* [15]. [^3H]Ins(1,4,5) P_3 was supplied by New England Nuclear and was shown to be $> 98\%$ pure by h.p.l.c.

Assay conditions for selective hydrolysis of Ins(1,3,4) P_3

The enzyme preparation was routinely prepared as follows. Cerebral cortex from male Wistar rats was dissected on ice and homogenized ($3 \times 15\ \text{s}$ bursts with a Polytron tissue homogenizer) in ice-cold $25\ \text{mM}$ -Hepes/ $2\ \text{mM}$ - MgCl_2 , pH 7.4, at a concentration of 25% (w/v). Homogenates were centrifuged at $100\,000\ g$ for 90 min at 4°C and the supernatant was used as enzyme source. Preliminary data revealed that identical results could also be obtained using whole brain, and although fresh preparations were used in the present studies, supernatants stored at -20°C for up to 48 h still displayed activity. Supernatant was incubated with the neutralized

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tissue extracts or standards prepared in a buffer containing 25 mM-Hepes and 5 mM-EDTA in such proportions as to give a final supernatant concentration of 5% (w/v). Heat-inactivated brain supernatant used for control incubations was prepared by boiling at 100 °C for 20 min. Reactions were terminated at the time points indicated with ice-cold trichloroacetic acid to give a final concentration of 1 M, and the supernatants were extracted with diethyl ether as described above prior to neutralization with 5 mM-NaHCO₃.

The inositol phosphates were separated using Dowex AG 1-X8 anion-exchange resin (200–400 mesh, formate form) according to Batty *et al.* [5]. [³H]Ins(1,4,5)P₃ and [³H]Ins(1,3,4)P₃ isomers were separated by h.p.l.c. using a Partisil SAX column and ammonium phosphate elution gradient as described by Challiss *et al.* [16].

RESULTS AND DISCUSSION

In a previous detailed study [9] we have established that incubation of [³H]Ins(1,3,4)P₃ with cerebral supernatants in the presence of EDTA in molar excess over buffer Mg²⁺ concentrations results in the exclusive yield of [³H]Ins(1,3)P₂, which is further partially degraded to [³H]Ins(1)P₁. Under identical conditions neither [³H]Ins(1,4,5)P₃ nor [³H]Ins(1,3,4,5)P₄ undergoes significant hydrolysis [9]. This is consistent with previous observations [8,15] that [³H]Ins(1,3,4)P₃ can proceed by Mg²⁺-dependent and -independent routes yielding Ins(3,4)P₂ and Ins(1,3)P₂ respectively. Data in Table 1 demonstrate that under conditions in which known mixtures of standard [³H]Ins(1,3,4)P₃ and [³H]Ins(1,4,5)P₃ are incubated with cerebral supernatant in the absence of Mg²⁺, a selective and quantitative hydrolysis of [³H]Ins(1,3,4)P₃ occurs. Although encouraging, these results needed to be extended to tissue extracts containing different proportions of [³H]Ins(1,4,5)P₃ and [³H]Ins(1,3,4)P₃ as well as other potential sources of inositol trisphosphates.

In the parotid gland, muscarinic receptor stimulation results in the formation of both Ins(1,4,5)P₃ and Ins(1,3,4)P₃, although the latter accumulates much more markedly, albeit with somewhat slower kinetics [7]. In the present experiments, extracts of rat parotid gland slices prelabelled with [³H]inositol and stimulated with the muscarinic agonist carbachol in the presence of

Fig. 1. Hydrolysis of [³H]InsP₃ in rat parotid gland

Parotid gland extracts were treated as described in the Materials and methods section with either 200 µl of active (●) or heat-inactivated (○) supernatant prepared from rat cerebral homogenate. Incubations were performed for 15, 30, 45 or 60 min at 37 °C and then terminated with 1 M-trichloroacetic acid. Data are means ± S.E.M. from three experiments. In the same sample, h.p.l.c. analysis demonstrated that in heat-inactivated controls, 88% of the trisphosphate fraction coeluted with internal Ins(1,3,4)P₃ standard whilst 12% ran with Ins(1,4,5)P₃. After treatment with active enzyme as above, all of the Ins(1,3,4)P₃ was hydrolysed, whilst the levels of Ins(1,4,5)P₃ were unaffected (results not shown).

10 mM-Li⁺ were subjected to anion-exchange h.p.l.c. and revealed that > 90% of trisphosphate eluted was Ins(1,3,4)P₃. Incubation of these extracts with cerebral supernatant in the absence of Mg²⁺ led to the complete loss of this trisphosphate isomer with no significant effect on the concentration of [³H]Ins(1,4,5)P₃. Samples from the same extract were also treated with cerebral supernatant and at various times examined for their content of [³H]InsP₃ by Dowex chromatography. The results reveal that a loss of 91% of this trisphosphate fraction closely parallels the 88% loss revealed by h.p.l.c. and provides an excellent estimate of the proportion of the [³H]Ins(1,3,4)P₃ and [³H]Ins(1,4,5)P₃ isomers (Fig. 1). A careful comparison between enzymic and h.p.l.c. methods of separation was also performed in bovine tracheal smooth muscle (Table 2). In this tissue there was also a close agreement between the h.p.l.c. and enzymic estimates of the proportion of [³H]Ins(1,3,4)P₃ in the total [³H]InsP₃ fraction.

In contrast with those tissues, cerebral cortical slices of the rat stimulated with the muscarinic agonist carbachol have previously been shown to accumulate a higher proportion of [³H]Ins(1,4,5)P₃ [5,9,14]. Data from the present experiments (Fig. 2) confirm this, in that h.p.l.c. analysis of extracts prepared from slices stimulated for 30 min with carbachol revealed that 36% of the total [³H]InsP₃ fraction eluted as [³H]Ins(1,3,4)P₃ and that selective loss of this peak was observed after treatment of crude extracts with cerebral supernatant in the absence of Mg²⁺. As shown, identical samples of this extract separated on Dowex columns revealed that 34.5 ± 4.8% of the trisphosphate fraction was hydrolysed. This again

Table 1. [³H]Ins(1,3,4)P₃ and [³H]Ins(1,4,5)P₃ standards: hydrolysis with enzyme treatment

Authentic tritiated standards prepared in a variety of mixtures were treated with active enzyme from rat cerebral homogenate for a period of 30 min at 37 °C as described in the Materials and methods section. Data are means ± S.E.M. from three experiments.

[³ H]Ins(1,3,4)P ₃ in mixture (%)	[³ H]Ins(1,4,5)P ₃ in mixture (%)	Observed hydrolysis (%)
100	0	96.4 ± 1.7
75	25	77.6 ± 3.3
50	50	48.7 ± 1.0
25	75	26.6 ± 1.6
0	100	3.6 ± 1.1

Table 2. Determination of [^3H]Ins(1,3,4) P_3 and [^3H]Ins(1,4,5) P_3 in bovine tracheal smooth muscle slices using h.p.l.c. and enzyme treatment

Aliquots (100 μl) of *myo*-[^3H]inositol-labelled, gravity-packed smooth muscle slices were stimulated for 30 min with 10^{-4} M-carbachol or buffer in a final volume of 300 μl . Neutralized tissue extracts were subjected to h.p.l.c. analysis or incubated with active or heat-inactivated rat cerebral supernatant followed by re-extraction and separation of total [^3H]Ins P_3 as described in the Materials and methods section. Data represent the means $\pm$ S.E.M. of three separate experiments.

(a) H.p.l.c. analysis

	[^3H]Ins(1,3,4) P_3 (d.p.m./2 ml of extract)	[^3H]Ins(1,4,5) P_3 (d.p.m./2 ml of extract)	[^3H]Ins(1,3,4) P_3 (%)
Control	14 $\pm$ 14	253 $\pm$ 58	4.3 $\pm$ 2.3
Carbachol (10^{-4} M)	6049 $\pm$ 72	1208 $\pm$ 24	83.4 $\pm$ 0.8

(b) Enzyme analysis

	[^3H]Ins P_3 (inactive enzyme) (d.p.m./2 ml of extract)	[^3H]Ins P_3 (active enzyme) (d.p.m./2 ml of extract)	[^3H]Ins(1,3,4) P_3 (%)
Control	324 $\pm$ 206	393 $\pm$ 73	0
Carbachol (10^{-4} M)	6441 $\pm$ 668	1257 $\pm$ 186	80.7 $\pm$ 1.0

provides strong evidence of the reliability and accuracy of this simple method.

Results from the present experiments using three different tissue extracts demonstrate that selective metabolism of [^3H]Ins(1,3,4) P_3 can be achieved using crude cerebral supernatants under Mg^{2+} -free conditions. This has provided an accurate assay of Ins(1,3,4) P_3 and Ins(1,4,5) P_3 isomers using simple Dowex chromatography that closely agrees with h.p.l.c. analyses. This alone provides confidence that our results are not complicated by the enzymic generation of trisphosphates from other sources.

One of these sources could be Ins(1,3,4,5) P_4 , since recent studies by Doughney *et al.* [18] have suggested that a 3-phosphatase present in human erythrocytes provides a novel mechanism for the generation of Ins(1,4,5) P_3 . Their data suggests that this reaction can proceed, albeit slowly, in the absence of Mg^{2+} . In contrast, Hoer *et al.* [19] have shown that the same enzyme activity in porcine cerebral cytosol is only observed in the presence

Fig. 2. H.p.l.c. analyses of [^3H]Ins(1,3,4) P_3 and [^3H]Ins(1,4,5) P_3 in rat cerebral cortex

Rat cortex extracts were treated as described in the Materials and methods section with either active (b) or heat-inactivated (a) supernatants prepared from rat cerebral homogenate. H.p.l.c. analysis of the trisphosphate region demonstrated that in this experiment, representative of several conducted, 64 % was present as Ins(1,4,5) P_3 and 36 % as Ins(1,3,4) P_3 and that after 30 min of incubation with active supernatant, complete removal of the latter was achieved. Under the same conditions the sample, when separated by Dowex anion-exchange chromatography, showed that $34.5 \pm 2.52\%$ of [^3H]Ins P_3 was hydrolysed by active supernatant within 30 min (results not shown).

of high concentrations of Mg^{2+} . In agreement with the latter observations, our routine elution of $[^3H]InsP_4$ from tissue extracts has never displayed statistically significant loss with enzyme treatment. Similarly, extracts of unlabelled cerebral slices 'spiked' with authentic $[^3H]Ins(1,3,4,5)P_4$ revealed, at most, 3% conversion into a trisphosphate when incubated for 30 min with cerebral supernatants in the absence of Mg^{2+} (results not shown).

Another potential source of trisphosphates that could complicate analyses are the higher phosphates, $InsP_5$ and $InsP_6$. Significant labelling of these higher phosphates is unlikely in short-term incubations with $[^3H]$ inositol described in this study. Indeed, $[^3H]InsP_5$ and $[^3H]InsP_6$ were not detected in cerebral cortical slices labelled and incubated under identical conditions to those reported here [9]. On the other hand, extracts from NG108-15 neuroblastoma-glioma cells incubated with $[^3H]$ inositol for 48 h incorporate label into $InsP_5$ and $InsP_6$ in approximately a 1:3 proportion (I. H. Batty, J. G. Baird & S. R. Nahorski, unpublished work). However, these higher phosphates were totally resistant to hydrolysis when extracts of NG108-15 cells were incubated with cerebral supernatants in the absence of Mg^{2+} (results not shown).

In conclusion, we describe a relatively simple enzymic method to separate $[^3H]Ins(1,3,4)P_3$ and $[^3H]Ins(1,4,5)P_3$ isomers in crude extracts of several tissues. The method produces data that closely correspond to that observed in the same extracts using h.p.l.c. The accuracy of the method, of course, depends upon the extent and relative proportion of labelling of the trisphosphates, and it would be clearly advisable initially to compare h.p.l.c. separations with the enzymic method in each new cellular system examined. Nevertheless, the present study suggests that the potential sources of trisphosphates that could complicate this analysis [$Ins(1,3,4,5)P_4$, $InsP_5$ and $InsP_6$] do not undergo significant hydrolysis under the defined conditions described here. This enzymic separation therefore provides a relatively sensitive method that allows the assay of multiple samples without the laborious (and expensive) h.p.l.c. approach.

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REFERENCES

1. Streb, H., Irvine, R. F., Berridge, M. J. & Schultz, I. (1983) *Nature* (London) **306**, 67–69
2. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
3. Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) *Nature* (London) **312**, 374–376
4. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature* (London) **320**, 631–634
5. Batty, I. H., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
6. Strupish, J., Cooke, A. M., Potter, B. V. L., Gigg, R. & Nahorski, S. R. (1988) *Biochem. J.* **253**, 901–905
7. Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
8. Inhorn, R. C., Bansal, V. S. & Majerus, P. W. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2170–2174
9. Batty, I. H., Letcher, A. J. & Nahorski, S. R. (1989) *Biochem. J.* **258**, 23–32
10. Brown, E., Kendall, D. A. & Nahorski, S. R. (1984) *J. Neurochem.* **42**, 1379–1387
11. Hanley, M. R., Lee, C. M., Jones, L. H. & Michell, R. H. (1980) *Mol. Pharmacol.* **18**, 78–83
12. Hall, I. P. & Hill, S. J. (1988) *Br. J. Pharmacol.* **95**, 1204–1212
13. Chilvers, E. R., Barnes, P. J. & Nahorski, S. R. (1982) *Br. J. Pharmacol.* **96**, 778P
14. Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
15. Downes, C. P., Hawkins, P. T. & Irvine, R. F. (1986) *Biochem. J.* **238**, 501–506
16. Challiss, R. A. J., Batty, I. H. & Nahorski, S. R. (1988) *Biochem. Biophys. Res. Commun.* **157**, 684–691
17. Batty, I. H. & Nahorski, S. R. (1987) *Biochem. J.* **247**, 797–800
18. Doughney, C., McPherson, M. A. & Dormer, R. L. (1988) *Biochem. J.* **251**, 927–929
19. Hoer, D., Kwiatkowski, A., Seib, C., Rosenthal, N., Schultz, G. & Oberdisse, E. (1988) *Biochem. Biophys. Res. Commun.* **154**, 668–675

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