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Accumulation of inositol polyphosphate isomers in agonist-stimulated cerebral-cortex slices

Comparison with metabolic profiles in cell-free preparations

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1. Basal and carbachol-stimulated accumulations of isomeric [^{3}H]inositol mono-, bis-, tris- and tetrakis-phosphates were examined in rat cerebral-cortex slices labelled with *myo*-[2-^{3}H]inositol. 2. In control samples the major [^{3}H]inositol phosphates detected were co-eluted on h.p.l.c. with Ins(1)*P*, Ins(4)*P* (inositol 1- and 4-monophosphate respectively), Ins(1,4)P_2 (inositol 1,4-bisphosphate), Ins(1,4,5)P_3 (inositol 1,4,5-trisphosphate) and Ins(1,3,4,5)P_4 (inositol 1,3,4,5-tetrakisphosphate). 3. After stimulation to steady state with carbachol, accumulation of each of these products was markedly increased. 4. Agonist stimulation, however, also evoked much more dramatic increased accumulations of a second [^{3}H]inositol trisphosphate, which was co-eluted on h.p.l.c. with authentic Ins(1,3,4)P_3 (inositol 1,3,4-trisphosphate) and of three further [^{3}H]inositol bisphosphates {[^{3}H]InsP_2(s)}. 5. Examination of the latter by chemical degradation by periodate oxidation and/or h.p.l.c. allowed identification of these as [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(3,4)P_2 and [^{3}H]Ins(4,5)P_2 (inositol 1,3-, 3,4- and 4,5-bisphosphates respectively), which respectively accounted for about 22%, 8% and 3% of total [^{3}H]InsP_2 in extracts from stimulated tissue slices. 6. By using a h.p.l.c. method which clearly resolves Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 (inositol 1,3,4,6-tetrakisphosphate), only the former isomer could be detected in extracts from either control or stimulated tissue slices. Similarly, [^{3}H]inositol pentakis- and hexakis-phosphates were not detectable either in the presence or absence of carbachol under the radiolabelling conditions described. 7. The catabolism of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 by cell-free preparations from cerebral cortex was also studied. 8. In the presence of Mg^{2+}, [^{3}H]Ins(1,4,5)P_3 was specifically dephosphorylated via [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(4)*P* to free [^{3}H]inositol, whereas [^{3}H]Ins(1,3,4)P_3 was degraded via [^{3}H]Ins(3,4)P_2 and, to a lesser extent, via [^{3}H]Ins(1,3)P_2 to D- and/or L-[^{3}H]Ins(1)*P* and [^{3}H]inositol. 9. In the presence of EDTA, hydrolysis of [^{3}H]Ins(1,4,5)P_3 was $\geqslant 95\%$ inhibited, whereas [^{3}H]Ins(1,3,4)P_3 was still degraded, but yielded only a single [^{3}H]InsP_2 identified as [^{3}H]Ins(1,3)P_2. 10. The significance of these observations with cell-free preparations is discussed in relation to the proportions of the separate isomeric [^{3}H]inositol phosphates measured in stimulated tissue slices.

INTRODUCTION

Agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) by phosphoinositidase C releases the two putative second messengers, diacylglycerol and Ins(1,4,5)P_3 [1]. It is now clear that metabolism of the inositol head-group is complex, proceeding by two different routes. One pathway involves initial removal of the 5-monoester phosphate [2–5], producing Ins(1,4)P_2, which is subsequently degraded to free inositol through a monophosphate intermediate, identified by conflicting reports as either Ins(1)*P* and/or Ins(4)*P* [6–12]. The alternative metabolic route for Ins(1,4,5)P_3 involves phosphorylation by a specific 3-kinase which generates Ins(1,3,4,5)P_4 [13]. Although both of these pathways may serve to inactivate the Ins(1,4,5)P_3 Ca^{2+}-mobilizing signal, the second may have an additional function(s), since Ins(1,3,4,5)P_4 appears able to modulate the actions of Ins(1,4,5)P_3 in several systems [14–16].

Degradation of Ins(1,3,4,5)P_4 occurs via initial dephosphorylation to Ins(1,3,4)P_3 [17–19]. The subsequent steps, however, remain uncertain since in some tissues Ins(1,3,4)P_3 may be re-phosphorylated to Ins(1,3,4,6)P_4 [20,21] or catabolized to InsP_2. Studies with broken-cell or partially purified enzyme preparations from several tissues suggest that the predominant dephosphorylation product is Ins(3,4)P_2 [22,8,9,23]. However, Irvine *et al.* [24] have also identified Ins(1,3)P_2 in extracts from GH_4 cells, and the same bisphosphate is a minor product of Ins(1,3,4)P_3 dephosphorylation by liver and GH_3-cell homogenates [25,23]. Similarly, Bansal *et al.* [10] have shown that Ins(1,3)P_2 can be

Abbreviations used: Ins, InsP_1, InsP_2, InsP_3, InsP_4, InsP_5 and InsP_6, *myo*-inositol and its mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphate derivatives, with assignment of phosphate locants where appropriate and specification of D or L-enantiomers where essential; Ins-1,2-cyclic(4,5)P_3, *myo*-inositol 1,2-cyclic(4,5)trisphosphate; PtdIns, PtdIns*P* and PtdInsP_2, phosphatidylinositol and its mono- and bis-phosphate derivatives, with assignment of phosphate locants where appropriate; Gro*P*Ins, Gro*P*Ins*P* and Gro*P*InsP_2, glycerophosphoinositol and its mono- and bis-phosphate derivatives.

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derived from Ins(1,3,4)P_3 on incubation with calf brain extracts, but only forms the predominant product under non-physiological conditions.

In view of the current uncertainty about the precise routes of metabolism for inositol polyphosphates and the present lack of information about accumulation of inositol phosphate isomers in brain, we have compared the catabolic profiles of several radiolabelled substrates after incubation with cell-free preparations from cerebral cortex with the relative proportions of the separate isomeric [^{3}H]inositol phosphates which accumulate in [^{3}H]inositol-labelled cerebral-cortex slices during muscarinic-receptor stimulation. The results suggest that the 5-phosphatase and 3-kinase pathways responsible for initial metabolism of receptor-generated Ins(1,4,5)P_3 ultimately yield mutually exclusive products. Separate estimation of these in stimulated tissue slices indicates a marked muscarinic-receptor activation of the tris-/tetrakis-phosphate pathway.

MATERIALS AND METHODS

Materials

D-*myo*[2-^{3}H]Inositol 1,3,4,5-tetrakisphosphate (1 Ci/mmol) and D-*myo*-[2-^{3}H]inositol 1,4,5-trisphosphate (3.3 Ci/mmol) were generously given by NEN/Du Pont. Phosphatidyl[1,2-^{3}H]inositol 4,5-bisphosphate (2 Ci/mmol), phosphatidyl[1,2-^{3}H]inositol 4-monophosphate (2 Ci/mmol) and *myo*-[2-^{3}H]inositol (12–20 Ci/mmol) were also obtained from NEN. L-*myo*-[U-^{14}C]Inositol 1-monophosphate (55 mCi/mmol), D-*myo*-inositol 1,4,5[5-^{32}P]trisphosphate (1000 Ci/mmol), *myo*-[U-^{14}C]-inositol (270 mCi/mmol) and phosphatidyl[2-^{3}H]inositol (17.1 Ci/mmol) were from Amersham. AG 1 X8 resin (200–400 mesh, formate form) was from Bio-Rad. Nucleotides were from Sigma. All other reagents were from Fisons, Aldrich or Sigma.

Studies with [^{3}H]inositol-labelled brain slices

Cerebral-cortex slices (350 μm × 350 μm) were prepared from male Wistar rats and preincubated in bulk for 60 min in modified Krebs–Henseleit buffer (containing 1.3 mM-$CaCl_2$) as previously described [26]. Samples (50 μl) of gravity-packed tissue were then labelled with *myo*-[2-^{3}H]inositol (5 μCi) for a further 60 min in a final volume of 300 μl of buffer and subsequently challenged with agonist and extracted for ^{3}H-containing products exactly as previously described [17,27]. For most experiments triplicate tissue extracts were pooled before analysis of the reaction products by h.p.l.c. to facilitate detection of low concentrations of some [^{3}H]inositol polyphosphates. For preparative purposes, this procedure was scaled up 10-fold, and crude inositol mono-, bis-, tris- and tetrakis-phosphate fractions were isolated by Dowex anion-exchange chromatography and subsequently desalted as described by Batty *et al.* [17] before further analysis.

Studies with cell-free preparations

Cerebral cortex from at least three male Wistar rats was dissected on ice, pooled and homogenized (3 × 15 s bursts with a Polytron tissue homogenizer) in 10 ml of buffer containing 100 mM-KCl, 20 mM-NaCl, 2 mM-$MgCl_2$ and 25 mM-Hepes, adjusted to pH 7.4 with KOH. The homogenate obtained was diluted to 60 ml with the buffer, and a crude supernatant fraction was isolated by centrifugation of 30 ml of this preparation at 100000 *g* for 90 min at 4 °C. Homogenates and supernatants were then stored on ice until use the same day.

Assays of inositol polyphosphate hydrolysis were performed in a final volume of 100 μl comprising 60–70 μl of the buffer (above), 20 μl of tissue homogenate or supernatant and 10–20 μl of radiolabelled substrate dissolved in buffer. Reactions were started by addition of tissue extract and continued for the indicated times before addition of 100 μl of ice-cold 1 M-trichloroacetic acid and 10 μl of 5% (w/v) bovine serum albumin. Precipitated protein was removed by centrifugation, and a portion (150 μl) of the supernatant freed of acid by washing with 4 × 1 ml of water-saturated diethyl ether, then adjusted to pH 6–7 by dilution to 2 ml with 5 mM-$NaHCO_3$. Radiolabelled reaction products were analysed by h.p.l.c.

Preparation of [^{3}H]inositol phosphate standards

[^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,3,4)P_3 were prepared from authentic [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 respectively by incubation of the latter substrates with human erythrocyte membranes as previously described [17]. [^{3}H]Ins(1)*P* was prepared in mixture with [^{3}H]Ins(2)*P* and [^{3}H]inositol by incubation of [^{3}H]PtdIns with a crude preparation of brain phospholipase C as previously described [28], followed by extraction of the products into trichloroacetic acid and neutralization as above. [^{3}H]Ins(1)*P* in the reaction mixture was identified by co-elution on h.p.l.c. with authentic [^{14}C]Ins(3)*P*. [^{3}H]Ins(4)*P* in mixture with [^{3}H]Ins(1,4)P_2 and [^{3}H]-Ins(2,4)P_2, and [^{3}H]Ins(4,5)P_2 in mixture with [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]Ins(2,4,5)P_3, were prepared by separate alkaline hydrolysis of authentic [^{3}H]PtdIns*P* and [^{3}H]PtdInsP_2 respectively as described by Grado & Ballou [29]. Identification of the ^{3}H-containing products was by analogy with ref. [29] and by a process of elimination from co-elution on h.p.l.c. of authentic standards prepared by the other methods above. [^{3}H]-Gro*P*Ins, [^{3}H]Gro*P*Ins*P* and [^{3}H]Gro*P*InsP_2 were prepared either by deacylation of the corresponding authentic [^{3}H]phosphoinositides or by deacylation of a total [^{3}H]inositol-labelled phospholipid extract from [^{3}H]inositol-labelled brain slices as described by Wells & Dittmer [30].

Ins(1,3,4,5[5-^{32}P])P_4 was prepared from authentic Ins(1,4,5[5-^{32}P])P_3 exactly as described by Irvine *et al.* [13]. [^{3}H]Ins(1,3,4,6)P_4 was prepared from authentic [^{3}H]-Ins(1,3,4)P_3 by a modification of the method described by Shears *et al.* [20]. Briefly, whole rat liver was excised and homogenized in 250 mM-sucrose/5 mM-Hepes, pH 7.2, to give a final concentration of 30% (w/v). [^{3}H]Ins(1,3,4)P_3 at approx. 40 nM was then incubated at 37 °C for 10 min with 20 μl of this preparation in a final volume of 200 μl of 50 mM-Tris/maleate buffer (pH 7.5) containing 10 mM-ATP and 20 mM-$MgCl_2$ as previously described for phosphorylation of Ins(1,4,5)P_3 [13]. The reaction was stopped by addition of ice-cold 1 M-trichloroacetic acid and a neutral extract obtained as described above. [^{3}H]InsP_4 present in the extract was identified by its retention on h.p.l.c. relative to standard Ins(1,3,4,5[5-^{32}P])P_4 and is identified as [^{3}H]Ins(1,3,4,6)P_4 by analogy with the results of Shears *et al.* [20].

Separation of [^{3}H]inositol phosphates by anion-exchange h.p.l.c.

H.p.l.c. analyses of [^{3}H]inositol phosphates were performed by a modification of the method described by Dean & Moyer [31]. Separation was achieved with a Partisil (10 μM) SAX analytical column (Technicol) equipped with a pre-column packed with Whatman pellicular anion-exchange resin and eluted with gradients comprising water and $(NH_4)H_2PO_4$, adjusted to pH 3.7 with H_3PO_4. After sample injection (2 ml), free [^{3}H]-inositol was eluted by washing the column for 5–15 min with water, depending on the extent of sample radiolabelling. [^{3}H]Inositol phosphates were then separated by applying three consecutive gradients at a flow rate of 1 ml/min. Gro*P*Ins and InsP_1(s) were resolved by applying a linear gradient of 0–60 mM-$(NH_4)H_2PO_4$ over 30 min. Gro*P*Ins*P* and InsP_2(s) were then separated by isocratic elution at 190 mM-$(NH_4)H_2PO_4$ for 15 min, followed by a linear increase in eluent concentration to 300 mM over a further 15 min. Gro*P*InsP_2 and InsP_3(s) were separated by isocratic elution for 35 min at 500 mM-$(NH_4)H_2PO_4$. Ins(1,3,4,5)-P_4 was then displaced from the column by a 15 min wash at 1.4 M-$(NH_4)H_2PO_4$. A further modification of this procedure used exclusively for the resolution of isomeric inositol tetrakisphosphates is described in the legend to Fig. 2.

Identification of sample [^{3}H]inositol phosphates was based on co-elution with authentic ^{3}H-labelled standards in separate runs and/or by co-elution with internal ^{14}C- or ^{32}P-labelled inositol phosphate standards (see below). [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(1,4)P_2, and [^{3}H]Ins(3,4)P_2 were also identified chemically as described below. For routine analysis of samples by h.p.l.c., fractions (0.5–1.0 min) were collected only across the chromatographic windows appropriate for Ins, InsP_1(s), InsP_2(s), InsP_3(s) and InsP_4. To overcome problems associated with variations in retention times either between columns or due to column aging, samples were routinely spiked with 50–100 nmol each of adenosine and guanosine mono-, di-, tri- and tetra-phosphates before injection. Nucleotides were detected by continuous u.v. monitoring of the column eluate at 254 nm. Typically InsP_1(s) were eluted at retention times intermediate to AMP and GMP, InsP_2(s) between ADP and GDP (except [^{3}H]Ins(4,5)P_2, which ran 1–2 min after GDP), InsP_3(s) between ATP and GTP, and InsP_4 with the tetra-phosphates, thus providing a means of standardizing each run. For a similar purpose, [^{3}H]inositol-labelled extracts were also routinely spiked with a mixture of [^{14}C]InsP_1(s), [^{14}C]InsP_2(s) and [^{14}C]InsP_3 prepared from carbachol-stimulated rat parotid glands as described below. The isomeric composition of the latter mixture was defined by prior co-elution of ^{14}C-labelled extracts with authentic [^{3}H]inositol phosphate standards prepared as above.

Quantification of ^{3}H and ^{14}C or of ^{3}H and ^{32}P in column eluates was performed by dual-label scintillation counting under conditions where cross-nuclide spill was $\leqslant 1\%$. Counting efficiency was about 35% for ^{3}H and $> 90\%$ for ^{14}C.

Preparation of [^{14}C]inositol phosphates

[^{14}C]Inositol phosphates were prepared from carbachol-stimulated rat parotid-gland fragments labelled with *myo*-[U-^{14}C]inositol and used both as internal chromatographic standards and as a source of [^{14}C]Ins(1,3,4)P_3 for studies of inositol polyphosphate hydrolysis. Rat parotid glands were chosen for this purpose because of the high proportion of Ins(1,3,4)P_3 generated [32,33]. Briefly, glands were dissected from three male Wistar rats and chopped at 150 μm intervals in three directions at 60° by using a McIlwain chopper as previously described [34]. Pooled tissue was preincubated in bulk in 100 ml of modified Krebs–Henseleit buffer for 60 min as described above for brain slices; then 1.5–2.0 ml of gravity-packed tissue was incubated for a further 90 min in a total volume of 6 ml of buffer supplemented with 4–5 μCi of *myo*-[U-^{14}C]inositol and medium freshly oxygenated with O_2/CO_2 (19:1) at 15 min intervals. Carbachol and LiCl were then added to final concentrations of 1 mM and 10 mM respectively, and tissue was incubated for 45 min. The reaction was terminated by addition of an equal volume of ice-cold 1 M-trichloroacetic acid, and tissue fragments were sedimented by centrifugation. The resultant supernatant was neutralized as described above, and [^{14}C]inositol, InsP_1(s), InsP_2(s), InsP_3(s) and InsP_4(s) fractions were isolated by Dowex chromatography as previously described [17]. The mono-, bis- and tris-phosphate fractions were desalted as described in ref. [18] and individually analysed by h.p.l.c. in the presence of appropriate ^{3}H-labelled standards.

Two similar preparations yielded approx. 1.3×10^6 d.p.m. of [^{14}C]InsP_1, $0.2(-0.25) \times 10^6$ d.p.m. of [^{14}C]InsP_2 and $0.15(-0.20) \times 10^6$ d.p.m. of [^{14}C]InsP_3. The [^{14}C]InsP_4 fraction contained $\leqslant 10\%$ of the radiolabel present as InsP_3. Analysis by h.p.l.c. revealed two major [^{14}C]InsP_1(s), which co-eluted with authentic [^{3}H]Ins(1)P ($\sim 85\%$) and [^{3}H]Ins(4)P ($\sim 15\%$), two major [^{14}C]-InsP_2(s) which co-eluted with standard [^{3}H]Ins(1,4)P_2 ($\sim 70\%$) and with a [^{3}H]InsP_2 extracted from brain slices and identified below as [^{3}H]Ins(1,3)P_2 ($\sim 30\%$), and a single major [^{14}C]InsP_3 which co-eluted with [^{3}H]Ins-(1,3,4)P_3 and accounted for $\geqslant 95\%$ of the total labelled InsP_3. Several hundred d.p.m. of each total fraction was routinely added to [^{3}H]inositol-labelled tissue extracts before h.p.l.c. analysis in order to provide internal standards.

Chemical degradation of inositol phosphates and identification of resultant alditols

The conditions used for periodate oxidation of [^{3}H]-inositol phosphates and for subsequent reduction and dephosphorylation to yield the corresponding [^{3}H]-alditols and the methods used for chromatographic identification of these products were exactly as previously described [24].

RESULTS AND DISCUSSION

Earlier studies from our laboratory have demonstrated that exposure of [^{3}H]inositol-labelled cerebral-cortex slices to carbachol evokes a marked and sustained muscarinic-receptor-mediated hydrolysis of [^{3}H]inositol phospholipids [35,27,36]. The initial phase of this response appears to involve phosphoinositidase C-catalysed cleavage of polyphosphoinositides, as characterized by a rapid accumulation of [^{3}H]Ins(1,4,5)P_3 and its metabolite, [^{3}H]Ins(1,3,4,5)P_4 [17]. In stimulated intact cells of various types it appears that metabolism of the latter molecules is complex, resulting in produc-

Table 1. Control and carbachol-stimulated steady-state accumulations of isomeric [^{3}H]inositol phosphates in cerebral-cortex slices

Cerebral-cortex slices were labelled with [^{3}H]inositol for 60 min, then further incubated with or without 1 mM-carbachol for 30 min before extraction and separation of the indicated [^{3}H]inositol phosphates as described in the Materials and methods section. Identification of reaction products was as described in the text. Results represent means ± S.E.M. derived from five independent experiments, and are expressed either as radioactivity accumulated as each ^{3}H-labelled product in the absence (−) or the presence (+) of agonist (given as d.p.m./50 μl tissue sample) or as the percentage of the total separate [^{3}H]InsP_1, [^{3}H]InsP_2, [^{3}H]InsP_3 or [^{3}H]InsP_4 fractions contributed by the individual isomers resolved.

		Radioactivity (d.p.m.)		Stimulation	Fraction composition (%)	
Ins$P$	Agonist...	(−)	(+)	(fold)	(−)	(+)
InsP_1(s)						
Ins(1)P		5199 ± 618	84211 ± 9103	16.2	70.5 ± 2.7	88.3 ± 0.3
Ins(2)P		587 ± 44	1380 ± 78	2.4	8.2 ± 0.6	1.5 ± 0.1
Ins(4)P		1515 ± 145	9773 ± 1104	6.5	21.3 ± 2.5	10.2 ± 0.2
InsP_2(s)						
Ins(1,3)P_2†		58 ± 32	8121 ± 927	140.0	1.5 ± 0.8	22.3 ± 0.2
Ins(1,4)P_2		3908 ± 249	24414 ± 2763	6.2	94.8 ± 2.3	67.1 ± 0.2
Ins(3,4)P_2†		146 ± 56	2754 ± 271	18.9	3.6 ± 1.4	7.7 ± 0.3
Ins(4,5)P_2†		6 ± 6	1083 ± 158	180.0	0.1 ± 0.1	2.9 ± 0.1
InsP_3(s)						
Ins(1,3,4)P_3†		56 ± 27	2938 ± 289	52.5	2.5 ± 1.1	27.3 ± 0.5
Ins(1,4,5)P_3		2652 ± 380	7486 ± 608	2.8	94.5 ± 2.1	70.0 ± 0.4
Ins(2,4,5)P_3		88 ± 41	287 ± 27	3.3	3.0 ± 1.4	2.7 ± 0.3
InsP_4(s)						
Ins(1,3,4,5)P_4*		1494 ± 217	23119 ± 1903	15.5	⩾ 90	⩾ 98

* [^{3}H]Ins(1,3,4,5)P_4 was the only detectable isomer (see the text).
† Basal labelling not detectable on some occasions.

tion of multiple, isomeric, lower inositol phosphates [24,31,23,37–39]. Similar complexity of inositol polyphosphate metabolism in brain is indicated by recent studies with cell-free preparations [8–10], but has not previously been demonstrated directly in intact tissue under conditions of receptor activation. We have therefore used a recently introduced and improved h.p.l.c. technique in combination with an established method of chemical degradation by periodate oxidation to achieve a detailed characterization of the water-soluble [^{3}H]phosphoinositide-hydrolysis products released in response to carbachol in [^{3}H]inositol-labelled brain slices.

When cerebral-cortical slices are labelled with [^{3}H]-inositol under the current conditions and exposed to 1 mM-carbachol, maximal stimulated accumulations of [^{3}H]InsP_1, [^{3}H]InsP_2, [^{3}H]InsP_3 and [^{3}H]InsP_4 are achieved within 5–15 min, and are subsequently maintained for up to at least 45 min, but rapidly return to near-basal values on addition of the muscarinic-receptor antagonist atropine [35,27]. These observations show that, over this time course, steady-state stimulated concentrations reflecting the continuous synthesis and degradation of each [^{3}H]-inositol phosphate fraction are achieved (see [27]). In order to obtain a preliminary estimate of the isomeric composition of these fractions under such conditions, we therefore chose to examine the effects of a 30 min stimulation with 1 mM-carbachol in the current study.

Studies in cerebral-cortex slices

The results of several experiments comparing control and agonist-stimulated [^{3}H]inositol phosphate accumulations are summarized in Table 1 and Fig. 1. The latter illustrates a typical h.p.l.c. separation of the acid-soluble radioactivity extracted from [^{3}H]inositol-labelled cerebral-cortex slices stimulated with carbachol, but also indicates the comparative retention times for a number of inositol phosphate standards. As this elution trace demonstrates, multiple isomeric [^{3}H]inositol mono-, bis- and tris-phosphates present in tissue-slice extracts could be resolved by the h.p.l.c. separation described. Table 1 allows comparison of the relative concentrations of each of these ^{3}H-labelled products identified in extracts from both control and stimulated brain slices; it also indicates the fold stimulation of each [^{3}H]inositol phosphate induced by carbachol and shows the proportion of each of the [^{3}H]InsP_1, [^{3}H]InsP_2 and [^{3}H]InsP_3 fractions contributed by the separate isomeric components resolved. These data reveal several novel features of stimulated inositol polyphosphate metabolism in brain.

Firstly, the [^{3}H]InsP_1 fraction is shown to comprise two major components, both of which showed markedly increased accumulation in response to carbachol and which are identified by co-elution with authentic standards on h.p.l.c. as [^{3}H]Ins(1)P and [^{3}H]Ins(4)P, with the former predominating over the latter by several-fold in both the presence and the absence of agonist. It should be emphasized that the analytical technique employed does not distinguish between enantiomers. As stimulated brain tissue has the potential to generate both D- and L-Ins(1)P via dephosphorylation of Ins(1,3,4,5)P_4 and Ins-(1,3,4)P_3 [17,10], it is important to recall that the [^{3}H]Ins(1)P fraction may also contain the enantiomeric [^{3}H]Ins(3)P. Previous mass measurements of separate

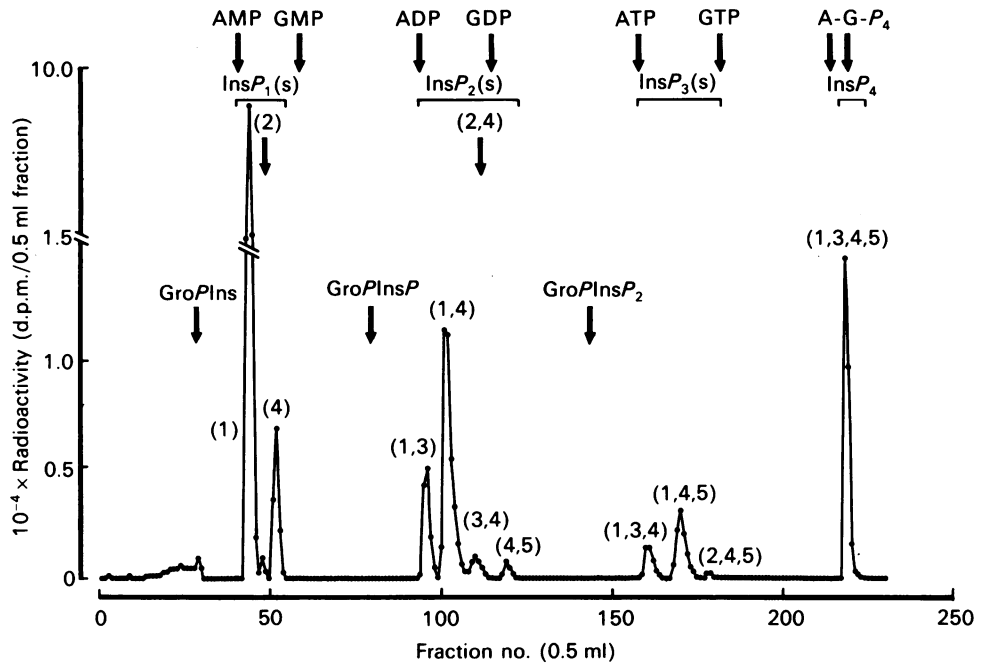

Fig. 1. H.p.l.c. separation of the [^{3}H]inositol phosphates present in an extract from carbachol-stimulated cerebral-cortex slices labelled with [^{3}H]inositol

Samples (50 μl) of cerebral-cortex slices were labelled with 5 μCi of [^{3}H]inositol for 60 min and then incubated for a further 30 min in the absence or presence of 1 mM-carbachol. Reactions were stopped by addition of trichloroacetic acid, and the supernatants (500 μl) obtained from three separate tissue incubations were pooled and neutralized as described in the Materials and methods section. A portion (2 ml) of the final extract (5 ml) was spiked with the indicated nucleotides and with [^{14}C]InsP_1(s), [^{14}C]InsP_2(s) and [^{14}C]InsP_3 and analysed by h.p.l.c. as described in the text. The Figure illustrates the separation of the ^{3}H-labelled products present in an extract from stimulated tissue slices. Data for control samples are given in Table 1. The relative elution positions of standard [^{3}H]-Gro*P*Ins, -Gro*P*Ins*P*, -Gro*P*InsP_2, -Ins(2)*P* and -Ins(2,4)P_2 determined in separate runs are also indicated by the arrows. Locants for isomers are shown in parentheses. Note that, before fraction 0, [^{3}H]inositol was eluted by washing the column with water for 15 min. Further abbreviations: A- and G-P_4, adenosine and guanosine tetraphosphate.

isomeric and enantiomeric inositol monophosphates in rat brain after treatment *in vivo* with muscarinic-receptor agonist would, however, indicate that, of the total Ins(1)*P*, the vast majority is counted for by the D-1 enantiomer [40,41]. In addition to [^{3}H]Ins(1)*P* and [^{3}H]Ins(4)*P*, a third ^{3}H-labelled product eluted intermediate to the major [^{3}H]inositol monophosphates was also detected in both control and stimulated brain extracts. Accurate quantification of the effects of carbachol on this ^{3}H-labelled product was difficult, owing to its closely similar retention to [^{3}H]Ins(1)*P* and its low concentration (< 2% of total [^{3}H]InsP_1 in extracts from stimulated tissue), but Table 1 shows that its accumulation increased relatively little compared with that of [^{3}H]-Ins(1)*P* and [^{3}H]Ins(4)*P* in the presence of carbachol. We tentatively identify this minor [^{3}H]InsP_1 as [^{3}H]-Ins(2)*P* on the basis of co-elution with an authentic standard, but cannot at present eliminate the possibility of Ins(5)*P*, low concentrations of which have been detected in rat brain [7], but for which we have no radiolabelled standard for chromatographic comparison.

H.p.l.c. analysis of the [^{3}H]inositol bisphosphates present in tissue extracts also revealed the presence of multiple isomers. The quantitatively major radiolabelled [^{3}H]InsP_2 under both control and stimulated conditions co-eluted with standard Ins(1,4)P_2. In basal samples this accounted for about 95% of total [^{3}H]InsP_2. Concentrations of [^{3}H]Ins(1,4)P_2 increased markedly after muscarinic-receptor stimulation, but still more dramatic effects of agonist were observed on three further labelled fractions. The first of these was eluted just before standard Ins(1,4)P_2, and typically accounted for 20–25% of total [^{3}H]InsP_2 in extracts from stimulated tissue. The other [^{3}H]InsP_2(s) were eluted at later retention times, which were closely similar to those expected for authentic [^{3}H]Ins(2,4)P_2 and [^{3}H]Ins(4,5)P_2 respectively. The latter InsP_2 has recently been identified in thyrotropin-releasing-hormone-stimulated GH_3 cells [38], but the former has not previously been reported to accumulate in stimulated tissues. In contrast, numerous recent studies have provided evidence for the formation of either or both Ins(1,3)P_2 and Ins(3,4)P_2 by dephosphorylation of Ins(1,3,4)P_3 [24,22,25,8,10,37,23]. Consequently we considered the possibility that the [^{3}H]InsP_2(s) eluted ahead of [^{3}H]Ins(1,4)P_2 and co-eluted with standard Ins(2,4)P_2 may represent these products. To establish the identities of these fractions, a bulk extract from carbachol-stimulated [^{3}H]inositol-labelled cerebral-cortex slices was prepared under conditions identical with those described in the legend to Table 1. The [^{3}H]InsP_2 fraction was isolated by Dowex chromatography, desalted and further fractionated by h.p.l.c. as described in Fig. 1. The fractions corresponding to [^{3}H]Ins(1,4)P_2 and those comprising the immediately neighbouring peaks were collected, desalted and individually subjected to periodate oxidation, followed by reduction with borohydride and

dephosphorylation with alkaline phosphatase to yield the corresponding [^{3}H]alditols as described by Irvine *et al.* [24]. The resultant ^{3}H-labelled products were analysed by paper chromatography and/or ionophoresis in the presence of appropriate standards exactly as previously described [32,24]. After this treatment, the [^{3}H]InsP_2 eluted ahead of Ins(1,4)P_2 exclusively yielded [^{3}H]ribitol, thus permitting its unequivocal identification as [^{3}H]Ins(1,3)P_2. The major [^{3}H]InsP_2 peak gave no radiolabelled alditols, consistent with its identification as [^{3}H]Ins(1,4)P_2, which is completely degraded under the described conditions [29,2,24]. The third [^{3}H]InsP_2 which co-eluted with [^{3}H]Ins(2,4)P_2 on h.p.l.c. yielded either [^{3}H]erythritol or [^{3}H]threitol, but the small amount of radiolabel recovered from this minor component of the [^{3}H]InsP_2 fraction was insufficient to allow the repeated analyses necessary to distinguish these two tetritols. However, the production of either [^{3}H]erythritol or [^{3}H]-threitol shows that this [^{3}H]InsP_2 is not [^{3}H]Ins(2,4)P_2, which would yield [^{3}H]arabitol. Further, the result obtained is consistent with only a limited number of structures. Both of the enantiomeric pair D-[^{3}H]Ins(1,2P_2 and D-[^{3}H]Ins(2,3)P_2 would yield [^{3}H]erythritol, but both structures are unlikely, as no appropriate precursors for either are labelled with [^{3}H]inositol under our conditions (see below). Similarly, both D- or L-[^{3}H]-Ins(3,4)P_2 and D- or L-[^{3}H]Ins(4,5)P_2 would give threitol, but, as the original radiolabel used was [2-^{3}H]inositol, only the former would yield threitol with retention of the ^{3}H label (see [24]). Additionally, Ins(4,5)P_2 is clearly resolved by our h.p.l.c. analysis in any case (see Fig. 1). On this basis we therefore identify the third [^{3}H]InsP_2 as either D- or L-[^{3}H]Ins(3,4)P_2, of which alternatives our current understanding of inositol polyphosphate metabolism would favour the D-enantiomer. Thus in carbachol-stimulated brain slices we can identify four separate isomeric [^{3}H]InsP_2(s), which, in order of elution on h.p.l.c. under the specified conditions, are [^{3}H]Ins(1,3)P_2, [^{3}H]-Ins(1,4)P_2, [^{3}H]Ins(3,4)P_2 and [^{3}H]Ins(4,5)P_2. At present the source of Ins(4,5)P_2 is uncertain, although two recent reports [38,42] suggest that this may derive from Ins(1,4,5)P_3 via the action of a 1-phosphatase. Such an indirect route is consistent with further preliminary data which indicate delayed formation of Ins(4,5)P_2 in response to carbachol under our conditions. However, the alternative possibility, that Ins(4,5)P_2 may arise directly from cleavage of PtdIns(4,5)P_2 by a phospholipase D similar to that shown to hydrolyse PtdIns in neutrophils [43], cannot be discounted. Comparison of the relevant data presented in Table 1 suggests that, although there is considerable basal labelling of [^{3}H]Ins(1,4)P_2, accumulation of each of the other [^{3}H]InsP_2(s) appears to be exquisitely stimulated by carbachol, particularly that of [^{3}H]Ins(1,3)P_2.

Examination of the effects of carbachol on the isomeric composition of the [^{3}H]InsP_3 fraction also reveals this latter phenomenon. Consistent with our previous studies [17,27], extracts from stimulated samples contained two major [^{3}H]inositol trisphosphates. These are identified as [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3 solely by co-elution with authentic standards, but we emphasize that both structures are fully consistent both with our previous identification of [^{3}H]Ins(1,3,4,5)P_4 in carbachol-stimulated brain slices [17] and with the current data presented for [^{3}H]InsP_2(s). In contrast with the situation in stimulated tissue slices, under basal conditions about 95% of total [^{3}H]InsP_3 was accounted for by [^{3}H]Ins(1,4,5)P_3 such that, although quantitatively similar increases in the concentrations of [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3 above control values are observed in response to carbachol, the percentage stimulation by agonist is very much greater for the former. This is pertinent, as concentrations of [^{3}H]Ins(1,3)P_2 and [^{3}H]Ins(3,4)P_2, both probable degradation products of [^{3}H]Ins(1,3,4)P_3 (see below), show similar dramatic sensitivity to muscarinic-receptor stimulation. As shown in Table 1, in addition to [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3, using the current h.p.l.c. technique we reproducibly observed a late-running shoulder to the [^{3}H]Ins(1,4,5)P_3 which typically contributed about 3% of total [^{3}H]InsP_3 and exhibited a similar retention time to authentic [^{3}H]Ins(2,4,5)P_3. Accurate quantification of this ^{3}H-labelled product was difficult, in view of the much higher labelling of [^{3}H]Ins(1,4,5)P_3, but one possibility is that it represents an acid-hydrolysis product of [^{3}H]Ins-1,2-cyclic(4,5)P_3, accumulation of which has been reported in carbachol-stimulated mouse pancreatic minilobules [44] and thrombin-stimulated platelets [45]. Further analyses are necessary to confirm this observation, which perhaps emphasizes the need for better extraction methods for inositol polyphosphates.

We have previously demonstrated that the major [^{3}H]-InsP_4 which accumulates in cerebral-cortex slices after muscarinic-receptor stimulation is the [^{3}H]Ins(1,3,4,5)P_4 isomer [17]. Recent studies, however, have demonstrated the accumulation of a second InsP_4, Ins(1,3,4,6)P_4, which is formed in stimulated cells by phosphorylation of Ins(1,3,4)P_3 [20,21]. The subsequent metabolism of Ins(1,3,4,6)P_4 is yet to be elucidated, but, since potential hydrolysis products of this molecule include D- and L-Ins(1,3,4)P_3, D- and L-Ins(3,4)P_2 and Ins(1,3)P_2, contamination of the [^{3}H]Ins(1,3,4,5)P_4 measured under the current conditions with [^{3}H]Ins(1,3,4,6)P_4 could complicate the identities and proposed sources of the lower [^{3}H]inositol phosphates described here. Additionally, InsP_5 and InsP_6 have now been identified in several cell types and tissues, including brain [46,18,47,48,21], and the accumulation of radiolabel within these products under our conditions could further complicate the present conclusions. Since the analyses described in Table 1 and Fig. 1 are not designed for the separation of isomeric [^{3}H]InsP_4(s) or quantification of [^{3}H]InsP_5 and [^{3}H]InsP_6, we performed a second series of experiments to investigate the occurrence of these molecules in control and stimulated brain slices.

When standard Ins(1,3,4,5[5-^{32}P])P_4 and [^{3}H]Ins-(1,3,4,6)P_4 were analysed by gradient-elution h.p.l.c. on a Partisil (10 μm) SAX column with either $(NH_4)H_2PO_4$ or ammonium formate as eluent, at pH 3.7 the two InsP_4(s) showed closely similar retention, with Ins(1,3,4,6)P_4 eluted marginally later than Ins(1,3,4,5)P_4, as previously reported [20] (results not shown). Clear resolution of the two isomers, however, could be achieved by elution with $(NH_4)H_2PO_4$ at higher pH, with optimal separation at pH 4.5–4.8. Fig. 2(*a*) illustrates the separation obtained. By using this method the isomeric composition of the [^{3}H]InsP_4 derived from control and carbachol-stimulated cerebral-cortex slices was examined. Samples were prepared as described above and were routinely spiked before analysis with Ins(1,3,4,5[5-^{32}P])P_4 and guanosine 5′-tetraphosphate to act as internal standards. Fig. 2(*b*) shows the elution trace obtained with an extract from stimulated brain slices, and reveals a single peak for

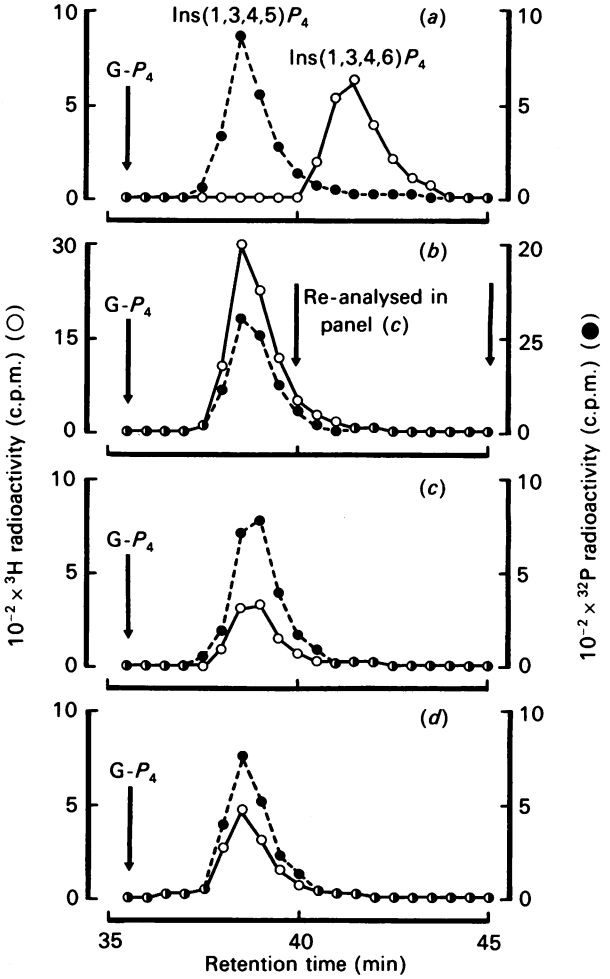

Fig. 2. Isomeric compositions of the [^{3}H]InsP_4 fraction from control and carbachol-stimulated brain slices

Extracts from control and carbachol-stimulated brain slices labelled with [^{3}H]inositol were prepared as described in the legend to Table 1, spiked with Ins(1,3,4,5[5-^{32}P])P_4 plus 200 nmol of guanosine 5′-tetraphosphate (G-P_4) and diluted to 20 ml. Samples or standards (both 20 ml) were pumped at a flow rate of 2 ml/min on to a Partisil (10 μm) SAX h.p.l.c. column equipped with a pre-column as described in the Materials and methods section. Sample application was followed by a 10 min water wash, and the column was then eluted at a flow rate of 1 ml/min with the following gradient, comprising water and $(NH_4)H_2PO_4$, adjusted to pH 4.75 with NH_3. Over 0–15 min the column was washed with water, then the eluent concentration was increased over 5 min to 0.6 M and held there for 8 min to displace labelled Ins$P$(s) up to and including InsP_3(s). Eluent concentration was then increased linearly over 10 min to 0.8 M, and held at this concentration for a further 10 min to displace the separate InsP_4(s). The column was then washed with 1.4 M-$(NH_4)H_2PO_4$ for 10 min and with water for 5 min before application of the next sample. Fractions (0.5 ml) were collected across the gradient and counted for radioactivity, but only those relevant to InsP_4 are shown. The figure illustrates elution traces for (*a*) Ins(1,3,4,5[5-^{32}P])P_4 and [^{3}H]Ins(1,3,4,6)P_4 standards, (*b*) a primary analysis of [^{3}H]InsP_4 from stimulated brain slices, (*c*) a secondary analysis of the indicated fractions from (*b*) after dilution to 20 ml (see the text), and (*d*) an analysis of [^{3}H]InsP_4 from control brain slices. Results shown are from a single experiment, but were reproduced on two further occasions.

[^{3}H]InsP_4 which co-eluted precisely with the internal Ins(1,3,4,5[5-^{32}P])P_4 standard. Fig. 2(*d*) illustrates an identical result for a corresponding control sample. Figs. 2(*a*), 2(*b*) and 2(*d*) show that, on each run, approx. 85–90% of both ^{3}H-labelled sample or standard [^{32}P]-InsP_4 were eluted over those fractions well resolved from those appropriate for Ins(1,3,4,6)P_4. Fig. 2(*a*), however, shows that there is a small degree (10–15%) of overlap between the two InsP_4 standards. Equally, with ^{3}H-labelled tissue extracts, although no second peak of radioactivity was observed, a similar 10–15% of total [^{3}H]InsP_4 overlapped the region appropriate to Ins-(1,3,4,6)P_4. Thus we considered the possibility that a minor proportion of [^{3}H]Ins(1,3,4,6)P_4 present in our extracts could be masked by the tail to the major [^{3}H]Ins(1,3,4,5)P_4 peak. Therefore, a secondary analysis was made in which these tail fractions (indicated in Fig. 2*b*) were pooled, diluted with water, spiked with standards and re-analysed. The results are illustrated in Fig. 2(*c*), which shows that the 10–15% of total [^{3}H]InsP_4 originally comprising the tail to the [^{3}H]-Ins(1,3,4,5)P_4 peak was re-eluted as a single compound which ran with the Ins(1,3,4,5[5-^{32}P])P_4 standard, with 85–90% of the recovered radioactivity again being eluted over those fractions clearly resolved from those expected for Ins(1,3,4,6)P_4.

On the basis of these analyses we can therefore be confident that [^{3}H]Ins(1,3,4,5)P_4 is by far the major, if not the only, [^{3}H]InsP_4 present in our stimulated tissue extracts, and that any [^{3}H]Ins(1,3,4,6)P_4 present must represent $\leqslant 2\%$ of the total. Owing to the lower radiolabelling of control samples, repeated analysis was not possible, although Fig. 2(*d*) provides no evidence for a second isomer of [^{3}H]InsP_4.

In further experiments, extracts from control and stimulated cerebral-cortex slices were also analysed for [^{3}H]InsP_5 and [^{3}H]InsP_6 by using an extension of the h.p.l.c. gradient described in Fig. 1 and which has recently been employed to demonstrate the presence of these [^{3}H]inositol polyphosphates in [^{3}H]inositol-labelled GH_3 cells [23]. These studies, however, reproducibly failed to reveal the presence of [^{3}H]inositol metabolites more polar than [^{3}H]Ins(1,3,4,5)P_4 even under conditions where any radiolabelled material comprising $\geqslant 2\%$ of the radioactivity present as [^{3}H]InsP_4 in extracts from carbachol-stimulated samples would have been detected (results not shown). These data suggest that, although InsP_5 and InsP_6 are present in brain [47], these polyphosphates do not achieve detectable labelling in either the presence or the absence of stimulus under the current incubation conditions with [^{3}H]inositol. Both this observation and the demonstration of the high purity of [^{3}H]Ins(1,3,4,5)P_4 in our experiments are important, as they indicate that the identifications of lower [^{3}H]inositol phosphates reported above are unlikely to be complicated by metabolism of [^{3}H]inositol through pathways not directly associated with receptor-mediated phosphoinositide turnover. Consequently, these results also indicate that, for those inositol polyphosphates where enantiomeric pairs exist, the predominating ^{3}H-labelled species accumulating under the present conditions are likely to be of the D-series.

Studies with cell-free preparations

In order to obtain further evidence in support of these conclusions and to aid identification of the probable

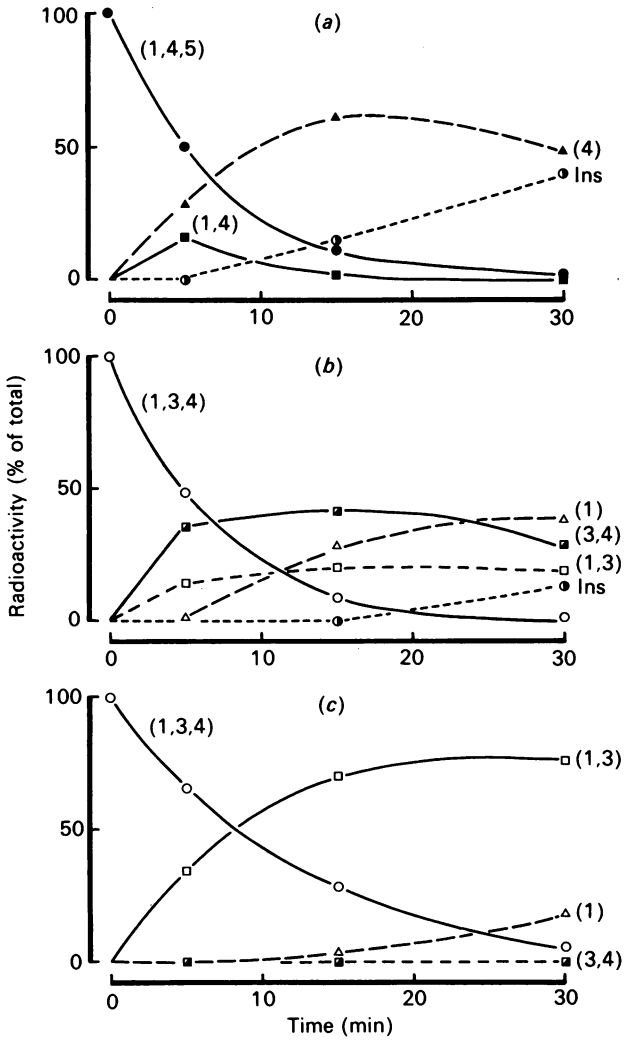

Fig. 3. Degradation of [^{3}H]inositol polyphosphates by cell-free preparations from rat cerebral cortex

[^{3}H]Ins(1,4,5)P_3 or [^{3}H]Ins(1,3,4)P_3, each at 10 nM, were incubated for the indicated times with rat cerebral-cortex supernatants or homogenates as described in the Materials and methods section before extraction of the reaction products and analysis by h.p.l.c. Panels (*a*) and (*b*) show the hydrolysis products derived from [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 respectively after incubation with a cerebral-cortex supernatant. Similar data were also obtained with homogenates. Panel (*c*) indicates the reaction products derived from incubation of [^{3}H]Ins(1,3,4)P_3 under identical conditions but in the presence of 5 mM-EDTA. Locants for isomers are shown in parentheses. Results shown are from single experiments, and were reproduced on several occasions (but see the text).

sources of the separate isomeric [^{3}H]inositol phosphates present in tissue extracts, we have also examined the hydrolysis of various radiolabelled inositol polyphosphates by cerebral-cortex homogenates and supernatants. These studies were performed either by separate incubation of [^{3}H]Ins(1,3,4)P_3, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4,5)P_3 or by co-incubation of these substrates with [^{14}C]Ins(1,3,4)P_3, with resolution of the resultant products by h.p.l.c. The advantage of this protocol is that evidence for the formation of similar or distinct ^{3}H- and ^{14}C-labelled products from separate tris- and tetrakis-phosphates could be established by virtue of both the chromatographic and radionuclide resolution achieved in a single experiment. The results of these studies are summarized in Figs. 3(*a*), 3(*b*) and 3(*c*). The first of these shows the time course for hydrolysis of [^{3}H]Ins(1,4,5)P_3 on incubation with a cerebral-cortex supernatant, and clearly demonstrates a sequential dephosphorylation of this substrate to free [^{3}H]inositol through transiently accumulating specific [^{3}H]inositol bis- and mono-phosphate intermediates. From their retention times on h.p.l.c., these intermediates are identified as [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(4)P respectively. Under the specified conditions, no evidence for production of [^{3}H]Ins(4,5)P_2 or [^{3}H]Ins(1)P was obtained, although concentrations $< 5\%$ that of the initial substrate would have been difficult to measure. These results, reproduced with both supernatant and homogenate preparations, are fully consistent with previous similar studies employing crude and purified enzyme preparations derived from both rat and bovine brain [5,12,8,10] but contrast with a recent report indicating specific hydrolysis of Ins(1,4)P_2 to Ins(1)P by a purified rat brain bisphosphatase [11]. The reason for the latter discrepancy is at present unclear.

Fig. 3(*b*) shows the corresponding results obtained from incubation of [^{3}H]Ins(1,3,4)P_3 with a cerebral-cortex supernatant, and indicates that hydrolysis of this substrate yielded two distinct [^{3}H]InsP_2(s). Both of these were clearly separable from [^{3}H]Ins(1,4)P_2 on h.p.l.c. and were eluted with retention times identical with those shown above to correspond to [^{3}H]Ins(1,3)P_2 and [^{3}H]-Ins(3,4)P_2. Both ^{3}H-labelled products are thus unequivocally identified, as no other [^{3}H]InsP_2(s) can be derived directly from [^{3}H]Ins(1,3,4)P_3. Fig. 3(*b*) also indicates that the sequential dephosphorylation of [^{3}H]Ins(1,3)P_2 and/or [^{3}H]Ins(3,4)P_2 through to free [^{3}H]inositol occurs specifically via D- or L-Ins(1)P without detectable accumulation of [^{3}H]Ins(4)P. Further similar experiments in which [^{3}H]Ins(1,3,4,5)P_4 was co-incubated with [^{14}C]Ins(1,3,4)P_3 revealed identical catabolic profiles for both substrates after incubation with either cerebral-cortex supernatants or homogenates (results not shown). In separate experiments, however, using both [^{3}H]- and [^{14}C]-Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4, we found considerable variability between the proportions of accumulated Ins(1,3)P_2 and Ins(3,4)P_2, such that on some occasions only the latter was clearly detected. However, using [^{3}H]Ins(1,3,4)P_3 as substrate, maximal accumulated concentrations of [^{3}H]Ins(1,3)P_2 never exceeded those indicated in Fig. 3(*b*). In contrast, incubation of the same substrate with a supernatant preparation in the presence of EDTA in molar excess of buffer Mg^{2+} concentrations reproducibly yield exclusively [^{3}H]Ins(1,3)P_3, which was further partially degraded to [^{3}H]Ins(1)P, as shown in Fig. 3(*c*). Alternatively, when [^{3}H]Ins(1,4,5)P_3 or [^{3}H]-Ins(1,3,4,5)P_4 was incubated in the presence of EDTA, hydrolysis of both substrates was inhibited by $\geqslant 95\%$ (results not shown).

Each of these results is consistent with earlier studies with brain. Firstly, the data re-inforce the previous suggestions [17,5,9] that catabolism of both Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 occurs initially by a Mg^{2+}-requiring 5-phosphatase similar to that found in other tissues [2–4]. Secondly, the results indicate that the Ins(1,3,4)P_3 produced by the action of this enzyme on Ins(1,3,4,5)P_4 is

further hydrolysed by both Mg^{2+}-dependent and -independent routes, which respectively yield Ins(3,4)P_2 and Ins(1,3)P_2, as suggested by Inhorn *et al.* [8] and Bansal *et al.* [10]. The latter authors have also demonstrated that dephosphorylation of these inositol bisphosphates respectively yields L-Ins(1)P and D-Ins(1)P. Consistent with this, we were unable to detect any Ins(4)P amongst the reaction products of Ins(1,3,4)P_3 hydrolysis. This is in direct contrast with the situation with Ins(1,4,5)P_3, which produced only Ins(4)P, derived via Ins(1,4)P_2. Thus it appears that the two inositol trisphosphates give mutually exclusive hydrolysis products at both the bis- and monophosphate levels.

Comparative metabolism in cell-free and tissue-slice preparations

In view of these observations, the relative concentrations of [^{3}H]inositol metabolites measured after muscarinic-receptor activation in cerebral-cortex slices raise some interesting points. Table 1 shows that, of the total stimulated accumulation of [^{3}H]InsP_2, about 22% and 8% respectively is accounted for by [^{3}H]Ins(1,3,)P_2 and [^{3}H]Ins(3,4)P_2. We emphasize that, although these data are for a single time point after agonist addition, our previous studies [35,27] would strongly suggest that these values are representative of a stimulated steady state. Thus there is a sharp contrast with the results shown in Fig. 3, where, in the presence of Mg^{2+}, the predominant product of [^{3}H]Ins(1,3,4)P_3 hydrolysis was [^{3}H]Ins(3,4)P_2. The two findings are not, however, necessarily incompatible since there will clearly be differences between the cell-free and tissue-slice preparations with respect to the concentrations of several factors potentially capable of influencing the relative rates of metabolism of the separate inositol phosphate isomers. Adenosine nucleotides have, for example, been shown to inhibit the dephosphorylation of various inositol polyphosphates [20,25], and the observation that ATP appears to inhibit differentially the hydrolysis of distinct InsP_2 isomers [25] may at least partly account for the opposing proportions of Ins(1,3,)P_2 and Ins(3,4)P_2 found here. An alternative or complementary explanation lies in the observation that a single enzyme appears to cleave the D-1 monoester phosphate of both Ins(1,3,4)P_3 and Ins(1,4)P_2 [10], so that competition with the latter may direct metabolism of the former via Ins(1,3)P_2 in stimulated tissue. As dephosphorylation of Ins(1,3)P_2 produces D-Ins(1)P [10], an important consequence of such a mechanism would be an enhanced accumulation of this product over the L-Ins(1)P enantiomer arising from Ins(3,4)P_2. In this respect, our current observation of the predominance of [^{3}H]Ins(1)P over that of [^{3}H]Ins(4)P in stimulated tissue slices, and the previous demonstration [40,41] that D-Ins(1)P concentrations exceed those of L-Ins(1)P in rat brain after administration of pilocarpine and/or Li^+ *in vivo*, are of interest. From Fig. 3, the most probable source of [^{3}H]Ins(4)P in cerebral-cortex slices is dephosphorylation of [^{3}H]Ins(1,4)P_2, itself derived from [^{3}H]Ins(1,4,5)P_3 {or possibly from receptor-mediated cleavage of [^{3}H]PtdIns(4)P}. In contrast, D-[^{3}H]Ins(1)P could arise by direct cleavage of [^{3}H]PtdIns by phosphoinositidase C, or by the sequential specific dephosphorylation of [^{3}H]Ins(1,3,4,5)P_4 through [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3)P_2. Hydrolysis of PtdIns rather than of polyphosphoinositides has previously been suggested to account for most of the D-Ins(1)P accumulated in rat brain after muscarinic-receptor activation [7], although the evidence for this was only indirect. The current data do not contradict this conclusion but, via our description of the stimulated accumulation of [^{3}H]Ins(1,3)P_2 in response to carbachol in cerebral-cortex slices and our definition of a metabolic pathway for formation of this product from [^{3}H]Ins(1,3,4,5)P_4, they do provide an alternative or complementary source of D-Ins(1)P. {We should note here that, although the present data strongly indicate that the [^{3}H]Ins(1,3)P_2 accumulating in stimulated tissue slices arises from [^{3}H]Ins(1,3,4)P_3, we cannot at present eliminate the possibility that a proportion might also derive from a [^{3}H]PtdIns(3)P similar to that very recently described in fibroblasts [49].} Clearly, further studies are required to establish the contribution made by dephosphorylation of Ins(1,3)P_2 and Ins(3,4)P_2 to D- and L-Ins(1)P accumulation in stimulated cerebral tissue, although the present study, demonstrating dramatic effects of receptor activation on the accumulation not only of these bisphosphates but also of the other metabolites of the inositol tris-/tetrakis-phosphate pathway, may indicate that this is likely to be considerable. Additional studies should help to clarify further the nature of the inositol phospholipid substrates hydrolysed in response to receptor stimulation in brain and reveal the extent to which Ins(1,4,5)P_3 is initially metabolized by the alternative 5-phosphatase and 3-kinase pathways. In establishing that the latter routes produce mutually exclusive products, and by identifying the nature of these for the first time in stimulated brain tissue, the current study represents a significant step towards this end.

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