Analysis of [³H]inositol phosphate formation and metabolism in cerebral-cortical slices

Evidence for a dual metabolism of inositol 1,4-bisphosphate

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Muscarinic-receptor-mediated phosphoinositide hydrolysis in rat cerebral cortex was investigated by analysis of the kinetics of [³H]inositol phosphate formation and degradation in *myo*-[2-³H]inositol-labelled tissue slices. Carbachol stimulated rapid (5 s) increases in the concentrations of [³H]Ins(1,4,5)P₃, [³H]Ins(1,3,4,5)P₄ and [³H]Ins(1,4)P₂. Stimulated accumulation of [³H]Ins(1,3,4)P₃, [³H]Ins(1,3)P₂ and [³H]Ins(3,4)P₂ and [³H]Ins(1/3)P or of [³H]Ins(4)P occurred only subsequently and with a sequence indicating formation by successive dephosphorylation of [³H]Ins(1,3,4,5)P₄ or of Ins(1,4)P₂ respectively. A similar sequence was inferred from the order of rapidity with which the accumulations of [³H]inositol polyphosphates, resulting from sustained (5 min) carbachol stimulation in the presence of LiCl, were reversed when muscarinic receptors were subsequently blocked with atropine. During this latter period of receptor blockade, radiolabel lost from [³H]inositol polyphosphates was quantitatively recovered as [³H]inositol monophosphates owing to effective inhibition of monophosphatase by Li⁺, and the rate of poly- into mono-phosphate conversion was similar to agonist-stimulated rates of monophosphate accumulation. This implies that, even during persistent stimulation, polyphosphoinositide, not PtdIns, is the substrate for phosphoinositidase C. Quantitative comparison of the degradation of [³H]inositol poly- to mono-phosphates after receptor blockade unexpectedly suggests the dual hydrolysis of [³H]Ins(1,4)P₂ to [³H]Ins(1)P and [³H]Ins(4)P. This result advises cautious interpretation of the origin of [³H]Ins(1)P in stimulated tissue, but, with other data presented, allows calculation from the observed ratio of [³H]Ins(1/3)P:[³H]Ins(4)P that a minimum of approx. 50 % of the [³H]Ins(1,4,5)P₃ produced during persistent muscarinic-receptor stimulation is metabolized by Ins(1,4,5)P₃ 3-kinase.

INTRODUCTION

The respective roles of the second messengers *sn*-1,2-diacylglycerol and Ins(1,4,5)P₃ in the regulation of protein kinase C activity and release of Ca²⁺ from intracellular sites are well documented [1]. Both signal molecules are released by phosphoinositidase C (PIC) on hydrolysis of PtdIns(4,5)P₂ after activation of many cell-surface receptors. Ins(1,4,5)P₃ is a unique product of this reaction, whereas diacylglycerol may potentially also be derived through hydrolysis of PtdIns and PtdIns(4)P or other non-inositol-containing glycerophospholipids. Thus the enhanced turnover of PtdIns and PtdIns(4)P which accompanies PtdIns(4,5)P₂ breakdown has variously been suggested to reflect either the compensatory re-synthesis of PtdIns(4,5)P₂ or the direct hydrolysis of these lipids to provide an alternative and perhaps quantitatively more significant source of diacylglycerol [2,3]. *In vitro*, most forms of PIC will hydrolyse all three phosphoinositides under appropriate conditions (for review see [4]), releasing Ins(1)P and Ins(1,4)P₂ from PtdIns and PtdIns(4)P respectively. However, as the same inositol phosphates are also products of Ins(1,4,5)P₃ degradation, it has proved difficult to establish the substrate specificity of receptor-activated PIC in stimulated cells. A limited number of studies have addressed this question by examining the rates of inositol phosphate turnover following receptor activation [5–8], and each has indicated that stimulated PIC activity is directed predominantly against PtdIns(4,5)P₂. Of these studies, however, only the last two post-

date the comparatively recent recognition of the complex pathways that exist for Ins(1,4,5)P₃ metabolism.

It is now clear that initial removal of Ins(1,4,5)P₃ occurs through the action of either a 5-phosphatase, producing Ins(1,4)P₂, or a 3-kinase, generating Ins(1,3,4,5)P₄ [9,10]. The former route is generally recognized as a means of inactivating the second messenger, as Ins(1,4)P₂ is unable to mobilize Ca²⁺ from internal stores. The second route may serve as an additional inactivation mechanism or may generate other signal molecules [11,12]. The re-cycling of Ins(1,4)P₂ and Ins(1,3,4,5)P₄ to free inositol for PtdIns re-synthesis appears to occur via distinct intermediates [13,14]. Ins(1,4)P₂ is degraded predominantly, if not exclusively, via Ins(4)P, whereas Ins(1,3,4,5)P₄ is dephosphorylated sequentially through Ins(1,3,4)P₃, Ins(1,3)P₂ and Ins(3,4)P₂, which in turn yield Ins(1)P and Ins(3)P. Although these pathways have been elucidated primarily through studies with broken-cell preparations, a limited number of studies on intact stimulated cells have presented kinetic data compatible with these sequences [7,15–19]. There is, however, little quantitative information on the extent to which different routes prevail during receptor stimulation [7]. Indeed, this and the precise substrate specificity of PIC are both difficult to establish, especially given the additional complexities imposed by related metabolic pathways associated with InsP₅ and InsP₆ synthesis and the isomeric heterogeneity of inositol lipid headgroups [14,20].

In the central nervous system many neurotransmitter receptors

Abbreviations used: Ins, InsP₁, InsP₂, InsP₃, InsP₄, InsP₅ and InsP₆; *myo*-inositol and its mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates with numbering of phosphates relative to the D-1 position as appropriate. PtdIns, PtdInsP and PtdInsP₂; phosphatidylinositol and its mono- and bis-phosphates with assignment of phosphate positions around the inositol ring as above. PIC, phosphoinositidase C.

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regulate phosphoinositide turnover [21,22], and the underlying mechanism, at least for muscarinic-receptor stimulation, appears similar to that in other tissues, including initial $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis and $\text{Ins}(1,4,5)\text{P}_3$ release [23–25]. However, the potential hydrolysis of PtdIns and/or $\text{PtdIns}(4)\text{P}$ under some circumstances has also been considered [26–28]. We have recently identified the major $[^3\text{H}]$ inositol phosphate metabolites produced in response to muscarinic-receptor stimulation in cerebral-cortex slices after brief $[^3\text{H}]$ inositol labelling [29]. In the present study we have applied this information to a detailed analysis of the kinetics of inositol phosphate turnover to establish the phosphoinositide substrate(s) utilized by PIC during acute and prolonged receptor stimulation, and obtain a quantitative estimate of the contributions made by 5-phosphatase and 3-kinase to regulation of $\text{Ins}(1,4,5)\text{P}_3$ concentrations.

MATERIALS AND METHODS

Materials

D-*myo*-[2- ^3H]Inositol 1,4-bisphosphate (4.5 Ci/mmol) and *myo*-[2- ^3H]inositol (12–20 Ci/mmol) were purchased from NEN/DuPont. D/L-*myo*-Inositol 1,4-bisphosphate was generously given by Dr. I. Ragan, Merck Sharpe & Dohme, Harlow, U.K. Other reagents were from Fisons, BDH, Sigma or other previously stated sources.

Studies with $[^3\text{H}]$ inositol-labelled tissue slices

Cerebral-cortex slices ($350\ \mu\text{m} \times 350\ \mu\text{m}$) were prepared from male Wistar rats and preincubated in bulk in modified Krebs–Henseleit buffer (containing 1.3 mM- CaCl_2) as described previously [29]. Tissue slices were then packed under gravity, and portions ($50\ \mu\text{l}$) were labelled with $[^3\text{H}]$ inositol ($5\ \mu\text{Ci}$ or $20\ \mu\text{Ci}$) for 60 min in a total volume of 280–290 μl of buffer before drug challenge. Reactions were stopped by addition of 300 μl of ice-cold 1 M-trichloroacetic acid. Samples were allowed to extract on ice for approx. 20 min, and tissue was then sedimented by centrifugation at 4°C . Neutralized $[^3\text{H}]$ inositol phosphate extracts were prepared from the pooled supernatants obtained from triplicate incubations [29] and analysed as described below.

For some experiments, $[^3\text{H}]$ inositol lipids were extracted from washed tissue pellets as described previously [30] and subsequently degraded sequentially by either deacylation [31] and deglyceration [32,33] or deacylation followed by alkaline hydrolysis [34] before analysis of the ^3H -labelled products by h.p.l.c.

Hydrolysis of $\text{Ins}(1,4)\text{P}_2$ by cell-free preparations

Homogenates and 100 000 g supernatants from rat cerebral cortex were prepared as described previously [29]. Assays of $\text{Ins}(1,4)\text{P}_2$ hydrolysis contained either 50 nM-D- $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ (50 000 d.p.m.) or the same substrate supplemented with unlabelled D/L- $\text{Ins}(1,4)\text{P}_2$ to give a final concentration of 100 μM . Reactions were performed in a final volume of 100 μl of buffer (20 mM or 30 mM- NaCl , 100 mM- KCl , 2 mM- MgCl_2 , 25 mM-Hepes, pH 7.4 with KOH) in the presence or absence of 5 mM-EDTA and/or 10 mM- LiCl . Where absent, LiCl was replaced with NaCl . Reactions were started by addition of tissue extract and stopped by addition of 100 μl of ice-cold 1 M-trichloroacetic acid followed by 10 μl of 5% (w/v) BSA. Protein was removed by centrifugation, and samples (175 μl) of the supernatants were neutralized as described previously [29]. Radiolabelled products were separated by h.p.l.c.

Separation of $[^3\text{H}]$ inositol metabolites by h.p.l.c.

This was achieved by gradient elution on a Partisil (10 μm) SAX column eluted with $(\text{NH}_4)_2\text{H}_2\text{PO}_4/\text{H}_3\text{PO}_4$, pH 3.7, either exactly as described previously [29] or by modifications as appropriate to sample origin. Thus samples derived by chemical degradation of $[^3\text{H}]$ inositol lipids were analysed with a gradient to 1.4 M eluent (B): 0 min, 0% B; 10 min, 0% B; 55 min, 54% B; 60 min, 54% B; 75 min, 100% B; 80 min, 100% B. Samples produced from assay of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ hydrolysis were analysed with a gradient to 0.3 M eluent: 0 min, 0% B; 5 min, 0% B; 35 min, 20% B; 50 min, 100% B; 60 min, 100% B. Sample volumes and flow rates were 2 ml and 1 ml/min in each case. $[^3\text{H}]$ inositol metabolites were identified as described previously [29].

Protein assay and expression of data

Concentrations of $[^3\text{H}]$ inositol metabolites are reported either as d.p.m./50 μl tissue aliquot (approx. 1.5 mg of protein) or, for some experiments where it was preferable to minimize inter-sample variation arising from minor differences in sample tissue content, as d.p.m./mg of protein. Measurements of sample protein were made using the trichloroacetic acid-insoluble material residual to $[^3\text{H}]$ inositol phosphate extraction. Tissue pellets were washed as described previously for subsequent lipid extraction [34], then solubilized in 3 ml of 1.0 M- NaOH and samples assayed for protein by the method of Lowry *et al.* [35] with BSA as standard.

RESULTS AND DISCUSSION

A large number of studies in brain have established the widespread importance of inositol lipid hydrolysis in the central nervous system and have indicated that this follows a similar sequence to that defined in less complex systems [21,22]. Thus muscarinic-receptor activation in rat cerebral cortex [23,25] and hippocampus [36] evokes a rapid hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, which is characterized by the stimulated accumulation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. More recent studies have further demonstrated both sustained hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ in response to persistent muscarinic-receptor stimulation in cerebral cortex [24] and the accumulation of multiple isomers of each InsP , InsP_2 and InsP_3 in response to several stimuli [28,29]. In contrast, no evidence for the stimulated production of additional $[^3\text{H}]\text{InsP}_4$ isomers nor for the presence of $[^3\text{H}]\text{InsP}_5$ or $[^3\text{H}]\text{InsP}_6$ was found in tissue slices labelled acutely with $[^3\text{H}]$ inositol [29]. Thus it is probable that the multiple inositol phosphates which accumulate after muscarinic-receptor stimulation in cerebral cortex arise as a consequence of the divergent routes of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, and so share $\text{PtdIns}(4,5)\text{P}_2$ as their common precursor. In the present work we have sought to examine this suggestion by detailed definition of the operative pathways of inositol polyphosphate metabolism, using several approaches. The first of these was to define in detail the early kinetics of carbachol-stimulated inositol phosphate accumulation.

Comparative kinetics of inositol phosphate accumulation

Fig. 1 summarizes the results of a number of experiments in which $[^3\text{H}]$ inositol-labelled cerebral-cortex slices were exposed to a maximally effective concentration of carbachol for various intervals up to 60 s, followed by analysis of the ^3H -labelled products by h.p.l.c. For each group of $[^3\text{H}]$ inositol phosphates at all levels of phosphorylation, the data are in close qualitative agreement with those of our previous, less comprehensive, study [24]. However, the detailed analysis of ^3H -labelled products afforded by the h.p.l.c. method currently employed allows greatly

Fig. 1. Early kinetics of carbachol-stimulated [³H]inositol phosphate accumulation

Samples (50 μl) of cerebral-cortex slices were labelled with 5 μCi of [³H]inositol for 60 min, then further incubated in either the absence (○) or the presence (●) of 1 mM-carbachol for the times indicated before addition of trichloroacetic acid to stop reactions. Samples from triplicate incubations were pooled, neutralized and analysed by h.p.l.c. Data represent means ± s.e.m. of 4-5 separate experiments. Stimulated values were compared with both zero-time and 60 s controls by Student's unpaired *t* test. Asterisks denote a significant stimulation (*P* < 0.05) above both control values.

enhanced resolution of the initial metabolic sequence after muscarinic-receptor stimulation and thereby reveals several important features of this response not previously demonstrated in brain. Fig. 1(a) shows that concentrations of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ increased sharply after carbachol addition, achieving an apparent new steady-state value about 2 times basal by the earliest time (5 s) monitored. Similarly, as illustrated in Fig. 1(b) and 1(c), concentrations of both $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ also rose markedly over the same interval. As the only established source of the former in this tissue is the phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ by 3-kinase (see below), the very early stimulated formation of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ confirms the high rate of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ turnover implied by the rapid approach of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to a new steady-state concentration. In turn, these observations may imply that the early rise in $[^3\text{H}]\text{InsP}_2$ accumulation, which the present data allow us to attribute to the 1,4-isomer for the first time in this system, arises from the equally rapid dephosphorylation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$. As $\text{Ins}(1,4)\text{P}_2$ is also the expected product of any PIC activity directed against $\text{PtdIns}(4)\text{P}$, the quantitative assignment of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ production to either potential origin is difficult. However, over the first 5–10 s, stimulated accumulation of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ is approximately equal to the sum total of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ + $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$, implying that, unless receptor-activated PIC exhibits little or not preference for $\text{PtdIns}(4,5)\text{P}_2$ over $\text{PtdIns}(4)\text{P}$, metabolism of $\text{Ins}(1,4,5)\text{P}_3$ probably occurs initially via both 3-kinase and 5-phosphatase at similar rates. Muscarinic-receptor-mediated accumulations of both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ of similar magnitude to those illustrated in Fig. 1(a) and 1(b) recently have been demonstrated in cerebral cortex by methods of mass assay [25,37]. This suggests that the very rapid changes in ^3H labelling of products reported here approximately reflect changes in concentration, although it should be noted that moderate increases in ^3H -labelled product specific radioactivity may occur under the current non-equilibrium $[^3\text{H}]\text{inositol}$ -labelling regime [24].

In marked contrast with, but immediately after, the rapid carbachol-stimulated accumulation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and its primary metabolites, there was a slower progressive accumulation of several additional ^3H -labelled products, as shown in Fig. 1(d)–1(h). Thus significant stimulated accumulation of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$, $[^3\text{H}]\text{Ins}(1,3)\text{P}_2$ and $[^3\text{H}]\text{Ins}(3,4)\text{P}_2$, the expected degradation products of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$, was not observed until 10 s. Increases in accumulation of $[^3\text{H}]\text{Ins}(1)\text{P}$ or $[^3\text{H}]\text{Ins}(3)\text{P}$ (an enantiomeric pair not resolved by the current approach) was not apparent before 30 s and did not achieve significance until 45 s after carbachol addition. Although the relatively higher basal labelling of this monophosphate may have masked early small accumulations, a lag preceding enhanced $[^3\text{H}]\text{Ins}(1/3)\text{P}$ is fully compatible with, and indeed strongly indicates, that its ultimate source is $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, and hence $[^3\text{H}]\text{PtdIns}(4,5)\text{P}_2$. This observation, however, also clearly suggests that $[^3\text{H}]\text{PtdIns}$ is unlikely to be an initial substrate for receptor-activated PIC. In contrast, a significantly elevated $[^3\text{H}]\text{Ins}(4)\text{P}$ concentration was readily measurable as early as 10 s, as would be expected from the marked accumulation of its presumed immediate precursor $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$.

The results in Fig. 1 therefore clearly support our earlier suggestion [24] that $\text{PtdIns}(4,5)\text{P}_2$ [and potentially $\text{PtdIns}(4)\text{P}$] rather than PtdIns is the primary substrate for muscarinic-receptor PIC in brain. Further, however, these data collectively also clearly imply a metabolic sequence in which $\text{Ins}(1,4,5)\text{P}_3$ released by $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis is removed first by conversion into $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4)\text{P}_2$, and in which these molecules are then successively dephosphorylated to free inositol via the mutually exclusive intermediates predicted on the basis of

studies with broken cells [13,14]. In this respect the present results also resemble those obtained in angiotensin-stimulated adrenal glomerulosa cells [15,16], thyrotropin-releasing-hormone-stimulated GH_3 cells [18], *N*-formylmethionyl-leucyl-phenylalanyl-stimulated leucocytes [17] and noradrenaline-stimulated cardiac myocytes [19]. It is also significant that the current conditions of acute muscarinic-receptor activation evoke accumulation of the same major $[^3\text{H}]\text{inositol}$ metabolites previously identified in cerebral-cortex slices after 30 min exposure to carbachol [29], implying the continuous operation of similar metabolic routes over prolonged periods of receptor stimulation.

Kinetics and routes of inositol polyphosphate degradation

In a further series of experiments we sought additional evidence for the metabolic sequence of stimulated inositol phosphate formation discussed above and for its persistent activation in the continuous presence of agonist. This was achieved by a strategy which takes combined advantage of the ability of Li^+ to inhibit inositol monophosphatase [38] and of an approach previously employed to examine the rates of inositol phosphate hydrolysis in several systems [5,7,24]. The procedure used was first to expose $[^3\text{H}]\text{inositol}$ -labelled tissue slices to a combination of carbachol and LiCl for a period sufficient to evoke substantial $[^3\text{H}]\text{inositol}$ (poly)phosphate accumulation and then to block rapidly the further stimulated release of ^3H -labelled products by using an excess of the muscarinic-receptor antagonist atropine (see [24]), and subsequently to follow the degradation of the pre-formed ^3H -labelled products. Since the enzymes responsible for the hydrolysis of inositol polyphosphates are either insensitive to Li^+ or markedly less so than inositol monophosphatase [13], the former decline toward control levels after receptor blockade, while any $\text{InsP}(s)$, either pre-formed during stimulation or subsequently derived from existing precursors, are trapped. Provided that inositol monophosphatase activity is suppressed, this protocol allows the loss of $[^3\text{H}]\text{inositol}$ polyphosphate after cessation of stimulated formation to be compared quantitatively with any corresponding gain of $[^3\text{H}]\text{inositol}$ monophosphates. Several assumptions about metabolism of these molecules in intact cells can therefore be directly examined. Details of the procedure used and the results obtained are presented in Figs. 2 and 3 and in Table 1.

Table 1 illustrates several important features. Firstly, it is evident that over a 5 min incubation carbachol evokes an approx. 10-fold increase in the accumulation of total $[^3\text{H}]\text{inositol}$ phosphates in the presence of Li^+ . These data also serve to illustrate that, under the conditions described, inositol monophosphatase is almost completely inhibited, since although further stimulated accumulation of ^3H -labelled products is rapidly and effectively inhibited after atropine addition, as previously reported [24], there is no decline in the concentration of pre-formed total $[^3\text{H}]\text{inositol}$ phosphate over a subsequent period of at least 10 min. Over the same interval, after addition of antagonist, however, there is a dramatic change in the distribution of ^3H label among different $[^3\text{H}]\text{inositol}$ metabolites. This is shown in Table 1(b), which illustrates the relative concentrations of the major $[^3\text{H}]\text{inositol}$ phosphates measured in both control and stimulated tissue slices and the effect of subsequent receptor blockade on these concentrations. A major feature of the response to agonist is the greater accumulation of $[^3\text{H}]\text{Ins}(1/3)\text{P}$ over that of $[^3\text{H}]\text{Ins}(4)\text{P}$. Similarly disproportionate accumulation of these metabolites was also observed in cerebral-cortex slices after persistent carbachol stimulation in the absence of Li^+ [29] and in rat brain after administration *in vivo* of muscarinic-receptor agonists and/or LiCl [39]. This implies that the inclusion of Li^+ does not dramatically alter the relative operation of distinct

Table 1. Effects of carbachol on the accumulation of $[^3\text{H}]$ inositol phosphates and of subsequent atropine blockade on the redistribution of ^3H radiolabel between metabolites in cerebral-cortex slices in the presence of LiCl

Cerebral-cortex slices were labelled with $[^3\text{H}]$ inositol and subsequently incubated with LiCl (5 mM) or with carbachol (1 mM) + LiCl for 5 min as described in the legend to Fig. 2. Reactions were then either stopped or continued for the indicated times in the presence of 10 μM atropine before extraction and analysis of $[^3\text{H}]$ inositol phosphates. Results in (a) are for the sum total of $[^3\text{H}]\text{Ins}(1/3)P + [^3\text{H}]\text{Ins}(4)P + [^3\text{H}]\text{Ins}(1,3)P_2 + [^3\text{H}]\text{Ins}(1,4)P_2 + [^3\text{H}]\text{Ins}(3,4)P_2 + [^3\text{H}]\text{Ins}(4,5)P_2 + [^3\text{H}]\text{Ins}(1,3,4)P_3 + [^3\text{H}]\text{Ins}(1,4,5)P_3 + [^3\text{H}]\text{Ins}(1,3,4,5)P_4$. $[^3\text{H}]\text{Ins}(3,4)P_2$ and $[^3\text{H}]\text{Ins}(4,5)P_2$ were minor products contributing < 500 d.p.m. each to the total under any of the described conditions.

(a) Effects on the accumulation of total $[^3\text{H}]$ inositol phosphates							
	Time after atropine blockade (min)	Total $[^3\text{H}]$ inositol phosphate (d.p.m./mg of protein)		Increase above basal (d.p.m./mg of protein)			
	0 Control	9330 $\pm$ 547		—			
	0 Carbachol	89368 $\pm$ 4418		80037 $\pm$ 4848			
	2.5 Control	8938 $\pm$ 679		—			
	2.5 Carbachol	91781 $\pm$ 3931		82843 $\pm$ 3873			
	5.0 Control	10134 $\pm$ 415		—			
	5.0 Carbachol	91398 $\pm$ 4290		81464 $\pm$ 4105			
	10.0 Control	13210 $\pm$ 818		—			
	10.0 Carbachol	93329 $\pm$ 1661		80119 $\pm$ 2478			
	20.0 Control	19814 $\pm$ 1620		—			
	20.0 Carbachol	93740 $\pm$ 4302		73925 $\pm$ 4834			

(b) Effects on major individual metabolites							
Time after atropine blockade (min)	$[^3\text{H}]$ Inositol phosphate (d.p.m./mg of protein)						
	(1/3)	(4)	(1,3)	(1,4)	(1,3,4)	(1,4,5)	(1,3,4,5)
0 Control	3712 $\pm$ 231	1589 $\pm$ 44	59 $\pm$ 4	2135 $\pm$ 177	123 $\pm$ 44	1134 $\pm$ 111	504 $\pm$ 61
0 Carbachol	33168 $\pm$ 1854	7431 $\pm$ 195	6265 $\pm$ 383	25121 $\pm$ 1105	2196 $\pm$ 131	3241 $\pm$ 172	11486 $\pm$ 993
10.0 Control	6331 $\pm$ 590	2600 $\pm$ 160	88 $\pm$ 4	2462 $\pm$ 141	114 $\pm$ 10	1169 $\pm$ 89	303 $\pm$ 55
10.0 Carbachol	61824 $\pm$ 1035	17607 $\pm$ 389	385 $\pm$ 5	9100 $\pm$ 311	225 $\pm$ 17	2605 $\pm$ 200	1084 $\pm$ 176

pathways for inositol polyphosphate metabolism over the current time scale, despite previous reports that prolonged exposure to Li^+ markedly attenuates the accumulation of $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,4,5)P_3$ resulting from persistent (> 10 min) exposure to carbachol [40,41]. However, we note that, under the present conditions, carbachol evoked much smaller increases in the accumulation of $[^3\text{H}]\text{Ins}(3,4)P_2$ than expected from previous data (Fig. 1) [29]. This presumably reflects the action of Li^+ on inositol polyphosphate 1-phosphatase [42], which degrades $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,4)P_2$ to $\text{Ins}(3,4)P_2$ and $\text{Ins}(4)P$ respectively. A consequence of this inhibitory action of Li^+ may be enhanced metabolism of $\text{Ins}(1,3,4)P_3$ via Li^+ -insensitive 4-phosphatase to $[^3\text{H}]\text{Ins}(1,3)P_2$. However, as $[^3\text{H}]\text{Ins}(1,3)P_2$ and $[^3\text{H}]\text{Ins}(3,4)P_2$ are hydrolysed to yield $[^3\text{H}]\text{Ins}(1)P$ and $[^3\text{H}]\text{Ins}(3)P$ respectively, this effect is not reflected as a change in the $[^3\text{H}]\text{Ins}(1/3)P : [^3\text{H}]\text{Ins}(4)P$ ratio.

Table 1(b) also identifies the major $[^3\text{H}]$ inositol polyphosphates produced in response to carbachol stimulation in the presence of Li^+ and reveals that, over a period of 10 min following receptor blockade, concentrations of each fell toward unstimulated values. The incomplete return to control concentrations most probably reflects an increase in specific radioactivity of ^3H -labelled products induced by prior carbachol stimulation as previously discussed [24]. In contrast with the fall in stimulated $[^3\text{H}]$ inositol polyphosphate concentrations, Table 1(b) shows that concentrations of $[^3\text{H}]\text{Ins}(1/3)P$ and $[^3\text{H}]\text{Ins}(4)P$ both increased after receptor blockade. Figs. 2 and 3 compare the relative changes in the concentrations of these different ^3H -labelled products.

Fig. 2 illustrates the accumulation over basal of the major ^3H -labelled products after exposure to carbachol and Li^+ for 5 min and the subsequent redistribution of $[^3\text{H}]$ inositol radiolabel over the ensuing 20 min period of receptor blockade. The data are

presented such that the decreasing concentrations of those $[^3\text{H}]$ inositol polyphosphates which can be identified on the basis of previous data discussed above either as products of $[^3\text{H}]\text{Ins}(1,4,5)P_3$ metabolism via initial 3-kinase or 5-phosphatase activity can be directly compared with the increasing concentrations of the corresponding $[^3\text{H}]$ inositol monophosphates which they would be predicted to yield. Hence the decremental loss in the sum total of major kinase-derived ^3H -labelled products $\{[^3\text{H}]\text{Ins}(1,3,4,5)P_4 + [^3\text{H}]\text{Ins}(1,3,4)P_3 + [^3\text{H}]\text{Ins}(1,3)P_2\}$ after receptor blockade is contrasted with the incremental gain in $[^3\text{H}]\text{Ins}(1/3)P$. Similarly, loss of $[^3\text{H}]\text{Ins}(1,4)P_2$ is compared with the increase in $[^3\text{H}]\text{Ins}(4)P$. Since $[^3\text{H}]\text{Ins}(1,4,5)P_3$ can yield either $[^3\text{H}]\text{Ins}(1/3)P$ or $[^3\text{H}]\text{Ins}(4)P$, the relatively small changes in its concentration are not included. These comparative data show that the patterns of change in $[^3\text{H}]\text{Ins}(1/3)P$ and $[^3\text{H}]\text{Ins}(4)P$ accumulation after atropine addition appear to mirror those of their expected precursors. After receptor blockade, concentrations both of 3-kinase-derived ^3H -labelled products and of $[^3\text{H}]\text{Ins}(1,4)P_2$ fell, but at different rates, with the rate of decline in the former exceeding that in the latter, perhaps reflecting either the relative intrinsic activity of these two routes or their different sensitivity to inhibition by Li^+ . Correspondingly, $[^3\text{H}]\text{Ins}(1/3)P$ accumulation continued at a rate very similar to that seen in the presence of carbachol for up to 2.5 min after atropine addition, whereas $[^3\text{H}]\text{Ins}(4)P$ accumulation continued unabated at its agonist-stimulated rate for up to 5 min past receptor blockade. In this respect, these results closely resemble those previously obtained after a similar procedure in carbachol-stimulated rat parotid acinar cells [7].

Despite the good qualitative correlation between loss of $[^3\text{H}]$ inositol polyphosphates and gain in $[^3\text{H}]\text{Ins}P(s)$, Fig. 2 also highlights an interesting quantitative discrepancy. This is revealed

Fig. 2. Accumulation of [^3H]inositol phosphate in response to carbachol + Li^+ and quantitative hydrolysis of [^3H]inositol poly- to mono-phosphates on subsequent receptor blockade

Cerebral-cortex slices were labelled with [^3H]inositol as in the legend to Fig. 1, except that LiCl (5 mM) was added to all samples after 55 min. After 60 min labelling (zero time), carbachol (1 mM) or Krebs-medium vehicle was added. Incubations were continued for 5 min and then either stopped or continued for the indicated times after addition of 10 μM -atropine. [^3H]Inositol phosphates were then extracted and analysed as described in the Materials and methods section. Results are means $\pm$ S.E.M. of 3 experiments and are expressed as d.p.m./mg of protein for each ^3H -labelled product after subtraction of corresponding control values. Changes in basal radioactivity over this time scale are evident from Table 1. Figs. 2(a)–2(d) show data for: (a) [^3H]Ins(1/3)P; (b) [^3H]Ins(4)P; (c) the sum of [^3H]Ins(1,3,4,5) P_4 + [^3H]Ins(1,3,4) P_3 + [^3H]Ins(1,3) P_2 ; (d) [^3H]Ins(1,4) P_2 . Numerical values shown compare the gain in [^3H]monophosphates or loss in [^3H]polyphosphates over the first 10 min after atropine addition.

by comparison of the data for loss of [^3H]Ins(1,4) P_2 and gain in [^3H]Ins(4)P, where at each interval after atropine addition the yield of [^3H]Ins(4)P was less than expected from the fall in concentration of [^3H]Ins(1,4) P_2 (gain as percentage of loss = 59 ± 8 , 58 ± 2 , 56 ± 3 and 42 ± 3 % respectively at 2.5, 5.0, 10.0 and 20.0 min after atropine; means $\pm$ S.E.M. of 3 experiments). This suggests that a significant proportion of [^3H]Ins(1,4) P_2 is degraded to [^3H]Ins(1)P, contrary to expectation from studies using broken-cell preparations (see above). In agreement with

this suggestion, the incremental gain in [^3H]Ins(1/3)P at each point after receptor blockade exceeded that lost from its predicted precursors. This point is illustrated by the values shown in Fig. 2. We would emphasize, however, that accurate quantification of the 'over-production' of [^3H]Ins(1/3)P and correlation of this with the 'under-production' of [^3H]Ins(4)P was difficult, given the substantially larger carbachol-stimulated accumulation of [^3H]Ins(1/3)P than of [^3H]Ins(4)P and the summation errors involved in predicting the expected increase in [^3H]Ins(1/3)P

from loss of its multiple precursors. The gain in total [³H]InsP(s) as a percentage of total [³H]polyphosphates lost at 2.5, 5.0, 10.0 and 20.0 min was 126 ± 18, 110 ± 15, 103 ± 9 and 88 ± 16 % respectively over three experiments. Since comparisons of [³H]Ins(1,4)P₂ losses and [³H]Ins(4)P gains, however, are more direct, we can feel confident of the disparity between these values, particularly as neither Table 1 nor Fig. 2 provides any evidence for continued inositol monophosphatase activity. We emphasize here that the loss of [³H]Ins(1,3,4)P₃ through metabolism other than by dephosphorylation is unlikely [43].

In view of this unexpected result regarding [³H]Ins(1,4)P₂ hydrolysis, we extended our previous studies of inositol polyphosphate hydrolysis by broken-cell preparations from cerebral cortex [29] to a more detailed examination of the degradation of [³H]Ins(1,4)P₂ as described in the Materials and methods section. In assays with either whole tissue homogenates or supernatant fractions in which [³H]Ins(1,4)P₂ hydrolysis was studied in both the presence and the absence of EDTA, 10 mM-LiCl and/or of 100 μM-D/L-Ins(1,4)P₂ carrier, the only product detected, even under conditions of virtually complete conversion into [³H]InsP, was [³H]Ins(4)P, as previously reported [29,44]. The present data suggesting the dual hydrolysis of Ins(1,4)P₂ by both 1- and 4-phosphatase in intact cells are, however, supported by kinetic studies of muscarinic-receptor-stimulated inositol phosphate turnover in parotid acinar cells [7]. Thus it may be that studies in broken-cell preparations fail to identify the full range of enzymic activities responsible for inositol polyphosphate metabolism. It is not unreasonable to assume that compartmentation of substrates and enzymes or regulation of the latter which may occur in intact cells is not observed in cell-free systems. Confirmation of the present observations will require further studies in intact stimulated cells, but may provide an explanation other than the direct hydrolysis of PtdIns to account for the observed accumulation of both Ins(1)P and Ins(4)P in circumstances [27,28] in which there is no detectable activity of Ins(1,4,5)P₃ 3-kinase.

The dual hydrolysis of Ins(1,4)P₂ to both Ins(1)P and Ins(4)P rather than exclusively to Ins(4)P has important implications for the study of inositol polyphosphate metabolism, since the ratio of Ins(1/3)P:Ins(4)P, which in the presence of Li⁺ would otherwise be expected to reflect the proportion of Ins(1,4,5)P₃ metabolized via 3-kinase and 5-phosphatase [assuming PtdIns(4,5)P₂ as the substrate for receptor-activated PIC] will be distorted to give an over-estimate of kinase activity. The present data clearly illustrate this point. Table 1 shows that, of the total increase in [³H]InsP after atropine treatment, about 74 % is accounted for by [³H]Ins(1/3)P. However, Fig. 2 shows that, of this, only about 70 % can be directly attributed to hydrolysis of [³H]Ins(1,3,4,5)P₄, [³H]Ins(1,3,4)P₃ and [³H]Ins(1,3)P₂, with the remainder deriving from [³H]Ins(1,4)P₂. Thus, of the total increase in [³H]InsP observed after receptor blockade, about 50 % originates from kinase-pathway intermediates and hence PtdIns(4,5)P₂, and the other 50 % from Ins(1,4)P₂ and thus ultimately from either PtdIns(4)P or PtdIns(4,5)P₂.

Since Fig. 2 also shows that the rates of accumulation of each [³H]Ins(1/3)P and [³H]Ins(4)P during the initial minutes after receptor blockade are closely similar to the prior rate measured in the presence of agonist alone, it is very probable that the metabolic origins of the [³H]inositol monophosphates are the same during both phases. Since formation of [³H]InsP(s) after atropine addition is quantitatively accounted for by [³H]polyphosphate hydrolysis, the accumulation of these [³H]InsP(s) observed in the continuous sustained presence of agonist is likely to be similarly derived and thus to arise ultimately from cleavage of [³H]PtdIns(4,5)P₂ and/or [³H]PtdIns(4)P. This suggestion is further supported by the observation that the rates

Fig. 3. Comparative rates of hydrolysis of [³H]inositol polyphosphates in carbachol-pre-stimulated cerebral-cortex slices

Tissue slices were incubated as described in the legend to Fig. 2. The Figure compares the rates at which concentrations of Ins(1,3,4,5)P₄ (●), [³H]Ins(1,3,4)P₃ (○), [³H]Ins(1,3)P₂ (△) and [³H]Ins(1,4)P₂ (■) declined after atropine addition. For each ³H-labelled product, 100 % represents the increase in concentration above basal achieved during the 5 min exposure to carbachol + LiCl; these values are apparent from Table 1. Increased concentrations of each ³H-labelled product above basal at the indicated times after atropine addition are expressed as a relative percentage. Data represent the mean values obtained in the three experiments described in Fig. 2 and Table 1.

of both [³H]Ins(1/3)P and [³H]Ins(4)P accumulation shown in Fig. 2 are maintained for at least 30 min in the continuous presence of carbachol and LiCl (I. H. Batty & S. R. Nahorski, unpublished work). This conclusion is in agreement with two other previous studies of muscarinic-receptor-mediated inositol lipid turnover in parotid acinar cells [7] and SK-N-SH neuroblastoma cells [8], which have used similar approaches to demonstrate that the accumulation of [³H]InsP in response to persistent receptor stimulation is likely to arise through sustained polyphosphoinositide hydrolysis.

Fig. 3 illustrates several final points established by the experiments described in Table 1 and Fig. 2. The data from these studies are presented to allow direct comparison of the rates at which the carbachol-pre-stimulated accumulation of distinct [³H]inositol polyphosphates decline after receptor blockade, thus giving an approximate measure of their comparative rates of hydrolysis in stimulated tissue. Note that the stimulated concentrations of [³H]Ins(1,4,5)P₃ were maximally reversed by atropine by the earliest time monitored (see [24] and Table 1). It is clear from Fig. 3 that after atropine addition concentrations of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ fell most rapidly and in tandem.

This is consistent with the rapid reversal of stimulated [^3H]Ins(1,4,5) P_3 accumulation, and suggests that [^3H]Ins(1,3,4) P_3 is degraded almost as rapidly as it is formed from Ins(1,3,4,5) P_4 . This result also indicates that, despite the probable inhibition of inositol polyphosphate 1-phosphatase by Li^+ under the current conditions, cerebral cortex exhibits a marked capacity to hydrolyse [^3H]Ins(1,3,4) P_3 via Li^+ -insensitive removal of the 4-phosphate to produce [^3H]Ins(1,3) P_2 , as might be expected from the high activity of Ins(1,4,5) P_3 4-phosphatase activity previously reported in brain [45]. These observations both support our previous study demonstrating rapid turnover of [^3H]Ins(1,3,4) P_3 in carbachol-stimulated brain slices [24] and may explain the failure of [^3H]Ins(1,3,4,6) P_4 to accumulate in response to muscarinic-receptor activation in this tissue [29]. These data show close agreement with results obtained in SK-N-SH neuroblastoma cells, where Ins(1,3,4) P_3 turnover appears to be similarly rapid [8], but contrast interestingly with a previous study in rat parotid acinar cells, where Ins(1,3,4) P_3 is metabolized very much more slowly [7]. The rate of decline in the stimulated concentration of [^3H]Ins(1,3) P_2 shown in Fig. 3 is also clearly compatible with its formation sequentially from [^3H]Ins(1,4,5) P_3 via Ins(1,3,4,5) P_4 and Ins(1,3,4) P_3 , and in this respect the results illustrated in Fig. 3 strongly support the early kinetic data presented in Fig. 1. In contrast with the rapid decline in the concentrations of each of the other [^3H]inositol polyphosphates on receptor blockade, hydrolysis of [^3H]Ins(1,4) P_2 is relatively slow. Indeed, comparison with a previous study indicates an approx. 2–3-fold slower rate of degradation of [^3H]Ins(1,4) P_2 than previously observed for total [^3H]Ins P_2 (~70% 1,4-isomer [24,29]) in the absence of Li^+ in cerebral cortex. This is again compatible with the inhibition of inositol polyphosphate 1-phosphatase by Li^+ under the present conditions.

In the preceding discussion we have assumed that PtdIns, PtdIns(4) P and PtdIns(4,5) P_2 are the only potential substrates for receptor-activated PIC. Recent studies, however, have identified a novel set of polyphosphoinositides, PtdIns(3) P , PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 [20,46]. We were previously unable to demonstrate the presence of [^3H]PtdIns P_3 in control or carbachol-stimulated cerebral-cortex slices acutely labelled with [^3H]inositol [23]. In view of recent observations, we have repeated and extended our previous analyses as described in the Materials and methods section, using tissue slices heavily labelled with [^3H]inositol. In [^3H]lipid extracts from controls or tissue slices exposed to 1 mM-carbachol for up to 1 min we were again unable to detect any [^3H]PtdIns P_3 , whereas [^3H]PtdIns P and [^3H]PtdIns P_2 were >99% isomerically homogeneous [^3H]PtdIns(4) P and [^3H]PtdIns(4,5) P_2 respectively. A ^3H -labelled material, which may be [^3H]PtdIns(3) P and which constituted ~0.5–1.0% of [^3H]PtdIns P in all samples, was, however, also detected (I. H. Batty & S. R. Nahorski, unpublished work). These observations strongly suggest that hydrolysis of 3-phosphorylated phosphoinositides is unlikely to contribute to or complicate the kinetic studies discussed above, particularly as [^3H]lipid labelling in cerebral cortex reflects the total heterogeneous cell population rather than the presumably limited subpopulation which responds to muscarinic-receptor stimulation. This view is reinforced by the previous observations that 3-phosphorylated phosphoinositides are poor substrates for purified PICs [47,48].

In summary, the present data confirm and greatly extend the results of our earlier studies [23,24,29], strongly suggesting that muscarinic-receptor-mediated inositol phospholipid turnover in cerebral cortex is initiated by stimulated PIC hydrolysis of PtdIns(4,5) P_2 releasing Ins(1,4,5) P_3 , which is rapidly metabolized to both Ins(1,3,4,5) P_4 and Ins(1,4) P_2 . The present results, however, also demonstrate that the sequential degradation of

Ins(1,3,4,5) P_4 to free inositol in intact cells occurs through Ins(1,3,4) P_3 , Ins(1,3) P_2 and Ins(3,4) P_2 and Ins(1/3) P , whereas that of Ins(1,4) P_2 proceeds, at least in the presence of Li^+ , via both Ins(4) P and Ins(1) P . The measured contributions of these alternative metabolic routes to the inositol monophosphate pool in stimulated cells indicates that PtdIns(4,5) P_2 [and perhaps PtdIns(4) P , but not PtdIns] is the predominant substrate for sustained PIC activity and that Ins(1,4,5) P_3 3-kinase plays a major role in removal of continuously released Ins(1,4,5) P_3 .

Financial support from the Medical Research Council (U.K.) is gratefully acknowledged.

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Received 9 April 1992/22 June 1992; accepted 3 July 1992