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Rapid accumulation and sustained turnover of inositol phosphates in cerebral-cortex slices after muscarinic-receptor stimulation

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The rapid kinetics of [^{3}H]inositol phosphate accumulation and turnover were examined in rat cerebral-cortex slices after muscarinic-receptor stimulation. Markedly increased [^{3}H]inositol polyphosphate concentrations were observed to precede significant stimulated accumulation of [^{3}H]inositol monophosphate. New steady-state accumulations of several ^{3}H-labelled products were achieved after 5–10 min of continued agonist stimulation, but were rapidly and effectively reversed by subsequent receptor blockade. The results show that muscarinic-receptor activation involves phosphoinositidase C-catalysed hydrolysis initially of polyphosphoinositides rather than of phosphatidylinositol. Furthermore, prolonged carbachol stimulation is shown not to cause receptor desensitization, but to allow persistent hydrolysis of [^{3}H]phosphatidylinositol bisphosphate and permit sustained metabolic flux through the inositol tris-/tetrakis-phosphate pathway.

INTRODUCTION

Stimulation of a large number of cell-surface receptors promotes hydrolysis of inositol phospholipids through a G-protein-mediated activation of a specific phosphoinositidase C [1]. The initial substrate for this receptor-coupled enzyme appears to be PtdIns(4,5)P_2, cleavage of which releases the two putative second messengers, Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol [2,3]. This primary response to agonist is accompanied by an increased turnover of PtdIns and PtdIns(4)P and a stimulated accumulation of InsP_1 and InsP_2. These latter events may reflect a compensatory re-synthesis of PtdIns(4,5)P_2 [4], with the lower inositol phosphates arising through InsP_3 dephosphorylation. Alternatively, more than one phosphoinositide may be cleaved in response to receptor activation, with each inositol phosphate deriving, in part, from its appropriate phospholipid precursor. The situation in brain remains unclear [5,6], since information relating to the routes and regulation of polyphosphoinositide and inositol polyphosphate metabolism in cerebral cells is as yet limited.

Further complication to this signalling system is now recognized, in that Ins(1,4,5)P_3 can be metabolized by dephosphorylation to Ins(1,4)P_2 or by phosphorylation to Ins(1,3,4,5)P_4, followed by successive dephosphorylation of this molecule through Ins(1,3,4)P_3 to free inositol. Whereas the former pathway may represent an important inactivation route, phosphorylation to Ins(1,3,4,5)P_4 may generate additional intracellular messengers (see [7,8]). In the present study we have attempted to clarify the order of events after muscarinic-receptor stimulation in rat cerebral cortex, by determining both the initial sequence of inositol phosphate production and, by using a receptor-blocking method, the subsequent turnover of these products under steady-state conditions.

MATERIALS AND METHODS

Materials

myo-[2-^{3}H]Inositol (12.6 Ci/mmol) was purchased from New England Nuclear. Analytical-grade anion-exchange resin was from Bio-Rad. All other materials were purchased from Sigma, BDH, Aldrich or Fisons.

Methods

Cerebral-cortex slices (350 μm × 350 μm) were prepared from male Wistar rats (175–250 g) and pre-incubated in bulk in modified Krebs–Henseleit buffer as described by Brown *et al.* [9]. Samples (50 μl) of gravity-packed tissue (approx. 1–2 mg of protein) were then incubated for a further 60 min in the same medium supplemented with 5 μCi of *myo*-[2-^{3}H]inositol before exposure to stimulus. Where present, drugs were added as samples (10 μl) of 30-fold-concentrated solutions to give the final concentrations indicated and a total 300 μl volume.

Reactions were stopped by addition of 300 μl of ice-cold 1 M-trichloroacetic acid. After 10–20 min on ice, tissue was sedimented by centrifugation, and portions (500 μl) of supernatants from triplicate incubations were pooled (see ref. [10]). Acid extracts were then neutralized and subsequently analysed by either Dowex anion-exchange chromatography for [^{3}H]InsP_1–[^{3}H]InsP_4 or by h.p.l.c. for [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3, exactly as previously described [10]. [^{3}H]Inositol phosphates were quantified by liquid-scintillation counting, with a counting efficiency for ^{3}H of approx. 25%.

RESULTS AND DISCUSSION

Several previous studies in brain [9–13] have demonstrated hydrolysis of (poly)phosphoinositides after stimulation of central-nervous-system neurotransmitter receptors, but have not characterized the rapid kinetics of these responses in sufficient detail to allow clear identification of the phospholipid substrate(s) for the receptor mechanisms involved. We have therefore addressed this problem, using two complementary approaches. The first of these is exemplified by Fig. 1, which compares both the initial and subsequent time course of [^{3}H]inositol phosphate accumulations in [^{3}H]inositol-labelled brain slices after muscarinic-receptor stimulation. At the earliest time studied (5 s), con-

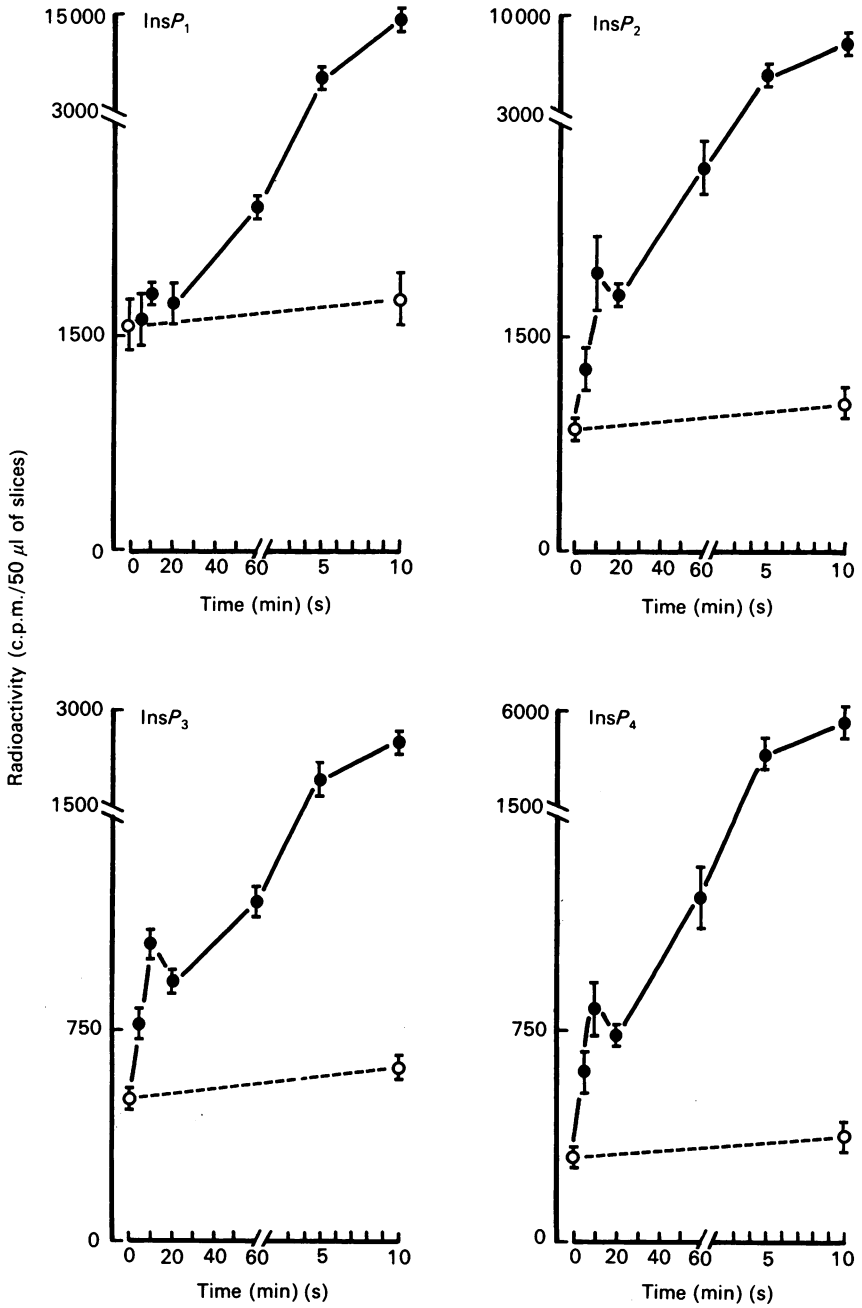

Fig. 1. Time courses of carbachol-stimulated [^{3}H]inositol phosphate accumulations

Cerebral-cortex slices were labelled with [^{3}H]inositol and incubated either without (○) or with (●) 1 mM-carbachol for the times indicated. [^{3}H]Ins*P*(s) were then extracted, separated and quantified as described in the Materials and methods section. Results are means ± S.E.M. for three separate experiments. Comparison of the zero-time control values with stimulated values by Student's paired *t* test showed that carbachol significantly ($P < 0.05$) increased concentrations of [^{3}H]InsP_4, [^{3}H]InsP_3 and [^{3}]InsP_2 by 5 s. Stimulated accumulation of [^{3}H]InsP_1 was not significant until after 20 s.

centrations of [^{3}H]InsP_4, [^{3}H]InsP_3 and [^{3}H]InsP_2 were markedly elevated over basal values, whereas a significant stimulated accumulation of [^{3}H]InsP_1 was not apparent until later than 20 s. In the continued presence of stimulus, concentrations of each [^{3}H]inositol phosphate progressively increased, approaching new steady-state values between 5 and 10 min as previously observed [14,15].

It should be emphasized that under identical conditions we have previously demonstrated very similar rapid kinetics of [^{3}H]InsP_3 and [^{3}H]InsP_4 accumulation in cerebral cortex in response to carbachol, and have established that the initial increase in [^{3}H]InsP_3 is accounted for almost exclusively by the Ins(1,4,5)P_3 isomer [10]. Thus our present and previous data together show that muscarinic-receptor activation in brain evokes an initial selective hydrolysis of polyphosphoinositide(s) rather than of PtdIns. Fig. 1, however, also shows that the initial stimulated accumulation of [^{3}H]InsP_3 is accompanied by quantitatively similar increased production of [^{3}H]InsP_4 and [^{3}H]InsP_2, whereas, after more prolonged exposure to stimulus, accumulations of lower

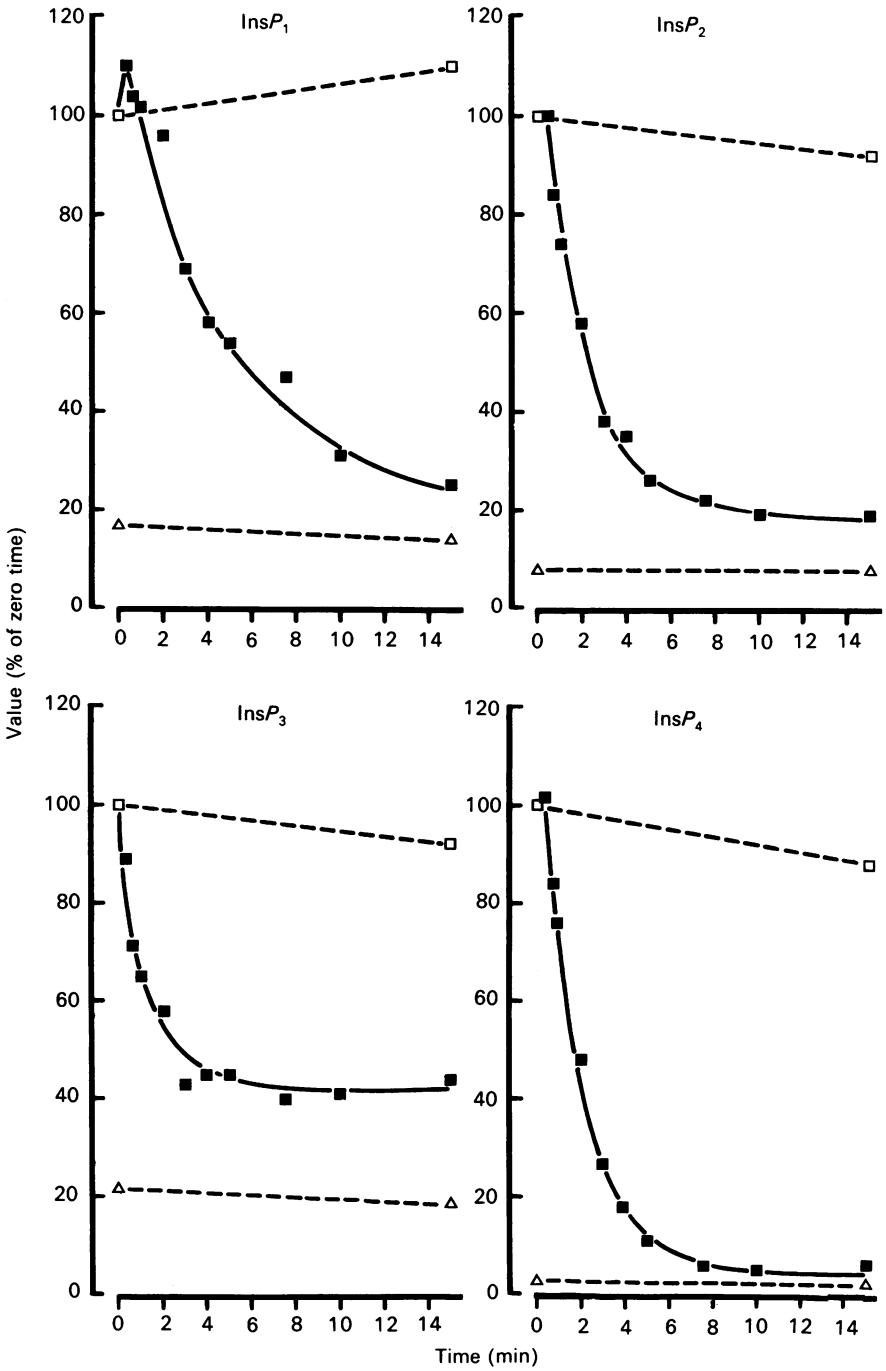

Fig. 2. Rates of [^{3}H]inositol phosphate decay from carbachol-stimulated steady state after receptor blockade

[^{3}H]Inositol-labelled cerebral-cortex slices were pre-stimulated with 1 mM-carbachol for 30 min (■, □) or run as controls (△). Reactions were then either stopped (zero time) or continued for the indicated periods after addition of 10 μM-atropine (■) or Krebs buffer vehicle (□, △) before extraction of [^{3}H]Ins$P$(s) and analysis by Dowex chromatography. Control values were not affected by atropine (result not shown). Results represent the mean values from four experiments and indicate [^{3}H]Ins$P$ concentrations relative to the mean steady-state values at zero time. Values (±S.E.M.) representing 100% are: [^{3}H]InsP_1 23828±3155, [^{3}H]InsP_2 9141±1618, [^{3}H]InsP_3 2896±542 and [^{3}H]InsP_4 6231±800 c.p.m./50 μl tissue sample.

[^{3}H]inositol phosphates exceed that of [^{3}H]InsP_3 by several-fold. Since the [^{3}H]InsP_4 measured under these conditions has been identified solely as the [^{3}H]Ins(1,3,4,5)P_4 isomer [10,16], which arises through the action of Ins(1,4,5)P_3 3-kinase, the first of these observations suggests an initial rapid metabolic turnover of [^{3}H]Ins(1,4,5)P_3 which, if subsequently maintained, may also account for the larger accumulations of the other, potentially more slowly metabolized, ^{3}H-labelled products. However, as the specific radioactivities and rates of hydrolysis of the [^{3}H]inositol phosphates measured are unknown, these data could also imply hydrolysis of other phosphoinositides in addition to PtdIns(4,5)P_2.

In order to define better the inositol phospholipid substrate(s) hydrolysed in response to muscarinic-receptor stimulation, we have therefore examined the turnover of the separate [^{3}H]inositol phosphates under conditions of steady-state stimulation, using a receptor-blocking approach [17]. [^{3}H]Inositol-labelled cerebral-cortex slices were either run as controls or exposed to 1 mM-carbachol for 30 min, at which time maximally stimulated steady-state accumulations of [^{3}H]InsP_1–[^{3}H]InsP_4 are achieved. A large excess ($> 5000 \times K_i$) of the muscarinic-receptor antagonist atropine was then added to terminate rapidly and prevent further response to agonist, and the remaining concentrations of each [^{3}H]inositol phosphate were then determined at various intervals. The rates at which the stimulated concentrations of the [^{3}H]inositol phosphates decay back toward control values after receptor blockade give an approximation of the rates at which these products are hydrolysed under conditions of steady-state stimulation. The decay curves for [^{3}H]InsP_1–[^{3}H]InsP_4 are illustrated in Fig. 2 and those for separate [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3 in Fig. 3. These data show that receptor blockade induced a rapid reversal of the response to carbachol, which was maximally decreased ($> 85\%$ for each [^{3}H]inositol phosphate except [^{3}H]Ins(1,4,5)P_3; see below) within 10–15 min of atropine addition. In contrast, stimulated samples not exposed to antagonist maintained relatively constant amounts of each [^{3}H]inositol phosphate across the same time course. Control values were unaffected by atropine (results not shown). Together these findings demonstrate that in the presence of agonist (i) there is a continuous turnover of each [^{3}H]inositol phosphate and (ii) there are only small changes in ^{3}H-labelled-product specific radioactivity under the defined radiolabelling conditions. Changes in specific radioactivity are revealed by that fraction of the response to agonist which is not reversible by antagonist, and are most obviously observed for [^{3}H]Ins(1,4,5)P_3, as this product accumulates to the smallest extent in proportion to control compared with all the other [^{3}H]inositol phosphates assayed. However, as receptor blockade results in a rapid return of the concentration of each [^{3}H]inositol phosphate close to unstimulated values, the present data also demonstrate that the responses to agonist predominantly reflect increases in inositol phosphate mass and not merely changes in specific radioactivity. Furthermore, the limited fraction of agonist-stimulated labelling that may be accounted for by increased specific radioactivity was approximately the same (approximately equivalent to $2 \times$ basal), relative to appropriate control values, for each [^{3}H]inositol phosphate, perhaps indicating a common [^{3}H]phosphoinositide cursor. Most probably agonist-stimulated increases in specific radioactivity are largely confined to the first few minutes of receptor activation rather than being progressive across the entire stimulation period, since after a prior 30 min exposure to carbachol no further increase in [^{3}H]inositol phosphate labelling was observed over the subsequent time courses shown in Figs. 2 and 3. This suggestion is supported by preliminary studies in which both tritiated and unlabelled Ins(1,4,5)P_3 have been measured under the current conditions (I. H. Batty, R. A. J. Challiss & S. R. Nahorski, unpublished work). The ability of antagonist effectively to reverse the response to agonist thus demonstrates that the steady-state stimulated accumulations of all [^{3}H]inositol phosphates reflect

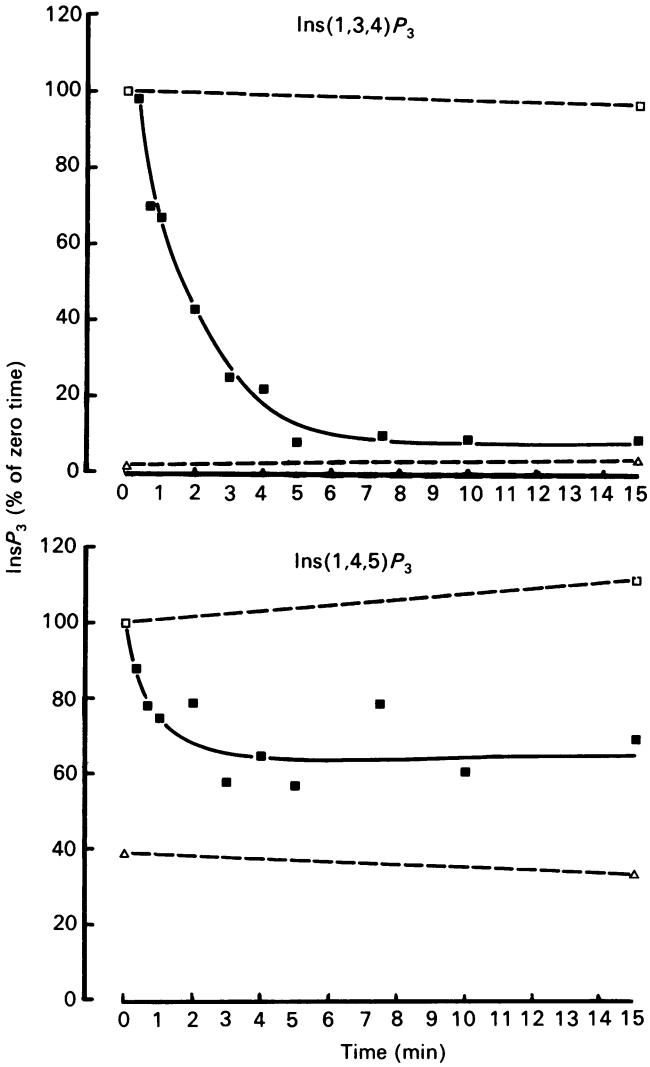

Fig. 3. Atropine-induced decay of [^{3}H]InsP_3 accumulations from carbachol-stimulated steady state

Experimental conditions were as described in the legend to Fig. 2, except that [^{3}H]InsP_3(s) were analysed by anion-exchange h.p.l.c. Data are expressed as for Fig. 2 and are derived from three of the experiments shown in Fig. 2: ■, □, stimulated samples; △, controls; ■, effects of atropine (10 μM). Values (±S.E.M.) representing 100% are: [^{3}H]Ins-(1,3,4)P_3 1049±238 and [^{3}H]Ins(1,4,5)P_3 1846±261 c.p.m./50 μl tissue sample.

their continuous synthesis and degradation (see [14,15,18]). It is therefore evident that, over the time course of these experiments, the muscarinic receptor does not desensitize to carbachol and that in the continued presence of this agonist there is a sustained hydrolysis of PtdIns(4,5)P_2, resulting in a persistent release of the two second messengers, Ins(1,4,5)P_3 and diacylglycerol, and their metabolic products.

The different rates of [^{3}H]inositol phosphate hydrolysis also shown here additionally indicate that a substantial proportion of agonist-stimulated [^{3}H]InsP_1 and [^{3}H]InsP_2 accumulation is likely to result from the continuing turnover of [^{3}H]Ins(1,4,5)P_3. After addition of atropine to carbachol-stimulated tissue, [^{3}H]Ins(1,4,5)P_3 was the first product to show a decreased accumulation. Subse-

quently, concentrations of [^{3}H]InsP_4, [^{3}H]Ins(1,3,4)P_3 and [^{3}H]InsP_2 were also found to decline at closely similar rates. In contrast, a marked fall in the concentration of [^{3}H]InsP_1 was not detected until atropine reversal of stimulated [^{3}H]Ins(1,4,5)P_3 concentrations was almost complete. These results are fully consistent with the rapid kinetics of [^{3}H]inositol polyphosphate accumulation discussed above, and suggest an initial phosphoinositidase C-catalysed cleavage of PtdIns(4,5)P_2 to release Ins(1,4,5)P_3, followed by metabolism of this molecule by both 5-phosphatase and 3-kinase enzymes and subsequent degradation of the resultant products by sequential dephosphorylation. A recent parallel study [16] has demonstrated that, under the conditions of steady-state stimulation employed here, the [^{3}H]InsP_4 measured comprises a single isomer, identified by chemical degradation studies and co-chromatography as [^{3}H]Ins(1,3,4,5)P_4 [10,16]. Alternatively, the [^{3}H]InsP_1 and [^{3}H]InsP_3 fractions can each be resolved into two major components which co-elute on h.p.l.c. with [^{3}H]Ins(1)P and [^{3}H]Ins(4)P and [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,4,5)P_3 respectively. The latter identities have yet to be confirmed by more rigorous methods, but are consistent both with our identification of [^{3}H]Ins(1,3,4,5)P_4 and with the isomeric composition of the [^{3}H]InsP_2 fraction in stimulated brain slices. The latter can be resolved into three major components identified by co-chromatography and chemical degradation as [^{3}H]Ins(1,3)P_2, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(3,4)P_2, and a fourth minor component, tentatively identified as [^{3}H]Ins(4,5)P_2 [16]. The accumulation of all the major isomeric components of each inositol phosphate fraction increases markedly on muscarinic-receptor stimulation under the present conditions [16]. Both the identities of these products in carbachol-stimulated brain slices and their metabolic relationships, also previously demonstrated in broken-cell preparations from brain [6,16,19], are compatible with and lend support to the metabolic sequence implied by the current data. However, the isomeric complexity of the lower inositol phosphate fractions also emphasizes the requirement for more detailed kinetic studies of inositol phosphate turnover than those attempted here before the metabolic pathways activated after muscarinic-receptor stimulation in brain can be conclusively defined.

In summary, the current data demonstrate that muscarinic-receptor stimulation in cerebral cortex promotes an initial, rapid phosphoinositidase C-mediated hydrolysis of polyphosphoinositides. Continued receptor activation evokes a persistent release of Ins(1,4,5)P_3, metabolism of which probably proceeds by both phosphorylation to Ins(1,3,4,5)P_4 and direct dephosphorylation to InsP_2. Since there is a large stimulated accumulation of Ins(1,3,4,5)P_4 coupled with a rapid metabolic turnover of this product, metabolism by the former route probably accounts for a substantial fraction of the continuous stimulated turnover of InsP_2 and InsP_1. Further, as Ins(1,3,4,5)P_4 derives directly from Ins(1,4,5)P_3, it is therefore suggested that muscarinic-receptor-stimulated phosphoinositide turnover in cerebral cortex is predominantly a consequence of PtdIns(4,5)P_2 [and possibly PtdIns(4)P] cleavage rather than of direct PtdIns hydrolysis. This conclusion differs from that proposed by Ackermann *et al.* [5] based on the observations that the major inositol monophosphate detected *in vivo* after muscarinic-receptor stimulation is D-Ins(1)P, whereas hydrolysis of Ins(1,4)P_2 by a soluble preparation of brain yields primarily Ins(4)P [20]. It should be noted, however, that in broken-cell preparations from brain both D- and L-Ins(1)P can be derived from Ins(1,3,4,5)P_4 via Ins(1,3,4)P_3, Ins(1,3)P_2 and Ins(3,4)P_2 [6,19], and recent studies from our laboratory [16] provide evidence for accumulation of the last two bisphosphates in carbachol-stimulated brain slices. A rapidly turning-over but limited pool of Ins(1,3)P_2 could generate substantial D-Ins(1)P without the necessity to invoke direct PtdIns cleavage to account for the accumulation of this monophosphate. Since the current data demonstrate a marked flux through the tris-/tetrakis-phosphate pathway without major accumulation of Ins(1,3,4)P_3 {i.e. [^{3}H]Ins(1,3,4)P_3 $< 20\%$ [^{3}H]Ins(1,3,4,5)P_4}, this possibility would appear worthy of further investigation.

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