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AGONIST-MEDIATED FORMATION OF INOSITOL MONOPHOSPHATE ISOMERS IN RAT CORTICAL PRISMS

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Abstract—The carbachol and adrenaline-mediated accumulation of inositol monophosphate isomers in rat cortical prisms has been studied using a commonly employed experimental protocol involving preincubation with myo-[2-^{3}H]-inositol and subsequent incubation with agonists in the presence of 10 mM LiCl. Inositol phosphate isomers have been analysed by HPLC and identified by comparison of their elution characteristics with those of commercially available standards and the degradation products of authentic Ins 1,3,4-P_3 and Ins 1,4,5-P_3. Incubation of prelabelled cortical prisms for 1 hr with 10 mM LiCl alone gives rise to accumulation of radioactivity in two inositol monophosphate peaks which co-elute with Ins 1-P and Ins 4-P and one major bisphosphate peak which co-migrates with Ins 1,4-P_2. Most of the monophosphate radioactivity is recovered in the Ins 4-P peak (Ins 1-P/Ins 4-P labelling ratio 0.68). Both carbachol and adrenaline produce dose-dependent increases in the labelling of Ins 1-P and Ins 4-P which are antagonized by atropine and prazosin respectively. However, carbachol produces a larger stimulation of accumulation of both monophosphates and also gives rise to a larger selective increase in the accumulation of Ins 1-P (Ins 1-P/4-P labelling ratio 1.40 in the presence of 1 mM carbachol, 0.98 in the presence of 1 mM adrenaline). Kinetic studies of the carbachol-stimulated increases in inositol mono- and bisphosphate labelling have revealed that, in the early period following carbachol addition (0–5 min), Ins 4-P and Ins 1,4-P_2 are labelled more rapidly than Ins 1-P, whereas the reverse is true at later periods (15–60 min) of the incubations. These observations, coupled with the low levels of labelling of the major Ins 1,3,4-P_3 breakdown products (Ins 1,3-P_2 and Ins 3,4-P_2) compared with that of Ins 1,4-P_2, suggest that large-scale production of Ins 1-P is a comparatively late feature of carbachol-mediated inositol phospholipid metabolism and that, if the Ins 1-P is derived from breakdown of Ins 1,3,4,5-P_4 via Ins 1,3,4-P_3, the turnover of Ins 1,3-P_2 + Ins 3,4-P_2 must be approximately one order of magnitude greater than that of the Ins 4-P precursor, Ins 1,4-P_2.

There is now a large amount of experimental evidence showing that activation of many types of receptor in the central nervous system can lead to enhanced formation of inositol phosphates (for recent reviews, see Refs 1 and 2). Brain slice preparations have been particularly popular for these studies and, although recent studies by a number of research groups have clearly demonstrated the agonist-mediated production of inositol polyphosphates [3–5] and of their isomers [6], by far the most popular approach has been the measurement of total inositol monophosphates produced during prolonged incubations in the presence of LiCl to inhibit inositol monophosphatase activity.

This approach has yielded a great deal of useful information but has been based on the assumption that all the inositol monophosphate is derived ultimately from the metabolism of the Ins 1,4,5-P_3* formed by the action of phospholipase C on $PtdInsP_2$. Recent advances in analytical techniques, chiefly using HPLC, have shed considerable light on the metabolic transformations of Ins 1,4,5-P_3 and have revealed two major metabolic routes. The first involves breakdown to Ins 1,4-P_2 which appears, in the brain, to be principally or exclusively metabolized to Ins 4-P [6–8]. The second involves conversion to Ins 1,3,4,5-P_4 with subsequent metabolism, via Ins 1,3,4-P_3 and Ins 1,3-P_2 or Ins 3,4-P_2, to Ins 1-P or Ins 3-P [6, 9]. In the light of these recent advances in knowledge, we have examined the effects of carbachol and adrenaline on the labelling of inositol phospholipids and the accumulation of inositol phosphate isomers in the presence of LiCl.

* Abbreviations: Ins, inositol; Ins 1-P, inositol 1-phosphate; Ins 2-P, inositol 2-phosphate; Ins 3-P, inositol 3-phosphate; Ins 4-P, inositol 4-phosphate; Ins 1,3-P_2, inositol 1,3-bisphosphate; Ins 1,4-P_2, inositol 1,4-bisphosphate; Ins 3,4-P_2, inositol 3,4-bisphosphate; Ins 1,3,4-P_3, inositol 1,3,4-trisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins 1,3,4,5-P_4, inositol 1,3,4,5-tetrakisphosphate; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol 4-phosphate; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate.

MATERIALS AND METHODS

Myo-[2-^{3}H]-inositol (22.8 Ci/mmol) was purchased from Amersham International plc (Amersham, Bucks, U.K.). Inositol phosphate standards (^{3}H-labelled Ins 1-P, Ins 4-P, Ins 1,4-P_2, Ins 1,3,4-P_3 and Ins 1,4,5-P_3) were purchased from NEN Research Products (Du Pont Ltd, Stevenage, Herts, U.K.). KH_2PO_4 (HPLC grade) was obtained from FSA Laboratory Supplies (Loughborough, U.K.) and HPLC water from BDH Ltd (Poole, U.K.). All other chemicals were analytical grade.

Labelling of cortical prisms and incubation with LiCl, *carbachol and adrenaline.* Adult Wistar rats were killed by cervical fracture and their brains removed and cortices freed from as much white matter as possible, then cross-chopped using a

McIlwain tissue chopper to give 350 μm prisms. The prisms were dispersed in 6 mL of Krebs–Henseleit buffer containing 50 μCi of myo-[2-^{3}H]-inositol (1 μCi/mg protein). Aliquots (0.9 mL, equivalent to 100–120 mg of cortical tissue) were then transferred to Warburg flasks and incubated at 37° for 1 hr with shaking under a stream of humidified 95% O_2–5% CO_2. LiCl (final concentration 10 mM) and agonists/antagonists were then added and the incubations continued for a further 1 hr. Reactions were terminated by addition of 0.7 mL of 15% TCA and homogenizing. After standing in ice for 20–30 min, the homogenates were centrifuged at 3000 *g* for 10 min and the supernatants were removed and washed three times with 4 vols water-saturated diethyl ether to remove TCA and then stored at −30° pending HPLC. The pellets were resuspended in 2 mL distilled water by sonication. An aliquot (50 μL) was used for protein estimation [10] and the remainder was used for lipid extraction and analysis as described previously [11]. Briefly, inositol phospholipids were separated by thin-layer chromatography on oxalate-impregnated silica-gel H plates, and visualized using iodine vapour. Bands corresponding to authentic inositol phospholipids were scraped and radioactivity determined by scintillation counting.

HPLC analysis. Resolution of Ins 1-P, Ins 4-P, Ins 1,4-P_2, Ins 1,3,4-P_3 and Ins 1,4,5-P_3 standards was achieved by HPLC using an SAX cartridge column fitted with a 20 mm × 2 mm diameter precolumn containing Whatman CoPell SAX (Whatman Partisphere, Laserchrom Ltd, Gravesend, U.K.). A sample (400–800 μL) of ether-washed TCA supernatant was applied to an SAX cartridge column and eluted at 1 mL/min with the following gradient of $(NH_4)_2PO_4$ (adjusted to pH 3.5 with phosphoric acid): 0–10 min, HPLC water wash; 10–28 min, 0–60 mM; 28–28.1 min, 60–140 mM; 28.1–38 min, 140–160 mM; 38–48 min, 160–250 mM; 48–48.1 min, 250–500 mM; 48.1–57 min, 500–600 mM; 57–62 min, isocratic elution with 600 mM; 62–62.1 min, return to HPLC water. All gradient steps were linear. The eluent from the column was then passed through an LKB fixed wavelength (254 nm) monitor to determine nucleotide concentrations. Radioactivity was estimated either by using a homogeneous radioactivity detection system (LKB) or by collecting fractions (0.33 mL or 0.50 mL) and scintillation counting after mixing with 2 mL of LKB Optiphase HiSafe 3 scintillant.

Preparation of inositol phosphates. At the time of this study, the only commercially available radiolabelled inositol mono- and bisphosphates were Ins 1-P, Ins 4-P and Ins 1,4-P_2. The complexity of inositol phosphate metabolism dictated that we establish the mobilities of certain other compounds, particularly those resulting from breakdown of Ins 1,3,4-P_3 (Ins 1,3-P_2 and Ins 3,4-P_2). These compounds were produced by preparing a 10% (w/v) homogenate of rat cerebral cortex in Krebs–Henseleit buffer lacking $CaCl_2$ and incubating this homogenate (0.1 mL) with 20 nCi of [^{3}H]Ins 1,3,4-P_3 in a total vol of 500 μL for 30 min at 37° in the presence or absence of EDTA (0.5 mM). The reaction was terminated by adding 0.3 mL of 15% TCA and centrifuging (2500 *g* for 10 min). The supernatant was washed with water-saturated ether before analysis by HPLC. Under these conditions, the major bisphosphate products in the presence and absence of EDTA are Ins 1,3-P_2 and Ins 3,4-P_2 respectively [6]. Ins 2-P was prepared by heating Ins 1-P with HCl [12].

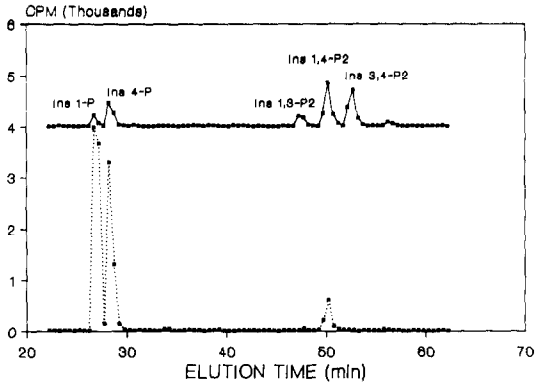

Fig. 1. HPLC separation of inositol mono- and bisphosphate isomers. The upper trace (displaced upwards by 4000 cpm for clarity) shows the elution pattern produced by a mixture of isomers derived from breakdown of Ins 1,3,4-P_3 and Ins 1,4,5-P_3 in the presence of rat cortical high-speed supernatant. Isomers were identified as previously described [6]. The lower trace was produced by incubating prelabelled slices for 1 hr in the presence of 10 mM LiCl plus 1 mM carbachol as described in the text. More than 95% of the recovered radioactivity was eluted in the three peaks shown on incubation with Li alone, Li plus carbachol or Li plus adrenaline.

RESULTS

Effects of carbachol and adrenaline on labelling of phosphoinositides and inositol phosphate isomers

In a number of preliminary experiments, we examined the effects of various commonly used procedures, including preincubation of slices in Krebs–Henseleit medium and washing with fresh medium before addition of myo-[2-^{3}H]-inositol, on slice energy charge and lipid labelling. In our hands, such preincubation/washing did not result in a significant improvement of either of these parameters.

After incubation of rat cortical prisms for 1 hr with 50 μCi of myo-[2-^{3}H]-inositol and subsequent incubation for 1 hr with 10 mM LiCl, the total level of radioactivity in inositol mono-, bis- and trisphosphates was 5200 ± 831 dpm/mg protein (mean ± SE, six separate experiments). About 95% of the labelling was in three peaks which co-migrated with Ins 1-P (or Ins 3-P, these two are not separated by HPLC). Ins 4-P and Ins 1,4-P_2 standards. The ratio of labelling in these peaks was 0.68:1.00:0.31 (Ins 1-P:Ins 4-P:Ins-1,4-P_2). There were also small amounts of radioactivity which co-migrated with two breakdown products of Ins 1,3,4-P_3, tentatively identified as Ins 1,3-P_2 and Ins 3,4-P_2 [6]. These each represented about 1.3% of total inositol mono-, bis- and trisphosphate radioactivity. A typical separation of these inositol mono- and bisphosphate isomers is shown in Fig. 1. The remaining 3% of

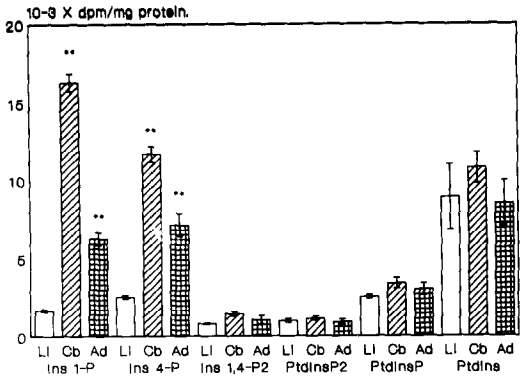

Fig. 2. Effects of carbachol and adrenaline on labelling of inositol phospholipids and inositol phosphates. Rat cortical prisms were prelabelled with myo-[2-^{3}H]-inositol as described in the text and subsequently incubated with adrenaline (1 mM) or carbachol (1 mM) plus LiCl (10 mM) or LiCl alone for 1 hr at 37°. Lipids and inositol phosphates were extracted and analysed by TLC or HPLC as described in the text. Data are means ± SE from five separate experiments. * and ** indicate significant differences (two-tailed t-test, $P < 0.0001$ and $P < 0.000001$ respectively) from controls (Li alone).

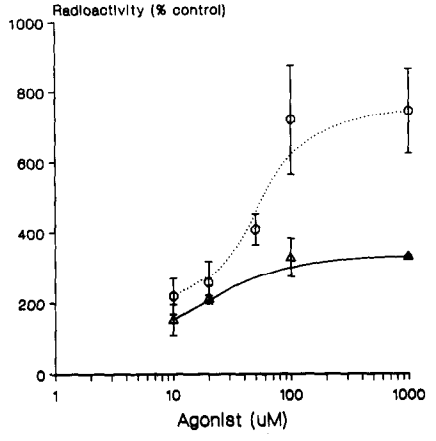

Fig. 3. Carbachol and adrenaline dose-dependence of Ins 1-P formation in rat cortical prisms. Rat cortical prisms were prelabelled with myo-[2-^{3}H]-inositol as described in the text and incubated with adrenaline or carbachol (up to 1 mM) in the presence of 10 mM LiCl for 1 hr at 37°. Inositol phosphates were isolated and analysed as described in the text. Data are mean values ± SE from 3–5 separate experiments. (△——△) Adrenaline; (○·····○) carbachol.

total radioactivity was present in two inositol trisphosphate peaks which co-migrated with Ins 1,3,4-P_3 and Ins 1,4,5-P_3 standards.

Incubation of the slices with carbachol (1 mM) or adrenaline (1 mM) in the presence of LiCl (10 mM) did not significantly change labelling of any of the phosphoinositides but did result in highly significant increases in the labelling of radioactive peaks which co-migrated with Ins 1-P, Ins 4-P and Ins 1,4-P_2 (Fig. 2). The increases in Ins 1-P, Ins 4-P and Ins

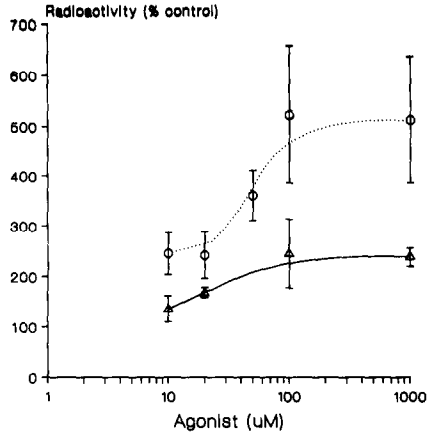

Fig. 4. Carbachol and adrenaline dose-dependence of Ins 4-P formation in rat cortical prisms. Details as for Fig. 2.

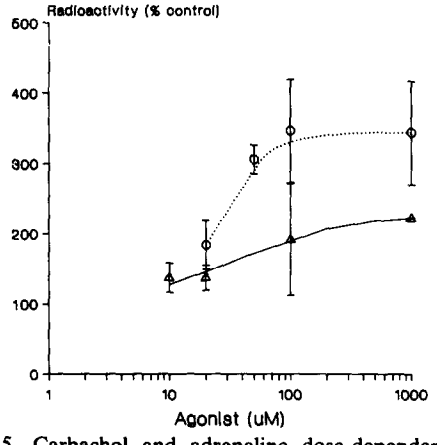

Fig. 5. Carbachol and adrenaline dose-dependence of Ins 1,4-P_2 formation in rat cortical prisms. Details as for Fig. 2.

1,4-P_2 labelling produced by both carbachol and adrenaline were dose-dependent (Figs 3–5). Experimental data were fitted by standard non-linear least squares techniques to hyperbolic curves of the form:

$$R = R_{max}[A]/EC_{50} + [A]$$

where R = response, R_{max} = maximum response and $[A]$ = agonist concentration.

The calculated parameters of the best-fit curves are indicated in Table 1. It can be seen that carbachol gave rise to a larger maximal increase in Ins 1-P, Ins 4-P and Ins 1,4-P_2 labelling than adrenaline ($P < 0.0001$, <0.0001 and <0.001, respectively, two-tailed t-tests). Both carbachol and adrenaline also produced significantly larger effects on the labelling of Ins 1-P than on that of Ins 4-P [P values 0.021 (carbachol) and 0.038 (adrenaline), two-tailed t-tests]. Furthermore, when Ins 1-P:Ins 4-P labelling ratios were calculated in the presence of LiCl (10 mM) alone and LiCl plus 1 mM agonist,

Table 1. Calculated parameters from dose–response curves of carbachol and adrenaline effects on inositol mono- and bisphosphate formation

Agonist	Inositol phosphate	EC_{50} (μM)	Max response (% of control)
Adrenaline	Ins 1-P	32.2 ± 14.8	350 ± 34
Adrenaline	Ins 4-P	35.6 ± 25.5	250 ± 32
Adrenaline	Ins 1,4-P_2	36.6 ± 9.7	226 ± 15
Carbachol	Ins 1-P	47.8 ± 21.8	821 ± 103
Carbachol	Ins 4-P	28.7 ± 12.0	551 ± 55
Carbachol	Ins 1,4-P_2	16.7 ± 9.8	360 ± 38

Parameters were calculated from best-fit curves to dose–response data (see Figs 3–5) consisting of 13 points from 3–4 experiments (adrenaline) or 18 points from 3–4 experiments (carbachol). Parameter values are estimates ± standard errors using the curve fitting method described in the text.

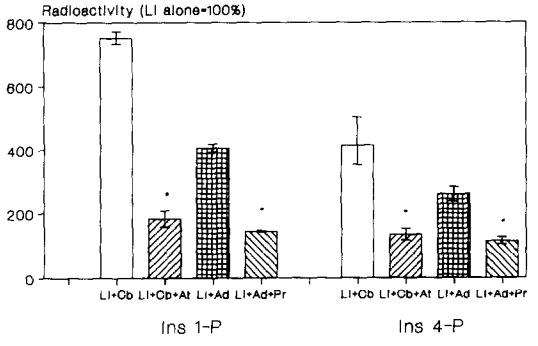

Fig. 6. Effects of antagonists on carbachol- and adrenaline-mediated increases in Ins 1-P and Ins 4-P radioactivity in rat cortical prisms. Prisms were labelled and incubated with Li alone, Li + agonists [Ad = adrenaline (1 mM), Cb = carbachol (1 mM)] or Li + agonists + antagonists [Pr = prazosin (1 μM), At = atropine (10 μM)] for 1 hr at 37°, before terminating reactions and isolating and analysing inositol phosphates by HPLC as described in the text. Data are mean ± SE from three separate experiments. Asterisks indicate significant differences ($P < 0.001$, two-tailed t-test) between agonists alone and agonists plus their respective antagonists. Prazosin and atropine alone, at the concentrations described above, had no significant effect on labelling of inositol phosphates.

these were 0.676 ± 0.133 (LiCl alone, N = 13), 0.983 ± 0.199 (Li plus adrenaline, N = 9) and 1.395 ± 0.167 (Li plus carbachol, N = 13, all values means ± SD). There were significant differences in this labelling ratio between both Li + carbachol and Li + adrenaline and Li alone (P values both <0.001, two tailed t-test). In addition, the ratios found with the two agonists were significantly different from each other ($P < 0.001$, two-tailed t-test).

Kinetics of inositol phosphate labelling in the presence of carbachol

Following these observations, we went on to demonstrate that agonist-mediated increases in the labelling of both inositol monophosphates could be abolished by addition of atropine (10 μM) or prazosin (1 μM) to incubations containing carbachol and adrenaline respectively (Fig. 6), suggesting that the responses were muscarinic and α_1-adrenergic in nature, confirming and extending previous findings on the unfractionated monophosphates [13].

The next stage of our investigation consisted of a study of the kinetics of labelling of the two inositol monophosphate peaks and of their possible inositol bisphosphate precursors (see Ref. 14). This study was only carried out using one of the agonists (carbachol), which produced larger responses and thus more accurate estimates of initial labelling rates. These rates were compared with those calculated at a later stage in the incubation (15–60 min) and the results are shown in Table 2. It can be seen that, in the first 5 min following addition of carbachol (1 mM) in the presence of 10 mM LiCl, the most rapid increase in radioactivity levels occurred in Ins 4-P and its presumed precursor Ins 1,4-P_2, which were labelled significantly more rapidly than Ins 1-P. Labelling of the other inositol bisphosphates was not great enough to yield reliable estimates of initial labelling rates. We were, however, able to measure Ins 1,3-P_2 and Ins 3,4-P_2 labelling at 15, 30, 45 and 60 min after adding carbachol (1 mM) and LiCl (10 mM) and to compare their labelling with that of Ins 1,4-P_2. The mean ratios of Ins 1,3-P_2:Ins 1,4-P_2:Ins 3,4-P_2 labelling were 0.04:1:0.07 (15 min), 0.04:1:0.09 (30 min), 0.05:1:0.09 (45 min) and 0.07:1:0.06 (60 min). Thus, at no stage did labelling of Ins 1,3-P_2 + Ins 3,4-P_2 exceed about 10% of total bisphosphate radioactivity. There is also a significant increase in the rate of Ins 1-P labelling between the initial period (0–5 min) and the later stages (15–60 min) and a significant decrease in the rate of labelling of Ins 1,4-P_2.

DISCUSSION

Measurement of total inositol monophosphate accumulation in the presence of Li^+ is commonly used as an index of receptor-mediated $PtdInsP_2$ breakdown (see above). This technique relies on the assumption that all the monophosphates which are "trapped" in the presence of Li^+ are derived from the Ins 1,4,5-P_3 formed on hydrolysis of $PtdInsP_2$. Some insight into the possible metabolic origins of the heterogeneous monophosphate pool can be gained by using HPLC to resolve the various

Table 2. Rates of accumulation of radioactivity in inositol mono- and bisphosphates in the presence of 1 mM carbachol and 10 mM LiCl after 1 hr preincubation of slices with myo-[2-^{3}H]-inositol

Inositol phosphate	Rate of change of labelling (cpm/min/700 μL TCA supernatant) 0–5 min	15–60 min
Ins 1-P + Ins 3-P	37.6 ± 2.6	187.5 ± 19.9‡
Ins 4-P	80.0 ± 13.0*	133.1 ± 20.3†
Ins 1,4-P_2	90.3 ± 17.6*	9.1 ± 5.0§
Ins 1,3-P_2	ND	1.2 ± 0.3
Ins 3,4-P_2	ND	0.3 ± 0.2

Data are slopes of linear regression lines ± standard errors derived from least squares fitting of labelling data at 0, 30 sec, 1, 2 and 5 min (0–5 min) or 15, 30, 45 and 60 min (15–60 min) after adding carbachol (1 mM) and LiCl (10 mM). Data are derived for 3–5 separate experiments (0–5 min) or three separate experiments (15–60 min). ND, levels of radioactivity not accurately distinguishable from background level. Initial labelling rates (0–5 min) significantly higher than that of Ins 1-P are denoted by an asterisk ($P < 0.01$). Significant increases in labelling rate from 0–5 min to 15–60 min are denoted by a dagger ($P < 0.05$) or a double dagger ($P < 0.0001$). A significant decrease in labelling rate from 0–5 min to 15–60 min is denoted by the symbol § ($P < 0.001$). All comparisons were made by two-tailed *t*-tests.

monophosphate isomers and those of their polyphosphate precursors.

Recent experimental evidence has shown that metabolism of Ins 1,4,5-P_3 by phosphorylation (to Ins 1,3,4,5-P_4) or phosphatase action (to Ins 1,4-P_2) results in the formation of distinct bis- and monophosphate products (see Refs 2, 14 and 15). These observations have resulted in a considerable number of studies of inositol phosphate isomer formation in different systems (for examples, see Refs 6, 12 and 16). The situation is somewhat complicated by the fact that Ins 1-P or Ins 3-P (which co-migrate on non-chiral HPLC systems) can arise *either* from Ins 1,3,4-P_3 catabolism *or* from direct hydrolysis of PtdIns, whereas the easily separable monophosphate isomer Ins 4-P can apparently only be formed by the breakdown of Ins 1,4-P_2, thus providing a direct measure of phospholipase C action on polyphosphoinositides.

Our present data clearly show that stimulation of muscarinic, cholinergic and α_1-adrenergic receptors in rat cortical prisms leads to the accumulation of radioactivity in both the major HPLC-separable inositol monophosphate isomers, Ins 1-P (+Ins 3-P) and Ins 4-P. However, it is also clear that there are certain differences in the patterns of monophosphate accumulation which occur on stimulation of the two receptor classes. Thus, while both carbachol and adrenaline cause preferential accumulation of Ins 1-P compared with LiCl alone, this preference is significantly higher in the presence of carbachol than in the presence of adrenaline. This finding implies that carbachol leads to a preferentially greater flux through an Ins 1-P/3-P generating route (PtdIns hydrolysis or formation and catabolism of Ins 1,3,4,5-P_4) than does adrenaline. Recently, Batty *et al.* [6] have also shown that 1 mM carbachol, in the absence of LiCl, also causes preferential accumulation of inositol monophosphate radioactivity in Ins 1-P. However, the findings of Batty *et al.* [6] indicated a considerably greater accumulation of Ins 1-P than that reported in the present study.

There is clear evidence for some Ins 1,3,4,5-P_4 metabolism in stimulated slices [2] and in an attempt to shed some light on the possible importance of this route compared with PtdIns hydrolysis, we have carried out a kinetic investigation of inositol mono- and bisphosphate labelling in the presence of 1 mM carbachol. Our data suggest (i) that immediately after adding carbachol, Ins 1,4,5-P_3 degradation via Ins 1,4-P_2 is quantitatively the most important route for accumulation of radioactivity in inositol monophosphate and (ii) that at subsequent stages of incubation, Ins 1-P labelling becomes quantitatively more important. In the period 15–60 min after carbachol addition, the radioactive pools of Ins 1,4-P_2 and Ins 1,3-P_2 + Ins 3,4-P_2 change only slowly and the former is much larger than the latter. Thus, if all the Ins 1-P (+Ins 3-P) is derived from catabolism through Ins 1,3,4,5-P_4 and its breakdown products, then our present data would suggest that the labelled Ins 1,3-P_2 plus Ins 3,4-P_2 pools must be turning over very rapidly compared with Ins 1,4-P_2. Moreover, it has been shown that LiCl markedly reduces carbachol-mediated $InsP_3/InsP_4$ accumulation in brain slices after more than 10 min incubation [5]. As we have shown that, during the same period, the rate of increase of Ins 1-P radioactivity rises dramatically, this suggests that sources other than $InsP_4$ might contribute substantially to Ins 1-P formation under these conditions.

It is also possible that Ins 1-P could arise by degradation of PtdIns. Sherman and co-workers have shown that this compound is the major inositol monophosphate isomer in rat brain and that its levels are increased by chronic treatment with LiCl [17, 18]. Interestingly, Ackermann *et al.* [19] have suggested that PtdIns is the major source of Ins 1-P in brain. It is technically difficult, at the present time, to unequivocally identify the relative contributions of

PtdIns hydrolysis and Ins 1,3,4,5-P_4 catabolism to the stimulated formation of Ins 1-P. Clearly, all possibilities need to be considered until this question is resolved. Finally, the difference in isomer labelling patterns produced by adrenaline and carbachol argue that this question must be addressed for each receptor type linked to enhanced phosphoinositide hydrolysis.

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