Determination of mass changes in phosphatidylinositol 4,5-bisphosphate and evidence for agonist-stimulated metabolism of inositol 1,4,5-trisphosphate in airway smooth muscle

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Stimulation of muscarinic receptors in bovine tracheal smooth muscle (BTSM) causes a sustained increase in muscle tone, but a transient increase in the second messenger $\text{Ins}(1,4,5)\text{P}_3$. To examine whether this brief increase in $\text{Ins}(1,4,5)\text{P}_3$ mass results from transient formation or is due to agonist-stimulation of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, we have studied the relationship between mass changes in $\text{PtdIns}(4,5)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ accumulation, and changes in $[^3\text{H}]\text{InsP}_3$, $[^3\text{H}]\text{PtdIns}$, $[^3\text{H}]\text{PtdInsP}_1$ and $[^3\text{H}]\text{PtdInsP}_2$ in carbachol-stimulated *myo*- $[^3\text{H}]\text{inositol}$ -prelabelled BTSM slices. Carbachol (0.1 mM) caused a rapid transient increase in $\text{Ins}(1,4,5)\text{P}_3$ concentration (basal, 12.9 ± 0.8 pmol/mg of protein; 5 s carbachol treatment, 27.1 ± 1.5 pmol/mg of protein), with values returning to basal levels by 30 s, but a sustained accumulation of total $[^3\text{H}]\text{InsP}_3$ s, with $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ being the predominant isomer present at later time points. In contrast, $\text{PtdIns}(4,5)\text{P}_2$ mass, determined by radioreceptor assay of $\text{Ins}(1,4,5)\text{P}_3$ in desalted alkaline hydrolysates of acidified chloroform/methanol tissue extracts, declined rapidly (basal, 941 ± 22 pmol/mg of protein; 120 s carbachol, 365 ± 22 pmol/mg of protein; $t_{1/2}$ 14 s) and remained at this new steady-state level for at least 20 min in the continued presence of carbachol. Addition of $10 \mu\text{M}$ -atropine 2 min after carbachol caused a prompt return of $\text{PtdIns}(4,5)\text{P}_2$ concentration to prestimulated values ($t_{1/2}$ 210 s). Ongoing resynthesis of $\text{PtdIns}(4,5)\text{P}_2$ after carbachol stimulation was demonstrated in $[^3\text{H}]\text{inositol}$ -labelled tissue by observing a persistent increase in the specific radioactivity of $[^3\text{H}]\text{PtdInsP}_2$, shown to be exclusively $[^3\text{H}]\text{PtdIns}(4,5)\text{P}_2$, over a 10 min period. These findings strongly suggest the occurrence of persistent receptor-mediated increases in $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis and $\text{Ins}(1,4,5)\text{P}_3$ formation which, in conjunction with the transient accumulation of $\text{Ins}(1,4,5)\text{P}_3$ observed, provide evidence that regulation of the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ is a major determinant of $\text{Ins}(1,4,5)\text{P}_3$ concentration in this tissue under agonist-stimulated conditions.

INTRODUCTION

$\text{Ins}(1,4,5)\text{P}_3$, the product of receptor-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, is widely recognized as an important intracellular second messenger in many cell types, due to its ability to mobilize Ca^{2+} from intracellular stores (Berridge, 1988; Berridge & Irvine, 1989). In keeping with its function as a second messenger, it is now clear that both the production and the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ are tightly regulated in cells.

In many tissues the formation, and hence accumulation, of $\text{Ins}(1,4,5)\text{P}_3$ following phosphoinositidase C-linked receptor activation is very transient. This may result from either receptor desensitization or metabolism of the neurotransmitter or hormone signal (Horstman *et al.*, 1988; Chuang, 1989; Perney & Miller, 1989; Pittet *et al.*, 1989). In addition, feedback inhibition by protein kinase C or cyclic AMP-dependent protein kinase at the level of the receptor, guanine-nucleotide-binding protein or phosphoinositidase C may serve to terminate $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis and hence attenuate the $\text{Ins}(1,4,5)\text{P}_3$ signal (Rittenhouse & Sasson, 1985; Watson & Lapetina, 1985; Chuang, 1989). In other cells, the formation and resultant increase in cellular $\text{Ins}(1,4,5)\text{P}_3$ concentration is sustained throughout the period of agonist stimulation (Hansen *et al.*, 1986; Dixon & Hokin, 1987; Challiss *et al.*, 1988; Stephens & Logan, 1989).

However, in a significant proportion of tissues, activation of Ca^{2+} -mobilizing receptors with stable agonists results in only brief increases in the $\text{Ins}(1,4,5)\text{P}_3$ concentration, despite evidence, albeit indirect, of ongoing phosphoinositide hydrolysis (Balla *et al.*, 1986; Merritt *et al.*, 1986; Chilvers *et al.*, 1989a).

In this situation, agonist-stimulated metabolism of $\text{Ins}(1,4,5)\text{P}_3$ has been proposed as the major mechanism responsible for controlling the $\text{Ins}(1,4,5)\text{P}_3$ concentration. The basis of this proposal has been the demonstration in cell-free systems that the activity of both the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase, which is responsible for conversion of $\text{Ins}(1,4,5)\text{P}_3$ into $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1986), and the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase, which controls the alternative route of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, may be independently regulated by factors such as Ca^{2+} /calmodulin (Biden & Wollheim, 1986; Shears, 1989), cyclic AMP-dependent protein kinase (Sim *et al.*, 1990) and protein kinase C (Connolly *et al.*, 1986; Molina y Vedia & Lapetina, 1986; King & Rittenhouse, 1989; Lin *et al.*, 1990; Sim *et al.*, 1990).

Understanding and dissecting the relative importance of factors that either regulate the formation of $\text{Ins}(1,4,5)\text{P}_3$ or influence its metabolism on the overall final pattern of $\text{Ins}(1,4,5)\text{P}_3$ accumulation is difficult, and is complicated by the possibility that the initial receptor-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ may be superseded, through a secondary increase

Abbreviations used: GroPIns, GroPIns P_1 and GroPIns P_2 , glycerophosphoinositol and its mono- and bis-phosphate derivatives; BTSM, bovine tracheal smooth muscle; KHB, Krebs–Henseleit buffer.

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in the intracellular Ca^{2+} concentration, by direct breakdown of PtdIns and PtdIns(4,5) P_2 (see Majerus *et al.*, 1988; Brammer *et al.*, 1988).

In bovine tracheal smooth muscle (BSTM), muscarinic receptor stimulation of tissue slices prelabelled with [^3H]inositol, in the presence of Li^+ , results in a linear accumulation of [^3H]Ins P_1 and a sustained increase in [^3H]Ins(1,3,4) P_3 over a 30 min incubation period (Chilvers *et al.*, 1989a, 1990b). Despite these findings, [^3H]Ins(1,4,5) P_3 accumulates only very transiently, and subsequent mass measurements of Ins(1,4,5) P_3 have confirmed that, in the continued presence of carbachol, the Ins(1,4,5) P_3 concentration returns to, and is maintained at, basal or sub-basal values between 30 s and 30 min (Chilvers *et al.*, 1989b). In addition, the contractile response of BSTM strips to carbachol stimulation is remarkably sustained, with no loss of tension even after many hours of incubation with the agonist (Park & Rasmussen, 1985, 1986). These data are suggestive of ongoing muscarinic receptor-mediated PtdIns(4,5) P_2 hydrolysis and Ins(1,4,5) P_3 formation; however, with basal and sub-basal concentrations of Ins(1,4,5) P_3 being maintained by the cell during all but the initial 30 s after agonist addition, this would imply dramatic and sustained increases in the activities of one or both enzymes responsible for the metabolism of this second messenger.

In order to explore how changes in Ins(1,4,5) P_3 formation and Ins(1,4,5) P_3 metabolism influence steady-state Ins(1,4,5) P_3 accumulation in this tissue, we have sought to establish the pattern of phosphoinositidase C-mediated PtdIns(4,5) P_2 hydrolysis occurring during stimulation by carbachol. To achieve this, we have utilized the specific radioreceptor assay for Ins(1,4,5) P_3 to measure PtdIns(4,5) P_2 mass, as well as Ins(1,4,5) P_3 concentration, and to therefore correlate time courses for changes in [^3H]PtdIns(4,5) P_2 and [^3H]Ins P_3 with actual mass changes of PtdIns(4,5) P_2 and Ins(1,4,5) P_3 .

MATERIALS AND METHODS

Materials

[^3H]Ins(1,4,5) P_3 (17–20 Ci/mmol) and [$^2\text{-}^3\text{H}$]inositol (17–20 Ci/mmol) were purchased or obtained as gifts from NEN (Du Pont). [$^5\text{-}^{32}\text{P}$]Ins(1,4,5) P_3 (1000 Ci/mmol) and Ins(1,4,5) P_3 were purchased from Amersham International. Dowex AG1-X8 resin (200–400 mesh, formate form) was from Bio-Rad, and all cation-exchange resins were from Sigma. All other reagents were obtained from Fisons, Aldrich or Sigma and were of analytical grade.

Preparation and stimulation of [^3H]inositol-labelled muscle slices

BSTM slices (300 $\mu\text{m} \times 300 \mu\text{m}$) were prepared and pre-incubated in bulk for 60 min in Krebs–Henseleit buffer (KHB) containing 1.3 mM- CaCl_2 as described previously (Chilvers *et al.*, 1989a). A 2 ml portion of gravity-packed slices was then transferred to 25 ml Erlenmeyer flasks containing 10 ml of buffer and incubated for a further 60 min at 37 °C in a shaking water-bath with regular oxygenation (O_2/CO_2 , 19:1). For [^3H]inositol labelling studies, 100 μCi of [^3H]inositol was present during this latter 60 min incubation period. Samples (50 μl) of gravity-packed tissue slices (approx. 1.5 mg of protein) were transferred to flat-bottomed vials containing 240 μl of KHB and challenged with agonists which were added in a 10 μl volume.

Extraction and measurement of [^3H]InsPs and Ins(1,4,5) P_3 mass

Incubations were terminated by addition of 300 μl of ice-cold 1 M-trichloroacetic acid, and after centrifugation (3000 g, 20 min, 4 °C) the supernatants were washed with 5 $\times$ 2 vol. of water-saturated diethyl ether or 1,1,2-trichlorotrifluoroethane/tri-n-octylamine (1:1, v/v) according to Downes *et al.* (1986). After

neutralization with 60 mM- NaHCO_3 , [^3H]Ins P_{1-4} fractions were separated by Dowex AG1-X8 anion-exchange chromatography as described previously (Batty *et al.*, 1985), and Ins(1,4,5) P_3 mass was determined using a radioreceptor assay method exactly as detailed previously (Challiss *et al.*, 1988).

Extraction and measurement of [^3H]phosphoinositides

Trichloroacetic acid-precipitated tissue pellets (see above) were sequentially washed with 1 ml of 5% (w/v) trichloroacetic acid/1 mM-EDTA and 1 ml of water (Griffin & Hawthorne, 1978) and [^3H]lipids were routinely extracted with chloroform/methanol/12 M-HCl (40:80:1, by vol.) as described by Downes & Wusteman (1983); samples of the lower phase were dried under N_2 and deacylated according to the method described by Wells & Dittmer (1965). The resulting [^3H]glycerophosphoinositol (phosphates) [[^3H]GroPIns(P_s)] were separated using Dowex AG1-X8 resin columns (Chilvers *et al.*, 1989a).

In experiments designed to examine potential isomeric heterogeneity of [^3H]polyphosphoinositides, lipids were extracted from washed tissue pellets by repeated treatment with acidified solvent and then further washed as described by Simpson *et al.* (1987) to ensure minimal contamination with water-soluble materials. Washed and N_2 -dried ^3H -labelled lipid was then deacylated according to Clark & Dawson (1981) and the resulting ^3H -labelled products were either separated directly by anion-exchange h.p.l.c. (Batty *et al.*, 1989), or similarly analysed following deglyceration (Brown & Stewart, 1966; Irvine *et al.*, 1986).

Measurement of PtdIns(4,5) P_2 mass

Dried lipid extracts prepared by routine extraction as described above were dissolved in 250 μl of 1 M-KOH and incubated in capped tubes at 100 °C for 30 min. Samples were cooled on ice and excess alkali was removed by filtration through columns [0.5 ml of 50% (w/v) slurry] of Dowex 50 (200–400 mesh; H^+ form). After a column wash with 2.25 ml of water, the combined eluates (pH 3–4) were extracted twice with butan-1-ol/light petroleum (b.p. 40–60 °C) (5:1, v/v) as described by Clark & Dawson (1981). Samples of 1 ml of the resultant lower phase were freeze-dried and redissolved in 2 ml of water and portions were assayed for Ins(1,4,5) P_3 (Challiss *et al.*, 1988).

Ins(1,4,5) P_3 5-phosphatase treatment

Human erythrocyte membranes, prepared according to Downes *et al.* (1982), were used to compare rates of hydrolysis of Ins(1,4,5) P_3 mass in deacylated lipid samples and the loss of authentic [^3H]Ins(1,4,5) P_3 'spiked' into parallel samples. These incubations were performed as described previously (Challiss *et al.*, 1990).

RESULTS AND DISCUSSION

Muscarinic-receptor-mediated inositol phospholipid turnover in BSTM slices, which is considered to be mediated by an M_3 receptor subtype (Roffel *et al.*, 1990), produces a sustained (> 60 min) linear accumulation of Ins P_1 in the presence of Li^+ , indicating that the receptor is not readily desensitized (Chilvers *et al.*, 1989a). In contrast, Fig. 1 confirms that, as in previous studies (Chilvers *et al.*, 1989a, 1990b), carbachol stimulation evokes a rapid yet short-lived increase in Ins(1,4,5) P_3 mass in this tissue, with stimulated concentrations of Ins(1,4,5) P_3 being maximal within 5 s of carbachol addition and returning to basal or sub-basal values by 30 s. A consistent finding in all experiments was a small, but significant, decrease in Ins(1,4,5) P_3 mass values below control levels at 60–300 s after carbachol addition; whether this decrease in the steady-state cellular Ins(1,4,5) P_3 concen-

Fig. 1. Carbachol-stimulated accumulation of total $[^3\text{H}]\text{InsP}_3$ s and $\text{Ins}(1,4,5)\text{P}_3$ mass in BTSM slices

Samples ($50\ \mu\text{l}$) of $[^3\text{H}]\text{inositol}$ -prelabelled BTSM slices ($300\ \mu\text{m} \times 300\ \mu\text{m}$) were incubated in the absence ($\circ$) or presence ($\bullet$) of $100\ \mu\text{M}$ -carbachol for the time periods indicated. $[^3\text{H}]\text{InsP}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ mass (insert) were determined in neutralized trichloroacetic acid extracts by Dowex anion-exchange chromatography and radioreceptor assay [using $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$] respectively. Data for $[^3\text{H}]\text{InsP}_3$ represent mean values and those for $\text{Ins}(1,4,5)\text{P}_3$ are means $\pm$ S.E.M. of three separate experiments, each performed in triplicate.

Fig. 2. H.p.l.c. separation of $[^3\text{H}]\text{inositol}$ bis- and tris-phosphates produced by alkaline hydrolysis of a lipid extract of $[^3\text{H}]\text{inositol}$ -labelled BTSM slices

Samples ($50\ \mu\text{l}$) of $[^3\text{H}]\text{inositol}$ -prelabelled BTSM slices were incubated with $100\ \mu\text{M}$ -carbachol for 10 min. Lipids were extracted from trichloroacetic acid-precipitated tissue pellets with chloroform/methanol/12 M-HCl (40:80:1, by vol.) and subjected to alkaline hydrolysis with 1 M-KOH at 100°C for 30 min. After desalting using Dowex 50 (H^+ form) and washing with butan-1-ol/light petroleum (see the Materials and methods section), the sample was lyophilized, reconstituted in water, spiked with the indicated nucleotides and analysed by h.p.l.c. as described in the text.

tration is dependent upon receptor occupation (i.e. is reversed by atropine addition subsequent to carbachol challenge) has not been established. Together with our previous observations (Chilvers *et al.*, 1989b), the transient nature of the $\text{Ins}(1,4,5)\text{P}_3$ signal raises several important alternatives concerning stimulated phosphoinositide metabolism in BTSM. Firstly, it is possible that stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis in this tissue is restricted to the early phase of receptor activation, but that resultant signals then trigger cleavage of the other phosphoinositides. Such a system has been suggested by Majerus *et al.* (1986) and may

have important implications regarding the major source of 1,2-diacylglycerol under stimulated conditions. Alternatively, $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis may be persistent, with the rapid decline in $\text{Ins}(1,4,5)\text{P}_3$ concentrations from an early peak occurring either through decreased phosphoinositidase C activity or through an activation of $\text{Ins}(1,4,5)\text{P}_3$ metabolism. Evidence in favour of these possibilities in tracheal smooth muscle is limited and inconclusive. Earlier reports, for example, have demonstrated either a rapid and maintained decrease in PtdInsP_2 concentration in response to carbachol in BTSM (Takuwa *et al.*, 1986) or little change in canine trachea (Baron *et al.*, 1989). Our own observation that total $[^3\text{H}]\text{InsP}_3$ concentrations remain markedly elevated up to 30 min after muscarinic receptor stimulation (Fig. 1) in $[^3\text{H}]\text{inositol}$ -prelabelled BTSM strongly implies a persistent stimulated cleavage of $\text{PtdIns}(4,5)\text{P}_2$. However, the $[^3\text{H}]\text{InsP}_3$ accumulated under these conditions is due predominantly to accumulation of the $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ isomer (Kennedy *et al.*, 1989; Chilvers *et al.*, 1990b); this product is considered to be derived indirectly from $\text{PtdIns}(4,5)\text{P}_2$ via $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, but recent demonstrations of the isomeric heterogeneity of polyphosphoinositides have questioned this assumption (Traynor-Kaplan *et al.*, 1989). Thus, as a persistent stimulated accumulation of neither of its assumed precursors has been demonstrated in BTSM, stimulated $\text{Ins}(1,3,4)\text{P}_3$ accumulation is not necessarily a conclusive index of sustained $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. In the present study we have sought appropriate correlative data concerning the isomeric homogeneity of $\text{PtdIns}(4,5)\text{P}_2$ in BTSM and the effects of muscarinic receptor stimulation on the concentration of this lipid in order to clarify both this situation and the basis for the transient nature of the $\text{Ins}(1,4,5)\text{P}_3$ signal in this tissue.

Measurement of $\text{PtdIns}(4,5)\text{P}_2$

To study the effects of muscarinic receptor stimulation specifically on $\text{PtdIns}(4,5)\text{P}_2$ concentrations, we have used a novel method in which this lipid is first converted to $\text{Ins}(1,4,5)\text{P}_3$, which is then measured using a radioreceptor assay (Challiss *et al.*, 1988; Palmer *et al.*, 1989). The advantage conferred on this approach by the previously defined isomeric specificity and high sensitivity of the $\text{Ins}(1,4,5)\text{P}_3$ assay is that it obviates the requirement for prior chromatographic isolation of $\text{PtdIns}(4,5)\text{P}_2$ from other lipids [including the potential $\text{PtdIns}(3,4)\text{P}_2$ isomer] as is essential for conventional measurement by phosphate or inositol determination (e.g. Sadler *et al.*, 1984; Traynor-Kaplan *et al.*, 1988).

Conversion of $\text{PtdIns}(4,5)\text{P}_2$ into $\text{Ins}(1,4,5)\text{P}_3$ was achieved by alkaline hydrolysis of crude lipid extracts under conditions previously shown to give quantitative cleavage of the phosphodiester bond (see Hawthorne & White, 1975). Extracts were then neutralized and assayed directly for $\text{Ins}(1,4,5)\text{P}_3$. To ensure that alkaline hydrolysates did not contain products which significantly interfered with this assay, samples from both control and carbachol-stimulated BTSM slices were incubated with human erythrocyte membranes [which contain an $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase] and the rate and extent of $\text{Ins}(1,4,5)\text{P}_3$ removal was monitored. Both the rate and extent ($> 93\%$) of loss of 'apparent' $\text{Ins}(1,4,5)\text{P}_3$ mass in these samples was identical with the loss of authentic $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ 'spiked' into parallel incubations and measured using Dowex chromatography (results not shown; see Challiss *et al.*, 1990). This result strongly suggests that the assay faithfully reflects the $\text{Ins}(1,4,5)\text{P}_3$ concentration in alkaline hydrolysates. It is important to note, however, that $\text{Ins}(1,4,5)\text{P}_3$ is not the sole inositol phosphate product of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis under the described conditions, but is produced in a mixture with $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(4,5)\text{P}_2$ (Brockerhoff & Ballou, 1961). The latter are minor products, and

Fig. 3. Effect of carbachol on PtdIns(4,5) P_2 mass in BTSM slices

Portions (50 μ l; approx. 1.5 mg of protein) of BTSM slices were incubated in the presence (●, ○) or absence (▲) of 100 μ M-carbachol for the time periods indicated. Reactions were terminated by addition of trichloroacetic acid and lipids were extracted and subjected to alkaline hydrolysis with 1 M-KOH as detailed in the Materials and methods section. Ins(1,4,5) P_3 mass was determined in the desalted butan-1-ol/light petroleum-washed products by radioreceptor assay. In some samples (○), 100 μ M-carbachol was added at t_0 , followed by 10 μ M-atropine at $t = 2$ min. Values represent the means $\pm$ S.E.M. of three separate experiments.

like Ins(1,3,4) P_3 they are detected by the Ins(1,4,5) P_3 assay at approx. 100–400-fold lower sensitivity than Ins(1,4,5) P_3 (Challiss *et al.*, 1988, 1990; Palmer *et al.*, 1989). The proportions of [3 H]Ins(1,4,5) P_3 , [3 H]Ins(2,4,5) P_3 and [3 H]Ins(4,5) P_2 produced from [3 H]PtdIns(4,5) P_2 were determined using extracts from [3 H]inositol-prelabelled tissue and h.p.l.c. to separately quantify the labelled products, as illustrated in Fig. 2. In over 12 determinations using extracts from three separate experiments, Ins(1,4,5) P_3 was found to constitute $65.8 \pm 0.7\%$ of PtdIns(4,5) P_2 -derived inositol phosphate [Ins(2,4,5) P_3 , $20.3 \pm 0.8\%$; Ins(4,5) P_2 , $13.9 \pm 0.5\%$]. Both this high reproducibility and the quality of other data obtained (see Fig. 3) lend confidence to the use of this approach to quantify PtdIns(4,5) P_2 . We did note, however, that the processing of alkaline hydrolysates by cation-exchange chromatography to yield neutral extracts resulted in a consistent loss of [3 H]Ins(1,4,5) P_3 , measured at $18.7 \pm 2.1\%$ ($n = 13$ for three experiments) when assessed using authentic [3 H]Ins(1,4,5) P_3 added to unlabelled alkaline hydrolysates. Data presented below are expressed in relative rather than absolute terms, but from the values obtained (and using a mean protein concentration of 1.5 mg/50 μ l of BTSM slices) the mass of PtdIns(4,5) P_2 in BTSM slices incubated under control conditions can be calculated at 941 ± 22 pmol/mg of protein ($n = 35$, 10 experiments). No previous values are available for direct comparison in BTSM, but similar processing of lipid from rat cerebral cortex slices gave a value of 3190 ± 190 pmol/mg of protein ($n = 6$, one experiment) for PtdIns(4,5) P_2 content. This value is in very close agreement with previously reported values for brain (Wells & Dittmer, 1965; Nishihara & Keenan, 1985), thus strongly supporting the validity of this method. The sensitivity of the method also appears to be similar to that recently described using monoclonal antibodies to PtdIns(4,5) P_2 (Fukami & Takenawa, 1989).

Effects of carbachol on PtdIns(4,5) P_2 mass

Using the above approach, we examined the effects of muscarinic receptor stimulation on the concentration of PtdIns(4,5) P_2 in BTSM slices. Fig. 3 shows that addition of 100 μ M-carbachol evoked a marked decrease in PtdIns(4,5) P_2 mass, which declined to 39% of the basal value within 60 s ($t_{1/2}$ 14 s) and was then maintained at this concentration for at least 20 min in the continued presence of carbachol. The rapid decrease

in PtdIns(4,5) P_2 concentration is consistent with the rapid agonist-induced rise in Ins(1,4,5) P_3 illustrated in Fig. 1 and, together with these data, demonstrate that activation of phosphoinositide C is an early consequence of muscarinic receptor stimulation in BTSM. The action of carbachol is in close qualitative agreement with the results of an earlier, similar study of PtdIns P_2 hydrolysis in BTSM (Takuwa *et al.*, 1986), but contrasts sharply with data obtained in canine trachealis muscle (Baron *et al.*, 1989), which did not demonstrate any change in PtdIns P_1 or PtdIns P_2 mass upon stimulation of muscle strips with 5.5 μ M-carbachol. Since this concentration of carbachol is very close to the EC_{50} (concn. causing half-maximal effect) for total [3 H]InsP and Ins(1,4,5) P_3 accumulation (Chilvers *et al.*, 1989a,b) and loss of PtdIns P_2 mass (Takuwa *et al.*, 1986) observed in bovine tissue, the findings of the study of Baron *et al.* (1989) cannot be readily explained. Fig. 3 also shows that addition of an excess of the muscarinic receptor antagonist atropine to tissue prestimulated for 2 min results in a rapid return of PtdIns(4,5) P_2 to control values, although the rate of lipid resynthesis appears somewhat slower than the initial hydrolytic rate ($t_{1/2}$ 210 s). The ability of atropine to reverse the response to carbachol is important, since it demonstrates that the sustained decrease in PtdIns(4,5) P_2 concentration, observed well beyond the time at which stimulated Ins(1,4,5) P_3 concentrations have returned to basal, reflects the continued activation of a receptor-mediated step. Furthermore, as we could find no evidence (see below) to suggest loss of PtdIns(4,5) P_2 through either dephosphorylation to PtdIns4 P_1 or phosphorylation to a putative PtdIns P_3 (Vadnal & Parthasarathy, 1989), this result clearly implies a persistent action of phosphoinositide C against this lipid. Thus the transient Ins(1,4,5) P_3 signal shown in Fig. 1 cannot be attributed to similarly limited PtdIns(4,5) P_2 hydrolysis. In addition, the common observation that high concentrations of KCl (Takuwa *et al.*, 1986; Grandordy *et al.*, 1986) and ionomycin (E. R. Chilvers, R. A. J. Challiss & S. M. Nahorski, unpublished work) fail to stimulate either the accumulation of Ins(1,4,5) P_3 mass or [3 H]Ins P_1 in [3 H]inositol-labelled BTSM slices in the presence of Li^+ indicates that the phosphoinositide C present in this tissue is not stimulated by physiological increases in intracellular Ca^{2+} , and hence that the sustained changes in PtdIns(4,5) P_2 mass observed are unlikely to result from any secondary effects of raised Ca^{2+} concentrations on phosphoinositide metabolism.

Effects of carbachol on [3 H]phosphoinositide labelling

One limitation of the above method for measurement of phosphoinositide hydrolysis is that it can only be used for quantification of PtdIns(4,5) P_2 mass. In two further series of experiments we therefore sought to (i) correlate receptor-stimulated changes in PtdIns(4,5) P_2 mass with the actions of agonist on all three phosphoinositides using [3 H]inositol-prelabelled tissue, and (ii) obtain preliminary data on the isomeric nature of these 3 H-labelled lipids in order to clarify the probable source of [3 H]Ins(1,3,4) P_3 in stimulated tissue.

Fig. 4 summarizes the results of the first of these experiments, in which tissue slices were prelabelled with [3 H]inositol for 60 min before agonist challenge. Previous studies from this laboratory have shown that under these conditions muscarinic-receptor stimulation evokes a dramatic increase in total phosphoinositide labelling, which achieves a new steady-state by 10 min (Chilvers *et al.*, 1989a). Fig. 4 allows this phenomenon to be examined in detail and demonstrates that while little change in [3 H]PtdIns labelling is seen over the first 60 s of exposure to carbachol, both [3 H]PtdIns P_1 and [3 H]PtdIns P_2 are profoundly depleted (42% and 50% of control values respectively). The attenuation of [3 H]PtdIns P_2 labelling is clearly very similar to

Fig. 4. Effect of carbachol on (a) $[^3\text{H}]\text{GroPIns}$, (b) $[^3\text{H}]\text{GroPInsP}_1$ and (c) $[^3\text{H}]\text{GroPInsP}_2$ in BTSM slices

Samples (50 μl) of $[^3\text{H}]\text{inositol}$ -prelabelled BTSM slices were incubated in the absence (open symbols) or presence (filled symbols) of 100 μM -carbachol for the time periods indicated. Lipids were extracted from trichloroacetic acid-precipitated tissue pellets, subjected to mild alkaline hydrolysis as detailed in the Materials and methods section and $[^3\text{H}]\text{GroPIns}$, $[^3\text{H}]\text{GroPInsP}_1$ and $[^3\text{H}]\text{GroPInsP}_2$ were separated by anion-exchange chromatography using Dowex AG1-X8 resin. Data represent the means of three experiments; S.E.M. values were $< 15\%$ for all points.

that shown above for $\text{PtdIns}(4,5)P_2$ mass (Fig. 3), strongly suggesting that both assays accurately measure the same lipid and that little change in $[^3\text{H}]\text{PtdInsP}_2$ specific radioactivity occurs during the initial 60 s stimulation. Since both $[^3\text{H}]\text{PtdInsP}_2$ and $[^3\text{H}]\text{PtdInsP}_1$ are decreased on agonist addition, these data further indicate that agonist-stimulated metabolism of $\text{PtdIns}(4,5)P_2$ is unlikely to occur via the degradative action of $\text{PtdIns}(4,5)P_2$ 5-phosphatase. After the initial decrease in $[^3\text{H}]\text{polyphosphoinositides}$, labelling of $[^3\text{H}]\text{PtdIns}$, $[^3\text{H}]\text{PtdInsP}_1$ and $[^3\text{H}]\text{PtdInsP}_2$ increased dramatically between 1 and 10 min, with each achieving new values 2–4-fold above basal values, with subsequent increases occurring in parallel with controls (Fig. 4). As the results presented above demonstrate that under agonist-stimulated conditions $\text{PtdIns}(4,5)P_2$ mass remains depleted over the same period, the later increase in lipid labelling is assumed to reflect increased specific radioactivity resulting from continued stimulated turnover. This effect of the stimulus is both quali-

Fig. 5. H.p.l.c. separation of (a) $[^3\text{H}]\text{glycerophosphoinositides}$ and (b) $[^3\text{H}]\text{inositol phosphates}$ derived from $[^3\text{H}]\text{inositol}$ -labelled phospholipids in BTSM slices

BTSM slices were labelled with 2.5 μCi of $[^3\text{H}]\text{inositol}$ for 60 min before stimulation with 100 μM -carbachol for 10 min. Lipids were extracted from trichloroacetic acid-precipitated tissue pellets by sequential acidified chloroform/methanol treatments and deacylated with monomethylamine reagent as described in the text. (a) H.p.l.c. analysis of the $[^3\text{H}]\text{GroPIns(Ps)}$ present in the deacylated sample. A aliquot of the same sample was deglycerated using mild NaIO_4 oxidation as described in Materials and methods section; (b) shows h.p.l.c. analysis of the resulting $[^3\text{H}]\text{InsPs}$ AT-4 and GP-4 are adenosine and guanosine tetraphosphates respectively.

tatively and quantitatively in close agreement with that anticipated from our previous studies (Chilvers *et al.*, 1989a) and shows clearly that PtdInsP_2 resynthesis is also persistent for at least 10 min after receptor stimulation.

The data presented in Fig. 4 derive from analysis of $[^3\text{H}]\text{phosphoinositide}$ deacylation products by conventional Dowex chromatography, a method which does not resolve potential isomers. In order to investigate the possible heterogeneity of the phosphoinositides in BTSM we therefore examined $[^3\text{H}]\text{inositol}$ -labelled lipid extracts from both control and stimulated tissue slices by h.p.l.c. after conversion of ${}^3\text{H}$ -labelled lipid to water-soluble glycerophosphoinositol (phosphates) by deacylation (Clark & Dawson, 1981) or further degradation by specific removal of the glycerol moiety (Brown & Stewart, 1966; Irvine *et al.*, 1985) to yield the corresponding inositol (poly)phosphate headgroups. Fig. 5 summarizes the results of this study. The data presented are for extracts from stimulated tissue, but were qualitatively similar to those obtained for control samples. Examination of $[^3\text{H}]\text{inositol}$ lipid deacylates by h.p.l.c. revealed three major labelled products whose retention times corresponded precisely with those of $[^3\text{H}]\text{GroPIns}$, $[^3\text{H}]\text{GroPInsP}_1$ and $[^3\text{H}]\text{GroPInsP}_2$ derived from prelabelled rat cerebral cortex lipid extracts. A fourth additional peak was eluted just before $[^3\text{H}]\text{GroPInsP}_1$. This was tentatively identified as $[^3\text{H}]\text{GroPInsP}_3$ both by comparison with previous studies

(Stephens *et al.*, 1989) and from the observation that traces of an [^3H]Ins P_2 with the chromatographic retention appropriate to [^3H]Ins(1,3) P_2 were detected in alkaline hydrolysates of ^3H -labelled lipid extracts (see Fig. 2). Further evidence of ^3H -labelled lipid identities was obtained by degradation of ^3H -labelled lipid deacylates to yield appropriate [^3H]inositol phosphates as described above. Comparison of Figs. 5(a) and 5(b) demonstrates that this resulted in virtually quantitative conversion of [^3H]GroPIns, [^3H]GroPIns3/4 P_1 and [^3H]GroPIns(4,5) P_2 into products with the appropriate chromatographic characteristics of authentic [^3H]Ins1 P_1 , [^3H]Ins(1,3)/(1,4) P_2 and [^3H]Ins(1,4,5) P_3 . Neither the original lipid deacylate nor the deglycerated samples (Fig. 5b) revealed any products consistent with the presence of [^3H]PtdIns(3,4) P_2 or more polar products. Furthermore, treatment of a deglycerated ^3H -labelled sample with human erythrocyte membrane Ins(1,4,5) P_3 5-phosphatase quantitatively degraded the [^3H]Ins P_3 derived from [^3H]PtdIns P_2 to [^3H]Ins(1,4) P_2 .

While it is emphasized that these data do not represent a conclusive structural analysis of the [^3H]inositol lipids present in BTSM, it is evident from Fig. 5 that the major labelled species are the anticipated [^3H]PtdIns, [^3H]PtdIns4 P_1 and [^3H]PtdIns(4,5) P_2 . Equally, these results indicate that, although a significant amount of a putative [^3H]PtdIns3 P_1 may be present both in the presence and absence of stimulus, no similar heterogeneity of [^3H]PtdIns P_2 was detectable. Further preliminary data (not shown) indicate that, unlike [^3H]PtdIns4 P_1 and [^3H]PtdIns(4,5) P_2 , [^3H]PtdIns3 P_1 concentrations in this tissue are not affected by muscarinic receptor stimulation. In addition, it has recently been demonstrated that polyphosphoinositides with a phosphate substitution at the 3-hydroxy group of the inositol ring are not readily cleaved by purified phospholipase C (Servian *et al.*, 1989). Together with the current data, this strongly suggests that the sustained muscarinic receptor-stimulated accumulation of [^3H]Ins(1,3,4) P_3 previously reported in BTSM (Chilvers *et al.*, 1990b), and illustrated as total [^3H]Ins P_3 in Fig. 1, arises from the persistent stimulated hydrolysis of [^3H]PtdIns(4,5) P_2 rather than through cleavage of a corresponding [^3H]PtdIns(3,4) P_2 .

These findings clearly indicate persistent receptor-mediated PtdIns(4,5) P_2 hydrolysis and hence Ins(1,4,5) P_3 formation which, in conjunction with the transient accumulation of Ins(1,4,5) P_3 , provides strong evidence that regulation of the metabolism of Ins(1,4,5) P_3 is a major determinant of Ins(1,4,5) P_3 concentration in carbachol-stimulated BTSM slices. Stimulated metabolism of Ins(1,4,5) P_3 may also be of importance in tissues where similar patterns of inositol polyphosphate accumulation have been observed [i.e. transient Ins(1,4,5) P_3 accumulation and sustained accumulation of exclusive products of Ins(1,4,5) P_3 metabolism (e.g. Merritt *et al.*, 1986)]. The mechanism(s) underlying this dramatic control of Ins(1,4,5) P_3 metabolism in this tissue have yet to be identified, but may involve the widely demonstrated Ca^{2+} /calmodulin activation of Ins(1,4,5) P_3 3-kinase or the protein kinase C-mediated phosphorylation and activation of Ins(1,4,5) P_3 5-phosphatase observed in human platelets (Connolly *et al.*, 1986). It is important, however, to note that protein kinase C regulation of 5-phosphatase may not be a general phenomenon, and two recent reports have suggested that protein kinase C may also inhibit 3-kinase activity (Lin *et al.*, 1990; Sim *et al.*, 1990). Although the regulation of 3-kinase or 5-phosphatase activities has not been examined in airway smooth muscle, it is of interest that one of the only two demonstrations of a Ca^{2+} -stimulated Ins(1,4,5) P_3 5-phosphatase was in smooth muscle (Sasaguri *et al.*, 1985). The other demonstration was in macrophages (Kukita *et al.*, 1986).

The functional consequences of this apparent strict control of

receptor-mediated Ins(1,4,5) P_3 accumulation in this tissue are unknown. The rapid agonist-stimulated increase in Ins(1,4,5) P_3 that precedes the development of tension and the dependence of contraction on intracellular Ca^{2+} (Giembycz & Rodgers, 1987), which can be released by Ins(1,4,5) P_3 (Hashimoto *et al.*, 1985), all suggest an important second messenger role for Ins(1,4,5) P_3 in initiating contraction of airway smooth muscle. Previous studies characterizing the Ins(1,4,5) P_3 receptor in this tissue (Chilvers *et al.*, 1990a) have indicated that, in contrast with the Ins(1,4,5) P_3 receptor in rat neuronal tissue (Worley *et al.*, 1987; Supattapone *et al.*, 1988), Ins(1,4,5) P_3 binding is not inhibited by Ca^{2+} , suggesting that in BTSM Ca^{2+} release is likely to parallel changes in Ins(1,4,5) P_3 concentration, whereas in neuronal tissues, where carbachol stimulation results in sustained Ins(1,4,5) P_3 accumulation, additional factors aside from the ambient Ins(1,4,5) P_3 concentration may regulate Ca^{2+} release. This differential regulation of Ins(1,4,5) P_3 binding by Ca^{2+} may explain the different patterns of agonist-stimulated accumulation of Ins(1,4,5) P_3 observed in these tissues (Challiss *et al.*, 1990). In addition, the ongoing formation of 1,2-diacylglycerol that results from persistent PtdIns(4,5) P_2 hydrolysis may, via an activation of protein kinase C, provide a mechanism for the sustained contractile response observed with muscarinic agonist stimulation in this tissue (Park & Rasmussen, 1986). Although agonist-stimulated metabolism of Ins(1,4,5) P_3 is undoubtedly a major factor regulating Ins(1,4,5) P_3 concentration in this tissue, the precise mechanisms underlying this effect, in particular the relative importance of the 5-phosphatase and 3-kinase pathways, require further study.

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