Characterization of agonist-stimulated incorporation of *myo*-[³H]inositol into inositol phospholipids and [³H]inositol phosphate formation in tracheal smooth muscle

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The effects of the muscarinic agonist carbachol, histamine and bradykinin on incorporation of [³H]inositol into the phosphoinositides and the formation of [³H]InsPs were examined in bovine tracheal smooth-muscle (BTSM) slices labelled with [³H]inositol. These agonists result in substantial and dose-related increases in the incorporation of [³H]inositol into the phospholipids. Carbachol and histamine stimulated the incorporation of [³H]inositol into the phospholipids to the same degree, despite histamine being only 35% as effective as carbachol on [³H]InsP accumulation. Histamine and carbachol, at maximal concentrations, were non-additive with respect to both the stimulated incorporation of [³H]inositol and [³H]InsP formation. For carbachol this effect on incorporation was found to occur to a similar extent in PtdInsP and PtdInsP₂ as well as PtdIns. The initial effect of carbachol on [³H]inositol incorporation was rapid (maximal by 10 min); however, with prolonged stimulation large secondary declines in PtdInsP and PtdInsP₂ labelling were observed, with depletion of the much larger PtdIns pool only evident in the presence of Li⁺. Lowering buffer [Ca²⁺] increased the incorporation of [³H]inositol under basal conditions, but did not attenuate the subsequent agonist-stimulated incorporation effect. The large changes in specific radioactivity of the phosphoinositides, and consequently the [³H]InsP products, after carbachol stimulation resulted in the apparent failure of atropine to reverse the [³H]InsP response completely. Labelling muscle slices with [³H]inositol in the presence of carbachol or labelling for longer periods (> 6 h) prevented subsequent carbachol-stimulated effects on incorporation without significantly altering the dose-response relationship for carbachol-stimulated [³H]InsP formation and resulted in steady-state labelling conditions confirmed by the ability of atropine to reverse fully the [³H]InsP response to carbachol. This study demonstrates the profound effects of a number of agonists on [³H]inositol incorporation into the phospho- and polyphosphoinositides in BTSM with important consequent changes in the specific radioactivity of these lipids and the resulting [³H]InsP products. In addition, a selective depletion of PtdInsP and PtdInsP₂ over PtdIns has been demonstrated with prolonged muscarinic-receptor stimulation.

INTRODUCTION

The initial contractile response to agonists in airway smooth muscle is mediated through an increase in cytosolic [Ca²⁺] released from intracellular stores (see Rodger, 1988) and appears to be independent of membrane potential or extracellular Ca²⁺ concentration (Small & Foster, 1986). A variety of spasmogens have been demonstrated to induce the hydrolysis of membrane phosphoinositides in this tissue (Grandordy *et al.*, 1986, 1988), and Ins(1,4,5)P₃, the immediate product of PtdIns(4,5)P₂ hydrolysis, has been shown to release Ca²⁺ from non-mitochondrial internal stores in permeabilized tracheal smooth-muscle cells (Hashimoto *et al.*, 1985). Diacylglycerol is also generated after receptor-stimulated hydrolysis of PtdInsP₂ (see Berridge, 1987; Takuwa *et al.*, 1986), and there is now growing evidence that the consequent activation of protein kinase C is important

in maintaining the contractile response (Rasmussen & Barrett, 1984; Park & Rasmussen, 1985).

Baron *et al.* (1984) have demonstrated that in dog trachealis muscle muscarinic receptor stimulation, in addition to causing a rapid decrease in PtdIns mass and increases in phosphatidic acid and diacylglycerol, markedly increases the incorporation of [³²P]P_i into PtdIns. A similar 'labelling effect' has been observed in a number of other tissues with both [³²P]P_i and [³H]inositol (see, e.g., Jones & Michell, 1974; Hanley *et al.*, 1980; Monaco, 1982). However, airway smooth muscle appears unusual in respect to the magnitude of this response, and we have therefore used this model to examine in detail the effect of agonists on the incorporation of [³H]inositol into the total and individual phosphoinositides, with special reference to the relationships between the 'labelling effect' and the [³H]InsP response in this tissue and the consequences of stimulated labelling on [³H]InsP formation.

Abbreviations used: GroPIns, GroPInsP, GroPInsP₂, glycerophosphoinositol, its phosphate and bisphosphate; BTSM, bovine tracheal smooth muscle; EC₅₀, concn. producing 50% of maximal response.

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MATERIALS AND METHODS

Tissue preparation

Fresh tracheas from 12–18-month-old cattle were obtained from the local abattoir and transported at 4 °C in modified Krebs–Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, CaCl₂ 1.3 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25 mM, glucose 12 mM). The trachealis muscle was dissected free of overlying epithelium and surrounding connective tissue, and 300 µm × 300 µm slices were prepared with a McIlwain tissue chopper. The slices were preincubated at 37 °C in 100 ml of the same buffer equilibrated with O₂/CO₂ (19:1) for 60 min in a shaking water bath with four intermediate changes of buffer. Tissue was then allowed to settle, and 2 ml of gravity-packed slices was transferred to conical flasks containing 10 ml of buffer and 20–100 µCi of *myo*-[2-³H]inositol and incubated for a further 60 min. The flasks were gassed regularly over this period with O₂/CO₂ (19:1). Samples (50 µl) of gravity-packed slices (1–1.2 mg of protein) and 240 µl of the supernatant were then transferred to flat-bottomed vials, and agonists were added in a 10 µl volume. In studies where Li⁺ was used, slices were preincubated with 5 mM-LiCl for 10 min before the addition of agonist. Reactions were terminated with either 0.94 ml of chloroform/methanol (1:2, v/v) as described by Berridge *et al.* (1982) for measurement of total [³H]inositol phosphates and [³H]inositol incorporation into PtdIns or 300 µl of ice-cold 1 M-trichloroacetic acid for measurement of individual phosphoinositides (see below).

Two alternative labelling protocols were used in later experiments. (i) For ‘pulse–chase’ labelling, smooth-muscle slices were prepared and incubated with *myo*-[³H]inositol as above and then incubated for a further 60 min in buffer containing 10 mM unlabelled *myo*-inositol with ten changes of the medium over this period. Subsequent stimulations were also performed in the presence of 10 mM-*myo*-inositol. (ii) To achieve steady-state labelling conditions (see the Results section), slices were labelled with [³H]inositol for 60 min in the presence of 1 µM-carbachol. The slices were then washed thoroughly over a 30 min period to remove the carbachol, and [³H]inositol label was replaced to the same initial concentration before samples were taken and stimulated as above.

Before use, [³H]inositol was first passed through a 250 µl column of Dowex 1-X8 anion-exchange resin (100–200 mesh, Cl[−] form) to remove polar contaminants.

Measurement of [³H]inositol phosphates and [³H]inositol phospholipids

Total labelled inositol phosphates were assayed as described by Rooney & Nahorski (1986), by using Dowex 1-X8 anion-exchange resin (100–200 mesh, Cl[−] form; 0.5 cm × 1.75 cm columns). After washes with 20 ml of water and 10 ml of 0.025 M-ammonium formate to remove [³H]inositol and [³H]GroPIns respectively, total inositol phosphates (> 90% InsP₁ in the presence of 5 mM-Li⁺) were eluted with 5 ml of 1 M-HCl, and radioactivity was determined by liquid-scintillation counting.

The incorporation of [³H]inositol into the phospholipids was estimated by drying a 200 µl sample of the lower chloroform layer and counting for radioactivity as above. This method largely quantifies the incorporation of [³H]inositol into PtdIns, since neutral chloroform/

methanol mixtures do not effectively extract PtdInsP or PtdInsP₂. For analysis of the separate [³H]-phosphoinositides, after terminating the reactions with 1 M-trichloroacetic acid, the smooth-muscle slices were sedimented by centrifugation and the tissue pellets washed with 1 ml of 5% (w/v) trichloroacetic acid/1 mM-EDTA followed by 1 ml of water (Griffin & Hawthorne, 1978). The phospholipids were extracted as described by Downes & Wusteman (1983) and deacylated as described by Wells & Dittmer (1965). The resulting [³H]glycerophosphoinositol (phosphate) diesters were separated on Dowex AG 1-X8 resin (200–400 mesh, formate form; 0.5 cm × 3.0 cm columns). After the removal of [³H]inositol with 10 ml of water the following ammonium formate (AF)/Na₂B₄O₇/formic acid (FA) buffers were used to elute the indicated ³H-labelled products: 0.18 M-AF/0.005 M-Na₂B₄O₇ (20 ml; [³H]-GroPIns), 0.4 M-AF/0.1 M-FA (20 ml; [³H]GroPInsP) and 1.0 M-AF/0.1 M-FA (10 ml; [³H]GroPInsP₂).

Materials

myo-[2-³H]inositol (20 Ci/mmol) was purchased from New England Nuclear. Anion-exchange resin was obtained from Bio-Rad. All other materials were from Sigma, BDH or Fisons.

RESULTS

Receptor-stimulated incorporation of [³H]inositol into phosphoinositides and [³H]InsP formation

For the purposes of most of these experiments, we have chosen to trap InsPs at the level of the monophosphate by using Li⁺ as an uncompetitive inhibitor. Under these conditions with neutral extraction > 90% of [³H]InsP accumulates as a monophosphate. In other studies (Chilvers *et al.*, 1988; E. R. Chilvers, P. J. Barnes & S. J. Nahorski, unpublished work) we have shown that carbachol induces a very rapid accumulation of [³H]Ins(1,4,5)P₃ and [³H]Ins(1,3,4)P₃, and we have no evidence to suggest that the monophosphate arises from sources other than dephosphorylation of these polyphosphates.

In agreement with previous workers, we show here that both carbachol, a muscarinic-receptor agonist, and histamine, in the presence of Li⁺ cause a marked and dose-related accumulation of [³H]InsPs in bovine tracheal smooth muscle (BTSM) (Fig. 1a) with EC₅₀ of 2 µM and 15 µM respectively. Li⁺ (5 mM) resulted in a 10.3 ± 0.5 (mean ± S.E.M.)-fold increase in [³H]InsPs accumulating in slices stimulated for 30 min with carbachol without significantly affecting the EC₅₀ (results not shown). In addition to the formation of [³H]InsPs, Table 1 demonstrates the marked effects of carbachol and histamine on [³H]inositol incorporation into the total phosphoinositide pool. The effect of bradykinin, a far weaker spasmogen, is also shown. It is apparent that histamine and bradykinin have a disproportionate effect on [³H]inositol incorporation compared with their effect on [³H]InsP formation, and this is particularly evident for bradykinin, which causes a relatively large ‘labelling effect’ despite a very small [³H]InsP response. Likewise, histamine was able to stimulate the incorporation of [³H]inositol to the same extent as carbachol, despite producing only 35% of the [³H]InsPs generated by carbachol. Table 1 and Fig. 1(b) also demonstrate that in the presence of Li⁺ stimulation of muscle slices for

Fig. 1. Dose-response relationships for carbachol- and histamine-stimulated $[^3\text{H}]\text{InsP}$ accumulation and incorporation of $[^3\text{H}]\text{inositol}$ into phospholipids

Muscle slices were labelled with $0.1 \mu\text{M}$ - $[^3\text{H}]\text{inositol}$ for 60 min, and samples were incubated with carbachol (●) or histamine (□) in the presence of 5 mM-Li^+ for 30 min. Reactions were terminated and $[^3\text{H}]\text{InsP}$ formation (a) and $[^3\text{H}]\text{inositol}$ incorporation into the phospholipids (b) assayed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. for five separate experiments, each performed in triplicate.

30 min with carbachol concentrations that are required for maximal $[^3\text{H}]\text{InsP}$ formation results in a significant secondary decline in phosphoinositide labelling. This fall in labelling is not seen with histamine (Fig. 1b) or with carbachol in the absence of Li^+ (Fig. 2). Furthermore, the effects of carbachol and histamine are not additive for either the stimulated incorporation of $[^3\text{H}]\text{inositol}$ or the $[^3\text{H}]\text{InsP}$ response (Table 1).

In slices stimulated for 30 min with carbachol or histamine there appeared to be differences between the EC_{50} values for the stimulated incorporation of $[^3\text{H}]\text{inositol}$ into the inositol phospholipids and the $[^3\text{H}]\text{InsP}$ response, with the incorporation effect being considerably more sensitive to agonist (EC_{50} for incorporation effect and for $[^3\text{H}]\text{InsP}$ formation respectively: histamine $3 \mu\text{M}$ and $15 \mu\text{M}$; carbachol $0.15 \mu\text{M}$ and $2 \mu\text{M}$ (Figs. 1a and 1b)). This difference was of similar magnitude for histamine and carbachol and was maintained for both agonists in the absence of Li^+ . However, when this experiment was performed at a much earlier

Table 1. Effect of agonists on $[^3\text{H}]\text{inositol}$ incorporation into the phosphoinositides and $[^3\text{H}]\text{InsP}$ accumulation

BTSM slices were labelled with $0.5 \mu\text{Ci}$ of $[^3\text{H}]\text{inositol}$ /50 μl of gravity-packed slices for 60 min and stimulated with carbachol, histamine or bradykinin as indicated in a final volume of 300 μl for 30 min. $[^3\text{H}]\text{inositol}$ incorporation into the phospholipids and $[^3\text{H}]\text{InsPs}$ were measured as detailed in the Materials and methods section.

Treatment	$[^3\text{H}]\text{inositol}$ incorporation (d.p.m./50 μl of BTSM slices)	$[^3\text{H}]\text{InsPs}$ (d.p.m./50 μl of BTSM slices)
Control	5944 ± 104	219 ± 5
Carbachol ($1 \mu\text{M}$)	30530 ± 1945	4184 ± 275
Carbachol (0.1 mM)	18168 ± 1578	12967 ± 1131
Histamine ($10 \mu\text{M}$)	27020 ± 264	1480 ± 180
Histamine (1 mM)	30305 ± 1020	4499 ± 229
Bradykinin ($1 \mu\text{M}$)	15753 ± 2346	577 ± 94
Cumulative effects:		
Carbachol ($1 \mu\text{M}$) + histamine (1 mM)	31551 ± 1259	6364 ± 410
Carbachol (0.1 mM) + histamine (1 mM)	22080 ± 1054	12907 ± 658

time point (5 min), when the labelling was not influenced by a ceiling effect created by the phosphoinositides being already labelled to equilibrium (see Fig. 2b), the dose-response curve for the incorporation effect matched that for $[^3\text{H}]\text{InsP}$ formation.

Effects of changes in phosphoinositide labelling on $[^3\text{H}]\text{InsP}$ formation

It is apparent that carbachol, through stimulating the incorporation of $[^3\text{H}]\text{inositol}$ into the phosphoinositide pool, causes an initial increase in the specific radioactivity of the phosphoinositides. In the presence of Li^+ higher carbachol concentrations cause a decline in labelling probably consequent upon depletion of the inositol phospholipid pool, and both these effects must invariably complicate the interpretation of the subsequent $[^3\text{H}]\text{InsPs}$ produced. Two important examples of how agonist-induced changes in the specific radioactivity in the phosphoinositides distort the pattern of $[^3\text{H}]\text{InsPs}$ formed after receptor stimulation are shown in Figs. 2(a) and 2(b). The initial exponential increase in $[^3\text{H}]\text{InsP}$ formation with time almost certainly reflects the early changes in phosphoinositide labelling shown in Fig. 2(b), with the expected linear time course for $[^3\text{H}]\text{InsP}$ formation only evident when the labelling effect reaches a plateau. Fig. 2(b) also shows that prolonged stimulation with 0.1 mM -carbachol in the presence of Li^+ leads to a secondary decrease in lipid labelling, to values well below those in unstimulated slices, which would suggest that the $[^3\text{H}]\text{inositol}$ does have access to a hormone-sensitive pool under basal conditions. Slices incubated with a much lower concentration of carbachol ($1 \mu\text{M}$) show an identical initial rise in phosphoinositide labelling, which declines only after a more prolonged period of stimulation ($> 60 \text{ min}$). In the absence of Li^+ this secondary fall is not seen. Surprisingly, a 'pulse-chase' labelling protocol in which slices were washed and incubated with

Fig. 2. Time course for carbachol-stimulated $[^3\text{H}]\text{InsP}$ accumulation and incorporation of $[^3\text{H}]\text{inositol}$ into phospholipids

Muscle slices were labelled with $0.1 \mu\text{M}$ - $[^3\text{H}]\text{inositol}$ for 60 min before incubation with carbachol (0.1 mM) or buffer for the time periods indicated. $[^3\text{H}]\text{InsPs}$ and $[^3\text{H}]\text{inositol}$ incorporation into the phospholipids were assayed as described in the Materials and methods section. (a) Time course for control (O) or carbachol (0.1 mM)-stimulated (●) $[^3\text{H}]\text{InsPs}$ in the presence of 5 mM-Li^+ , and (b) time course showing effect of carbachol (0.1 mM) on incorporation of $[^3\text{H}]\text{inositol}$ into the phospholipids in the absence (O) or presence (●) of 5 mM-Li^+ (control values shown by broken lines). Data are means $\pm$ S.E.M. of three experiments, each performed in triplicate.

excess unlabelled inositol after the initial labelling period did not attenuate the stimulated incorporation of $[^3\text{H}]\text{inositol}$ into the phosphoinositides in response to carbachol, although it did result in a slightly greater fall in lipid labelling with stimulation periods $> 20 \text{ min}$ (results not shown).

The importance of the consequent changes in the specific radioactivity in the receptor-linked phosphoinositide pool induced by agonist are further demonstrated by the apparent inability of atropine to reverse completely the $[^3\text{H}]\text{InsPs}$ generated by carbachol in the absence of lithium (Fig. 3a). Total muscarinic-receptor blockade would be expected to prevent further $[^3\text{H}]\text{InsP}$ formation and allow the $[^3\text{H}]\text{InsPs}$ that accumulated before the addition of atropine to decrease to basal

Fig. 3. Atropine reversal of carbachol-stimulated $[^3\text{H}]\text{InsPs}$ responses

Muscle slices were labelled with $0.1 \mu\text{M}$ - $[^3\text{H}]\text{inositol}$ in the absence (a) or presence (b) of carbachol ($1 \mu\text{M}$) for 60 min. After extensive washing in unlabelled buffer, followed by replacement of $[^3\text{H}]\text{inositol}$, slices were divided into batches and incubated with carbachol (0.1 mM) (●, ○) or buffer (□) for 30 min in the absence of Li^+ . At 30 min atropine ($10 \mu\text{M}$) (●, □) or buffer (○) was added, and reactions were terminated as detailed in the Materials and methods section at the time points indicated. Atropine fully reversed the carbachol-stimulated $[^3\text{H}]\text{InsPs}$ response only after steady-state labelling conditions had been achieved by initially labelling in the presence of carbachol. Data are from a single experiment, with identical results obtained in a single further experiment.

values. The inability of atropine to do this can be accounted for by the hidden changes in lipid labelling caused by carbachol before receptor blockade, and if the 'true' new basal $[^3\text{H}]\text{InsP}$ value is calculated by multiplying the observed basal $[^3\text{H}]\text{InsP}$ value at 60 min by the ratio of lipid labelling in the slices stimulated by carbachol to that in the unstimulated slices it becomes clear that a $> 85 \%$ reversibility of the $[^3\text{H}]\text{InsP}$ response has in fact been achieved.

Attempts to achieve steady-state labelling conditions

It is clear that to allow any valid interpretation of receptor-generated $[^3\text{H}]\text{InsP}$ products in this tissue it is essential to achieve steady-state labelling conditions to minimize these marked concentration- and time-de-

Fig. 4. Effect of different buffer Ca^{2+} concentrations on basal and carbachol-stimulated $[^3\text{H}]$ inositol incorporation into the phosphoinositides

Muscle slices were labelled for 60 min with $0.1 \mu\text{M}$ - $[^3\text{H}]$ inositol in a modified Krebs-Henseleit buffer containing ($\square$) 0.6 mM -, ($\bullet$) 1.2 mM - or ($\circ$) 2.4 mM - CaCl_2 . These different buffer conditions were maintained throughout the subsequent 30 min incubations with carbachol or buffer (C). $[^3\text{H}]$ inositol incorporation into the phosphoinositides was measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three separate experiments, each performed in triplicate.

Fig. 5. Effect of labelling muscle slices in the presence of carbachol on subsequent agonist-stimulated incorporation of $[^3\text{H}]$ inositol into the phosphoinositides

Slices were labelled with $[^3\text{H}]$ inositol for 60 min in the absence ($\blacksquare$) or presence ($\square$) of $1 \mu\text{M}$ -carbachol. After extensive washing and replacement of $[^3\text{H}]$ inositol, slices were stimulated with 0.1 mM -carbachol or buffer (C) in the presence of 5 mM - Li^+ for the time periods indicated. $[^3\text{H}]$ Phosphoinositides were extracted and measured as detailed in the Materials and methods section. Data represent means $\pm$ S.E.M. of three separate experiments, each performed in triplicate.

pendent changes in phosphoinositide labelling produced by agonists.

The above experiments were all performed with slices that had been labelled with relatively low concentrations of *myo*- $[^3\text{H}]$ inositol and for a short time period (60 min). With longer prelabelling periods the agonist-sensitive phosphoinositide pool can be more fully labelled with $[^3\text{H}]$ inositol, decreasing the subsequent agonist-stimulated incorporation effect. Prelabelling slices for 0.5, 2, 4 or 6 h and then stimulating them with $1 \mu\text{M}$ -carbachol for 30 min in the presence of 5 mM - Li^+ resulted respectively in 5.7 ± 0.4 -, 2.6 ± 0.7 -, 1.1 ± 0.2 - and 1.0 ± 0.15 -fold increases in $[^3\text{H}]$ PtdIns labelling, with corresponding fold increases in $[^3\text{H}]$ InsP accumulation of 14.4 ± 0.4 -, 7.8 ± 1.0 -, 5.25 ± 0.3 and 4.25 ± 0.4 respectively (means $\pm$ S.E.M., six values from three separate experiments). This demonstrates the important consequence of steady-state labelling on the apparent fold stimulations of the $[^3\text{H}]$ InsPs, with a major component of the $[^3\text{H}]$ InsP response under non-steady-state labelling conditions simply reflecting agonist-induced changes in lipid labelling and hence altered specific radioactivity of the $[^3\text{H}]$ InsPs. This effect with $1 \mu\text{M}$ -carbachol is even more evident with maximal carbachol concentrations (0.1 mM), where stimulating slices that had been labelled for 1 h in the presence of Li^+ for 30 min results in a 50-fold stimulation in $[^3\text{H}]$ InsPs; however, with longer labelling periods ($> 4 \text{ h}$) a truer value of 10–15-fold is seen.

Further attempts to achieve steady-state labelling conditions were made by using different buffer Ca^{2+} concentrations ($[\text{Ca}^{2+}]_e$), in particular by lowering $[\text{Ca}^{2+}]_e$ in an attempt to stimulate inositol incorporation into phosphatidylinositol, since the inositol headgroup

exchange mechanism and the CDP-diacylglycerol: *myo*-inositol phosphatidyltransferase reactions are both inhibited by Ca^{2+} (Takenawa & Egawa, 1977; Egawa *et al.*, 1981). As in rat cerebral-cortical slices (Kendall & Nahorski, 1984), decreasing the $[\text{Ca}^{2+}]_e$ progressively increased the basal incorporation of $[^3\text{H}]$ inositol into the phosphoinositides, but this did not ameliorate the magnitude of the subsequent carbachol-stimulated incorporation of $[^3\text{H}]$ inositol into the phosphoinositides (Fig. 4). When slices were labelled in buffer with no added Ca^{2+} , the basal labelling was similar to that achieved with 0.6 mM - Ca^{2+} , and no change in lipid labelling was seen with carbachol stimulation (10 nM – 0.1 mM) (results not shown). However, this was probably a consequence of the marked inhibition ($> 85\%$) of the $[^3\text{H}]$ InsP response at this very low $[\text{Ca}^{2+}]_e$ (Baird *et al.*, 1989).

Finally, an attempt to achieve steady-state labelling was made by labelling the slices with $[^3\text{H}]$ inositol in the presence of $1 \mu\text{M}$ -carbachol. The slices were then thoroughly washed to remove the agonist, and the $[^3\text{H}]$ inositol label was replaced before repeat carbachol stimulation. This protocol allowed steady-state labelling conditions during subsequent carbachol incubations (Fig. 5), and resulted in a truly linear time course for $[^3\text{H}]$ InsP formation in the presence of Li^+ (results not shown). There was no evidence for desensitization of the subsequent $[^3\text{H}]$ InsP response to carbachol by using this labelling protocol, and the EC_{50} for carbachol was likewise unaffected. When slices labelled by this method were stimulated with carbachol in the absence of Li^+ and then exposed to atropine, a near-complete ($> 90\%$) inhibition of the $[^3\text{H}]$ InsP response was observed (Fig. 3b), which would be consistent with true steady-state labelling.

Table 2. Agonist-stimulated incorporation of [³H]inositol into polyphosphoinositides, and effect of Li⁺ on pool size with prolonged carbachol stimulation

Muscle slices were labelled with 0.25 μ M-[³H]inositol for 60 min in the presence (stimulated labelling) or absence (unstimulated labelling) of 1 μ M-carbachol. After extensive washing and replacement of [³H]inositol, slices were divided into portions and stimulated with 1 μ M- or 0.1 mM-carbachol or buffer control in the absence or presence of 5 mM-Li⁺ for 30 min. The individual [³H]phosphoinositides were extracted, separated and quantified as described in the Materials and methods section. Data represent means $\pm$ S.E.M. from three separate experiments.

Labelling	Incorporation (d.p.m./50 μ l of BTSM slices)					
	Control		Carbachol			
	No Li ⁺	5 mM-Li ⁺	1 μ M, no Li ⁺	1 μ M, 5 mM-Li ⁺	0.1 mM, no Li ⁺	0.1 mM, 5 mM-Li ⁺
PtdIns						
Unstimulated	34 828 $\pm$ 6093	41 091 $\pm$ 4332	100 372 $\pm$ 1224	112 845 $\pm$ 2220	97 881 $\pm$ 2120	73 240 $\pm$ 7173
Agonist-stimulated	87 493 $\pm$ 7214	91 175 $\pm$ 4597	90 467 $\pm$ 5912	97 007 $\pm$ 2831	82 006 $\pm$ 9338	61 282 $\pm$ 5202
PtdInsP						
Unstimulated	1559 $\pm$ 233	1657 $\pm$ 139	4656 $\pm$ 204	4905 $\pm$ 96	2760 $\pm$ 313	2245 $\pm$ 303
Agonist-stimulated	3678 $\pm$ 140	3781 $\pm$ 259	4304 $\pm$ 131	4394 $\pm$ 73	2200 $\pm$ 83	1978 $\pm$ 145
PtdInsP₂						
Unstimulated	3038 $\pm$ 600	3135 $\pm$ 285	11 273 $\pm$ 389	12 724 $\pm$ 797	5423 $\pm$ 510	4958 $\pm$ 349
Agonist-stimulated	10 090 $\pm$ 364	10 132 $\pm$ 130	10 706 $\pm$ 127	11 079 $\pm$ 184	4768 $\pm$ 335	3851 $\pm$ 416

Effect of carbachol on [³H]inositol incorporation into the polyphosphoinositides

Since measurement of radioactivity in the chloroform phase of the tissue extract only allows assessment of the incorporation of [³H]inositol into the total lipid pool (largely PtdIns), further studies were undertaken to detail the effect of carbachol on the individual phosphoinositide pools. We were particularly interested to see if the observed stimulated incorporation of [³H]inositol occurred to a similar extent in PtdIns, PtdInsP and PtdInsP₂, and specifically what the effect of prolonged stimulation with maximal carbachol concentrations was on the polyphosphoinositide pools, especially in the presence of Li⁺.

Table 2 shows that carbachol (1 μ M) stimulated the incorporation of [³H]inositol into all three phosphoinositide pools, with a slightly greater effect on PtdInsP₂ (mean fold stimulations PtdIns 2.9, PtdInsP 2.9, PtdInsP₂ 3.7). The degree of stimulation for PtdIns is slightly lower than would be inferred from Table 1 and Fig. 2; however, this reflects the increased concentration of [³H]inositol label used in these experiments, resulting in higher basal values. Table 2 shows, as in the previous experiments, that prolonged stimulation (30 min) with concentrations of carbachol that generate the maximal [³H]InsP response (0.1 mM) in the absence of Li⁺ do not cause a secondary decline in [³H]PtdIns. However, under identical conditions carbachol causes a marked fall in the radioactivity in the PtdInsP and PtdInsP₂ pools. This would imply that, under these conditions, the conversion of PtdIns into PtdInsP by the 4-kinase is rate-limiting with prolonged maximal phospholipase C stimulation. Table 2 also shows the results of further studies on slices labelled in the presence of carbachol to achieve steady-state labelling conditions and subsequently stimulated with 1 μ M- or 0.1 mM-carbachol for 30 min in the presence or absence of 5 mM-Li⁺. Again, labelling in the presence of 1 μ M-carbachol prevents changes in incorporation with

further carbachol administration, but does not influence the decline in PtdInsP and PtdInsP₂ labelling observed in the absence of Li⁺ under non-steady-state labelling conditions. The addition of 5 mM-Li⁺ during the period of carbachol stimulation now results in a significant decline in [³H]PtdIns at 0.1 mM-carbachol, with much smaller effects on [³H]PtdInsP and [³H]PtdInsP₂ (PtdIns 94 $\pm$ 2% versus 63 $\pm$ 4%; PtdInsP 51 $\pm$ 2% versus 45 $\pm$ 4%; PtdInsP₂ 44 $\pm$ 3% versus 35 $\pm$ 3%; per cent of incorporation with 1 μ M-carbachol, no Li⁺ versus 5 mM-Li⁺). This demonstrates that under conditions of prolonged carbachol stimulation the presence of 5 mM-Li⁺ can lead to the depletion of the PtdIns pool without significantly influencing PtdInsP and PtdInsP₂ labelling.

DISCUSSION

The present study confirms that, in common with other smooth-muscle preparations, stimulation of muscarinic and histamine receptors in BTSM leads to a marked hydrolysis of phosphoinositides and accumulation of inositol phosphates. An earlier study of BTSM by Grandordy *et al.* (1986) reported similar findings, although the effect of carbachol on [³H]InsPs was much smaller, and the EC₅₀ of 38 μ M was considerably different from that obtained in the present and other recent studies (Hall & Hill, 1988). Almost certainly the major substrate, at least over the first few seconds of agonist stimulation, is PtdInsP₂ (Takuwa *et al.*, 1986), with the production of Ins(1,4,5)P₃, which then undergoes dephosphorylation to Ins(1,4)P₂ or phosphorylation to Ins(1,3,4,5)P₄ and subsequent dephosphorylation to appropriate bis- and mono-phosphates (Chilvers *et al.*, 1988; E. R. Chilvers, I. H. Batty & S. R. Nahorski, unpublished work). This would be consistent with a role for Ins(1,4,5)P₃ and perhaps Ins(1,3,4,5)P₄ in tracheal smooth-muscle Ca²⁺ homeostasis, and would form the basis of pharmacomechanical coupling in this tissue

(Somlyo *et al.*, 1988). However, the emphasis in the present work was to examine the relationship between muscarinic-receptor occupation and the accumulation of [3 H]inositol phosphates in the presence of Li^+ , which prevents their dephosphorylation to inositol (Allison & Blisner, 1976; Berridge *et al.*, 1982). Furthermore, we were intrigued to observe the magnitude of the effects of carbachol and histamine and to a lesser degree bradykinin on stimulating the incorporation of [3 H]inositol into the phosphoinositides, especially as this had not been previously reported in this tissue (Grandordy *et al.*, 1986; Duncan *et al.*, 1987).

Although stimulated labelling of the inositol lipids would be expected as a consequence of agonist-induced hydrolysis, and indeed formed the basis of the first observation of a 'phosphoinositide response' by Hokin & Hokin (1953) and many other early studies (see Michell, 1975), the extent of agonist-induced [3 H]inositol labelling in tracheal smooth muscle is considerable. Although Aub & Putney (1984) have reported a 4-fold increase in [3 H]inositol labelling by the α_1 -adrenoceptor agonist phenylephrine in the parotid gland, agonist effects on [3 H]inositol incorporation into the phosphoinositides are generally relatively small, e.g. in rat salivary glands (Hanley *et al.*, 1980; and see Irvine *et al.*, 1982), or not demonstrable, e.g. in rat cerebral cortex (I. H. Batty & S. R. Nahorski, unpublished work). The extent of the labelling effect in BTSM may reflect either a relatively discrete pool of phosphoinositides associated with the agonist-sensitive phospholipase C or very low turnover of this pool under basal conditions. Certainly, even in the absence of Li^+ , prolonged maximal carbachol stimulation depletes the labelling of PtdInsP and PtdInsP_2 in this tissue, and when Li^+ is present this effect is also seen with the much larger PtdIns pool. Since these changes were also observed after steady-state labelling had been achieved, they almost certainly reflect actual changes in phosphoinositide mass, and are in agreement with previous mass measurements of the polyphosphoinositides in this tissue (Takuwa *et al.*, 1986). These data also suggest that in the absence of Li^+ the conversion of PtdIns into PtdInsP by the PtdIns kinase may be rate-limiting under conditions of maximal receptor stimulation and hence maximal PtdInsP_2 hydrolysis. This is in contrast with the parotid gland, where during prolonged agonist stimulation the PtdInsP_2 pool size is maintained at the expense of PtdIns even in the presence of Li^+ (Downes & Stone, 1986). The probability of a relatively small phospholipase C-linked phosphoinositide pool within this tissue or a pool that turns over slowly under basal conditions is supported by the very low levels of [3 H]InsPs accumulating in the unstimulated slices.

A few studies have suggested that agonist effects on phosphoinositide metabolism may be more complex than previously suggested, i.e. a simple compensatory re-synthesis of the phosphoinositides consequent upon receptor-mediated hydrolysis (Downes *et al.*, 1983). In BTSM slices labelled with [3 H]inositol for 60 min and stimulated with agonist for 30 min, the EC_{50} for the effects of carbachol and histamine on [3 H]inositol incorporation and [3 H]InsP accumulation appeared significantly different, with the dose-response curve for both carbachol and histamine incorporation lying an order of magnitude to the left of that reflecting breakdown of the phosphoinositides. This difference was not due to the

uncompetitive effect of Li^+ on the [3 H]InsP response, since the position of both curves was unchanged in the absence of Li^+ . However, the true position of the incorporation response became apparent when the effect of agonist was examined at early time points before maximal labelling of the phosphoinositides had occurred. Removal of this ceiling effect on the phosphoinositide labelling resulted in a shift of the labelling dose-response curve to the right, to a position identical with that for the [3 H]InsP response, hence eliminating the apparent differences in dose-response relationships between the incorporation effect and [3 H]InsP response seen after longer stimulation periods. It is also interesting that histamine is as effective as carbachol in stimulating the incorporation of [3 H]inositol into the phospholipids, despite being only 30% as effective as carbachol in respect to [3 H]InsP accumulation. A similar disparity between the incorporation effect and the [3 H]InsP response is also apparent for bradykinin. The carbachol and histamine [3 H]InsP responses are partially additive at submaximal concentrations only, and therefore, in common with other studies, we are unable to support the proposal of separate agonist-specific phosphoinositide pools in airway smooth muscle.

The practical consequences of this agonist-induced stimulation of incorporation of [3 H]inositol into the phosphoinositides, which was of similar magnitude for PtdIns , PtdInsP and PtdInsP_2 , are well illustrated in these studies. Although agonist stimulation may evoke an increase in PtdIns and polyphosphoinositide concentration through stimulating a net synthesis of the phosphoinositides (see Downes & Michell, 1985), the marked effect on phosphoinositide labelling is likely to result predominantly from changes in [3 H]inositol incorporation and hence the specific radioactivity of these lipids. This is clearly demonstrated by the inability of the muscarinic antagonist atropine to reverse completely the accumulation of [3 H]InsPs in the absence of Li^+ . Since any change in the specific radioactivity of the phosphoinositides necessarily results in similar changes in the specific radioactivity of the [3 H]InsPs, the interpretation of [3 H]InsP responses in tissues not labelled to steady state are extremely complex. It is therefore critical to label this tissue for several hours (> 6 h) before the addition of agonist, or to perform the labelling under conditions of receptor stimulation (Aub & Putney, 1984; and see Hokin, 1974). Furthermore, [3 H]inositol should be present throughout the period of receptor stimulation to prevent subsequent falls in specific radioactivity resulting from depletion of radiolabelled substrate.

Finally, it should be noted that, even under conditions of steady-state labelling, the EC_{50} for carbachol-stimulated [3 H]InsP accumulation in BTSM is clearly displaced from the occupation curve for carbachol at the muscarinic receptor. This has been observed in rat and guinea-pig parotid gland (Jacobson *et al.*, 1985; Ek & Nahorski, 1988), and differs from cerebral cortex, in which there is a near-linear relationship between muscarinic-receptor occupation and [3 H]InsP accumulation (Ek & Nahorski, 1988). Consistent with this phenomenon, partial agonists such as oxotremorine produce relatively much larger [3 H]InsP responses in parotid than in cerebral cortex (Ek & Nahorski, 1988; Brown *et al.*, 1984) and are nearly full agonists in this respect in BTSM (E. R. Chilvers, unpublished work). It is unlikely that this phenomenon is a consequence of the agonist-induced labelling effect seen

in BTSM and parotid (Aub & Putney, 1984), since it was also observed under conditions of steady-state labelling. However, it may suggest that there are important differences in receptor-occupation response relationships in these tissues that may relate to their function in pharmacomechanical/secretory coupling.

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