Pergamon

Biochemical Pharmacology, Vol. 47, No. 12, pp. 2171–2179, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0081-U

EFFECTS OF MEMBRANE DEPOLARIZATION AND CHANGES IN INTRA- AND EXTRACELLULAR CALCIUM CONCENTRATION ON PHOSPHOINOSITIDE HYDROLYSIS IN BOVINE TRACHEAL SMOOTH MUSCLE

EDWIN R. CHILVERS, BARBARA J. LYNCH, GRAHAM J. OFFER* and R. A. JOHN CHALLISS*†

Respiratory Medicine Unit, Department of Medicine (RIE), Rayne Laboratory, City Hospital, Greenbank Drive, Edinburgh EH10 5SB; *Department of Cell Physiology and Pharmacology, PO Box 138, Medical Sciences Building, University of Leicester, Leicester LE1 9HN, U.K.

(*Received* 6 *December* 1993; *accepted* 11 *February* 1994)

Abstract—Agonist-stimulated phosphoinositide metabolism plays a central role in pharmacomechanical coupling in airways smooth muscle (ASM). In many other tissues and cells, most noteably excitable cells, membrane depolarization or an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) generated by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)-induced Ca^{2+} release or agonist-mediated Ca^{2+}influx is able to trigger or augment phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) hydrolysis and/or initiate PtdIns4P/PtdIns hydrolysis by direct stimulation of PIC. To assess the importance of these mechanisms in ASM the effects of KCl-induced membrane depolarization, extracellular Ca^{2+}($[Ca^{2+}]_e$) chelation, and addition of ionomycin to elevate $[Ca^{2+}]_i$ on basal and agonist-stimulated $Ins(1,4,5)P_3$ concentration and [^{3}H]-$InsP_x$ accumulation have been examined. Reducing $[Ca^{2+}]_e$ from 1.8 mM to 6 or 0.8 μM caused a progressive inhibition of agonist-stimulated [^{3}H]inositol polyphosphate accumulation over 30 min with the histamine-stimulated response being significantly more sensitive to $[Ca^{2+}]_e$ chelation than the response to carbachol. In contrast, the initial accumulation of $Ins(1,4,5)P_3$ was completely unaffected by such reductions in $[Ca^{2+}]_e$. Incubation of [^{3}H]inositol-prelabelled BTSM slices with buffer containing 80 mM KCl failed to stimulate [^{3}H]$InsP_x$ accumulation, causing instead a small inhibition of carbachol-stimulated [^{3}H]$InsP_x$ accumulation with a similar effect seen with respect to $Ins(1,4,5)P_3$ accumulation. Addition of 5 μM ionomycin to BTSM slices similarly did not stimulate $Ins(1,4,5)P_3$ generation and only increased [^{3}H]$InsP_x$ accumulation after prolonged stimulation in the presence of high (mM) $[Ca^{2+}]_e$. These data indicated that in ASM, membrane depolarization or physiological increases in $[Ca^{2+}]_i$ did not result in either independent activation of PIC or augmentation of initial agonist-stimulated $PtdIns(4,5)P_2$ hydrolysis. However, while the initial agonist-stimulated generation of $Ins(1,4,5)P_3$ was not dependent on $[Ca^{2+}]_e$, a normal plasmalemmal Ca^{2+} gradient was required to sustain maximal rates of agonist-stimulated $PtdIns(4,5)P_2$ hydrolysis.

Key words: airways smooth muscle; inositol phosphates; $Ins(1,4,5)P_3$; muscarinic cholinoceptors

It is now well recognized that in neuronal cells and other excitable tissues an increase in $[Ca^{2+}]_i$,‡ mediated either by the release of Ca^{2+} from intracellular stores or by Ca^{2+} influx across the plasma membrane, can result in direct activation of PIC or facilitation of agonist-stimulated PIC activity [1–3]. In such tissues it has been suggested that this effect may afford an important agonist-independent mechanism for regulating PIC activity. It has also been proposed that $[Ca^{2+}]_i$ may have an important influence on the substrate specificity of PIC; in particular, facilitating the hydrolysis of PtdIns and/or PtdIns(4)P in addition to $PtdIns(4,5)P_2$ [1, 4, 5; but see 6]. This would have the important consequence of partially dissociating diacylglycerol generation (and hence PKC activation) from $Ins(1,4,5)P_3$ formation. It is also clear from studies undertaken in brain tissue that membrane depolarization or an elevation of $[Ca^{2+}]_i$ immediately prior to agonist addition can have a profound influence on the subsequent accumulation and metabolism of $Ins(1,4,5)P_3$ particularly with regard to $Ins(1,3,4,5)P_4$ generation [7, 8].

Despite this, the ability of Ca^{2+} to regulate PIC activity in a similar manner in other tissues is less certain. In smooth muscle, for example, major tissue and species differences appear to exist with respect to the effects of $[Ca^{2+}]_i$ and transmembrane potential (E_m) on $PtdIns(P_x)$ hydrolysis [9, 10]. Furthermore, despite the undoubted importance of this signalling pathway to pharmacomechanical coupling in ASM [11, 12], little is known about the possible influence of changes in membrane potential or $[Ca^{2+}]_i$ on phosphoinositide metabolism in this tissue. Likewise, while it has been suggested that the varying ability

† Corresponding author: Tel. 0533 522920; FAX (031) 0533 523996.

‡ Abbreviations: ASM, airway smooth muscle; PIC, phosphoinositidase C; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $InsP_x$, inositol phosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_e$, extracellular calcium concentration; PKC, protein kinase C; BTSM, bovine tracheal smooth muscle.

of β_2-adrenoceptor agonists to inhibit spasmogen-stimulated phosphoinositide hydrolysis in ASM may relate to a differential dependence of the contractile agonist responses to Ca^{2+} influx [13–16], the potential influence of $[Ca^{2+}]_e$ on agonist-stimulated phosphoinositide metabolism has not been fully examined, particularly with respect to the generation of the pathway second messengers.

In the current study undertaken in bovine tracheal smooth muscle (BTSM) it was sought to investigate the influence of $[Ca^{2+}]_e$ on both the initial and sustained phases of the phosphoinositide response observed following muscarinic cholinoceptor and histaminergic stimulation and to assess whether membrane depolarization or an increase in $[Ca^{2+}]_i$ alone can directly influence PIC activity.

MATERIALS AND METHODS

Materials. *myo*-[^{3}H]inositol (12–20 Ci/mmol) and [^{3}H]inositol 1,4,5-trisphosphate (17–20 Ci/mmol) were purchased from DuPont (Stevenage, U.K.). Before use, the *myo*-[^{3}H]inositol was cleaned of polar contaminants by passing it through a 0.25 mL bed-volume Dowex 1-X8 (100–200 mesh, Cl^- form) column. D-Ins(1,4,5)P_3 and ionomycin were purchased from Boehringer (Mannheim, Germany). Dowex 1-X8 (100–200 mesh, Cl^- form) and Dowex 1-X8 (200–400 mesh, formate form) were purchased from BioRad Laboratories Ltd (Watford, U.K.). Tissue culture supplies and M199 medium were obtained from GIBCO. All other reagents were obtained from commercial sources and were of analytical grade.

Measurement of [^{3}H]*inositol phosphate accumulation.* BTSM slices (300 × 300 μm) were prepared using a McIlwain tissue-chopper as detailed previously [17] and incubated at 37° for 60 min in oxygenated (95% O_2/5% CO_2) modified Krebs–Henseleit (KH) buffer (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7). The BTSM slices were then washed in M199 tissue culture medium and labelled with *myo*-[^{3}H]inositol by adding 50 μL aliquots of gravity packed slices to 930 μL M199 ($[Ca^{2+}] = 1.8$ mM) containing 1–2.5 μCi *myo*-[^{3}H]inositol in 24 well tissue culture plates for 24 hr in a 5% CO_2 incubator [18]. This method permitted near steady-state radiolabelling of the agonist-sensitive phosphoinositide pool (data not shown), and was used specifically to avoid problems relating to the effects of changes in $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ on [^{3}H]phosphoinositide labelling implicit in the use of short-term labelling protocols [19].

The effects of changes in $[Ca^{2+}]_e$ on carbachol and histamine-stimulated [^{3}H]InsP_{1-3} accumulation were measured by adding 20 μL buffer or 20 μL of 100 or 200 mM EGTA (adjusted to pH 7.0) to the labelled slices 30 sec prior to addition of carbachol (100 μM). Preliminary experiments using a Ca^{2+}-sensitive electrode [20] indicated that such additions performed in the presence of tissue slices caused a rapid (<30 sec) and stable adjustment of the $[Ca^{2+}]_e$ from 1.8 mM to 6 μM (with 100 mM EGTA) and 0.8 μM (with 200 mM EGTA). The effects of membrane depolarization, and elevation in $[Ca^{2+}]_i$ on total [^{3}H]InsP accumulation ([^{3}H]InsP_x) were examined by the addition of KCl (final concentration 80 mM) or ionomycin (final concentration 5 μM) to prelabelled BTSM slices for 30 min, as detailed in Table 1.

Final incubations were all performed in the presence of 10 mM LiCl and terminated after a 30 min agonist/agent-stimulation period by the addition of 200 μL 3 M ice-cold trichloroacetic acid (final concentration 0.5 M). After leaving the samples to extract on ice for 20 min, the contents of each well were transferred to a series of plastic insert vials, vortexed and centrifuged at 4° for 20 min at 3000 *g*. A 1 mL aliquot of the supernatant was added to 250 μL of 10 mM EDTA and neutralized using 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine and $NaHCO_3$ as detailed previously [17]. Total [^{3}H]InsPs ([^{3}H]InsP_x) or [^{3}H]InsP mono-, bis- or trisphosphates were separated by anion exchange chromatography using Dowex Cl^- or formate form columns as described by Chilvers *et al.* [17].

Measurement of Ins(1,4,5)P_3 *concentration.* To examine the effects of membrane depolarization and elevated $[Ca^{2+}]_i$ on basal and muscarinic-cholinoceptor-stimulated Ins(1,4,5)P_3 mass, 50 μL aliquots of gravity packed BTSM slices that had been pre-incubated for 60 min in oxygenated KH buffer, as detailed above, were transferred to plastic insert vials containing 240 μL KH buffer and stimulated with either KH buffer, KCl (final concentration, 80 mM), the Ca^{2+} ionophore ionomycin (5 μM), the muscarinic agonist carbachol (carbachol; 100 μM) or a combination of carbachol and 80 mM KCl. Reactions were terminated over a 0–300 sec time–course using ice-cold 1 M trichloroacetic acid, and Ins(1,4,5)P_3 mass measured in 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine neutralized extracts as detailed previously [21]. The tissue pellets were washed in 0.9% NaCl, 20 mM HEPES (pH 7.4), dissolved in 1 M NaOH and the protein concentration determined to allow expression of results as pmol Ins(1,4,5)P_3/mg protein.

Data analysis. [^{3}H]InsP_x and [^{3}H]InsP_{1-3} accumulations were calculated as dpm/50 μL BTSM slices or dpm/mg protein and Ins(1,4,5)P_3 values expressed in pmol/mg protein. All values are presented as means ± SE means for triplicate determinations performed in at least N = 3 separate experiments. Statistical comparisons of values obtained were performed using Student's *t*-test for unpaired observations.

RESULTS

Effects of changes in $[Ca^{2+}]_e$ *on agonist-stimulated phosphoinositide responses*

In agreement with previous studies [15, 18, 22], carbachol and histamine caused concentration-dependent stimulations of [^{3}H]InsP_x accumulation in BTSM, with half-maximal increases in [^{3}H]-InsP_x accumulations (EC_{50}s) being observed at approximately 3 and 30 μM for carbachol and histamine, respectively (Fig. 1).

Over a 30 min period, the rates of [^{3}H]InsP_x accumulation stimulated by either agonist were linear, and at maximally effective concentrations,

Table 1. Effect of 30 min carbachol, high KCl and ionomycin incubation on [^{3}H]InsP accumulation in BTSM slices

	[^{3}H]InsP$_x$ accumulation (expressed relative to control values)
Carbachol	19.5 ± 1.3
KCl	0.93 ± 0.06
Ionomycin	5.11 ± 0.67
EGTA	0.83 ± 0.06
Carbachol + KCl	14.9 ± 1.1*
Carbachol + EGTA	14.2 ± 1.2*
Ionomycin + EGTA	0.78 ± 0.03*

BTSM slices were pre-incubated in bulk for 60 min at 37° before transferring 50 μL portions of gravity-packed slices to multiwells, each containing 1 mL M199 and 1 μCi [^{3}H]inositol. Multiwell plates were then incubated for 24 hr at 37° in a 5% CO_2 incubator. After this labelling period LiCl (final concentration 10 mM) was added to all samples which were then challenged with carbachol (100 μM), KCl (80 mM) or ionomycin (5 μM) for 30 min as indicated. EGTA (final concentration 2 mM) was added as detailed in Materials and Methods. For the dual addition experiments KCl or EGTA were added 30 sec prior to carbachol or ionomycin. Reactions were terminated with ice-cold trichloroacetic acid and samples processed for separation of [^{3}H]InsP$_x$ as described in Materials and Methods.

All values represent means ± SEM of 3–6 experiments performed in triplicate and are expressed relative to the [^{3}H]InsP$_x$ accumulation under control conditions (5961 ± 582 dpm/50 μL BTSM slices).

* Represents $P < 0.05$ in comparison to values obtained with carbachol or ionomycin alone.

histamine (1 mM) evoked a [^{3}H]InsP$_x$ response which was about 40% of that evoked by carbachol (100 μM).

Omission of $[Ca^{2+}]_e$ (reducing $[Ca^{2+}]_e$ in KHB buffer from 1.2 mM to 5–10 μM) dramatically attenuated [^{3}H]InsP$_x$ accumulation evoked by a maximally-effective concentration of histamine by 70–80%, without shifting the EC_{50} for this response. In contrast, carbachol-stimulated [^{3}H]InsP$_x$ accumulation was less affected by $[Ca^{2+}]_e$ omission, with a maximal attenuation of 30–40% being observed at any of the carbachol concentrations used (Fig. 1).

The differential sensitivities of carbachol- and histamine-stimulated responses to decreasing $[Ca^{2+}]_e$ in BTSM were investigated further. Resolution of [^{3}H]InsP$_{1-3}$ from the [^{3}H]InsP$_x$ fraction, showed that after 30 min stimulation with 100 μM carbachol the increases in [^{3}H]InsP$_1$, [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ were 13.3-, 11.6- and 2.1-fold, respectively, over basal levels. Comparable values for stimulation with 1 mM histamine were 7.8-, 2.3- and 1.6-fold, respectively, over basal values for [^{3}H]InsP$_1$, [^{3}H]-InsP$_2$ and [^{3}H]InsP$_3$. Reducing $[Ca^{2+}]_e$ from 1.8 mM to either 6 or <1 μM immediately prior to agonist challenge decreased [^{3}H]InsP$_{1-3}$ accumulation, with the extent of the decrease being dependent upon the isomer fraction, agonist and extent of reduction in $[Ca^{2+}]_e$ (Fig. 2). Thus, for both carbachol and histamine, reduction in $[Ca^{2+}]_e$ caused a greater decrease in [^{3}H]InsP$_3$ compared to [^{3}H]InsP$_2$, which in turn was more affected than [^{3}H]InsP$_1$. For all [^{3}H]InsP$_{1-3}$ fractions, decreases in $[Ca^{2+}]_e$ affected histamine-stimulated responses to a much greater extent than those evoked by carbachol (Fig. 2), consistent with the results shown in Fig. 1, and reducing $[Ca^{2+}]_e$ to <1 μM had a generally greater effect than that caused by reducing $[Ca^{2+}]_e$ to 6 μM.

Although measurement of [^{3}H]InsP$_x$ accumulation in the presence of lithium blockade of inositol phosphate recycling provided a simple assay of agonist-stimulated PIC activity [3], it gave no information on the tissue concentration of the pathways second messenger Ins(1,4,5)P$_3$. As reported previously [15, 23–25] in BTSM, both carbachol and histamine evoked transient increases in Ins(1,4,5)P$_3$ mass, with Ins(1,4,5)P$_3$ returning to basal or sub-basal levels within 0.5–5 min of agonist challenge. Thus, the major isomer in the [^{3}H]InsP$_3$ fraction accumulating over 30 min of agonist challenge (Fig. 2) was not Ins(1,4,5)P$_3$ but Ins(1,3,4)P$_3$ [24]. To investigate the effects of manipulating $[Ca^{2+}]_e$ on Ins(1,4,5)P$_3$ directly, mass levels of this messenger were assessed using a radioreceptor assay [21].

In agreement with previous work [23, 25], carbachol (100 μM) evoked a rapid increase in Ins(1,4,5)P$_3$ concentration in BTSM which was maximal at 5 sec and declined to sub-basal levels after 5 min (Fig. 3). Incubation of BTSM slices with 2 mM EGTA for 30 sec (data not shown) or 30 min (Fig. 3) had no effect on basal Ins(1,4,5)P$_3$ accumulation, and failed to influence the magnitude of the initial increase in Ins(1,4,5)P$_3$ following carbachol challenge. Indeed, the only statistically significant effect of decreasing $[Ca^{2+}]_e$ was found 5 min after carbachol addition (Fig. 3), where, in contrast to the Ins(1,4,5)P$_3$ response in the presence of normal $[Ca^{2+}]_e$, no decrease in Ins(1,4,5)P$_3$ mass to sub-basal levels was observed.

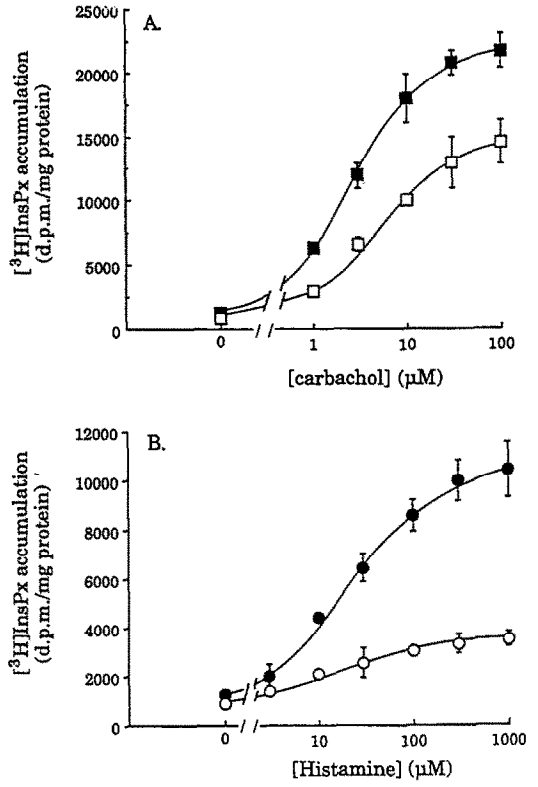

Fig. 1. Effect of nominal removal of $[Ca^{2+}]_e$ on the magnitude and concentration-dependency of total $[^3H]$-$InsP_x$ accumulation stimulated by carbachol or histamine. BTSM slices were prepared, incubated and radiolabelled in the presence of 1 μCi/mL [^{3}H]inositol, as described previously [15]. At the end of the labelling period slices were either extensively washed (6 × 20 mL) over a 15 min period) in normal KH buffer ($[Ca^{2+}] = 1.2$ mM) or nominally Ca^{2+}-free ($[Ca^{2+}] = 5$–10 μM) KH buffer. Gravity-packed slices (75 μL) were incubated in a final volume of 500 μL KH buffer (±Ca^{2+}) containing 0.5 μCi [^{3}H]inositol and 5 mM LiCl. Incubations were initiated by addition of the indicated concentrations of carbachol (panel A) or histamine (panel B) to slices maintained under normal $[Ca^{2+}]$ (closed symbols) or nominally Ca^{2+}-free (open symbols) conditions. Incubations were terminated after 30 min by addition of 500 μL 1 M trichloroacetic acid and processed for separation of the total $[^3H]InsP_x$ fraction as described previously [15]. Values are shown as means ± SE mean for three separate experiments performed in triplicate.

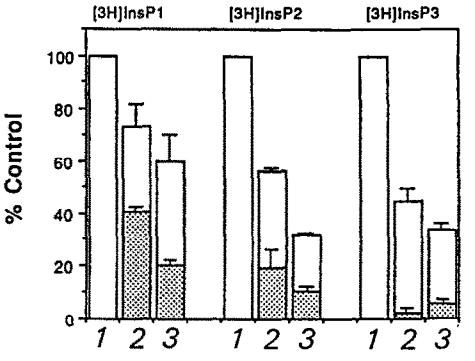

Fig. 2. Effect of changes in $[Ca^{2+}]_e$ on carbachol- and histamine-stimulated accumulations of $[^3H]InsP_1$, $[^3H]InsP_2$ and $[^3H]InsP_3$. BTSM slices were preincubated in bulk for 60 min at 37° before transferring 50 μL portions of gravity-packed slices to multiwells, each containing 1 mL M199 and 2.5 μCi [^{3}H]inositol. Multiwell plates were incubated for 24 hr at 37° in a 5% CO_2 incubator. After this labelling period LiCl (final concentration 10 mM) was added to all samples and, where indicated, $[Ca^{2+}]_e$ was maintained at normal levels (1.8 mM, column 1) or adjusted to approximately 6 μM (column 2) or 0.8 μM (column 3) by addition of EGTA (see Materials and Methods). Phosphoinositide turnover was stimulated by addition of carbachol (100 μM) or histamine (1 mM) for 30 min. Reactions were terminated and samples processed for separation of $[^3H]InsP_{1-3}$ as described in Materials and Methods. In all cases data are expressed relative to the respective responses to carbachol (open bars) or histamine (stippled bars) in the presence of 1.8 mM $[Ca^{2+}]_e$ (and set to 100%). These values were, respectively, for carbachol- and histamine-stimulated accumulations: 78,728 ± 8546 and 46,041 ± 7568 for $[^3H]InsP_1$, 34,249 ± 6365 and 6848 ± 1014 for $[^3H]InsP_2$, and 2257 ± 228 and 1723 ± 134 for $[^3H]InsP_3$. Values are shown as means ± SE mean for three separate experiments performed in triplicate.

Effects of depolarization and other manipulations which changed $[Ca^{2+}]_i$ on basal and carbachol-stimulated phosphoinositide responses

In a number of cells and tissues, including neuronal preparations increasing $[K^+]_e$ [7, 8], or adding Ca^{2+} ionophore [26], could evoke a phosphoinositide response *per se*, or act to potentiate agonist-stimulated phosphoinositide turnover. However, in BTSM slices increasing $[K^+]_e$ from 4.7 to 80 mM did not stimulate a $[^3H]InsP_x$ response (Table 1). Furthermore, in the presence of 80 mM $[K^+]_e$, the $[^3H]InsP_x$ accumulation evoked by carbachol (100 μM) was significantly attenuated (34%, $P < 0.05$, Table 1).

The effects of raising $[K^+]_e$ *per se* on BTSM $Ins(1,4,5)P_3$ mass levels were also investigated. Addition of sufficient KCl to increase the extracellular K^+ concentration from 4.7 to 80 mM caused an immediate 40% decrease in $Ins(1,4,5)P_3$ concentration which remained significantly decreased below time-matched control values over the 5 min duration of the experiment (Fig. 4A). Because of the possible added complexity caused by maintaining osmolarity [27], in these experiments, the KCl additions were not compensated for by equimolar decreases in NaCl. Therefore, in control experiments [NaCl] was increased from 118 to 193 mM. This manipulation caused a small decrease in basal $Ins(1,4,5)P_3$ levels, however, $[K^+]$ addition caused a significantly greater effect at all time-points investigated (Fig. 4A). In addition to suppressing basal $Ins(1,4,5)P_3$ accumulation, the initial (5 sec) increase in $Ins(1,4,5)P_3$ was also significantly attentuated in the presence of 80 mM $[K^+]_e$ (Fig. 4B), but not in the presence of 193 mM $[Na^+]_e$ (data not shown).

In contrast to the effects of elevating $[K^+]_e$,

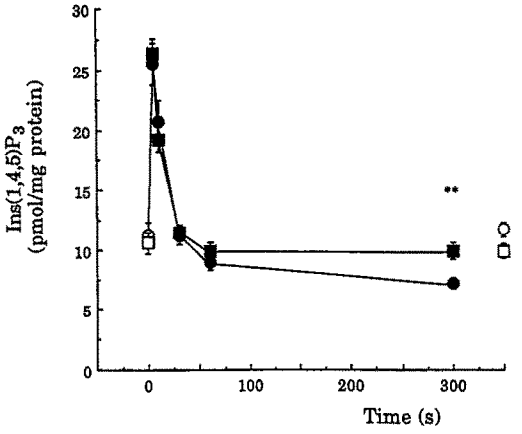

Fig. 3. Effect of decreasing $[Ca^{2+}]_e$ on carbachol-stimulated Ins(1,4,5)P_3 mass accumulation. BTSM slices were pre-incubated in bulk in KH buffer ($[Ca^{2+}] = 1.2$ mM) for 60 min at 37°, before adding aliquots of gravity-packed slices (50 μL) to 230 μL KH buffer. Where indicated, EGTA (pH 7.0) was added to reduce $[Ca^{2+}]_e$ to approximately 6 μM (□, ■) 30 min before addition of 100 μM carbachol (●, ■) or vehicle (○, □). Incubations were terminated at the times indicated after carbachol addition with 300 μL 1 M trichloroacetic acid and processed for determination of Ins(1,4,5)P_3 as described in Materials and Methods. Values are shown as means ± SE mean for three separate experiments performed in triplicate. Statistical analysis (Student's *t*-test) of the differences in carbachol-stimulated changes in Ins(1,4,5)P_3 accumulation in the presence and absence of EGTA revealed only a single significant difference (**$P < 0.01$) 5 min after agonist challenge.

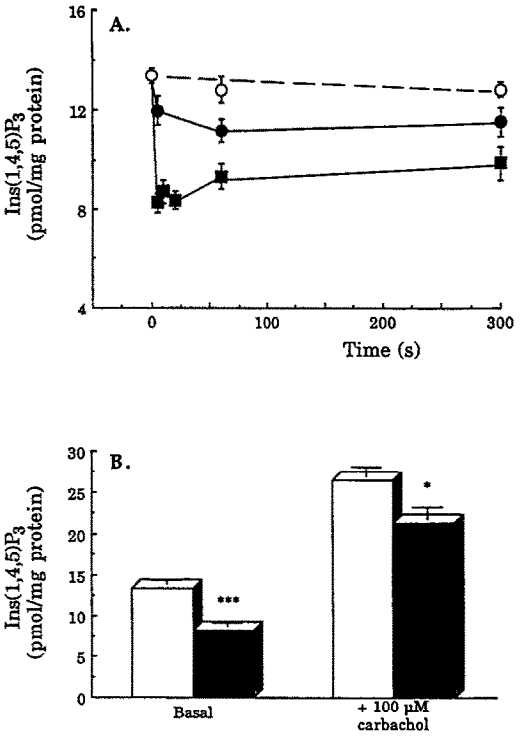

Fig. 4. Effect of K^+-evoked depolarization on basal and carbachol-stimulated Ins(1,4,5)P_3 mass accumulation. BTSM slices were prepared as for Fig. 3. *Panel A*: samples were challenged with 80 mM KCl (■), 80 mM NaCl (●) or vehicle (○) for the times indicated. Panel B: samples were challenged with carbachol (100 μM) or vehicle for 5 sec in the absence (open bars) or presence (closed bars) of 80 mM KCl. In all cases, incubations were terminated by addition of 300 μL 1M trichloroacetic acid and processed for determination of Ins(1,4,5)P_3 as described in Materials and Methods. Values are shown as means ± SE mean for three separate experiments performed in triplicate. Indications of significance have been omitted from panel A for clarity, however, statistical analysis (Student's *t*-test) demonstrated highly significant ($P < 0.001$ at 5, 10 and 20, and $P < 0.01$ at 60 sec) decreases in basal Ins(1,4,5)P_3 accumulations following addition of KCl, while equimolar NaCl addition caused only a significant effect ($P < 0.05$) at 60 sec. Significant differences in basal or carbachol-stimulated changes in Ins(1,4,5)P_3 accumulation in the presence and absence of 80 mM KCl are indicated as; *$P < 0.05$, and ***$P < 0.001$.

ionomycin (5 μM) evoked a 5.1 ± 0.7-fold increase in [^{3}H]InsP_x accumulation, which was completely suppressed by reducing $[Ca^{2+}]_e$ from 1.8 mM to 6 μM (Table 1). Ionomycin (5 μM) addition did not significantly affect basal Ins(1,4,5)P_3 accumulation, nor did it affect the subsequent response to carbachol (Fig. 5).

DISCUSSION

In this study the effects membrane depolarization and other manipulations which affected $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ on agonist-stimulated phosphoinositide metabolism in BTSM were examined. A specific objective was to compare the effects of changes in membrane potential, $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ on the immediate versus sustained hydrolysis of inositol phospholipids induced by the muscarinic cholinoceptor agonist carbachol.

One of the main findings of this study was that while the basal and initial carbachol-stimulated accumulation of Ins(1,4,5)P_3 appeared to be extremely resistant to any decrease in $[Ca^{2+}]_e$, the sustained phase of the agonist-stimulated phosphoinositide response monitored using total [^{3}H]InsP_x accumulation in the presence of Li^+ was significantly attenuated by lowering $[Ca^{2+}]_e$. These data are in agreement with reports in A10 smooth muscle cells [28] and also hepatocytes [29], where reduction of the $[Ca^{2+}]_e$ to 30 nM failed to inhibit the initial (0–60 sec) increase in EGF or angiotensin II-stimulated [^{3}H]Ins(1,4,5)P_3 accumulation, but thereafter resulted in severe attenuation of the ongoing response. It suggested that in certain tissues, ASM included, the presence of a normal plasmalemmal Ca^{2+} gradient was required to sustain maximal agonist-stimulated phosphoinositide hydrolysis.

Further evidence in support of an initial "protected" phase of PIC-mediated PtdIns(4,5)P_2 hydrolysis in BTSM comes from the recent demonstration that the ATP-regulated K^+ channel opener BRL 38227, which induced membrane hyperpolarization in this

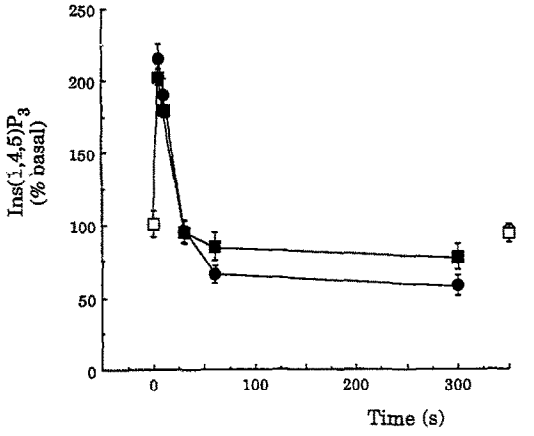

Fig. 5. Effect of the Ca^+ ionophore ionomycin on basal and carbachol-stimulated $Ins(1,4,5)P_3$ mass accumulation. BTSM slices were prepared as for Fig. 3. Where indicated, ionomycin (5 μM final concentration) (□, ■) was added 5 min before challenge with carbachol (●, ■; 100 μM) or vehicle (□, ○) for the indicated periods. Incubations were terminated by addition of 300 μL 1 M trichloroacetic acid and processed for determination of $Ins(1,4,5)P_3$ as described in Materials and Methods. Values are shown, relative to the control $Ins(1,4,5)P_3$ accumulation in the absence of ionomycin or carbachol (11.3 ± 1.6 pmol/mg protein), as means ± SE mean for three separate experiments performed in triplicate.

tissue and thereby inhibited Ca^{2+} influx, failed to influence early (0–300 sec) histamine-stimulated $Ins(1,4,5)P_3$ accumulation despite exerting a major inhibitory effect on the sustained phase of histamine-induced $PtdIns(4,5)P_2$ hydrolysis [15]. Similarly, detailed examination in BTSM of the inhibitory action of β_2-adrenoceptor agonists on histamine-stimulated $[^3H]InsP_x$ accumulation (which is now thought to relate at least in part to their ability to inhibit the function of the Ca^{2+}-dependent K^+ channel [30, 31]) revealed a distinct lag period before this effect became apparent. Such a delay was not seen, however, when these agents were added 10 min post histamine addition [22].

The mechanism underlying this apparent difference in the dependency of the "early" and "late" agonist-induced $InsP_x$ response on $[Ca^{2+}]_e$, particularly in tissues such as BTSM where the muscarinic cholinoceptor-mediated hydrolysis of $PtdIns(4,5)P_2$ does not desensitize and where physiological increases in $[Ca^{2+}]_i$ may not stimulate PIC directly, remains uncertain. Whether such an observation relates to an effect of $[Ca^{2+}]_e$ on substrate supply (i.e. the generation of $PtdIns(4,5)P_2$ from PtdIns and PtdIns4P), or a sequential, time-dependent activation of different PIC isoform(s) at later agonist-stimulation times that are relatively more dependent on a normal cross-membrane Ca^{2+} gradient or Ca^{2+}-influx is currently unknown.

One further important finding of this study was the far greater susceptibility of the histamine-induced $[^3H]InsP_x$ response to a reduction in $[Ca^{2+}]_e$ compared to that seen with carbachol. A similar variable sensitivity of agonist-induced $[^3H]InsP_x$ responses to changes in $[Ca^{2+}]_e$ have been reported in a number of other tissues, including rat cerebral cortex (carbachol v histamine [32]), rat aortic smooth muscle (angiotensin II v ATP [33]) and striatal neurons (quisqualate v carbachol [34]). Furthermore, it has been demonstrated in mouse cerebral cortex that an elevation of $[Ca^{2+}]_e$ from 1.3 to 2.4 mM, or co-incubation of agonist with the Ca^{2+} ionophore A23187 in the presence of 1.3 mM $[Ca^{2+}]_e$, significantly enhanced histamine but not carbachol-stimulated $[^3H]InsP_x$ accumulation [35]. These findings would also concur with contractile data obtained in human bronchial smooth muscle where histamine-induced contractions appear to be considerably more dependent on the influx of $[Ca^{2+}]_e$ than those caused by methacholine [36, 37] and the demonstration that both β_2-adrenoceptor agonists, BRL 38229 and nitrendipine, which inhibit Ca^{2+} influx in ASM, all cause a differentially greater inhibition of histamine stimulated $[^3H]InsP_x$ accumulation compared to their effects on the carbachol response [15, 18, 22]. Again, the possible mechanisms for the differential sensitivity of the histamine and carbachol $[^3H]InsP_x$ responses in BTSM to $[Ca^{2+}]_e$ are unknown, especially since in contrast to a number of the examples cited above, both responses are insensitive to pertussis toxin treatment up to 500 ng/mL for 24 hr [Chilvers and Offer, unpublished observations] and are non-additive implying utilization of a common hormone-sensitive phosphoinositide pool.

The second objective of this study was to ascertain whether membrane depolarization and/or increases in $[Ca^{2+}]_i$ could activate PIC-mediated $PtdIns(4,5)P_2$ directly or induce a switch in the substrate specificity of PIC to allow breakdown of PtdIns and/or PtdIns(4)P. In the first instance it became clear that, in contrast to previous data reported in lung parenchymal tissue [38], application of 80 mM KCl for 30 min failed to stimulate $[^3H]InsP_x$ accumulation or augment the carbachol response, but rather caused a significant inhibition of both the basal and immediate carbachol-stimulated accumulation of $Ins(1,4,5)P_3$. The concentration of KCl used was chosen specifically as it has been shown previously to initiate a prompt contractile response in this tissue associated with both membrane depolarization and an elevation in $[Ca^{2+}]_i$ secondary to Ca^{2+} influx [39]. While it is recognized that in some cells application of high KCl containing solutions may in addition release Ca^{2+} from intracellular stores and lead to mobilization of extracellularly bound Ca^{2+}, this did not appear to occur to any great extent in BTSM, as the high KCl-induced contractile effect and aequorin signal was completely abolished in the presence of nimodipine or in the absence of $[Ca^{2+}]_e$ [39]. It is important also to note that our high KCl incubations were performed specifically without equimolar reduction in the buffer NaCl content since in other smooth muscle types this manoeuvre alone could induce $[^3H]InsP_x$ formation (via activation of an amiloride-sensitive Na^+/Ca^{2+} exchange mechanism [27]), and hence a set of controls employing high (80 mM) NaCl were used to exclude a purely osmotic effect of the high KCl solutions.

The inability of high KCl to induce $[^3H]InsP_x$ accumulation in BTSM not only differed from data obtained in most excitable tissues but was also in marked contrast to previous reports in other smooth muscle types, including the guinea pig ileum where an identical stimulation with KCl provided a near-maximal stimulus for $[^3H]InsP_x$ accumulation [40]. Furthermore, in rat cerebral cortex preparations application of ionomycin or depolarizing concentrations of KCl have been shown to both stimulate $PtdIns(4,5)P_2$ hydrolysis directly and augment agonist-stimulated increases in $Ins(1,4,5)P_3$ [7, 8], and perhaps also to initiate PtdIns and PtdIns(4)P breakdown [26, 41]. This ability of Ca^{2+} to regulate PIC activity in neuronal cells has been proposed as an important mechanism allowing enhancement of the initial agonist-mediated $InsP_x$ signal and redirection of PIC activity away from $PtdIns(4,5)P_2$. Increasing $[Ca^{2+}]_i$ in these tissues also had the effect of increasing the amount of $Ins(1,4,5)P_3$ metabolized via the 3-kinase pathway [7, 8]. It should be noted however that in a number of tissues co-incubation of PIC-linked agonists with high KCl resulted in a purely additive $InsP_x$ signal implying that physiological increases in $[Ca^{2+}]_i$ may have been insufficient to initiate such independent PIC activation. In this context, it was observed that addition of 5 μM ionomycin in the presence of 1.2 mM $[Ca^{2+}]_e$ resulted in a significant increase in $[^3H]InsP_x$ levels, to approximately 27% of those seen with a maximally effective concentration of carbachol. Since ionomycin may have resulted in a far greater increase in Ca^{2+} influx compared to that induced by the high KCl containing buffer, the ionomycin incubations were repeated in the presence of 2 mM EGTA to reduce the $[Ca^{2+}]_e$ to approximately 6 μM and hence prevent or restrict such "supra" physiological increases in $[Ca^{2+}]_i$. Under these conditions (which caused only a small decrease in 30 min carbachol-stimulated $[^3H]InsP_x$ accumulation) the previously observed ionomycin-induced increase in $[^3H]InsP_x$ accumulation was completely abolished. Although our data suggest that physiological increases in $[Ca^{2+}]_i$ do not induce phosphoinositide hydrolysis in ASM, a Ca^{2+} requirement for maximal PIC activation clearly exists in this tissue [42]. However, this requirement would appear to be fully accommodated by the level of Ca^{2+} normally present under resting conditions [42]. It remains to be determined whether the small increases in total $[^3H]InsP_x$ observed with ionomycin in the presence of mM concentrations of $[Ca^{2+}]_e$ in the absence of any immediate stimulatory effect on $Ins(1,4,5)P_3$ mass reflects stimulated hydrolysis of PtdIns(4)P or PtdIns.

While the KCl-induced inhibition of basal and carbachol-stimulated $Ins(1,4,5)P_3$ levels observed in BTSM clearly contrast to the dramatic elevations in $Ins(1,4,5)P_3$ seen in rat cerebral cortex slices following the addition of KCl [7, 8], a somewhat similar observation has been reported in fura-2 loaded human ASM cells where an elevation in $[K^+]_e$ inhibits the $[Ca^{2+}]_i$ transient induced by histamine [43]. While the mechanism underlying this effect is unclear, it is possible that this reflects a Ca^{2+}/calmodulin-mediated activation of the $Ins(1,4,5)P_3$ 3-kinase which would result in enhanced $Ins(1,4,5)P_3$ metabolism. Of further interest are the recent data of Biden *et al.* [6] who, working with rat pancreatic acinar tissue, demonstrated a direct correlation between the calculated E_m and the degree of carbachol-induced PtdIns [although not $PtdIns(4,5)P_2$] hydrolysis. While unlike ASM in that high $[K^+]_e$ clearly induced an InsP response in these cells, this result points to the possibility that alterations in membrane potential alone may play some role in modulating PIC activity and that such a direct effect of E_m on basal and agonist-mediated $Ins(1,4,5)P_3$ generation in ASM cannot be excluded.

With regard to depolarization-induced $[^3H]InsP_x$ accumulations reported in other smooth muscle preparations (in particular ileal smooth muscle), Sasaguri and Watson [27] have suggested that this response was unlikely to be due to a direct effect of membrane depolarization, since nifedipine was able to inhibit fully the formation of $[^3H]InsP_x$ induced by high KCl, despite having little effect on the observed changes in membrane potential. More importantly, these authors have reported [44] that the majority of the atropine-insensitive high KCl-induced $[^3H]InsP_x$ response observed in guinea-pig ileal smooth muscle is probably neuronal in origin. These data would therefore concur with our own data in ASM and with those in vas deferens and A10 smooth muscle cells where high KCl solutions also fail to initiate $[^3H]InsP$ accumulation [4, 28], and imply that depolarization-induced increases in $[Ca^{2+}]_i$ do not initiate independent phosphoinositide hydrolysis in smooth muscle, as first suggested [9, 40]. Likewise, the Ca^{2+} ionophore-stimulated increases in $PtdIns(4,5)P_2$ hydrolysis, first observed in rabbit iris smooth muscle, have now been reported to be blocked by the α_1-adrenoceptor antagonist prazosin, again indicating an indirect effect mediated via catacholamine release from nerve endings contained within the preparation [45].

Taken together, these data provide evidence suggesting that $[Ca^{2+}]_e$ plays an important role in maintaining agonist-stimulated inositol phospholipid hydrolysis in BTSM, despite having little influence on the initial rate of $PtdIns(4,5)P_2$ breakdown and that membrane depolarization or a physiological increase in $[Ca^{2+}]_i$ is unable to activate PIC directly. The apparent differential sensitivity of agonist-induced $[^3H]InsP_x$ responses to $[Ca^{2+}]_e$ may be an important factor underlying agonist-dependent inhibition of phosphoinositide hydrolysis by agents such as β_2-adrenoceptor agonists and K_{ATP} channel openers.

Acknowledgements—The secretarial assistance of Mrs J MaMahon is gratefully acknowledged. This work was supported by the MRC (U.K.), Chest, Heart and Stroke Association and The Wellcome Trust.

REFERENCES

1. Eberhard DA and Holz RW, Intracellular Ca^{2+} activates phospholipase C. *Trends Neurosci* **11**: 517–520, 1988.
2. Fisher SK, Domask LM and Roland RM, Muscainic receptor regulation of cytoplasmic Ca^{2+} concentrations in human SK-N-SH neuroblastoma cells: Ca^{2+}

requirements for phospholipase C activation. *Mol Pharmacol* **35**: 195–204, 1989.
3. Wojcikiewicz RJH, Tobin AB and Nahorski SR, Desensitization of cell signalling mediated by phospholipase C. *Trends Pharmacol Sci* **14**: 279–285, 1993.
4. Claro E, Wallace MA, Lee H-M and Fain JN, Carbachol in the presence of guanosine 5′-O-(3-thiotriphosphate) stimulates the breakdown of exogenous phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate and phosphatidylinositol by rat brain membranes. *J Biol Chem* **264**: 18288–18295, 1989.
5. Hiramatsu Y, Horn VJ, Baum BJ and Ambudkar IS, Characterization of polyphosphoinositide-specific phospholipase C in rat parotid gland membranes. *Arch Biochem Biophys* **297**: 368–376, 1992.
6. Biden TJ, Davison AGM and Prugue ML, Regulation by membrane potential of phosphatidylinositol hydrolysis in pancreatic islets. *J Biol Chem* **268**: 11065–11072, 1993.
7. Baird JG and Nahorski SR, Dual effects of K^+ depolarization on inositol polyphosphate production in rat cerebral cortex. *J Neurochem* **53**: 681–685, 1989.
8. Challiss RAJ and Nahorski SR, Depolarization and agonist-stimulated changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulation in rat cerebral cortex. *J Neurochem* **57**: 1042–1051, 1991.
9. Jafferji SS and Michell RH, Muscarinic cholinergic stimulation of phosphatidylinositol turnover in the longitudinal smooth muscle of guinea pig ileum. *Biochem J* **154**: 653–657, 1976.
10. Egawa K, Sacktor B and Takenawa T, Ca^{2+}-dependent and Ca^{2+}-independent degradation of phosphatidylinositol in rabbit vas deferens. *Biochem J* **194**: 129–136, 1981.
11. Schramm CM and Grunstein MM, Assessment of signal transduction mechanisms regulating airway smooth muscle contractility. *Am J Physiol* **262**: L119–L139, 1992.
12. Chilvers ER, Lynch BJ and Challiss RAJ, Phosphoinositide metabolism in airway smooth muscle. *Pharmacol Ther* in press.
13. Grandordy BM, Cuss FM, Sampson AS, Palmer JB and Barnes PJ, Phosphatidyl-inositol response to cholinergic agonists in airway smooth muscle: relationship to contraction and muscarinic receptor occupancy. *J Pharmacol Exp Ther* **238**: 273–279, 1986.
14. Takuwa Y, Takuwa N and Rasmussen H, Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositides in bovine tracheal smooth muscle: measurements of the mass of polyphosphoinositides, 1,2-diacylglycerol, and phosphatidic acid. *J Biol Chem* **261**: 4670–4675, 1986.
15. Challiss RAJ, Patel N and Arch JRS, Comparative effects of BRL 38227, nitrendipine and isoprenaline on carbachol- and histamine-stimulated phosphoinositide metabolism in airway smooth muscle. *Br J Pharmacol* **105**: 997–1003, 1992.
16. Challiss RAJ, Adams D, Mistry R and Boyle JP, Second messenger and ionic modulation of agonist-stimulated phosphoinositide metabolism in airway smooth muscle. *Biochem Soc Trans* **21**: 1138–1145, 1993.
17. Chilvers ER, Barnes PJ and Nahorski SR, Characterisation of agonist-stimulated incorporation of *myo*-[^{3}H]inositol into inositol phospholipids and [^{3}H]inositol phosphate formation in tracheal smooth muscle. *Biochem J* **262**: 739–746, 1989.
18. Offer GJ, Chilvers ER and Nahorski SR, Beta-adrenoceptor induced inhibition of muscarinic receptor-stimulated phosphoinositide metabolism is agonist specific in bovine tracheal smooth muscle. *Eur J Pharmacol (Mol Pharmcaol)* **207**: 243–248, 1991.
19. Baird JG, Chilvers ER, Kennedy ED and Nahorski SR, Changes in extracellular calcium within the physiological range influence inositol phosphate responses in cerebral cortex and tracheal smooth muscle. *Naunyn-Schmiedebergs Arch Pharmacol* **339**: 247–251, 1989.
20. Wojcikiewicz RJH, Safrany ST, Challiss RAJ, Strupish J and Nahorski SR, Coupling of muscarinic receptors to the mobilization of intracellular Ca^{2+} stores in permeabilized SH-SY5Y human neuroblastoma cells. *Biochem J* **272**: 269–272, 1990.
21. Challiss RAJ, Batty IH and Nahorski SR, Mass measurements of inositol(1,4,5)-trisphosphate in rat cerebral cortex slices using a radioreceptor assay. *Biochem Biophys Res Commun* **157**: 684–691, 1988.
22. Hall IP and Hill SJ, β-Adrenoceptor stimulation inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br J Pharmacol* **95**: 1204–1212, 1988.
23. Chilvers ER, Challiss RAJ, Barnes PJ and Nahorski SR, Mass changes of inositol 1,4,5-trisphosphate in trachealis muscle following agonist stimulation. *Eur J Pharmacol* **164**: 587–590, 1989.
24. Chilvers ER, Batty IH, Barnes PJ and Nahorski SR, Formation of inositol polyphosphates in airway smooth muscle after muscarinic receptor stimulation. *J Pharmacol Exp Ther* **252**: 786–791, 1990.
25. Chilvers ER, Batty IH, Challiss RAJ, Barnes PJ and Nahorski SR, 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. *Biochem J* **275**: 373–379, 1991.
26. Baird JG and Nahorski SR, Increased intracellular calcium stimulates [^{3}H]inositol polyphosphate accumulation in rat cerebral cortical slices. *J Neurochem* **54**: 555–561, 1990.
27. Sasaguri T and Watson SP, Lowering of the extracellular Na^+ concentration enhances high-K^+-induced formation of inositol phosphates in the guinea-pig ileum. *Biochem J* **252**: 883–888, 1988.
28. Xuan YT, Whorton AR and Watkins WD, Inhibition by nicardipine of endothelin-mediated inositol phosphate formation and Ca^{2+} mobilization in smooth muscle cells. *Biochem Biophys Res Commun* **160**: 758–764, 1989.
29. Johnson RM and Garrison JC, Epidermal growth factor and angiotensin II stimulate formation of inositol 1,4,5- and 1,3,4-trisphosphate in hepatocytes. *J Biol Chem* **262**: 17285–17293, 1987.
30. Kume H, Takai A, Tokuno H and Tomita T, Regulation of Ca^{2+}-dependent and K^+-channel activity in tracheal myocytes by phosphorylation. *Nature* **341**: 152–154, 1989.
31. Kume H, Graziano MP and Kotlikoff MI, Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc Natl Acad Sci USA* **89**: 11051–11055, 1992.
32. Kendall DA and Nahorski SR, Inositol phospholipid hydrolysis in rat cerebral cortical slices: II. Calcium requirement. *J Neurochem* **42**: 1388–1394, 1984.
33. Nabika T, Velletri PA, Lovenberg W and Beaven MA, Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and [Arg]-vasopressin in vascular smooth muscle cells. *J Biol Chem* **260**: 4661–4670, 1985.
34. Ambrosini A and Meldolesi J, Muscarinic and quisqualate receptor-induced phosphoinositide hydrolysis in primary cultures of striatal and hip-

pocampal neurons. Evidence for differential mechanisms of activation. *J Neurochem* **53**: 825–833, 1989.
35. Alexander SPH, Hill SJ and Kendall DA, Differential effects of elevated calcium ion concentrations on inositol phospholipid responses in mouse and rat cerebral cortical slices. *Biochem Pharmacol* **40**: 1793–1799, 1990.
36. Raeburn D, Roberts JA, Rodger IW and Thomson NC, Agonist-induced contractile responses of human bronchial muscle *in vitro*: effects of Ca^{2+} removal, La^{2+} and PY108068. *Eur J Pharmacol* **121**: 251–255, 1986.
37. Giembycz MA and Rodger IW, Electrophysiological and other aspects of excitation-contraction coupling and uncoupling in mammalian airway smooth muscle. *Life Sci* **41**: 111–132, 1987.
38. Kardasz AM, Langlands J, Rodger IW and Watson J, Inositol lipid turnover in isolated guinea pig trachealis and lung parenchyma. *Biochem Soc Trans* **15**: 474, 1987.
39. Takuwa Y, Takuwa N and Rasmussen H, Measurement of cytosolic free Ca^{2+} concentration in bovine tracheal smooth muscle using aequorin. *Am J Physiol* **253**: C817–C827, 1987.
40. Best L and Bolton TB, Depolarisation of guinea-pig visceral smooth muscle causes hydrolysis and contractility in guinea-pig visceral longitudinal smooth muscle. *Naunyn-Schmiedebergs Arch Pharmacol* **333**: 78–82, 1986.
41. Brammer M and Weaver K, Kinetic analysis of A23187-mediated polyphosphoinositide breakdown in rat cortical synaptosomes suggests that inositol bisphosphate does not arise primarily by degradation of inositol trisphosphate. *J Neurochem* **53**: 399–407, 1989.
42. Yang CM, Chou S-P, Wang Y-Y, Hsieh J-T and Ong R, Muscarinic regulation of cytosolic free calcium in canine tracheal smooth muscle cells: Ca^{2+} requirement or phospholipase C activation. *Br J Pharmacol* **110**: 1239–1247, 1993.
43. Murray RK and Kotlikoff MI, Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol* **435**: 123–144, 1991.
44. Watson SP, Lai J and Sasaguri T, K^+-stimulation of the phosphoinositide pathway in guinea-pig ileum longitudinal smooth muscle is predominantly neuronal in origin and mediated by the entry of extracellular Ca^{2+}. *Br J Pharmacol* **99**: 212–216, 1990.
45. Akhtar RA and Abdel-Latif AA, Carbachol causes rapid phosphodiesteric cleavage of phosphatidylinositol 4,5-bisphosphate and accumulation of inositol phosphates in rabbit iris smooth muscle: prazosin inhibits noradrenaline- and ionophore A23187-stimulated accumulation of inositol phosphates. *Biochem J* **224**: 291–300, 1984.