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BRADYKININ STIMULATES PHOSPHOLIPASE D IN PRIMARY CULTURES OF GUINEA-PIG TRACHEAL SMOOTH MUSCLE

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Abstract—Conditions were established for the primary culture of guinea-pig tracheal smooth muscle cells, the identity of which was confirmed by the presence of smooth muscle α-actin by western blotting. Cells were preincubated with [^{3}H]palmitate which was incorporated, almost exclusively, into phosphatidylcholine. When these cells were stimulated by either bradykinin or phorbol 12-myristate 13-acetate (PMA), in the presence of butan-1-ol, the non-metabolizable product [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) accumulated by virtue of the phosphatidyltransferase activity of phospholipase D. The activation of phospholipase D by bradykinin was inhibited by 86 ± 11% (N = 3 experiments) in the presence of the protein kinase C inhibitor, staurosporine (1 μM) and by 88 ± 11% (N = 3 experiments) in cells that had been chronically treated with PMA to down-regulate their protein kinase C. PMA-stimulated phospholipase D was similarly affected (92 ± 2% inhibited by staurosporine, 87 ± 6% inhibited by protein kinase C down-regulation). Removal of extracellular Ca^{2+} markedly reduced the bradykinin-stimulated phospholipase D response (by 73 ± 10%, N = 3 experiments) but had only a limited effect upon PMA-stimulated phospholipase D activity (by 23 ± 6%, N = 3 experiments). $[AlF_4]^-$-stimulation of the cells also resulted in the activation of phospholipase D, indicating the involvement of a G-protein. However, this was not G_i since pertussis-toxin pretreatment of the cells failed to abolish either bradykinin-stimulated inositol (1,4,5)trisphosphate formation or [^{3}H]PtdBut accumulation. Western blotting revealed the presence of G_q/G_{11} which couples to the inositol lipid-directed phospholipase C. Indomethacin (10 μM) was without effect upon bradykinin-stimulated phospholipase D activity, suggesting that the bradykinin effects were not mediated indirectly by cyclo-oxygenase products. The role of phospholipase D activation in tracheal smooth muscle may be to, indirectly, produce diacylglycerol for the activation of protein kinase C which has been implicated in sustained contraction. However, the immediate product of phospholipase D, phosphatidate, has been proposed to have a number of second messenger roles and may itself, by an undefined mechanism, be involved in the sustained contraction of airway smooth muscle.

Bradykinin is a nonapeptide which is produced in the tracheo-bronchial tree and in plasma from kininogen by the action of kallikrein and kininogenase [1]. It is generated during the inflammatory response and may be an important mediator in diseases such as asthma. For example, asthmatic patients exhibit elevated levels of bradykinin in plasma and in nasal and bronchoalveolar lavage after antigen challenge and undergo bronchoconstriction in response to bradykinin [2]. However, in guinea-pig airways *in vivo*, bradykinin-induced bronchoconstriction can be markedly inhibited by indomethacin, suggesting an indirect effect of bradykinin (mediated by prostaglandins) and atropine, suggesting the involvement of a cholinergic reflex [3]. Nevertheless, bradykinin receptors are present in airway smooth muscle [4–6].

A number of molecular mechanisms exist by which bradykinin exerts its effects [7]. These include the activation of phospholipase C-catalysed phosphatidylinositol(4,5)bisphosphate hydrolysis, mediated by a G-protein, with subsequent Ca^{2+} mobilization and protein kinase C activation, for example in human A431 carcinoma cells [8], neural cells [9], Madin–Darby canine kidney cells [10] and human fibroblasts [11]. In addition, bradykinin activates phospholipase A_2-catalysed production of arachidonate and, thereby, the production of prostaglandins, for example in Madin–Darby canine kidney cells [10] and Swiss 3T3 fibroblasts [12]. More recently, bradykinin-stimulated phosphatidylcholine hydrolysis by phospholipases D and C has been demonstrated in endothelial cells [13], human fibroblasts [14] and PC12 cells [15, 16]. Bradykinin also induces the opening of receptor-operated Ca^{2+} channels in human airway smooth muscle [17].

Agonist-stimulation of phospholipase D is common to a large number of agonists which also activate inositol lipid-directed phospholipase C [18, 19]. The mechanism of activation of phospholipase D remains unclear and has been reported to be both downstream of inositol lipid hydrolysis and subsequent protein kinase C activation and, in some tissues, an independent receptor-mediated event [18, 19].

To date, there are no reports of phospholipase D activation in airway smooth muscle. Therefore, the aims of this study were to identify and characterize bradykinin-stimulated phospholipase D-catalysed phosphatidylcholine hydrolysis with regard to protein kinase C, Ca^{2+} and G-protein involvement using

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primary cultures of guinea-pig tracheal smooth muscle cells.

MATERIALS AND METHODS

[^{3}H]Palmitate (sp. act. 40–60 Ci/mmol) and [^{3}H]-inositol (1,4,5)trisphosphate (Ins(1,4,5)P_3†; sp. act. 20–60 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). Tissue culture reagents and plasticware were obtained from Gibco BRL (Paisley, U.K.) and ICN Flow (High Wycombe, U.K.). Bradykinin was purchased from Calbiochem (Nottingham, U.K.). TLC plates (LK5D) were obtained from Whatman (Maidstone, U.K.). Collagenase (Type II), elastase (Type IV), soya bean trypsin inhibitor and mouse monoclonal antibody raised to smooth muscle α-actin was purchased from the Sigma Chemical Co. (Poole, U.K.) whereas rabbit polyclonal antibody raised to the C-terminal decapeptide of G_q/G_{11} (QLNLKEYNLV) [20] was a generous gift from Dr G. Milligan (University of Glasgow). Horseradish peroxidase-linked anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG were obtained from the Scottish Antibody Production Unit (Carluke, U.K.). All other reagents were of the highest purity commercially available.

Cell culture. Preparation of the primary smooth muscle cells was based on the method of Panettieri [6]. Working under sterile conditions in an air flow-controlled cabinet, the trachea was removed from a male guinea-pig (Dunkin–Hartley, 450–500 g) and dissected free of cartilage and the epithelium to leave the tracheal smooth muscle cell strip. This was titrated in 4 mL of Dulbecco's Modified Eagle's medium (DME) containing collagenase (Type II, 1 mg/mL), elastase (Type IV, 0.2 mg/mL) and soya bean trypsin inhibitor (50 μg/mL) to detach any remaining epithelial cells. The tissue was transferred into a further 1 mL of the medium and incubated at 37° for 1.5–2 hr until fully digested. The cell suspension was diluted (1:5) with DME containing 10% (v/v) foetal calf serum (FCS) and 10% (v/v) donor horse serum (DHS) and transferred to an 80 cm^2 tissue culture flask which was incubated at 37° in air/CO_2 (95:5, v/v). The medium was replaced with DME containing 10% (v/v) FCS and 10% (v/v) DHS after 48 hr. The cells were routinely passaged twice, using trypsin, prior to experiment. Cells for experiments were grown to confluence on 24-well plates and used for experiments at 13–15 days after the initial preparation.

SDS–PAGE and immunoblotting. A homogenate (in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) of the tracheal smooth muscle cells was mixed with an equal volume of Laemmli buffer before boiling for 3 min prior to separation of the proteins by SDS–PAGE in Laemmli buffer on a 10% acrylamide gel [21]. The resolved proteins were subsequently transferred to a nitrocellulose sheet which was blocked with 3% gelatin (w/v) in 20 mM Tris–HCl (pH 7.4)/0.5 M NaCl (Tris-buffered saline, TBS) at 37° for 1 hr. The sheet was washed repeatedly in distilled water and then incubated with the primary antibody (anti-smooth muscle α-actin antibody at 1:400 dilution or anti-G_q/G_{11} antibody at 1:200 dilution) in 1% gelatin (w/v, in TBS) at 30° for 12 hr. The nitrocellulose sheet was sequentially washed in TBS/0.05% Tween-20 and TBS before incubation with horseradish peroxidase-linked anti-mouse IgG (1:200 dilution) or anti-rabbit IgG (1:200 dilution), as appropriate, in 1% gelatin (w/v, in TBS) for 1.5 hr at 23°. After sequential washing as before, the immunological reactive peptide was detected in the presence of 10 mM Tris–HCl (pH 7.4), *O*-dianisidine (10 mg/mL) and hydrogen peroxide (0.75%, v/v).

Incubation of cells with [^{3}H]palmitate; identification of [^{3}H]palmitate-labelled lipids. Primary tracheal smooth muscle cells, grown to approx. 90% confluency, were preincubated with [^{3}H]palmitate (2 μCi/mL) in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24–48 hr.

Organic extracts of [^{3}H]palmitate-labelled cells were prepared by removal of the labelling medium and the addition of 200 μL ice-cold methanol. Samples were kept on ice for 10 min prior to scraping the precipitated cells and transferring the mixture to 2 mL glass centrifuge tubes. The wells were rinsed with a further 200 μL ice-cold methanol which was combined with the first extract prior to the addition of 200 μL $CHCl_3$. Lipids were allowed to extract at room temperature for 30 min prior to their evaporation to dryness in peparation for subsequent TLC analysis.

The distribution of radioactivity between the major phospholipids was achieved by redissolving the dried lipid extracts in $CHCl_3$:CH_3OH (19:1, v/v) and TLC on EDTA-impregnated TLC plates using a solvent system of $CHCl_3$:CH_3OH:acetic acid:H_2O (75:45:3:1, by vol.) [22]. The lanes were divided into 0.5 cm strips, each of which was excised and the associated radioactivity determined. Samples of the major phospholipids were run in parallel and visualized by I_2 staining in order to identify the radioactive phospholipids detected.

Phospholipase D assay. Briefly, this assay employs [^{3}H]palmitate-labelled cells which, when stimulated in the presence of butan-1-ol, produce [^{3}H]-phosphatidylbutan-1-ol ([^{3}H]PtdBut) by virtue of the transphosphatidylation activity of the phospholipase D enzyme [23]. The formation of the non-metabolizable [^{3}H]PtdBut, which can be resolved from other lipids by TLC, is a direct indicator of phospholipase D activity [24].

Primary tracheal smooth muscle cells were incubated with [^{3}H]palmitate as described above. The tissue culture medium was removed and the cells rinsed once with 500 μL Krebs–Ringer–bicarbonate buffer (KRB; 118 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1 mM K_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM glucose, pH 7.4) containing bovine serum albumin (Fraction V) 1% (w/v) prior to preincubation in air/CO_2 (95:5, v/v) at 37° for

† Abbreviations: DME, Dulbecco's Modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FCS, foetal calf serum; DHS, donor horse serum; TBS, Tris-buffered saline; IgG, immunoglobulin G; KRB, Krebs–Ringer–bicarbonate buffer; Ins(1,4,5)P_3, inositol (1,4,5)trisphosphate.

30 min in 250 μL KRB. This medium was removed and replaced with 250 μL KRB containing 0.3% (v/v) butan-1-ol for 5 min. Additions of buffer, bradykinin, phorbol 12-myristate 13-acetate (PMA) or $[AlF_4]^-$ (a mixture of $AlCl_3$/NaF, 1:2000) were then made as required. In experiments where extracellular Ca^{2+} was depleted, the medium was supplemented with 2.5 mM EGTA throughout the preincubation and experimental periods.

Incubations were terminated by removal of the medium and organic extracts prepared as described above. Dried lipid extracts were redissolved in 100 μL $CHCl_3$:CH_3OH (19:1, v/v) and the lipid components resolved by TLC on LK5D plates using a solvent of the upper phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:H_2O (110:50:20:100, by vol.) [24]. [^{3}H]PtdBut, which routinely migrated with an $R_f = 0.35$, was quantified by excising the appropriate area from each lane and counting the associated radioactivity.

For the experiments involving cyclooxygenase inhibition, cells were pretreated with indomethacin (10 μM) for 10 min prior to stimulation. For experiments involving the protein kinase C inhibition, cells were preincubated with staurosporine (1 μM) for 10 min prior to stimulation. Protein kinase C down-regulation was achieved by including PMA (100 nM) in the labelling medium for at least 24 hr. In specified experiments, the labelling medium was supplemented with pertussis toxin (100 ng/mL) for 18 hr prior to experiment which completely ADP-ribosylated G_i (data not shown) [25].

Measurement of Ins(1,4,5)P_3. Measurement of Ins(1,4,5)P_3 mass was performed as described previously [26]. The tissue culture medium was removed and the cells rinsed once with 500 μL KRB prior to preincubation for 30 min in 250 μL KRB. This medium was replaced with 100 μL of KRB in the presence and absence of bradykinin (1 μM) for 10 sec. Incubations were terminated by the addition of 25 μL of ice-cold perchloric acid (10%, w/v) and the samples placed on ice. The acid extracts were harvested and neutralized by the addition of approx. 25 μL 1.5 M KOH containing 60 mM Hepes in the presence of a trace quantity of Universal Indicator. After centrifugation, the Ins(1,4,5)P_3 content of each supernatant was determined using an Ins(1,4,5)P_3-specific binding assay [26]. Briefly, the Ins(1,4,5)P_3 content of the extracts is measured by radioligand binding assay using [^{3}H]Ins(1,4,5)P_3 and a crude adrenocortical microsomal fraction as a binding protein preparation. A standard curve of 25 fmol–25 pmol was conducted in parallel.

Fig. 1. Immunoblotting of smooth muscle α-actin from primary cultured tracheal smooth muscle cells. Proteins were resolved on a 10% polyacrylamide gel by SDS–PAGE, transferred to nitrocellulose and reacted with a mouse monoclonal antibody raised to smooth muscle α-actin. A second antibody, raised to mouse IgG and coupled to horseradish peroxidase was used to visualize the α-actin immunoprecipitate. Lane 1, primary tracheal smooth muscle cells; lane 2, guinea-pig liver. The position of molecular mass markers is illustrated. Results are from a single, representative experiment.

RESULTS

Confirmation of smooth muscle cell identity

The identity of the cell preparation was confirmed to be smooth muscle, as previously successfully isolated by Farmer *et al.* [4], by the presence of smooth muscle α-actin, detected using a specific monoclonal antibody (Fig. 1). The antibody, raised to mouse smooth muscle α-actin, immunoblotted a 43 kDa protein in the primary cultured tracheal smooth muscle cells (Fig. 1, lane 1) which was not detected in guinea-pig liver (Fig. 1, lane 2). Epithelial cells which were occasionally isolated upon digestion of the tracheal smooth muscle strip did not survive the passage procedure and were absent from the cultured cell preparation at the time of the experiments (passage 3).

[^{3}H]*Palmitate-labelling of cells*

Agonists which activate inositol lipid hydrolysis have been demonstrated to also induce phosphatidylcholine hydrolysis in a variety of systems (reviewed in Refs 18 and 19). The inositol lipids and phosphatidylcholine exhibit a different distribution of fatty acids [27]: inositol lipids are enriched in arachidonate whereas phosphatidylcholine is enriched in palmitate. Thus, the fatty acids provide a means of selectively labelling a particular phospholipid.

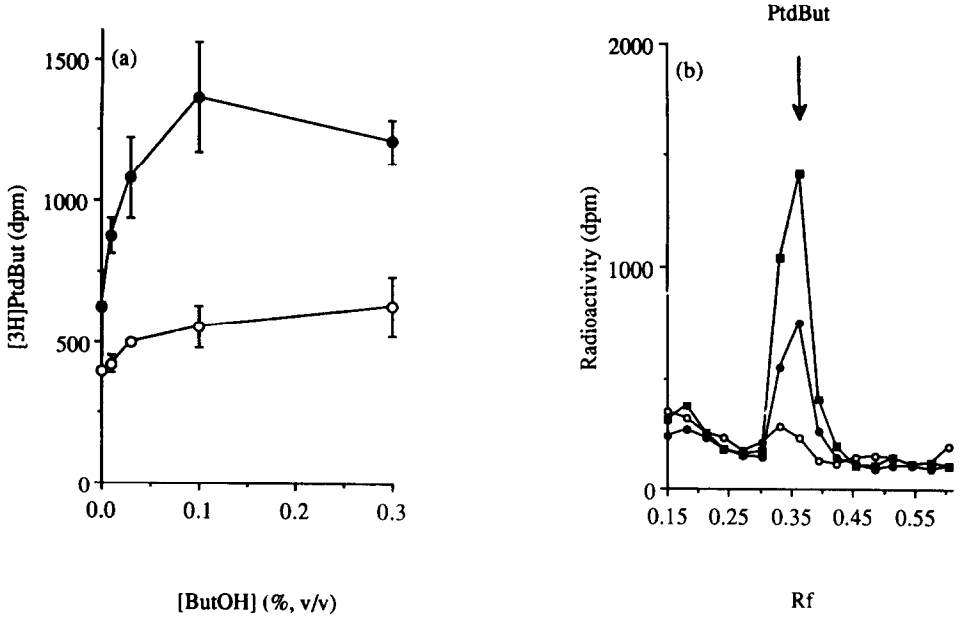

Fig. 2. Formation of [^{3}H]PtdBut in agonist-stimulated cells; effect of butan-1-ol concentration and TLC profile. (a) [^{3}H]Palmitate-labelled tracheal smooth muscle cells were preincubated for 5 min with varying concentrations (0–0.3%, v/v) of butan-1-ol prior to the addition of buffer (○) or bradykinin (●, 1 μM). An organic extract was prepared and [^{3}H]PtdBut was resolved by TLC and its radioactivity determined as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment. (b) Organic extracts from [^{3}H]palmitate-labelled cells that had been treated with buffer (○), bradykinin (●, 1 μM) or PMA (■, 100 nM) were subjected to TLC to resolve [^{3}H]PtdBut as described in Materials and Methods. Sections (0.5 cm) of each lane of the TLC plate were excised and their radioactivity determined. [^{3}H]PtdBut migrated with an R_f of approx. 0.35. Results are from individual samples from a single, representative experiment.

Incubation of the primary cultured tracheal smooth muscle cells with [^{3}H]palmitate (2 μCi/mL DME/1% FCS/1% DHS) resulted in the incorporation of radioactivity into the organic-soluble lipid fraction of cell extracts which was maximal and remained unchanged between 24 and 48 hr (data not shown). Under these conditions, approximately 82% of the radioactivity associated with the major phospholipids was in phosphatidylcholine and this did not change significantly during the period of the experiment (data not shown). Thus, changes in the levels of [^{3}H]palmitate-labelled compounds produced during the experiment directly reflect changes in their chemical amount. In addition, approximately 12% of the radioactivity was associated with phosphatidate and only 6% with phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine combined.

Bradykinin-stimulated [^{3}H]PtdBut formation: effect of butan-1-ol concentration

Initial experiments were conducted to maximize the amount of [^{3}H]PtdBut that could be detected upon bradykinin stimulation. This was achieved by preincubation of the [^{3}H]palmitate-labelled cells for 5 min in the presence of a range of concentrations (0–1%, v/v) of butan-1-ol prior to stimulation with bradykinin (1 μM) for 10 min (Fig. 2a). Bradykinin-stimulated accumulation of [^{3}H]PtdBut was maximal at 0.1–0.3% (v/v) butan-1-ol whereas higher concentrations resulted in elevated [^{3}H]PtdBut in the absence of any stimulation, suggesting that the cells may have lost their integrity at greater than 0.3% (v/v) butan-1-ol (data not shown). Butan-1-ol (0.3%, v/v) was without effect upon basal and bradykinin-stimulated Ins(1,4,5)P_3 levels (data not shown). A typical TLC profile of the [^{3}H]palmitate-labelled lipids from control, bradykinin- and PMA-stimulated tracheal smooth muscle cells (Fig. 2b) illustrates the agonist-stimulated production of [^{3}H]PtdBut, which migrated with an R_f of approx. 0.35. Bradykinin also elicited the production of [^{3}H]choline in [^{3}H]choline-labelled cells, thus confirming the hydrolysis of phosphatidylcholine (data not shown).

Bradykinin- and PMA-stimulated phospholipase D activity

Bradykinin- or PMA-stimulation of the [^{3}H]-palmitate-labelled cells in the presence of butan-1-ol (0.3%, v/v) resulted in the time-dependent formation of [^{3}H]PtdBut (Fig. 3). The formation of [^{3}H]PtdBut was absolutely dependent upon the presence of butan-1-ol in the incubation medium (see Fig. 2a). The response to bradykinin (1 μM) was significant at 10 sec, the earliest time point studied (data not shown) whereas the response to PMA was not significant until 60 sec. The

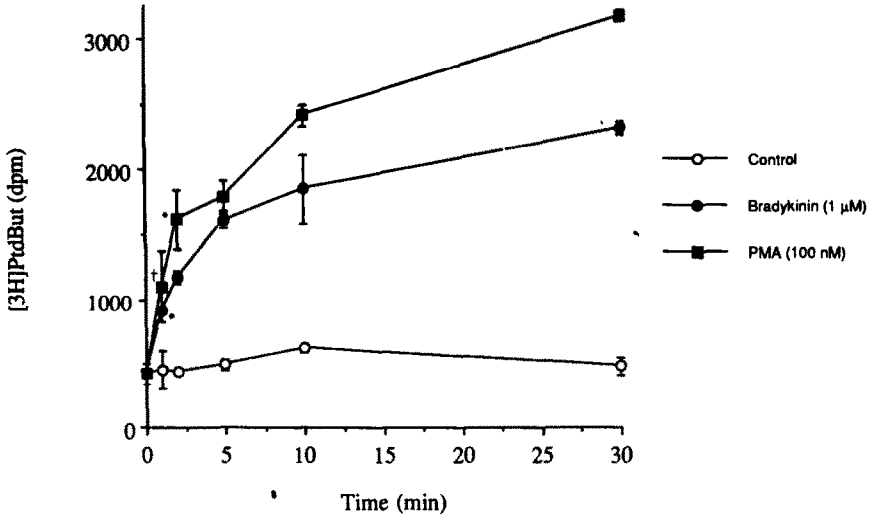

Fig. 3. Time course of bradykinin- and PMA-stimulated [^{3}H]PtdBut formation. [^{3}H]Palmitate-labelled tracheal smooth muscle cells were preincubated for 5 min with 0.3% (v/v) butan-1-ol prior to the addition of buffer (○), bradykinin (1 μM, ●) or PMA (100 nM, ■) for the times indicated. Organic extracts were prepared and [^{3}H]PtdBut resolved and counted as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment. †$P < 0.01$, *$P < 0.005$ compared to control, Student's *t*-test, unpaired.

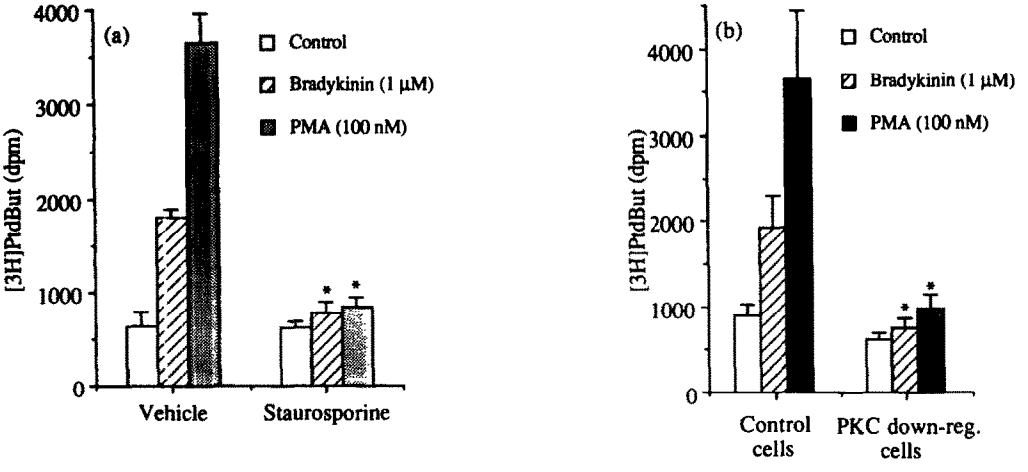

Fig. 4. Role of protein kinase C in the activation of phospholipase D. (a) [^{3}H]Palmitate-labelled tracheal smooth muscle cells were pretreated with vehicle or staurosporine (1 μM) for 10 min and with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer (□), bradykinin (1 μM, ▨) or PMA (100 nM, ▩) for 10 min. Organic extracts were prepared and [^{3}H]PtdBut resolved and counted as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment. *$P < 0.0005$ vs response in control cells, Student's *t*-test, unpaired. (b) Tracheal smooth muscle cells, labelled with [^{3}H]palmitate for 24 hr in the presence of vehicle or PMA (100 nM) were incubated with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer (□), bradykinin (1 μM, ▨) or PMA (100 nM, □) for 10 min. Radioactivity associated with [^{3}H]PtdBut was determined as in (a). Results are means ± SD (N = 3) from a single, representative experiment. *$P < 0.0005$ vs response in control cells, Student's *t*-test, unpaired.

accumulation of [^{3}H]PtdBut to both agonists appeared to be biphasic with a rapid initial accumulation of [^{3}H]PtdBut to between 2 and 5 min followed by a reduced rate of accumulation throughout the 30 min time course. This suggests that agonist-stimulated phospholipase D activation may undergo a partial desensitization in these cells. Bradykinin induced increases in [^{3}H]PtdBut of

approximately 2–3-fold at 10 min and by approximately 3–4-fold at 30 min whereas PMA (100 nM), a protein kinase C activating phorbol ester [28] induced a larger response (3–4-fold at 10 min and 5–6-fold at 30 min).

The B_1 agonist, *des*-Arg^9-bradykinin failed to elicit either a phospholipase C or phospholipase D response (data not shown). In addition, bradykinin-stimulated Ins(1,4,5)P_3 formation and [^{3}H]PtdBut accumulation were differentially inhibited by the B_2 antagonist *des*-Arg-[Hyp^3, D-Phe^7]-bradykinin (data not shown) suggesting the possible involvement of both B_2 and B_3 receptors in mediating the observed responses [4].

Inhibition of protein kinase C: effect on phospholipase D activation

The mechanism of activation of phospholipase D remains unclear but both protein kinase C and Ca^{2+} have been implicated [18, 19]. To investigate the role of protein kinase C in this process, cells were either treated with a protein kinase C inhibitor or protein kinase C was down-regulated by chronic pretreatment of the cells with PMA.

Pretreatment of the cells for 10 min with a protein kinase C inhibitor, staurosporine (1 μM) resulted in the inhibition of the bradykinin- and PMA-stimulated [^{3}H]PtdBut accumulation (Fig. 4a). The bradykinin response was inhibited by 86 ± 11% (N = 3 experiments) and that to PMA by 88 ± 11% (N = 3 experiments). This suggests that both the bradykinin- and PMA-stimulated phospholipase D activities are regulated by protein kinase C.

This was confirmed by experiments in which protein kinase C was down-regulated by the inclusion of 100 nM PMA for at least 24 hr in the labelling medium (Fig. 4b). Under these conditions, basal phospholipase D activity was reduced to approx. 70% of that in control cells. Bradykinin-stimulated accumulation of [^{3}H]PtdBut in protein kinase C down-regulated cells was reduced to 12 ± 11% (N = 3 experiments). Similarly, PMA-stimulated phospholipase D activity was reduced to 13 ± 6% (N = 3 experiments) in the protein kinase C down-regulated tracheal smooth muscle cells. These results suggest that bradykinin-stimulated phospholipase D activation may be down-stream of protein kinase C.

Removal of extracellular Ca^{2+}: effect on phospholipase D activation

There is some evidence to suggest that the activation of phospholipase D by bradykinin is partially dependent upon extracellular Ca^{2+} (e.g. Ref. 14). In addition, bradykinin has been reported to open receptor-operated Ca^{2+} channels in human airway smooth muscle [17]. Preincubation of tracheal smooth muscle cells in a Ca^{2+}-depleted medium was used to assess the effect of extracellular Ca^{2+} upon bradykinin- and PMA-stimulated phospholipase D activities. Under these conditions, bradykinin-stimulated accumulation of [^{3}H]PtdBut was inhibited by 73 ± 10% (N = 3 experiments, Fig. 5), confirming that extracellular Ca^{2+} does play a role in the agonist-stimulated response. In contrast, the PMA-stimulated phospholipase D response was less sensitive to depletion of extracellular Ca^{2+}, being

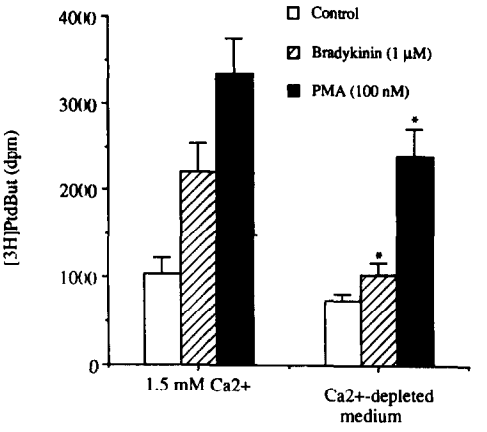

Fig. 5. Role of extracellular Ca^{2+} in the activation of phospholipase D. [^{3}H]Palmitate-labelled tracheal smooth muscle cells were preincubated in the presence of 1.5 mM Ca^{2+} or with 2.5 mM EGTA for 30 min and with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer (□), bradykinin (1 μM, ▨) or PMA (100 nM, ▩) for 10 min. Organic extracts were prepared and [^{3}H]PtdBut resolved and counted as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment. *P < 0.0005 vs response in control cells, Student's *t*-test, unpaired.

inhibited by only 23 ± 6% (N = 3 experiments, Fig. 5). This result suggests that there is a component of both the bradykinin- and PMA-stimulated responses which involves extracellular Ca^{2+}. This component is approx. 73% of the bradykinin-stimulated phospholipase D whereas it is only approx. 23% of the PMA-stimulated phospholipase D. However, the PMA-stimulated phospholipase D response is greater than that evoked by bradykinin and the inhibition of bradykinin- and PMA-stimulated [^{3}H]PtdBut accumulation upon removal of extracellular Ca^{2+} are the same in real terms, i.e. a reduction of 874 ± 139 dpm (N = 3 experiments) for bradykinin and of 660 ± 327 dpm (N = 3 experiments) for PMA. Since both the bradykinin- and PMA-stimulated phospholipase D responses appear to be down-stream of protein kinase C activation (Fig. 4), this result may indicate that additional isoforms of protein kinase C that are insensitive to extracellular Ca^{2+} are involved in the PMA activation of phospholipase D.

Preincubation with pertussis toxin: effect on phospholipase D activation

Bradykinin-stimulation of phospholipase C and A_2 has been shown to involve a pertussis toxin-sensitive G-protein in several tissues [7], i.e. pretreatment of the tissue with pertussis toxin, which catalyses the ADP-ribosylation of G_i (and G_o in neural tissue) attenuates the subsequent bradykinin-stimulated response. However, exceptions to this have also been observed suggesting that an alternative G-protein may be involved [8, 29]. In addition, activation of phospholipase D has been reported to involve a G-protein in several tissues [18, 19].

Direct activation of G-proteins in whole cells can be achieved by treatment with $[AlF_4]^-$ which, in combination with GDP, mimics GTP and thereby activates G-proteins and their effector enzymes [25]. Stimulation of [^{3}H]palmitate-labelled tracheal smooth muscle cells with $[AlF_4]^-$ (10 μM) for 10 min resulted in the accumulation of [^{3}H]PtdBut, suggesting the involvement of a G-protein in the activation of phospholipase D in these cells (Fig. 6a).

However, pertussis toxin-pretreatment of the cells [100 ng/mL, 18 hr, which completely ADP-ribosylated G_i (data not shown)] was without effect upon subsequent bradykinin-stimulated phospholipase D activation (Fig. 6b), suggesting that the G-protein involved is not G_i. If phospholipase D activation is down-stream of protein kinase C activation, it would be expected that bradykinin-stimulated formation of Ins(1,4,5)P_3, and, by implication, *sn*-1,2-diacylglycerol formation, should also be pertussis toxin-insensitive. Indeed, this was demonstrated to be the case (Fig. 6c). Furthermore, in systems where agonist-stimulated inositol lipid hydrolysis is pertussis toxin-insensitive, the identity of the G-protein involved in the activation of the phospholipase C has been shown to be G_q/G_{11} [30, 31]. A polyclonal antibody raised to the C-terminal decapeptide of G_q/G_{11} was used to confirm the presence of this G-protein in the primary guinea-pig tracheal smooth muscle cells by western blotting (Fig. 7). Thus, $[AlF_4]^-$-activation of phospholipase D may be an indirect effect of the activation of G_q/G_{11} and, thereby, inositol lipid hydrolysis and subsequent protein kinase C activation.

Inhibition of cyclooxygenase: effect on phospholipase D activation

Pretreatment of the cells for 10 min with indomethacin (10 μM) results in the inhibition of cyclooxygenase and, therefore, inhibition of bradykinin-stimulated production of prostanoids [4]. Bradykinin-stimulated [^{3}H]PtdBut formation was unaffected by cyclooxygenase inhibition (Fig. 8). Therefore, bradykinin-stimulated phospholipase D activation must be a direct consequence of bradykinin receptor occupation and independent of prostaglandin synthesis.

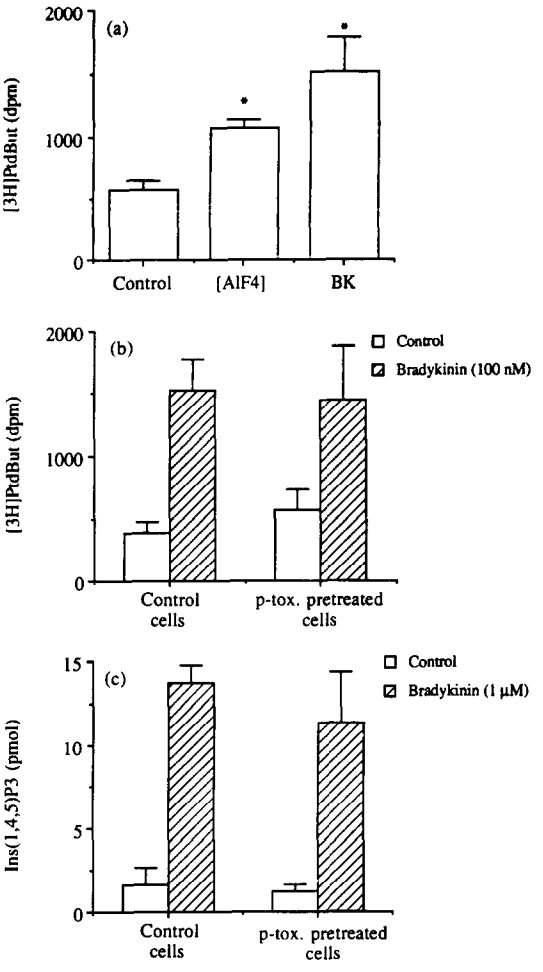

Fig. 6. G-protein involvement in the activation of phospholipase D. (a) [^{3}H]Palmitate-labelled tracheal smooth muscle cells were incubated with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer, bradykinin (1 μM) or $[AlF_4]^-$ (10 μM) for 10 min. Organic extracts were prepared and [^{3}H]PtdBut resolved and counted as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment. *$P < 0.005$ vs response in control cells, Student's *t*-test, unpaired. (b) Tracheal smooth muscle cells, labelled with [^{3}H]palmitate for 24 hr in the presence of vehicle or pertussis toxin (100 mg/mL, 18 hr), were incubated with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer (□) or bradykinin (1 μM, ▨) for 10 min. Radioactivity associated with [^{3}H]PtdBut was determined as in (a). Results are means ± SD (N = 3) from a single, representative experiment. (c) Tracheal smooth muscle cells were incubated with vehicle or pertussis toxin (100 ng/mL, 18 hr) prior to the addition of buffer (□) or bradykinin (▨) for 10 sec. An acid extract was prepared, neutralized and assayed for Ins(1,4,5)P_3 content using a radioligand binding assay as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment.

DISCUSSION

This study provides the first demonstration of a phospholipase D activity in airway smooth muscle. The phospholipase D assay employed, i.e. the formation of a non-metabolizable phosphatidylalcohol, phosphatidylbutan-1-ol, is definitive for the activation of phospholipase D since only phospholipase D possesses a phosphatidyltransferase activity. Alternative methods to assess phospholipase D activity e.g. measurement of the formation of phosphatidate can be misleading since this compound can also be produced by phosphorylation of 1,2-diacylglycerol, the hydrolysis product of phospholipase C. In addition, phosphatidate can be dephosphorylated by phosphatidate phosphohydrolase to diacylglycerol. Therefore, measurement of phosphatidate formation could either under- or over-estimate the phospholipase D activity depending upon the relative activities of diacylglycerol kinase and phosphatidate phosphohydrolase.

Phospholipase D-catalysed [^{3}H]PtdBut formation can be assumed to be derived from phospha-

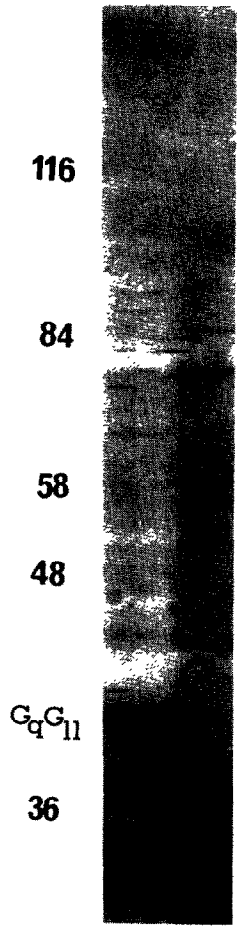

Fig. 7. Immunoblotting of G_q/G_{11} from primary cultured tracheal smooth muscle cells. Proteins were resolved on a 10% polyacrylamide gel by SDS–PAGE, transferred to nitrocellulose and reacted with a rabbit polyclonal antibody raised to the C-terminal decapeptide of G_q/G_{11}. A second antibody, raised to rabbit IgG and coupled to horseradish peroxidase was used to visualize the G_q/G_{11} immunoprecipitate. Lane 1, primary tracheal smooth muscle cells; lane 2, no sample. The position of molecular mass markers is illustrated. Results are from a single, representative experiment.

tidylcholine since incubation of the cells in the presence of [^{3}H]palmitate resulted in incorporation of radioactivity almost exclusively into phosphatidylcholine. In addition, bradykinin-stimulation of [^{3}H]choline-labelled cells resulted in the production of [^{3}H]choline (data not shown). However, other possible substrates of the phospholipase D cannot be excluded. For example, phosphatidylethanolamine has been shown to be hydrolysed by phospholipase D in a variety of systems [18, 19].

The phospholipase D was activated by both bradykinin and PMA. Accumulation of [^{3}H]PtdBut was detected at 10 sec in response to bradykinin whereas the PMA response was not significant until 60 sec after stimulation. This probably reflects the time taken for the PMA to permeate the plasma membrane and activate protein kinase C. The mechanism of activation of phospholipase D by bradykinin and PMA appears to involve both protein kinase C and extracellular Ca^{2+} since inhibition and down-regulation of protein kinase C almost completely abolished the accumulation of [^{3}H]-PtdBut in response to both bradykinin and PMA and removal of extracellular Ca^{2+} severely inhibited bradykinin-stimulated and attenuated PMA-stimulated phospholipase D activation. This is in common with observations made with a variety of agonists in many tissues [18, 19]. The difference in the sensitivities of the bradykinin- and PMA-stimulated responses to removal of extracellular Ca^{2+} may suggest that, unlike bradykinin, PMA activates isoforms of protein kinase C which have either a low or no requirement of extracellular Ca^{2+}. The bradykinin-stimulated phospholipase D response, which is down-stream of protein kinase C, is predominantly dependent upon extracellular Ca^{2+}. Whether this is at the level of the protein kinase C or the phospholipase D enzyme itself remains unclear.

The activation of phospholipase D may involve a G-protein since $[AlF_4]^-$-stimulation of the primary tracheal smooth muscle cells resulted in [^{3}H]PtdBut accumulation. However, since the activation of phospholipase D in these cells appears to be down-stream of protein kinase C, it is likely that the G-protein involved is coupled to inositol lipid-directed phospholipase C rather than to phospholipase D directly as has been proposed in, for example, granulocytes and hepatocytes [18, 19]. The pertussis toxin sensitivity of bradykinin-stimulated responses appears to vary between cell types, being insensitive in, for example, A431 carcinoma cells [8] and fibroblasts [29] but pertussis toxin sensitive in, for example, NG108-15 neuroblastoma-glioma hybrid cells and NIH3T3 fibroblasts [7]. However, in primary guinea-pig tracheal smooth muscle cells, the identity of the G-protein involved is not G_i since pertussis toxin pretreatment of the cells was without effect upon both phospholipase D and phospholipase C activation. Furthermore, these cells were demonstrated to contain G_q/G_{11} by western blotting and it is probably activation of this G-protein that, indirectly, activates phospholipase D.

Some of the effects of bradykinin in airway smooth muscle have been reported to be mediated by prostanoids [7]. However, the activation of phospholipase D by bradykinin was shown to be independent of cyclooxygenase products. Bradykinin-stimulated prostacyclin production has also been shown to be independent of bradykinin-stimulated phospholipase D activity in bovine pulmonary artery epithelial cells [32].

Phosphatidylcholine hydrolysis, by phospholipases C and D, has been suggested to serve as an alternative source to inositol lipids for the sustained production of diacylglycerol and, thereby, the activation of protein kinase C. However, the diacylglycerol species generated from inositol lipids hydrolysis and phosphatidylcholine hydrolysis differ substantially and may activate distinct isoforms of protein kinase C [18, 19]. Furthermore, the immediate product of phospholipase D activation, phosphatidate, has been

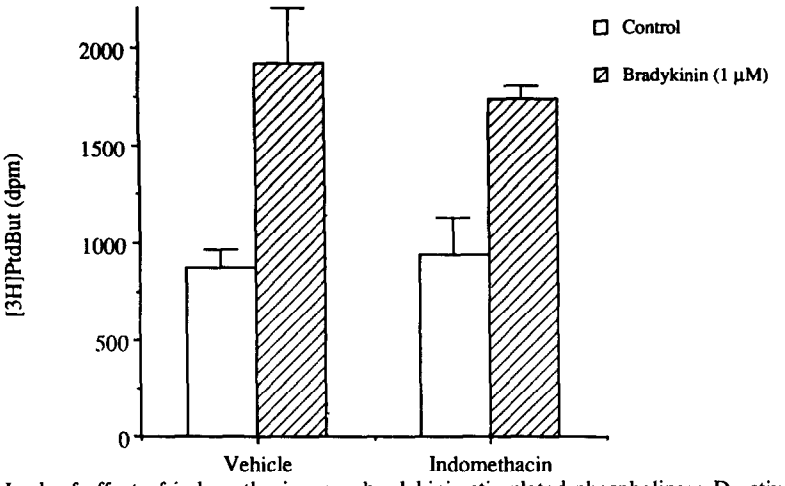

Fig. 8. Lack of effect of indomethacin upon bradykinin-stimulated phospholipase D activation. [^{3}H]-Palmitate-labelled tracheal smooth muscle cells were pretreated with vehicle or indomethacin (10 μM) for 10 min and with 0.3% (v/v) butan-1-ol for 5 min prior to the addition of buffer (□) or bradykinin (1 μM, ▨) for 10 min. Organic extracts were prepared and [^{3}H]PtdBut resolved and counted as described in Materials and Methods. Results are means ± SD (N = 3) from a single, representative experiment.

implicated as a second messenger in a wide variety of processes including the activation of protein kinase C [33–35]. Thus, phosphatidate derived from phospholipase D-stimulated phosphatidylcholine hydrolysis may play a role in the desensitization of agonist-stimulated inositol lipid hydrolysis, catalysed by phospholipase C, since the activation of protein kinase C has been implicated in this process [36]. Other potential roles of phosphatidate include the promotion of Ca^{2+} entry [37–40], the mobilization of intracellular Ca^{2+} [41, 42] and the autophosphorylation and activation of muscle phosphorylase kinase [43]. Furthermore, phospholipase D has been detected in vascular smooth muscle [44] and sustained contraction correlates better with phosphatidate formation rather than diacylglycerol formation [45]. More recently, phosphatidate-dependent protein phosphorylation has been observed in a cytosolic fraction of a variety of tissues including lung, catalysed either by a phosphatidylserine/diolein-independent isoform of protein kinase C or by a novel protein kinase [46]. This is particularly interesting in view of the possible role of phosphorylation of contractile and regulatory proteins in airway smooth muscle contraction [47, 48].

In summary, the role of phospholipase D-catalysed phosphatidylcholine hydrolysis in airway smooth muscle remains to be defined. However, it may be to provide diacylglycerol indirectly, for the activation of protein kinase C, or to provide phosphatidate, which may have multiple functions and may, by an undefined mechanism, be involved in the sustained contraction of airway smooth muscle.

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