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Differential effects of B_2 receptor antagonists upon bradykinin-stimulated phospholipase C and D in guinea-pig cultured tracheal smooth muscle

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1 Guinea-pig tracheal smooth muscle cells were isolated and maintained in culture for 14–21 days prior to the study of the effect of a selective bradykinin B_1 agonist and B_2 antagonists upon bradykinin-stimulated phospholipase C and D activities.

2 Bradykinin-stimulated phospholipase C activity was determined by mass measurement of inositol (1,4,5)trisphosphate (Ins(1,4,5)P_3) in unlabelled cells, whereas phospholipase D activity was assayed by the accumulation of [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBut) in [^{3}H]-palmitate-labelled cells, which were stimulated in the presence of butan-1-ol (0.3%, v/v).

3 Bradykinin elicited the rapid and transient formation of Ins(1,4,5)P_3, in a concentration-dependent manner (log $EC_{50} = -7.55 \pm 0.1$ M, $n = 3$). Bradykinin also rapidly activated the concentration-dependent (log $EC_{50} = -8.3 \pm 0.4$ M, $n = 3$) phospholipase D-catalysed accumulation of [^{3}H]-PtdBut; the accumulation of [^{3}H]-PtdBut was sustained. These effects were not inhibited by pretreatment of the cells with indomethacin (1 μM).

4 The bradykinin B_1 agonist, desArg^9-bradykinin (1 μM) was without effect upon phospholipase C or phospholipase D activity. Bradykinin-stimulated (10 nM, EC_{40}) Ins(1,4,5)P_3 formation was inhibited by B_2 receptor antagonists, D-Arg-[Hyp^3,D-Phe^7]-bradykinin (NPC 567) and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (NPC 349), with log IC_{50} values of -6.3 ± 0.5 M and -6.3 ± 0.4 M, respectively. However, bradykinin-stimulated (10 nM, EC_{100}) [^{3}H]-PtdBut accumulation was poorly inhibited and with low potency by each B_2 receptor antagonist and bradykinin-stimulated phospholipase D activity persisted at concentrations of antagonist that completely blocked bradykinin-stimulated Ins(1,4,5)P_3 formation (30 μM).

5 These observations suggest that the activation of phospholipase C by bradykinin may be mediated through a bradykinin B_2 receptor population, whereas bradykinin-stimulated phospholipase D may be activated via a distinct population of bradykinin receptors that do not appear to be either B_1 or B_2 receptor types, based upon pharmacological specificity. The mechanism of the activation of phospholipase D by bradykinin and the role of the putative B_3 bradykinin receptor are discussed.

Keywords: Bradykinin; phospholipase; receptor; smooth muscle; trachea; B_3 receptor

Introduction

Bradykinin is a potent nonapeptide which is generated in the tracheo-bronchial tree and in plasma during the inflammatory response (Proud & Kaplan, 1988) and may be an important mediator in diseases such as asthma. For example, asthmatics undergo bronchoconstriction in response to bradykinin and exhibit elevated levels of bradykinin in plasma and bronchoalveolar lavage after antigen challenge (Christiansen *et al.*, 1987). Whether the effects of bradykinin via B_2 receptors upon smooth muscle tone are direct or indirect remains ill-defined (Ichinose *et al.*, 1990). However, bradykinin receptors are present in airway smooth muscle (Farmer *et al.*, 1989; 1991; Panettieri *et al.*, 1989; Pyne & Pyne, 1993; Marsh & Hill, 1992).

The molecular mechanism whereby agonists induce airway smooth muscle contraction remains ill-defined. However, the initiation and development of contraction is believed to be due to agonist-stimulated phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) hydrolysis and the production of inositol (1,4,5)trisphosphate (Ins(1,4,5)P_3) which mobilizes Ca^{2+} from intracellular stores (Somlyo *et al.*, 1988). This leads to the activation of myosin light chain kinase and tension development (Chilvers *et al.*, 1989; Murray *et al.*, 1989). Bradykinin has been shown to elicit phosphoinositide hydrolysis in bovine cultured tracheal smooth muscle (Marsh & Hill, 1992) and the opening of receptor-operated Ca^{2+} channels in human cultured airway smooth muscle (Murray & Kotlikoff, 1991). Protein kinase C has been implicated in the process of sustained contraction since activators of protein kinase C, e.g. phorbol 12-myristate 13-acetate (PMA), synergize with Ca^{2+} to mimic agonist-stimulated contraction (Park & Rasmussen, 1985) and treatment of airway smooth muscle with PMA induces contraction in the absence of an intracellular Ca^{2+} signal (Kotlikoff *et al.*, 1987).

In addition, bradykinin elicits other biochemical responses including the activation of phospholipases A_2 and D (Farmer & Burch, 1992). We have recently demonstrated a bradykinin-stimulated phospholipase D activity in primary cultures of guinea-pig tracheal smooth muscle (Pyne & Pyne, 1993). These cultured cells have been shown to express large numbers of B_2 receptors mediating, for example, prostaglandin synthesis, but are also believed to contain a novel B_3 receptor type, coupled to bradykinin-induced Ca^{2+} efflux (Farmer *et al.*, 1991). This latter receptor type is believed to be involved in airway smooth muscle contraction since B_1 and B_2 antagonists have only a weak effect upon bradykinin-induced contraction of guinea-pig trachealis (Farmer *et al.*, 1989). Furthermore, bradykinin B_2 antagonists failed to displace [^{3}H]-bradykinin from freshly prepared guinea-pig tracheal smooth muscle membranes (Farmer *et al.*, 1989). However, the role of the putative B_3 receptor sub-type in airway smooth muscle contraction and the molecular mechanism involved remain unclear.

In this study, we aimed to characterize the bradykinin receptor type involved in the activation of phospholipase C

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and D in guinea-pig cultured tracheal smooth muscle by the use of bradykinin B_2 antagonists and a bradykinin B_1 agonist. We demonstrate that whilst neither phospholipase C nor D is activated by a B_1 agonist, activation of phospholipase C by bradykinin can be completely inhibited by B_2 antagonists whereas bradykinin-stimulated phospholipase D activity is poorly inhibited and persists in the absence of a measurable phospholipase C response.

Methods

Cell culture

Tracheal smooth muscle cells were cultured from male guinea-pigs (Dunkin-Hartley, 450–500 g) by the method of Panettieri *et al.* (1989) and maintained in Dulbecco's Modified Eagle's medium (DME) containing 10% (v/v) foetal calf serum (FCS)/10% (v/v) donor horse serum (DHS) at 37°C in air/CO_2 (95:5, v/v) (Pyne & Pyne, 1993). Cells were passaged by use of trypsin and grown to confluence on 24 well plates for experiments at 14–21 days after the initial preparation (passage 3). Identity of the smooth muscle cells was confirmed by the presence of smooth muscle α-actin using a mouse monoclonal antibody (Pyne & Pyne, 1993).

Measurement of inositol(1,4,5)trisphosphate

Ins(1,4,5)P_3 mass measurement was performed as described previously (Palmer & Wakelam, 1990; Pyne & Pyne, 1993). The cells were washed and preincubated in 250 μl Krebs Ringer bicarbonate buffer containing (mM): NaCl 118, $NaHCO_3$ 25, KCl 5, K_2HPO_4 1, $MgSO_4$ 1, $CaCl_2$ 1.5, glucose 10, pH 7.4) and supplemented with bovine serum albumin (Fraction V, 1% (w/v)) (KRB) for 30 min at 37°C in air/CO_2 (95:5, v/v). This medium was replaced with 100 μl of KRB in the presence and absence of bradykinin as required. In all cases bradykinin was present during the entire time-course of the incubation. Incubations were terminated by the addition of 25 μl of ice-cold perchloric acid (10%, w/v) and the samples placed on ice. Acid extracts were harvested, neutralized by the addition of approx. 25 μl (mM) 1500 KOH, 60 HEPES in the presence of a trace quantity of Universal Indicator and the resulting supernatants assayed for Ins(1,4,5)P_3 by an Ins(1,4,5)P_3-specific radioligand binding assay (Palmer & Wakelam, 1990) employing a crude adrenocortical microsomal fraction as a binding protein preparation. A standard curve of 25 fmol–25 pmol was conducted in parallel.

Incubation of cells with [^{3}H]-palmitate; phospholipase D assay

Confluent tracheal smooth muscle cells were preincubated with [^{3}H]-palmitate (2 μCi ml^{-1}) in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24–48 h. This procedure has previously been shown to result in the selective incorporation of radioactivity into phosphatidylcholine (PtdCh, which contained 82% of total radioactivity associated with major phospholipids in comparison with only 6% associated with phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine combined (Pyne & Pyne, 1993)).

Phospholipase D activity was determined by a transphosphatidylation assay (Pai *et al.*, 1988). Briefly, [^{3}H]-palmitate-labelled cells were washed and preincubated for 30 min with 500 μl KRB at 37°C in air/CO_2 (95/5, v/v) prior to a further preincubation for 5 min with 250 μl KRB containing butan-1-ol (0.3%, v/v). Bradykinin was added as required and was present during the entire time-course of the incubation. The incubations were terminated by removal of the medium and the addition of ice-cold methanol (200 μl). Organic extracts were prepared and the non-metabolizable [^{3}H]-PtdBut formed determined by its resolution upon thin layer chromatography (t.l.c.) using a solvent of the upper phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:H_2O (110:50:20:100, v/v) as described previously (Pyne & Pyne, 1993). [^{3}H]-PtdBut, which is a direct indicator of phospholipase D activity (Pai *et al.*, 1988), routinely migrated with an $R_F = 0.35$ and was quantified by excising the appropriate area from each lane and counting the associated radioactivity.

Preincubation of cells with indomethacin or bradykinin B_2 antagonists

For the experiments involving cyclo-oxygenase inhibition, cells were pretreated with indomethacin (1 μM) for 10 min prior to stimulation. In experiments involving the bradykinin B_2 antagonists, D-Arg-[Hyp^3,D-Phe^7]-bradykinin (NPC 567) and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (NPC 349), the cells were preincubated with the appropriate concentration for 2 min prior to the addition of bradykinin (10 nM).

Statistical analysis

All data are expressed as means ± s.d. and significance was determined by Student's *t* test.

Materials

[^{3}H]-palmitate (sp. act. 40–60 Ci mmol^{-1}) and [^{3}H]-inositol (1,4,5)trisphosphate (sp. act. 20–60 Ci mmol^{-1}) 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, bradykinin B_2 antagonists D-Arg-[Hyp^3,D-Phe^7]-bradykinin (NPC 567) and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (NPC 349) and the bradykinin B_1 agonists, des-Arg^9-bradykinin were purchased from Calbiochem (Nottingham, U.K.). Thin layer chromatography plates (LK5D) were obtained from Whatman (Maidstone, U.K.). All other reagents were of the highest purity commercially available.

Results

Bradykinin-stimulated Ins(1,4,5)P_3 formation

Bradykinin stimulated the rapid formation of Ins(1,4,5)P_3 in guinea-pig cultured tracheal smooth muscle cells. Basal levels of Ins(1,4,5)P_3 were 1.82 ± 0.35 pmol/0.25 × 10^6 cells ($n = 13$) and stimulation for 10s with a maximal concentration of bradykinin (100 nM) produced a 10.4 ± 6.1 ($n = 13$) fold increase in Ins(1,4,5)P_3 mass. A representative time course is illustrated in Figure 1a. The Ins(1,4,5)P_3 signal was transient, returning to close to basal levels by 30–60 s stimulation. The bradykinin-stimulated generation of Ins(1,4,5)P_3, measured at 10 s was concentration-dependent (Figure 1b) with a log EC_{50} of −7.55 ± 0.1 M ($n = 3$).

Bradykinin-stimulated phospholipase D

Bradykinin stimulated the activation of phospholipase D-catalysed PtdCh hydrolysis as evidenced by the accumulation of [^{3}H]-PtdBut in [^{3}H]-palmitate-labelled tracheal smooth muscle cells (Pyne & Pyne, 1993). Significant [^{3}H]-PtdBut formation was detected at 1 min and continued to be formed throughout the 10 min time course (Figure 2a). Stimulation with 100 nM bradykinin typically resulted in a 2.68 ± 1.1 ($n = 25$) fold increase in [^{3}H]-PtdBut above basal at 10 min. The bradykinin-stimulated phospholipase D response, measured at 10 min, was concentration-dependent (Figure 2b) with a log EC_{50} of −8.3 ± 0.4 M, which is significantly lower than that for bradykinin-stimulated phospholipase C activation.

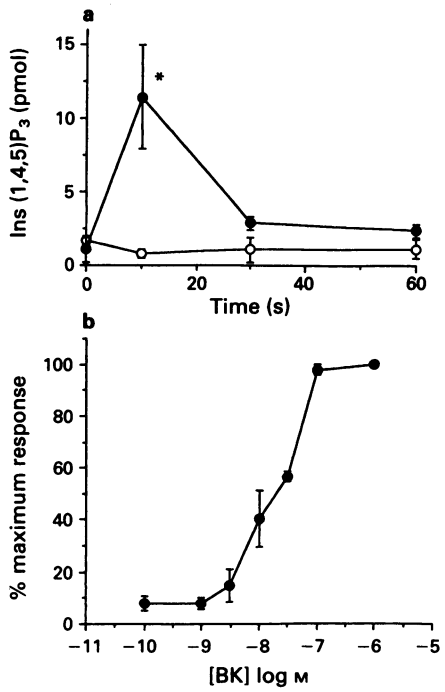

Figure 1 (a) Timecourse of bradykinin-stimulated inositol(1,4,5) trisphosphate (Ins(1,4,5)P_3) mass formation in guinea-pig cultured tracheal smooth muscle cells: (○) control; (●) bradykinin (100 nM). Results are mean ± s.d. ($n = 3$). (b) Bradykinin concentration-dependent Ins(1,4,5)P_3 formation at 10 s stimulation; means ± s.d. ($n = 3$) as % of maximal response. *$P < 0.025$ vs control (Student's t test).

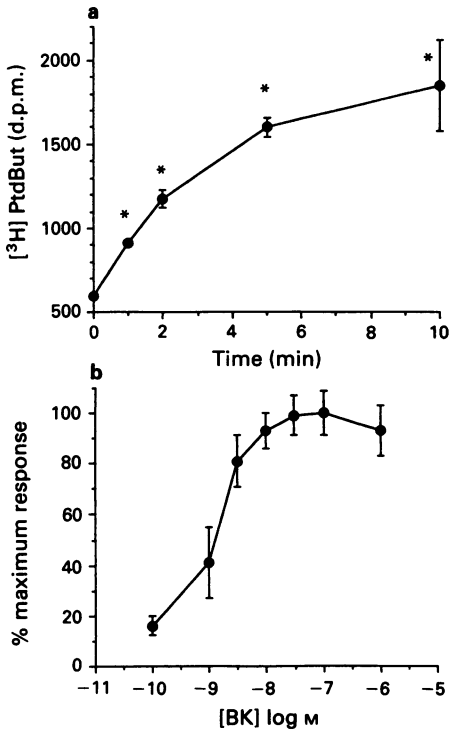

Figure 2 (a) Timecourse of bradykinin-stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBut) accumulation in guinea-pig cultured tracheal smooth muscle cells: (●) bradykinin (100 nM). Results are mean ± s.d. ($n = 3$). (b) Bradykinin concentration-dependent [^{3}H]-PtdBut accumulation at 10 min stimulation; means ± s.d. ($n = 3$ incubations) as % of maximal response. *$P < 0.005$ vs zero timepoint (Student's t test).

Effects of indomethacin and bradykinin antagonists

Bradykinin-stimulated phospholipase C was not inhibited by pretreatment of the cells with 1 μM indomethacin, suggesting that the observed effects of bradykinin were not secondary to bradykinin-stimulated prostaglandin synthesis (Figure 3). Similar data have previously been obtained for the bradykinin-stimulated phospholipase D response (Pyne & Pyne, 1993).

The identity of the bradykinin receptor sub-type(s) involved in the activation of these responses was investigated with a bradykinin B_1 agonist, desArg^9-bradykinin, and two bradykinin B_2 antagonists, NPC 567 and NPC 349. Stimulation of the tracheal smooth muscle cells with desArg^9-bradykinin (1 μM) elicited neither a phospholipase C nor phospholipase D response (Figure 3). In contrast, preincuba-

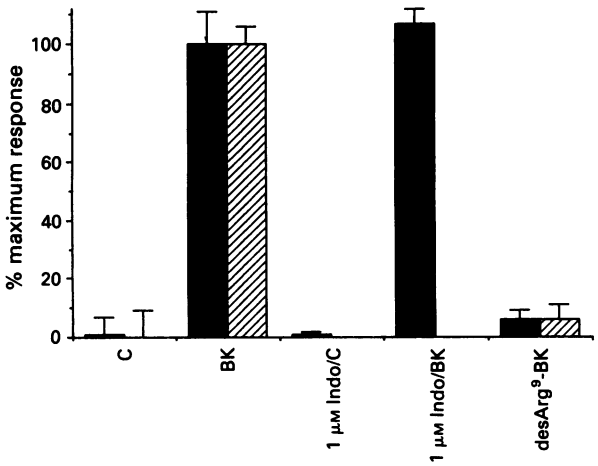

Figure 3 Lack of effect of indomethacin (Indo, 1 μM) upon 100 nM bradykinin (BK)-stimulated inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) formation at 10 s (solid columns) and the lack of effect of desArg^9-bradykinin (1 μM) upon [^{3}H]-phosphatidylbutanol ([^{3}H]PtdBut) accumulation (hatched columns) at 10 min and Ins(1,4,5)P_3 accumulation (solid columns) at 10 s. Results are means ± s.d. ($n = 3$) as % of maximal response.

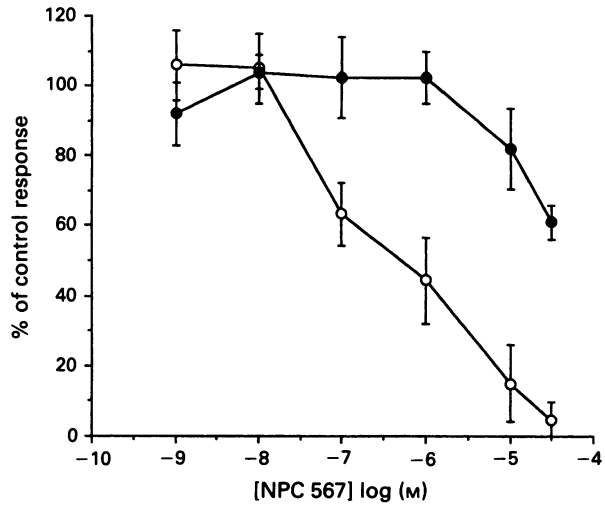

Figure 4 The effect of the bradykinin B_2 antagonist NPC 567 (D-Arg-[Hyp^3,D-Phe^7]-bradykinin) on bradykinin-stimulated (10 nM) inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) formation (10 s, ○) and [^{3}H]-phosphatidylbutanol ([^{3}H]PtdBut) accumulation (10 min, ●) in guinea-pig cultured tracheal smooth muscle cells. Control values were 0.81 ± 0.26 pmol Ins(1,4,5)P_3/0.25 × 10^6 cells and 750 ± 106 d.p.m. [^{3}H]PtdBut/0.25 × 10^6 cells. Results are means ± s.d. ($n = 3$).

tion of the cells with the bradykinin B_2 antagonist NPC 567 for 2 min prior to stimulation with bradykinin (10 nM) resulted in the inhibition of both Ins(1,4,5)P_3 formation and, at high concentrations (> 10 μM), [^{3}H]-PtdBut accumulation (Figure 4). A second B_2 antagonist, NPC 349, displayed essentially the same characteristics.

The IC_{50} for inhibition of the phospholipase C and phospholipase D responses by these antagonists differed markedly. Log IC_{50} values for the Ins(1,4,5)P_3 response to 10 nM bradykinin were -6.3 ± 0.5 M ($n = 3$) for NPC 567 and -6.3 ± 0.4 M ($n = 3$) for NPC 349. The Ins(1,4,5)P_3 response to 10 nM bradykinin was abolished by 30 μM NPC 567 (Figure 4) or NPC 349 (data not shown). In contrast, preincubation of the cells for 2 min with either bradykinin B_2 receptor antagonist resulted in only limited inhibition of bradykinin-stimulated (10 nM) phospholipase D activation (Figure 4). At 30 μM B_2 antagonist, a concentration which abolished the phospholipase C response, approx. 60% of the phospholipase D response remained and the IC_{50} for bradykinin-stimulated phospholipase D was estimated to be in excess of 100 μM. At these high concentrations of antagonist, non-specific interactions with bradykinin-stimulated phospholipase D may occur.

Discussion

This study provides the first demonstration of bradykinin-stimulated changes in Ins(1,4,5)P_3 mass in airway smooth muscle, although Marsh & Hill (1992) have recently shown bradykinin-stimulated accumulation of [^{3}H]-inositol phosphates. However, this latter technique is not sufficiently sensitive to detect agonist-stimulated changes in phosphoinositide metabolism at functionally important early times. The kinetics of the Ins(1,4,5)P_3 response are similar to those observed for carbachol in bovine cervical trachealis muscle slices (Chilvers *et al.*, 1989) and correlate with the kinetics of bradykinin-stimulated [Ca^{2+}] transients in Fura-2 loaded monolayers of human primary cultured airway smooth muscle (Panettieri *et al.*, 1989). Bradykinin-stimulated Ins(1,4,5)P_3 mass increased rapidly (within seconds) and was transient. This may be due to enhanced metabolism of Ins(1,4,5)P_3 by Ins(1,4,5)P_3 5-phosphatase and 3-kinase (Shears, 1991) or desensitization of agonist-stimulated PtdIns(4,5)P_2 hydrolysis (Palmer *et al.*, 1991) or both. Desensitization of phospholipase C during prolonged bradykinin-stimulation is suggested by the plateau in [^{3}H]-inositol phosphates accumulation in bovine cultured tracheal smooth muscle (Marsh & Hill, 1992).

Bradykinin was a less potent stimulator of Ins(1,4,5)P_3 formation in guinea-pig tracheal smooth muscle when compared to its effect upon [^{3}H]-inositol phosphate accumulation in bovine cultured tracheal smooth muscle cells (Marsh & Hill, 1992). However, both effects were inhibited by B_2 antagonists, suggesting that sensitivity of the bradykinin B_2 receptor coupling to phospholipase C may differ between species. In addition, desArg^9-bradykinin failed to elicit an Ins(1,4,5)·P_3 response (the present study) whereas it weakly activates [^{3}H]-inositol phosphate accumulation in bovine cultured tracheal smooth muscle cells (Marsh & Hill, 1992), indicating that B_1 bradykinin receptors may be differentially expressed between species.

In contrast to the transient Ins(1,4,5)P_3 signal, [^{3}H]-PtdBut accumulation was sustained in response to bradykinin due to the inability of the cells to metabolize phosphatidylalcohols. However, partial desensitization of bradykinin-stimulated phospholipase D activity may occur since the rate of accumulation of [^{3}H]-PtdBut declined after 2–5 min stimulation.

Interestingly, bradykinin-stimulated phospholipase D activity displayed significantly higher potency than that for bradykinin-stimulated phospholipase C activity. This may suggest that distinct bradykinin receptor types are involved in mediating the two responses. However, we cannot exclude from these studies the possibility that receptor-G-protein fidelities are different or that a receptor reserve exists for the phospholipase D response. However, our results from the antagonism experiment would argue against these latter hypotheses.

For example, if one receptor mediates both PLD and PLC responses via distinct G-proteins then, since the affinity of the receptor for antagonist is not dependent upon G-protein association (i.e. guanine nucleotides do not modify the affinity of the receptor for antagonist), both PLC and PLD responses should be blocked with exactly the same concentration-dependence for the antagonist. This clearly does not occur. Thus, if both responses were mediated by the same receptor, we would predict that the PLD response would be inhibited by at least 40% and with an identical antagonist concentration-dependence to that observed for PLC inhibition (in response to an EC_{40} concentration of agonist).

The conditions used in the antagonist experiments reduce the effect of a receptor reserve for PLD to a minimum, i.e. the concentration of agonist used (10 nM) is at the threshold for maximal activation of the PLD response. Thus, if one receptor mediates both responses, then, the occupancy of the 'spare receptors' will be minimal. Under these conditions, the antagonist concentration-dependence for inhibition of both PLD and PLC should be virtually identical since, by the law of mass action, receptor occupancy for both responses will be virtually the same. The fact that the IC_{50} values differ by approximately two orders of magnitude clearly precludes the possibility that a receptor reserve for PLD exists to account for these results.

We conclude that the only reasonable explanation for the large shift in antagonist concentration-dependence for the two responses is to invoke the possibility of a novel receptor mediating PLD activation. Furthermore, phospholipase D activation was detected in the absence of phospholipase C activation, which was completely blocked by B_2 antagonists. This suggests that, in this case, bradykinin-stimulated phospholipase D is not down-stream of phospholipase C activation, as has been suggested for several other agonists (Billah & Anthes, 1991).

We have previously shown that bradykinin-stimulated phospholipase D is down-stream of protein kinase C activation in guinea-pig cultured tracheal smooth muscle cells (Pyne & Pyne, 1993). Bradykinin-stimulated [^{3}H]-PtdBut accumulation is abolished in protein kinase C down-regulated cells or in the presence of a protein kinase C inhibitor, staurosporine. Since phospholipase D can be activated in the absence of PtdIns(4,5)P_2 hydrolysis, protein kinase C must be activated independently of *sn*-1,2-diacylglycerol derived from this source. Multiple isoforms of protein kinase C exist which can be variously activated by 1,2-diacylglycerol, phosphatidate, arachidonate and Ca^{2+} (Bell & Burns, 1991). The identify of the isoform(s) mediating activation of phospholipase D are unknown. However, 1,2-diacylglycerol and phosphatide can be produced by bradykinin-stimulated phospholipase C-catalysed hydrolysis of PtdCh in guinea-pig tracheal smooth muscle cells, independent of prior activation of protein kinase C. This mechanism may provide the source of protein kinase C activator necessary for phospholipase D activation (Pyne & Pyne, unpublished). Furthermore, bradykinin activates the influx of extracellular Ca^{2+} in human cultured airway smooth muscle (Murray & Kotlikoff, 1991), and we have previously demonstrated a requirement for extracellular Ca^{2+} for activation of phospholipase D (Pyne & Pyne, 1993). The arachidonate-activated protein kinase C isoform is unlikely to play a role, since bradykinin-stimulated prostanoid production is also abolished at concentrations of B_2 antagonist at which phospholipase D activity persists (Farmer *et al.*, 1991 and the present study).

Thus, bradykinin-stimulated phospholipase D activation appears to be independent of B_1 and B_2 receptor types. Farmer *et al.* (1989) have proposed the existence of a B_3 receptor which may mediate bradykinin-stimulated contrac-

tion of guinea-pig trachealis (Farmer *et al.*, 1989) and Ca^{2+} efflux from guinea-pig cultured airway smooth muscle (Farmer *et al.*, 1991). The EC_{50} for bradykinin-stimulated phospholipase D activation (the present study) is similar to that observed for Ca^{2+} efflux in guinea-pig cultured tracheal smooth muscle cells (Farmer *et al.*, 1991). Furthermore, the IC_{50} values for inhibition of these responses by NPC 567 are also similar (the present study and Farmer *et al.*, 1991). Therefore, we suggest that the putative B_3 bradykinin receptor may mediate bradykinin-stimulated phospholipase D activation in these cells. However, specific B_3 antagonists are required to prove that this is the case.

In conclusion, bradykinin induces airway smooth muscle contraction (Farmer *et al.*, 1989) and maintains the allergic inflammation of airways in guinea-pigs (Farmer *et al.*, 1992). Whilst the inflammatory response induced by bradykinin is mediated by B_1 and B_2 receptor types *in vivo* (Farmer *et al.*, 1992) and the bronchoconstriction induced by bradykinin *in vivo* is mediated by B_2 receptors (Jin *et al.*, 1989), the direct effects of bradykinin upon sustained smooth muscle contraction *in vitro* are not accounted for by these receptors. In this context, Farmer *et al.* (1991) have clearly shown that B_2 antagonists do not displace bradykinin from freshly prepared tracheal smooth muscle membranes thus identifying that B_2 receptors are unlikely to mediate direct effects of bradykinin upon contraction *in vitro*. However, in the same study, B_2 receptors appear to be 'up-regulated' in culture and this is consistent with the present study and that of Marsh & Hill (1992). The putative B_3 receptor appears to allow Ca^{2+} efflux and is present in both fresh and cultured guinea-pig tracheal smooth muscle cell membranes. Given the ability of PLD to generate activators of PKC and the possible B_3 mediated regulation of extracellular Ca^{2+} flux in these cells, we suggest that the physiological relevance of this receptor may be related to sustained contraction. However, this will only be resolved with the advent of specific B_3 receptor antagonists.

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