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Bradykinin-stimulated phosphoinositide metabolism in cultured canine tracheal smooth muscle cells

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1 Stimulation of bradykinin (BK) receptors coupled to phosphoinositide (PI) hydrolysis was investigated in canine cultured tracheal smooth muscle cells (TSMCs). BK, kallidin, and des-Arg^9-BK, stimulated [^{3}H]-inositol phosphates (IPs) accumulation in a dose-dependent manner with half-maximal responses (EC_{50}) at 20 ± 5, 13 ± 4, and 2.3 ± 0.7 nM, ($n = 5$), respectively.

2 D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, BK B_2 receptor antagonists, were equipotent in blocking the BK-induced IPs accumulation with $pK_B = 7.1$ and 7.3, respectively.

3 Short-term exposure of TSMCs to phorbol 12-myristate 13-acetate (PMA, 1 μM), attenuated BK-stimulated IPs accumulation. The concentrations of PMA that gave half-maximal and maximal inhibition of BK-induced IPs accumulation were 15 ± 4 nM and 1 μM, $n = 3$, respectively. The inhibitory effect of PMA on BK-induced response was reversed by staurosporine, a protein kinase C (PKC) inhibitor, suggesting that the inhibitory effect of PMA was mediated through the activation of PKC.

4 Prolonged incubation of TSMCs with PMA for 24 h, resulted in a recovery of receptor responsiveness which may be due to down-regulation of PKC. The inactive phorbol ester, 4α-phorbol 12, 13-didecanoate at 1 μM, did not inhibit this response.

5 The site of this inhibition was further investigated by examining the effect of PMA on AlF_4^--induced IPs accumulation in canine TSMCs. AlF_4^--stimulated IPs accumulation was inhibited by PMA treatment, suggesting that the G protein(s) can be directly activated by AlF_4^-, which is uncoupled from phospholipase C by PMA treatment.

6 Incubation of TSMCs in the absence of external Ca^{2+} or upon removal of Ca^{2+} by addition of EGTA, caused a decrease in IPs accumulation without changing the basal levels. Addition of Ca^{2+} (3–620 nM) to digitonin-permeabilized TSMCs stimulated IPs accumulation was obtained by inclusion of either guanosine 5′-O-(3-thiotriphosphate) (GTPγS) or BK. The combination of GTPγS and BK caused an additive effect on IPs accumulation.

7 Pretreatment of TSMCs with cholera toxin enhanced BK-stimulated IPs accumulation, whereas there was no effect with pertussis toxin.

8 These data suggest that BK-stimulated PI metabolism is mediated by the activation of BK B_2 receptors coupling to a G protein which is not blocked by cholera toxin or pertussis toxin treatment and dependent on external Ca^{2+}. The transduction mechanism of BK coupled to PI hydrolysis is sensitive to feedback regulation by PKC.

Keywords: Bradykinin; phorbol ester; protein kinase C; inositol phosphates; G protein; canine tracheal smooth muscle cells

Introduction

Bradykinin (BK) is a classic mediator of inflammatory diseases of the airways and may be implicated in allergic asthma (Christiansen *et al.*, 1987). In the airways, BK causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). Most of the biological actions of BK are mediated through at least two different receptors, termed BK B_1 and B_2 receptors, which have been pharmacologically characterized using different kinin analogues with agonist and antagonist properties (Regoli *et al.*, 1990). In many cell types, including the neuroblastoma-glioma hybrid NG108-15 (Osugi *et al.*, 1987), glioma C6-4-2 (Reiser *et al.*, 1990), astrocytoma 1321N1 cells (Helper *et al.*, 1987), and bovine tracheal smooth muscle cells (TSMCs) (Marsh & Hill, 1992), BK receptors activate phospholipase C (PLC) mediated phosphoinositide (PI) hydrolysis in the plasma membrane. The resultant increase in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) release Ca^{2+} from internal stores and activate protein kinase C (PKC), respectively (Nishizuka, 1988; Berridge & Irvine, 1989).

Binding and functional studies have provided evidence for subtypes of B_2 receptors in guinea-pig ileum and lung and in rat myometrium membranes and vas deferens (Manning *et al.*, 1986; Braas *et al.*, 1988; Plevin & Owen, 1988; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991). The mechanisms involved in smooth muscle contractile response are not completely understood particularly in the airways. One possible mechanism of BK-induced bovine smooth muscle contraction is an increase in PI hydrolysis mediated by B_2 receptors (Marsh & Hill, 1992). There are several lines of evidence that PLC is coupled to a variety of cell surface receptors via a guanine nucleotide binding protein (Sternweis & Smrcka, 1992). The stable analogue of GTP, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), has been shown to increase IPs accumulation in permeabilized tracheal smooth muscle of rabbit (Rosenberg *et al.*, 1991) and man (Murray *et al.*, 1989). In some studies, PI hydrolysis has been shown to be dependent on calcium, presumably due to the requirement of this ion for PLC activity (Fisher *et al.*, 1989; Eberhard & Holz, 1991). In other work, however, no dependence on calcium has been observed (Berridge & Irvine, 1989).

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Many studies have also suggested that BK B_2 receptors are coupled to G proteins in other cell types (Higashida *et al.*, 1986; Burch & Axelrod, 1987; Murayama & Ui, 1987). For example, BK-induced inositol phosphates (IPs) accumulation was found to be sensitive to guanine nucleotide analogues in NG108 and 3T3 cells (Higashida *et al.*, 1986; Murayama & Ui, 1987). It has been reported that many signal transduction processes which require GTP may not be affected by pertussis or cholera toxin (Martin *et al.*, 1985; Aub *et al.*, 1986). Indeed, in NG108 cells, pertussis toxin (PTX)-insensitive G_q protein(s) may account for kinin receptor coupling to PLC (Gutowski *et al.*, 1991).

Agonist-activation of PI hydrolysis also leads to the formation of DAG. The ability of phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) which mimics the effect of DAG, to activate PKC (Castagna *et al.*, 1982), has provided a useful tool to examine the role of the DAG pathway related to smooth muscle contraction. By using PMA it was shown that stimulation of PI hydrolysis induced by α_1-adrenoceptor activation on DDT_1MF_2 cells (Leeb-Lundberg *et al.*, 1985) was inhibited by PMA, probably through phosphorylation of the receptors by PKC. Because DAG is one of products of PI hydrolysis, it is believed that PKC may be involved in 'desensitization' or 'down-regulation' of the muscarinic receptors in astrocytoma cells and could exert a feedback regulation of PI breakdown (Orellana *et al.*, 1985).

The purpose of the present study was to investigate the effect of BK on IPs accumulation and its regulation in canine TSMCs, using PTX, cholera toxin (CTX), PKC activator, and AlF_4^-, an activator of the regulatory proteins. These results conclude that BK induces PLC-mediated PI hydrolysis through the activation of BK B_2 receptors in canine TSMCs and that this reaction is negatively regulated by PKC activation. Evidence is also provided that BK B_2 receptors may be coupled to PLC via a PTX-and CTX-insensitive Gq protein.

Methods

Animals

Mongrel dogs, 10–20 kg, of either sex were purchased from a local supplier and used throughout this study. Dogs were housed in our animal facilities under automatically controlled temperature and light cycle and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.v.) and the lungs were ventilated mechanically via an orotracheal tube. The tracheae were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods of Yang *et al.* (1991). The trachea was cut longitudinally through the cartilage rings and the smooth muscle was dissected. The muscle was minced and transferred to the dissociation medium containing 0.1% collagenase IV, 0.025% DNase I, 0.025% elastase IV, and antibiotics in physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, $CaCl_2$ 1.1, $NaHCO_3$ 20, NaH_2PO_4 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were gently agitated at 37°C in a rotary shaker for 1 h. The released cells were collected and the residual was again digested with fresh enzyme solution for an additional hour at 37°C. The released cells were washed twice with DMEM/F-12 medium. The cells, suspended in DMEM/F-12 containing 10% FBS, were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cell number was counted and the suspension diluted with DMEM/F-12 to 2×10^5 cells ml^{-1}. The cell suspension (1 ml/well) was plated onto 12-well culture plates. The medium was changed after 24 h and then every 3 days. After a 5-day culture, cells were shifted to DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were cultured in DMEM/F-12 containing 1% FBS supplemented with IGF-I (10 ng ml^{-1}) and insulin (1 μg ml^{-1}) for 12–14 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by indirect immunofluorescence using a monoclonal antibody of light chain myosin (Gown *et al.*, 1985). Over 95% of the cell preparation was composed of smooth muscle cells.

Accumulation of inositol phosphates

Effect of BK on the hydrolysis of PI was assayed by monitoring the accumulation of ^3H-labelled IPs as described by Berridge *et al.* (1983). Cultured TSMCs were incubated with 5 μCi ml^{-1} of *myo*-[2-^3H]-inositol at 37°C for 2 days. TSMCs were washed two times with PBS and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM): NaCl 117, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 2.4, glucose 1, HEPES 20 and LiCl 10 at 37°C for 30 min. After BK was added at the concentration indicated, incubation was continued for another 60 min in the presence of 10 μM phosphoramidon. Reactions were terminated by addition of 5% perchloric acid followed by sonication and centrifugation at 3,000 *g* for 15 min.

The percholic acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied for a column of AG1-X8, formate form, 100–200 mesh (Bio-Rad). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free *myo*-[^3H]-inositol and glycerophosphoinositol, respectively. Total IPs was eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^3H]-IPs was determined in a radiospectrometer (Beckamn LS5000TA, Fullerton, CA, USA).

Permeabilized cells

After the prelabelling period, TSMCs were washed twice with PBS and then permeabilized in potassium glutamate-EGTA-HEPES buffer (KGEH) containing (mM): K^+ glutamate 139, ATP 2, $MgCl_2$ 4, LiCl 10, EGTA 2, HEPES 20 (pH 7.4), and 10 μM digitonin, as described by Eberhard & Holz (1987). Cells were allowed to permeabilize for 5 min at 37°C. The permeabilizing buffer was discarded and cells were washed twice with KGEH buffer without digitonin. Incubations were continued for 15 min at 37°C. The required free Ca^{2+} concentrations ($[Ca^{2+}]_f$) were achieved by the addition of various amounts of $CaCl_2$ to the KGEH buffer containing 2 mM EGTA. The $[Ca^{2+}]_f$ was determined by the addition of 10 μM fura-2 (K^+-salt) using a SLM spectrofluorometer (Aminco SLM, Urbana, IL, U.S.A.). BK at the indicated concentration was added and incubation continued for 60 min. Reactions were terminated by the addition of 5% perchloric acid. The procedures for extraction, separation, and quantification of IPs were the same as those used for intact cells.

ADP-ribosylation

Cultured TSMCs were incubated with PTX (100 ng ml^{-1}) or CTX (10 μg ml^{-1}) at 37°C for 4 h. Each plate was rinsed with cold PBS and once with buffer A (mM) containing Tris-HCl 20, pH 7.5, NaCl 10, EGTA 1 and phenylmethylsulphonyl fluoride 1 (PMSF). Cells were scraped from the dish into buffer A and homogenized with a polytron (set 5 for 30 s). After removing the nuclei and unbroken cells by centrifugation at 400 *g* for 10 min, the supernatant was centrifuged at 10,000 *g* for 30 min. The final pellet was suspended in 25 mM Tris-HCl, pH 7.4 containing 2.5 mM $MgCl_2$. Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

ADP-ribosylation was performed as described (Katada & Ui, 1982) with minor modifications: 100 μg of total protein

from the membrane-rich fraction was diluted with an equal volume of ribosylation buffer (Tris-HCl 25 mM, pH 7.4, thymidine 10 mM, GTP 1 mM, $MgCl_2$ 2.5 mM, EDTA 1 mM), and 5 mM dithiothreitol (DTT). PTX was activated by incubation with 50 mM DTT in 25 mM Tris-HCl, pH 7.4 at 37°C for 1 h and added to a final concentration of 5 μg ml^{-1}. [^{32}P]-NAD^+ (5 μCi/sample) was added with cold NAD^+ to a final concentration of 10 μM and the reaction mixture was incubated at 37°C for 30 min. ADP ribosylation reaction was terminated by 5% trichloroacetic acid precipitation, and the samples were resolved by SDS-polyacrylamide (10%)-gel electrophoresis (Laemmli, 1970). Gels were stained with Commassie Brilliant Blue, dried, and autoradiographed. For CTX labelling, 5 μg CTX/sample was included in the ADP-ribosylation mixture.

Analysis of data

The EC_{50} of BK for stimulating IPs accumulation was estimated by Graph Pad Program (Graph Pad, San Diego, CA, U.S.A.). The dissociation constants (K_B) of BK antagonists were estimated by the method of Furchgott (1972), using their ability to antagonize BK-mediated IPs accumulation.

The data were expressed as the mean ± s.e.mean of at least four experiments with statistical comparisons based on a two-tailed Student's *t*-test at a $P<0.01$ level of significance.

Chemicals

DMEM/F-12 medium and FBS were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Insulin and IGF-I were from Boehringer Mannheim (GmbH, Germany). Fura-2 was from Molecular Probes Inc (Eugene, OR, U.S.A.). *Myo*-[^{3}H]-inositol (18 Ci $mmol^{-1}$) and [^{32}P]-NAD^+ (65 Ci $mmol^{-1}$) were from Amersham (Buckinghamshire, England). D-Arg[Hyp^3, D-Phe^7]-BK, D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, des-Arg^9-BK and kallidin were from Bachem California (Torrance, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.).

Results

Agonist-induced IPs accumulation

To study agonist-stimulated IPs accumulation, canine cultured TSMCs were labelled for 48 h with [^{3}H]-inositol, treated with agonist and extracted. The aqueous phase of cell extracts was used to determine the amount of [^{3}H]-IPs. Carbachol (100 μM), histamine (100 μM), 5-hydroxytryptamine (5-HT, 100 μM), and BK (1 μM), produced IPs accumulation of 9.1 ± 0.5, 2.6 ± 0.2, 10.5 ± 0.2, and 7.1 ± 0.3 fold, respectively, greater than the basal level (8600 ± 500 d.p.m./well; $P<0.001$, $n=3$). BK-induced IPs accumulation increased rapidly up to 10 min of incubation and reached a maximal value (52700 ± 2500 d.p.m./well, $n=3$) at 60 min in the presence of 10 mM LiCl. LiCl alone caused no significant accumulation of IPs within 60 min. The effects of BK, kallidin, and des-Arg^9-BK on IPs accumulation was dose-dependent over the range of 1 nM to 10 μM with EC_{50} values of 20 ± 5, 13 ± 4, and 2.3 ± 0.7 nM, ($n=5$), respectively (Figure 1). Among these agonists, des-Arg^9-BK at maximally effective concentration caused the smallest increase in IPs accumulation.

Effects of BK B_2 antagonists on BK-induced IPs accumulation

To determine which type of BK receptor is involved in the activation of PLC in canine TSMCs, we examined the effects of D-Arg[Hyp^3,D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, selective BK B_2 receptor antagonists, on BK-induced IPs accumulation. As shown in Figure 2, preincubation of TSMCs with these antagonists inhibited the BK-induced IPs accumulation. The concentration-effect relationship of BK was shifted to the right in a nearly parallel fashion, without changing the maximal response, upon addition of 5 μM D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8}, D-Phe^7]-BK. The EC_{50} values of BK were increased from 20 ± 5 nM to 2.1 ± 0.4 and 1.4 ± 0.3 μM, $n=4$, in the presence of D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, respectively. The dissociation constants (pK_B) were calculated from the dose-ratios and were 7.1 and 7.3 for D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, respectively. In contrast, the BK B_1 receptor antagonist, [D-Arg^9, Leu^8]-BK, did not change the BK-induced response (data not shown). These results indicated that the receptors mediating BK-induced IPs accumulation had similar pharmacological properties to BK B_2 receptors (Regoli *et al.*, 1990; Marsh & Hill, 1992).

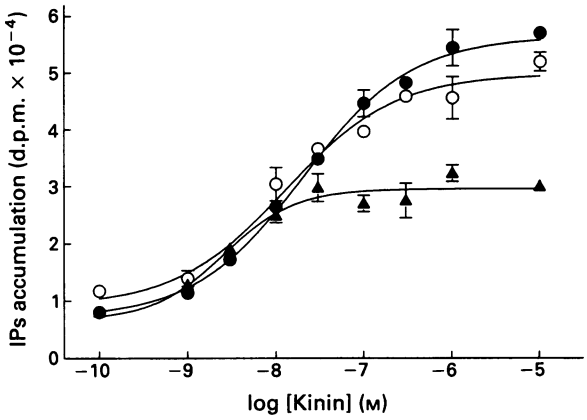

Figure 1 Dose-response curve for bradykinin (BK), kallidin-, and des-Arg^9-BK-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Agonist (1 nM–10 μM) was added and the reaction was stopped after 60 min incubation. [^{3}H]-IPs were determined as described under Methods. Each point represents the mean ± s.e.mean of five separate experiments determined in triplicate. (●) BK; (○) kallidin; (▲) des-Arg^9-BK.

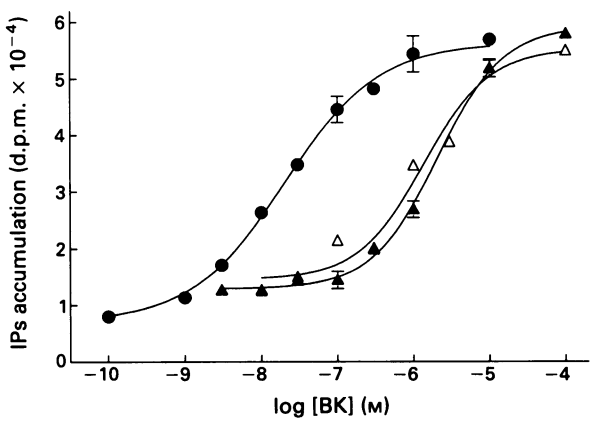

Figure 2 Inhibition of bradykinin (BK)-induced inositol phosphates (IPs) accumulation in TSMCs by D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK. [^{3}H]-IPs were determined as described under Methods. Each point represents the mean ± s.e.mean of four experiments determined in triplicate. BK-stimulated IPs accumulation was measured in the absence (●) and presence of D-Arg[Hyp^3,D-Phe^7]-BK (△, 5 μM) and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK (▲, 5 μM).

Effect of phorbol esters on BK-induced IPs accumulation

In order to determine whether PKC activation by phorbol esters causes an inhibition on BK-induced PI response, the concentration-response relationship for PMA inhibition of BK-stimulated IPs accumulation was examined in cultured TSMCs. As shown in Figure 3, half-maximal and maximal inhibition of PMA on BK-stimulated IPs accumulation occurred at 15 ± 4 nM and 1 μM, $n = 3$, respectively. PMA had no effect on the basal levels of IPs accumulation at any of the concentrations tested. Furthermore, [^{3}H]-inositol-labelled TSMCs were treated with PMA and 4α-PDD (1 μM) and then stimulated with 1 μM BK in the continuous presence of phorbol esters (Figure 4). Treatment of TSMCs with 1 μM PMA for 30 min led to an inhibition of BK-stimulated IPs accumulation by 48% ($P<0.001$, $n = 4$, as compared with the control). However, when TSMCs were treated with 1 μM PMA for 24 h, the extent of BK-stimulated IPs accumulation was greater than that of TSMCs of the control group ($P<0.01$, $n = 4$). The inactive phorbol ester, 4α-phorbol 12, 13-didecanoate (4α-PDD, 1 μM), did not block BK-induced IPs accumulation (Figure 4). When TSMCs were pretreated with staurosporine (1 μM), a potent PKC inhibitor, the inhibitory effect of PMA on BK-stimulated IPs accumulation was reversed (Figure 4).

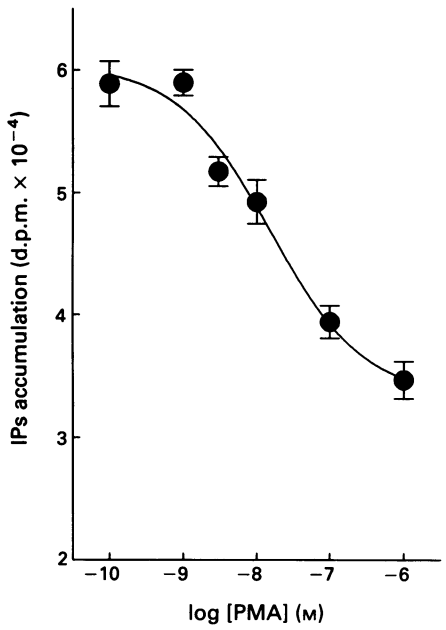

Figure 3 Concentration dependence of phorbol 12-myristate 13-acetate (PMA) inhibition of bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells prelabelled with [^{3}H]-inositol were washed with KHS, treated with various concentrations of PMA for 30 min and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of three separate experiments determined in triplicate. The basal level and BK-induced IPs accumulation were 11690 ± 1170 and 61290 ± 2790 d.p.m./well, respectively.

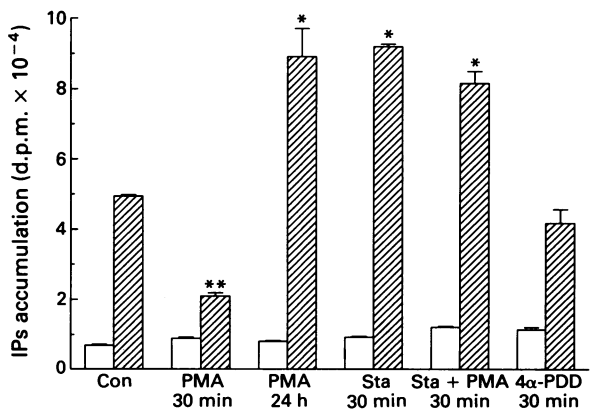

Figure 4 Effects of phorbol ester and staurosporine treatment on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. [^{3}H]-inositol-labelled TSMCs were pretreated with phorbol 12-myristate 13-acetate (PMA, 1 μM), staurosporine (Sta, 1 μM), staurosporine plus PMA, or 4α-phorbol 12,13-didecanoate (4α-PDD, 1 μM) for the indicated time and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of four separate experiments determined in triplicate. $*P<0.01$; $**P<0.001$, as compared with non-treated cells (Con) stimulated by BK. Open column, basal level; hatched column, stimulated by BK.

Effect of PTX and CTX on BK-induced IPs accumulation

To gain insight into the identity of the coupling G protein in the BK signal transduction mechanism, cultured TSMCs

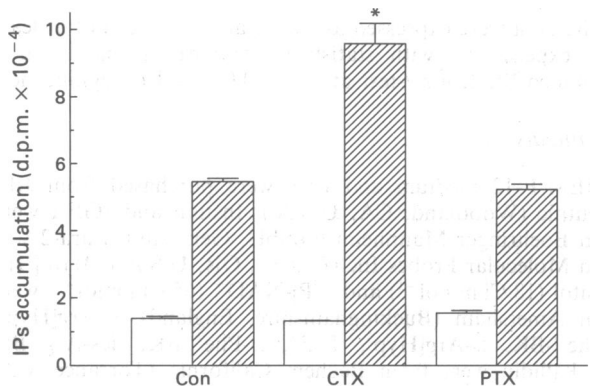

Figure 5 Effects of cholera toxin and pertussis toxin on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells were treated with either cholera toxin (CTX, 10 μg ml^{-1}) or pertussis toxin (PTX, 100 ng ml^{-1}) for 4 h and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of five separate experiments determined in triplicate. $*P<0.001$, as compared with control (Con) cells. Open column, basal level; hatched column, stimulated by BK.

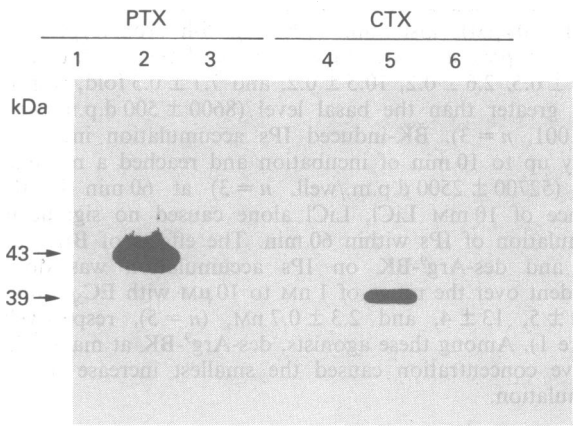

Figure 6 ADP-ribosylation of the G proteins. Cultured TSMCs were pretreated with either pertussis toxin (PTX, 100 ng ml^{-1}, lane 3), or cholera toxin (CTX, 10 μg ml^{-1}, lane 6) for 4 h. Control and toxin-treated samples were then assayed for ADP-ribosylation of the remaining available substrates in the presence of [^{32}P]-NAD$^+$ and PTX (Lanes 2–3) or CTX (Lanes 5–6) as described in Methods. Lane 1, control for PTX assay; Lane 4, control of CTX assay.

were preincubated with either CTX or PTX, and IPs accumulation in response to BK was examined. As shown in Figure 5, pretreatment of TSMCs with PTX (100 ng ml^{-1}, 4 h) had no effect on the basal or BK-stimulated IPs accumulation. In contrast, incubation of TSMCs with CTX (10 μg ml^{-1}, 4 h) enhanced BK-induced IPs accumulation by 75% ($P<0.001$, $n=5$, as compared with control).

ADP-ribosylation of G protein

To characterize the alteration of the α-subunit of G proteins by PTX and CTX, cultured TSMCs were incubated in the presence or absence of these toxins, and then assayed for ADP-ribosylation of any remaining unmodified G protein in the presence of [^{32}P]-NAD$^+$ (Figure 6). Control cultures of TSMCs showed a major substrate for PTX at 43 kDa, which was completely ribosylated under the experimental conditions used here. The major substrate for CTX was evident with a molecular weight of 41 kDa. Pretreatment of TSMCs with PTX or CTX for 4 h resulted in complete abolition of label incorporation into this substrate. These results confirmed that pretreatment of TSMCs with these two toxins is adequate to determine the roles of various G proteins involved in the BK signal transduction.

Effect of PMA on direct stimulation of G proteins

To determine the site of PMA treatment-related decrease in IPs accumulation induced by BK, AlF_4^- was used to stimulate directly G proteins to generate IPs. Figure 7 reveals that 1 μM BK or 10 μM AlF_4^- elicited essentially identical IPs accumulation in intact TSMCs. Pretreatment of TSMCs with 1 μM PMA for 30 min, inhibited the IPs accumulation induced by BK or AlF_4^- ($P<0.001$, $n=3$, as compared with non-treated cells). Parallel experiments were performed with digitonin-permeabilized TSMCs and GTPγS to activate G protein directly. IPs accumulation induced by GTPγS was also inhibited by PMA treatment (data not shown). These results suggest that the inhibitory effect of PMA on IPs accumulation may be due to activation of PKC and subsequently uncouple G proteins to PLC.

Dependence of BK-stimulated IPs accumulation on extracellular Ca^{2+}

To determine whether Ca^{2+} influx is required for the activation of PLC, BK-induced IPs accumulation was performed in Ca^{2+}-free KHS buffer. Results in Figure 8 illustrate the dependence of BK-induced IPs accumulation on Ca^{2+} influx. TSMCs preincubated in Ca^{2+}-free KHS or in Ca^{2+}-free KHS plus 0.5 mM EGTA for 5 min, led to an attenuation of the BK-induced response. Furthermore, BK-induced IPs accumulation was slightly affected by pretreatment with the Ca^{2+}-channel blockers diltiazem and verapamil at a concentration of 10 μM (Figure 9). However, Ni^{2+} (5 mM) significantly inhibited BK-induced IPs accumulation (Figure 9; $P<0.001$, $n=3$, as compared with control).

To assess more directly the role played by physiologically relevant Ca^{2+} concentrations in the regulation of the activity of PLC, [^{3}H]-inositol-labelled TSMCs were first permeabilized in digitonin-containing KGEH buffer and then exposed to Ca^{2+}-EGTA buffer, in which the $[Ca^{2+}]_f$ had been measured directly by the addition of fura-2 (free acid). IPs accumulation was measured after 60 min incubation. Elevation of $[Ca^{2+}]_f$ from 3 to 620 nM resulted in a 35% increase in IPs accumulation, with an EC_{50} of 163 ± 18 nM, $n=3$ (Figure 10). The addition of 50 μM GTPγS further potentiated this Ca^{2+}-dependent IPs accumulation with an EC_{50} for Ca^{2+} of about 25 ± 7 nM ($n=3$). The effect of BK was potentiated by the inclusion of 50 μM GTPγS, such that, under these conditions, the $[Ca^{2+}]_f$ required to elicit a half-maximal increase in IPs accumulation was reduced from 125 ± 15 nM to 3.9 ± 1.2 nM ($n=3$). When BK and GTPγS were added simultaneously, the stimulated accumulation of IPs was 313 ± 26% of control (n = 3), a value that compares favourably with the degree of stimulation obtained in intact cells. Therefore, GTPγS and BK together elicit an additive increase in IPs accumulation.

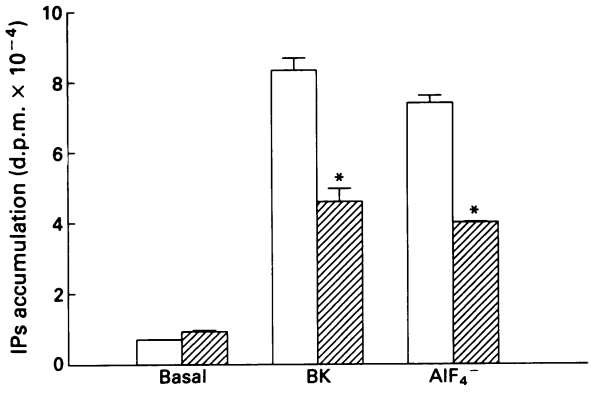

Figure 7 Effects of phorbol 12-myristate 13-acetate (PMA) on direct stimulation of G proteins coupling to phosphoinositide (PI) hydrolysis in intact TSMCs. Cells prelabelled with [^{3}H]-inositol were washed with KHS and incubated in the presence or absence of 1 μM PMA for 30 min. The cells were then exposed to 1 μM bradykinin (BK) or 10 μM AlF_4^- (10 μM $AlCl_3$ + 10 mM NaF) for 60 min. The accumulation of inositol phosphates (IPs) was determined as described under Methods. Values are expressed as the mean ± s.e.mean from three separate experiments determined in triplicate. *$P<0.001$, as compared with untreated cells stimulated by BK. Open column, control; hatched column, treated with PMA.

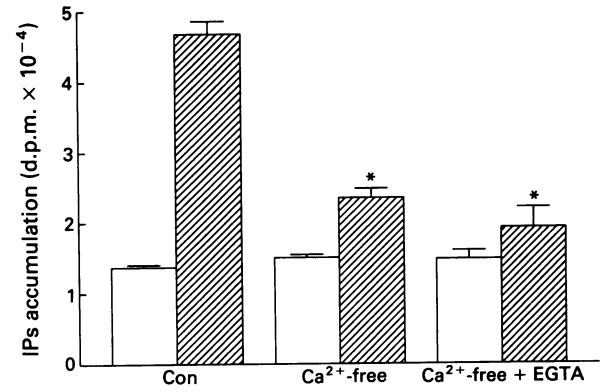

Figure 8 Calcium dependence of bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells were prelabelled with [^{3}H]-inositol for 2 days. Labelled cells were incubated in either KHS, Ca^{2+}-free KHS or Ca^{2+}-free KHS plus 0.5 mM EGTA. Then BK (1 μM) was added and continuously incubated for 60 min. [^{3}H]-IPs were determined as described under Methods. Results are the mean ± s.e.mean of three separate experiments determined in triplicate. *$P<0.001$, as compared with that of cells induced by BK alone in KHS buffer (Con). Open column, basal level; hatched column, stimulated by BK.

Discussion

It is well established that receptor activation by neurotransmitters, growth factors, hormones, and light causes rapid PI hydrolysis (Rana & Hokin, 1990). In the present study, we have demonstrated that BK induces PI turnover in canine TSMCs by interacting with its receptors coupled to PLC via a G protein. BK-stimulated IPs accumulation is time- and dose-dependent, and regulated by $[Ca^{2+}]_f$ and GTPγS, a non-hydrolyzable analogue of GTP. BK-stimulated inositol

phospholipid-specific PLC may be regulated by PKC stimulation via the formation of DAG. Short-term treatment of TSMCs with PMA dramatically inhibits BK-stimulated IPs accumulation. One possible consequence for the activation of this arm of the second messenger pathway is the presence of a short inhibitory feedback loop in which DAG formation and PKC activation cause an attenuation of agonist-stimulated PLC activity. This mechanism has been proposed for the regulation of agonist-stimulated PLC in a number of cell types (Castagna *et al.*, 1982; Leeb-Lungberg *et al.*, 1985; Orellana *et al.*, 1985).

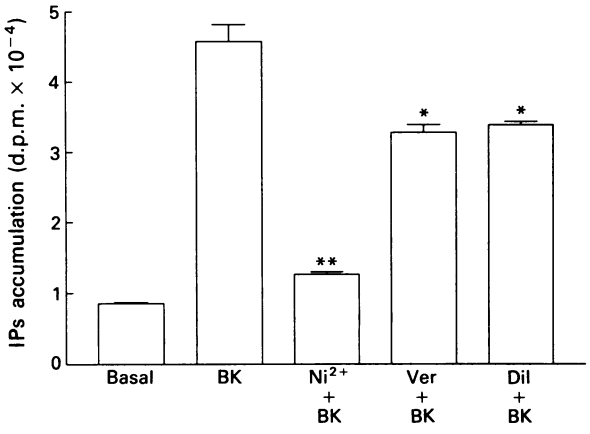

Figure 9 Effects of Ca^{2+}-channel blockers on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. [^{3}H]-inositol-labelled cells were preincubated with diltiazem (Dil, 10 μM), verapamil (Ver, 10 μM), or Ni^{2+} (5 mM) for 30 min and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results are the mean ± s.e.mean of three separate experiments determined in triplicate. $*P<0.01$; $**P<0.001$, as compared with control.

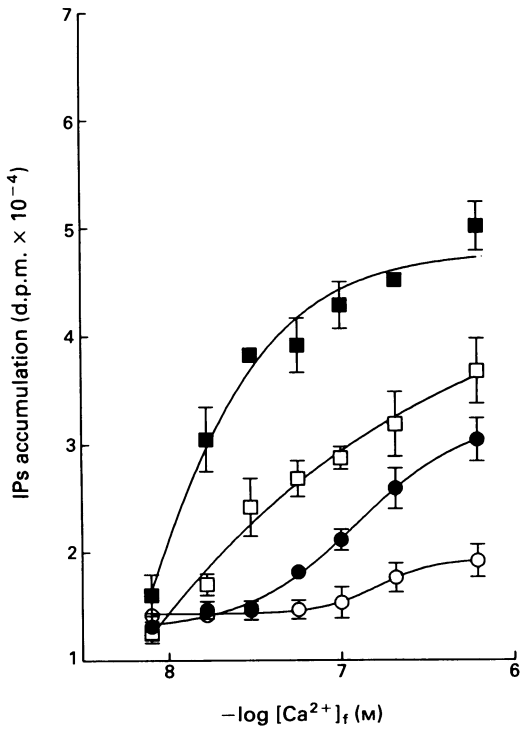

Figure 10 Increase in $[Ca^{2+}]_f$ causes the release of inositol phosphates (IPs) from digitonin-permeabilized TSMCs. [^{3}H]-inositol-prelabelled cells were permeabilized and incubated in KGEH buffer with added Ca^{2+} to achieve the required $[Ca^{2+}]_f$ (as measured by the addition of fura-2). Cells were incubated for 60 min in the absence (○) or presence of either bradykinin (BK, ●, 1 μM), guanosine 5'-O-(3-thiotriphosphate) (GTPγS, □, 50 μM), or BK plus GTPγS (■). The EC_{50} values for basal, BK, GTPγS, and BK plus GTPγS were 163, 125, 25, and 3.9 nM, respectively. Results are expressed as means ± s.e.mean from three separate experiments determined in triplicates. $[Ca^{2+}]_f$ values of 7.93, 16.61, 39.89, 56.35, 100.4, 207.3 and 616.8 nM were obtained at Ca^{2+}/EGTA molar ratios of 0.25, 0.38, 0.50, 0.63, 0.75, 0.88 and 1, respectively, with EGTA maintained at a concentration of 2 mM.

The discrepancy in the dose-response relationship for the effects of BK, kallidin, and des-Arg^9-BK on the IPs accumulation in canine TSMCs is consistent with those reported by others using different tissues and cell preparation (Regoli *et al.*, 1990). Our data obtained from the IPs accumulation induced by these agonists show that BK and kallidin produce a larger response than des-Arg^9-BK (Figure 1). The EC_{50} value of des-Arg^9-BK is very low, *circa* 2 nM. This may imply that there is a significant population of B_1 receptors in cultured TSMCs. It has been suggested that BK induces its effects through at least two types of receptors, which have been characterized as B_1 and B_2 receptors (Regoli *et al.*, 1990). BK and kallidin have high affinity for B_2 receptors and low affinity for the B_1 receptors (Regoli *et al.*, 1990). In contrast, des-Arg^9-BK has low affinity for B_2 receptors but high affinity for the B_1 receptors (Regoli *et al.*, 1990). Therefore, our findings may reflect the presence of both B_1 and B_2 receptors in canine TSMCs.

As an alternative approach to define the receptor subtypes, the determination of antagonist affinities can provide more accurate information than the use of agonist relative potencies. From the experiments, the effects of discriminating antagonists have been analysed at the level of the biochemical response to BK. The results obtained with D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, differentiated the receptor subtype mediating IPs accumulation (Figure 2). The pK_B values for the B_2 antagonists D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK were 7.1 and 7.3, respectively, consistent with the involvement of B_2 receptors in the IP response to BK. These results are in good agreement with those reported by others in the rabbit jugular vein (Regoli *et al.*, 1990) and in bovine TSMCs (Marsh & Hill, 1992).

Because PKC activation is associated with several cellular responses, phorbol ester-mediated inhibition of IP_3 formation might occur at one or more different sites. In a number of cell types, elevation of $[Ca^{2+}]_i$ by Ca^{2+}-mobilizing agonists known to act by receptor-mediated stimulation of PI turnover, has been shown to be inhibited by phorbol esters (Castagna *et al.*, 1982; Leeb-Lundberg *et al.*, 1985; Orellana *et al.*, 1985). It has been suggested that protein phosphorylation mediated by interaction of phorbol ester with PKC may be the mechanism by which PMA modulates hormone-sensitive PI metabolism. One proposed mechanism by which phorbol ester might attenuate a rise in IP_3 is due to its increasing degradation mediated by activation of a phosphomonoesterase specific for IP_3 (Watson & Lapetina, 1985; Connolly *et al.*, 1986). The activity of this cytosolic enzyme increases after phosphorylation by PKC which provides a mechanism for the inhibition of the agonist-induced rise in IP_3 concentration in platelets. We observed that PMA blocks both BK-stimulated IPs accumulation and also AlF_4^--mediated IPs formation in canine TSMCs (Figures 4 and 7). After prolonged treatment of TSMCs with PMA, BK induced a fast rise in IPs accumulation, which reaches a plateau greater than that of the control level. We therefore propose that DAG, which is formed during stimulation with BK, prevents any additional increase in IPs accumulation via PKC activation. Our results indicate that PMA acts through the activation of PKC with subsequent protein phosphorylation, since staurosporine, a potent PKC inhibitor, blocks the inhibitory effect of PMA.

BK-stimulated IPs accumulation is almost completely dependent on the presence of extracellular Ca^{2+} (Figure 8). This calcium dependence is similar to PI response to agonists in several types of cells (Fisher *et al.*, 1989; Eberhard & Holz,

1991; Yang & Chou, 1992). In the present study, TSMCs prelabelled with [^{3}H]-inositol for 2 days and then exposed to BK, caused a rapid release of IPs in the presence of external Ca^{2+} (1.8 mM). However, Ca^{2+}-free buffer or inclusion of 0.5 mM EGTA almost completely attenuates the BK-induced increase in IPs accumulation. Furthermore, the BK-induced effect is slightly affected by the presence of the Ca^{2+}-channel blockers, diltiazem and verapamil (Figure 9). However, the non-selective Ca^{2+}-channel blocker, Ni^{2+}, did inhibit the BK-stimulated response (Figure 9), indicating that Ca^{2+} influx may be required for the activation of PLC through an unidentified pathway.

Further indication for the requirement of Ca^{2+} for PI hydrolysis has been obtained from experiments with digitonin-permeabilized TSMCs (Figure 10). In the presence of BK and GTPγS, the extent of PI breakdown in permeabilized TSMCs appears to be the same as that obtained in intact cells stimulated by BK alone. Since GTPγS alone could also stimulate IPs accumulation in permeabilized cells, a guanine nucleotide binding protein might be involved in the transduction process (Evans *et al.*, 1985; Merritt *et al.*, 1986; Jones *et al.*, 1988). Although BK does enhance IPs accumulation without the addition of GTPγS, this ability presumably reflects the presence of residual endogenous guanine nucleotides within the permeabilized cells. However, the combination of BK and GTPγS has an additive effect on IPs accumulation. GTPγS may play a role in the breakdown of PI that reduces the requirement of $[Ca^{2+}]_f$ for PLC activity (Bradford & Rubin, 1986; Smith *et al.*, 1986). The availability of $[Ca^{2+}]_f$ is required for stimulation of PI hydrolysis. A half-maximal (EC_{50}) increase of IPs accumulation induced by BK alone occurs at a $[Ca^{2+}]_f$ greater than 125 nM, while in the presence of GTPγS, only 3.9 nM $[Ca^{2+}]_f$ is required for BK to induce a half-maximal increase of IPs accumulation. Therefore, guanine nucleotides appear to sensitize the transduction process such that BK effectively initiates PI hydrolysis at a $[Ca^{2+}]_f$ that is encountered in quiescent cells.

G proteins are involved in receptor coupling to PLC activity for many agonists (Sternweis & Smrcka, 1992). Although we have shown that the presence of an ADP-ribosylated protein of molecular weight at 43 and 41 kDa is demonstrated in PTX- and CTX-treated cells (Figure 6), respectively, our results demonstrate that BK stimulation of PLC activity is not sensitive to inhibition by these two toxins (Figure 5). This suggests that a coupling process occurs in these cells through a mechanism which is not mediated through a PTX- or CTX-sensitive G protein. Similar results have been reported for several cell types by others (Etscheid & Villereal, 1989; Galron *et al.*, 1990; Gutowski *et al.*, 1991). BK-stimulated IPs accumulation in TSMCs is enhanced by short-term (4 h) treatment with CTX (Figure 5). Signal transduction processes have been reported that require GTP but are not affected by toxins (Martin *et al.*, 1985; Aub *et al.*, 1986). Furthermore, it has been shown that treatment of cells with CTX or PTX enhances PI turnover in rat heart myocytes and human foreskin fibroblasts (Etscheid & Villereal, 1989; Galron *et al.*, 1990). The precise mechanism of action of these toxins is not known.

In conclusion, our results demonstrate that BK-stimulated IPs accumulation is mediated by the activation of PLC coupling to a G protein which is not blocked by CTX or PTX treatment and activated by external Ca^{2+}. Short-term PMA treatment results in a negative feedback regulation on agonist-induced IPs accumulation. Long-term PMA treatment might be associated with augmenting responses to agonists, perhaps due to down-regulation of PKC and the loss of its inhibitory function. These results suggest that physiological activation of PKC might serve as a modulator of cellular responses induced by IP_3. The site of PMA inhibition appears to be at a postreceptor level. Determination of how DAG and other second messengers modulate agonist-induced cellular responses is important in order to clarify the mechanisms underlying bronchial hyperreactivity of the airway in asthma.

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