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Pharmacological and functional characterization of bradykinin receptors in canine cultured tracheal epithelial cells

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1 A direct [^{3}H]-bradykinin ([^{3}H]-BK) binding assay has been used to characterize the BK receptors in canine cultured tracheal epithelial cells (TECs). Based on receptor binding assay, TECs have specific, saturable, high-affinity binding sites for [^{3}H]-BK.

2 The specific [^{3}H]-BK binding was time- and temperature-dependent. Equilibrium of association of [^{3}H]-BK with the BK receptors was attained within 30 min at room temperature and 1 h at 4°C, respectively.

3 Analysis of binding isotherms yielded an apparent equilibrium dissociation constant (K_D) of 1.5 ± 0.2 nM and a maximum receptor density (B_{max}) of 53.2 ± 5.2 fmol mg^{-1} protein. The Hill coefficient for [^{3}H]-BK binding was 1.00 ± 0.02. The association (K_1) and dissociation (K_{-1}) rate constants were $(7.6 \pm 1.1) \times 10^6$ M^{-1} min^{-1} and $(9.2 \pm 1.5) \times 10$ M^{-3} min^{-1}, respectively. K_D, calculated from the ratio of K_{-1} and K_1, was 1.2 ± 0.3 nM, a value close to that calculated from Scatchard plots of binding isotherms.

4 Neither a B_1 receptor selective agonist (des-Arg^9-BK, 0.1 nM - 10 μM) nor antagonist ([Leu^8, des-Arg^9]-BK, 0.1 nM - 10 μM) significantly inhibited [^{3}H]-BK binding to TECs, which excludes the presence of B_1 receptors in canine TECs.

5 The specific binding of [^{3}H]-BK to canine TECs was inhibited by the B_2 receptor selective antagonists ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Hoe 140, 0.1 nM - 10 μM) and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK, 0.1 nM - 10 μM) and agonists (BK and kallidin, 0.1 nM - 10 μM) with a best fit by a one-binding site model. The order of potency for the inhibition of [^{3}H]-BK binding was kallidin = BK = Hoe 140 > [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK.

6 BK and kallidin significantly induced concentration-dependent accumulation of IPs with a half-maximal response (EC_{50}) at 17.6 ± 3.5 and 26.6 ± 5.3 nM, respectively, while the B_1-selective agonist, des-Arg^9-BK did not stimulate IPs accumulation and the B_1-selective antagonist [Leu^8, des-Arg^9]-BK did not inhibit BK-induced IPs accumulation. Two B_2-selective antagonists, Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK, inhibited BK-stimulated IPs accumulation with apparent pK_B values of 8.8 ± 0.3 and 7.0 ± 0.3, respectively.

7 It is concluded that the pharmacological characteristics of the BK receptors in canine cultured TECs are primarily of the B_2 receptor subtype which might regulate the function of tracheal epithelium through the activation of this receptor subtype coupling to PI hydrolysis.

Keywords: Bradykinin receptor; canine tracheal epithelial cells; inositol phosphates; kinins

Introduction

Kinins are potent vasoactive peptides that display a range of powerful proinflammatory properties. Kinin generation in upper airway secretions has been demonstrated during experimental models of allergic rhinitis (Proud *et al.*, 1983; Naclerio *et al.*, 1985), as well as during experimental (Naclerio *et al.*, 1988) and natural (Proud *et al.*, 1990) rhinovirus colds. Bradykinin (BK), one of the kinin family, is a classical mediator of inflammatory diseases of the airways and is implicated in allergic asthma (Christiansen *et al.*, 1987; Farmer *et al.*, 1991). In the airways, BK causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). It is well established that the kinins, BK, kallidin, and des-Arg^9-BK, interact with two BK receptor subtypes, which have been classified as B_1 and B_2 (Regoli *et al.*, 1990). On the basis of the relative potencies of these three agonists, preparations with higher affinity for BK than for des-Arg^9-BK are considered to express B_2 receptor activity, while preparations having opposite affinity are considered to express B_1 receptor activity (Proud & Kaplan, 1988; Regoli *et al.*, 1990; Yang *et al.*, 1995). The use of B_2-selective antagonists has further strengthened the existence of such a receptor subtype (Regoli *et al.*, 1990). In addition, studies with B_2 receptor selective antagonists have revealed that further heterogeneity exists among BK receptors (Farmer *et al.*, 1989; Regoli *et al.*, 1990; Stewart & Vavrek, 1990). The existence of a B_3 receptor subtype has been proposed in guinea-pig trachea and lung, principally due to a lack of activity of B_2 receptor-selective antagonists (Farmer *et al.*, 1989).

Binding and functional studies have provided evidence for the B_2 receptor subtype in guinea-pig ileum and lung and in rat myometrium membranes and vas deferens (Manning *et al.*, 1986; Plevin & Owen, 1988; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991). We have previously reported that BK induces an increase in phosphoinositide (PI) hydrolysis and a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in canine cultured tracheal smooth muscle cells which appears to be mediated via activation of the B_2 receptors (Yang *et al.*, 1994a,b). The existence of B_2 receptor subtype in tracheal smooth muscle cells was further confirmed by a direct binding assay (Yang *et al.*, 1995). However, there is no information on the distribution of BK receptor subtypes in canine tracheal epithelial cells (TECs). Therefore, the pharmacological characteristics of the BK receptor subtypes present in canine cultured TECs need to be

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defined. Characterization and localization of these receptors are of importance in understanding how physiological responses are mediated at the BK receptor level.

BK has been shown to stimulate chloride secretion in native canine tracheal epithelium (Leikauf *et al.*, 1985), but the mechanism by which it stimulates secretion is not well understood. In many cell types, including the neuroblastoma-glioma hybrid NG108-15 (Osugi *et al.*, 1987), human astrocytoma cell line D 384 (Balmforth *et al.*, 1992), and canine tracheal smooth muscle cells (Yang *et al.*, 1994a), BK receptors activate phospholipase C-mediated PI hydrolysis in the plasma membrane. Subsequently, the resultant IP_3 releases Ca^{2+} from its intracellular stores (Marsh & Hill, 1993; Yang *et al.*, 1994b). Thus, in terms of second messenger generation, enhancement of tracheal secretory function may be mediated by IP_3-induced Ca^{2+} mobilization from its internal stores.

The purpose of this study was to characterize the BK receptors in canine cultured TECs by a radioligand [^{3}H]-BK binding assay by measurement of PI metabolism. To determine whether BK receptor subtypes are present in canine TECs, competitive inhibition of [^{3}H]-BK binding was performed with B_1 and B_2 receptor selective agonists and antagonists being used as competing ligands. Moreover, we investigated the effects of BK and its analogues on PI hydrolysis in canine TECs. The BK receptor subtype coupled to PI hydrolysis was differentiated by use of selective BK receptor agonists and antagonists.

Methods

Animals

Mongrel dogs, 10–20 kg, both male and female were purchased from a local supplier. Dogs were housed indoors in the animal facilities under automatically controlled temperature and light cycle conditions and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthesized with ketamine (20 mg kg^{-1}, intramuscularly) and pentobarbitone (30 mg kg^{-1}, intravenously). The tracheae were surgically removed.

Isolation and culture of tracheal epithelial cells

Cells were isolated essentially as described by Wu *et al.* (1985). The trachea was cut longitudinally through the cartilage rings, and a strip epithelium was pulled off the submucosa, rinsed with phosphate-buffered saline (PBS) containing 5 mM dithiothreitol, and digested with 0.05% protease XIV in PBS at 4°C for 24 h; after vigorous shaking of the strips at room temperature, 5 ml of foetal calf serum (FCS) was added to terminate the digestion. The released cells were collected and washed twice with 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's nutrient F-12 medium that contained 5% FCS, nonessential amino acids, penicillin (100 u/ml^{-1}, streptomycin (100 μg ml^{-1}), gentamicin (50 μg ml^{-1}), and fungizone (2.5 μg ml^{-1}). Cell number was counted and diluted with DMEM/F-12 to 2×10^6 cells ml^{-1}. The cells were plated onto (0.5 ml/well) 24-well and (1 ml/well) 12-well culture plates coated with collagen for receptor binding assay and IPs accumulation, respectively. The culture medium was changed after 24 h and then changed every two days.

In order to characterize the isolated and cultured TECs, an indirect immunofluorescent staining was performed as described by O'Guin *et al.* (1985) using AE1 and AE3 mouse monoclonal antibodies and fluorescein isothiocyanate (FITC)-labelled goat antimouse IgG. Over 96% of the cells were epithelial cells.

[^{3}H]-BK binding assay

Binding assays were performed with confluent TECs on 24-well culture plates in DMEM/F-12 containing 1% FCS for 24 h prior to the binding experiments, as described by Yang *et al.* (1994c; 1995). Culture medium was removed and 1 ml of binding buffer (20 mM HEPES, pH 7.4, 17 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.21 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 110 mM N-methylglucamine, 0.1% (w/v) BSA and 2 mM bacitracin) was added to each well. Cells were equilibrated on ice for 10 min, after which the binding buffer was replaced with 0.25 ml of binding buffer containing the appropriate concentration of [^{3}H]-BK in the absence or presence of unlabelled BK (10 μM). After 4 h incubation at 4°C, the binding buffer was removed and cells were washed three times with 2 ml of binding buffer at 4°C. Cells were suspended in 0.25 ml of 0.1 N NaOH and counted in a radiospectrometer. The amount of specific binding was calculated as the total binding minus the binding in the presence of 10 μM unlabelled BK. Total receptor density (B_{max}) and dissociation constant (K_D) were calculated by Ligand programme, as described previously (Yang *et al.*, 1991). Protein concentration was measured by the method of Bradford (1976).

Kinetic assays

The kinetic studies were performed at room temperature and at 4°C. For the association rate constant and time course, 250 μl of [^{3}H]-BK (3 nM) was added to the cells at different time intervals. The dissociation rate constant was determined by first equilibrating the cells with 3 nM [^{3}H]-BK at room temperature and 4°C for 1 h and 2 h, respectively. At this time (time 0), 10 μM unlabelled BK was added and determinations were made at various time intervals over a 2 h period.

Analysis of binding data

Equilibrium dissociation constant (K_D) and maximal receptor density (B_{max}) were calculated by the Graph Pad Programme (Graph Pad, San Diego, U.S.A.) in a linear regression analysis of the transformed data. Half-maximal inhibitory concentration (IC_{50}) values were calculated from competition experiments by Graph Pad Programme. IC_{50} values were transformed to apparent inhibitory constant (K_i) values. Subtype analysis was performed by fitting the competitive inhibition curves with either a one- or a two-binding site model using an iterative least-squares fit by Graph pad Programme that corrected for occupancy of [^{3}H]-BK with statistical significance established by Fisher's F test (Zar, 1974).

Accumulation of inositol phosphates

The effect of BK on the hydrolysis of PI was assayed by monitoring the accumulation of [^{3}H]-IPs as described by Berridge *et al.* (1983). Cultured TECs were incubated with 5 μCi ml^{-1} of *myo*-[2-^{3}H]-inositol at 37°C for 24 h. TECs were washed twice 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 2 μM indomethacin and 10 μM phosphoramidon. BK receptor antagonists, when used, were added 30 min before the addition of BK. Reactions were terminated by addition of 5% perchloric acid followed by sonication and centrifugation at 3000 *g* for 15 min.

The perchloric acid-soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied to 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 [^{3}H]-inositol and glycerophosphoinositol, respectively. Total IPs was eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^{3}H]-IPs was determined in a radiospectrometer (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Analysis of data

The EC_{50} values were estimated by the Graph Pad Programme (Graph Pad, San Diego, CA, U.S.A.). The concentration-effect curves were constructed by non-cumulative addition of BK and its analogues and fitted by sigmoid curve (log scale). The dissociation constants (K_B) of BK antagonists were estimated by the method developed by Furchgott (1972), from their ability to antagonize BK-induced IPs accumulation. The equation used to derive pK_B estimates is as follows:

$$pK_B = \log(DR - 1) - \log[\text{antagonist}]$$

Dose-ratio (DR) is the ratio of EC_{50} values for BK in the presence and absence of antagonist.

The data were expressed as the mean ± s.e. mean of at least four experiments with statistical comparisons based on Student's two-tailed t test at a $P<0.05$ level of significance.

Chemicals

DMEM/F-12 medium and FCS were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Insulin and IGF-I were from Boehringer Mannheim (GmbH, Germany). [^{3}H]-BK (67 Ci mmol^{-1}) was from Du pont NEN (Boston, MA, U.S.A.). [^{3}H]-*myo*-inositol (18 Ci mmol^{-1}) was from Amersham (Buckinghamshire, England). BK, des-Arg^9-BK, [Leu^8, des-Arg^9]-BK, [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK and Hoe 140 ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK) were from Peninsula Laboratories (Belmont, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.). Indomethacin was dissolved in dimethyl sulphoxide as a 1 mM stock solution. All the other reagents were prepared as 1 mM stock solutions and diluted with de-ionized water to the appropriate concentration.

Results

Kinetic constants of [^{3}H]-BK binding to TECs

Specific [^{3}H]-BK binding to TECs was time- and temperature-dependent. Binding of [^{3}H]-BK to TECs reached an apparent equilibrium within 30 min and 1 h at room temperature and 4°C (Figure 1a). Half maximal bindings occurred within 5 and 10 min at room temperature and 4°C, respectively. At 4°C, no [^{3}H]-BK dissociation from TECs was observed (Figure 1). After 60 min association at room temperature, dissociation was initiated by addition of 10 μM unlabelled BK which resulted in a reduction of specifically bound [^{3}H]-BK (Figure 1b). Half dissociation occurred within 20 min. The observed association rate constant (K_{obs}) was 0.032 ± 0.009 min^{-1} and the dissociation rate constant (K_{-1}) was (9.2 ± 1.5) × 10^{-3} min^{-1}. The value of the association rate constant (K_1) calculated from the equation $K_1 = (K_{obs} - K_{-1})$/[radioligand] was (7.6 ± 1.1) × 10^6 M^{-1} min^{-1}. A K_D value of 1.2 ± 0.3 nM calculated from the ratio of the rate constants, K_{-1}/K_1, agreed reasonably well with the K_D (1.5 ± 0.2 nM) determined by Scatchard analysis of saturation isotherms, as shown in Figure 2b.

Saturability of [^{3}H]-BK binding

The saturability of [^{3}H]-BK binding was measured by incubating the cultured TECs with varying concentrations of [^{3}H]-BK from 0.1 to 12 nM (Figure 2a). The saturation isotherm is a rectangular hyperbola, suggesting that a single population of saturable high affinity BK binding sites exists. Nonspecific binding, on the other hand, increased linearly with increasing [^{3}H]-BK concentration up to 14 nM. Scatchard plot analysis of specific bound [^{3}H]-BK in the cultured TECs gave an apparent dissociation constant (K_D) of 1.5 ± 0.2 nM and a maximal receptor density (B_{max}) of 53.2 ± 5.2 fmol mg^{-1} protein, $n=6$, respectively (Figure 2b). A plot of binding data for [^{3}H]-BK according to the Hill equation gave a straight line with a Hill coefficient of 1.00 ± 0.02, suggesting the existence of one population of binding sites.

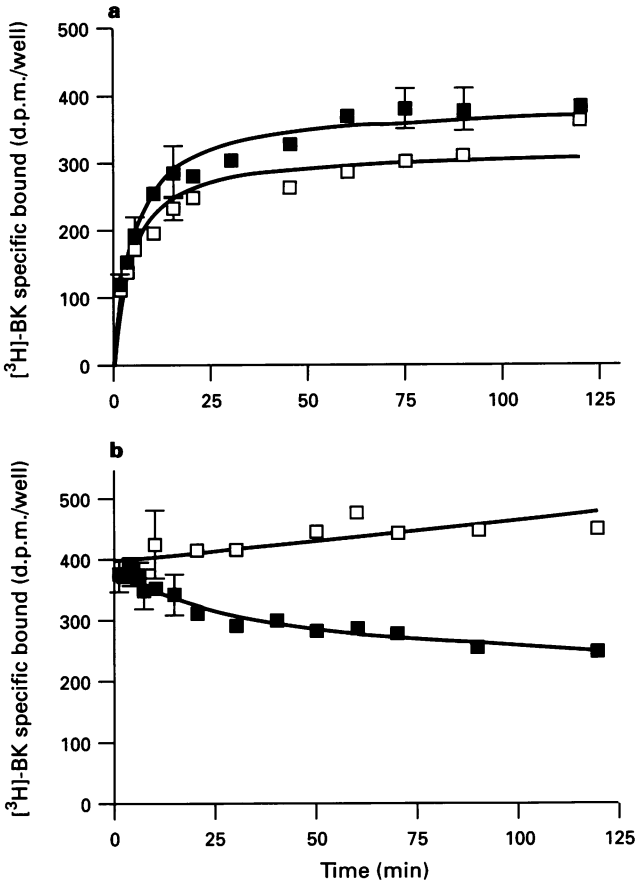

Figure 1 Time course of association and dissociation of specific [^{3}H]-BK binding to cultured TECs. For association (a), the cells were incubated with 3 nM [^{3}H]-BK for the various times indicated at room temperature (■) and 4°C (□). For determination of the dissociation rate constant (b), unlabelled BK at a final concentration of 10 μM was added after incubation with [^{3}H]-BK at room temperature (■) and 4°C (□) for 1 and 2 h, respectively. The data shown are the average of triplicate determinations from one experiment representative of three separate experiments.

Pharmacological specificity of [^{3}H]-BK binding

The pharmacological specificity of binding was derived by studying the inhibition of [^{3}H]-BK binding by BK receptor agonists and antagonists. Figure 3 shows that all of the drugs tested inhibited [^{3}H]-BK binding to BK receptors in a concentration-related manner except the B_1 receptor-selective agonist, des-Arg^9-BK and its antagonist [Leu^8, des-Arg^9]-BK, even at the highest concentration used (10 μM). The concentration of each drug required to inhibit 50% of the specific binding (IC_{50}) and their inhibition constant (K_i) in competition with [^{3}H]-BK are listed in Table 1. The order of potency for the inhibition of [^{3}H]-BK was kallidin = BK = Hoe 140 > [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK > > des-Arg^9-BK, [Leu^8, des-Arg^9]-BK. The competition curves were steep and could be adequately described by a one binding-site model (Figure 3). The pharmacological properties of the BK receptors are quite consistent with those of the B_2 receptors (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994; Yang *et al.*, 1995).

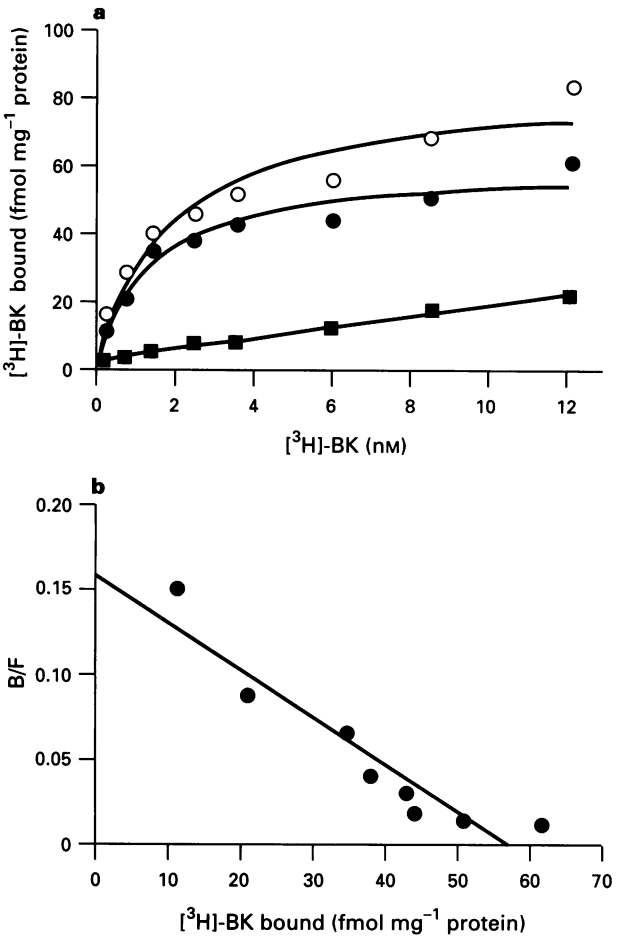

Figure 2 (a) Saturation isotherms of [^{3}H]-BK binding to canine cultured TECs incubated in triplicates with 0.1 to 12 nM [^{3}H]-BK at 4°C for 4 h as described in Methods. Specific binding (●) was experimentally determined as the difference between total binding (○) and nonspecific binding (■) in the absence and presence of 10 μM unlabelled BK. (b) Scatchard plots of specific [^{3}H]-BK binding data in (a). X-intercept of least-squares fit to Scatchard plot is a measure of maximal receptor density (B_{max}) and negative reciprocal of slope is the dissociation constant (K_D). Data are from one experiment representative of five separate experiments.

Table 1 Relative potencies of drugs for [^{3}H]-BK binding to canine cultured TECs.

Drug	*IC_{50} (nM)*	*K_i (nM)*
Agonist		
des-Arg^9-BK	>10000	>10000
Kallidin	14.9±3.7	3.2±0.8
Bradykinin	11.8±2.8	2.1±0.4
Antagonist		
[Leu^8, des-Arg^9]-BK	>10000	>10000
[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK	288±87	83.4±13.9
Hoe 140	15.8±2.4	2.8±0.6

[^{3}H]-BK (3 nM) binding was determined as described in Methods in the presence of increasing concentrations of drugs. Values are the mean±s.e.mean of three separate experiments determined in triplicate.

BK-induced IPs accumulation

Because a prominent action of BK in several cell types is to increase accumulation of IPs, we tested its coupling to the receptor present in canine TECs in this second messenger pathway. Canine cultured TECs were labelled for 24 h with

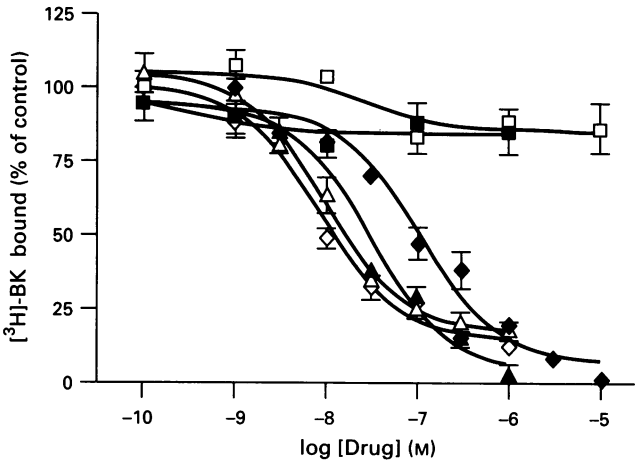

Figure 3 Competition for [^{3}H]-BK binding to canine cultured TECs by BK receptor agonists: (□) des-Arg^9-BK; (▲) kallidin; (△) bradykinin and antagonists, (■) [Leu^8, des-Arg^9]-BK: (◆) [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe]-BK; (◇) Hoe 140. Cells were incubated with various concentrations of the competing ligand in the presence of 3 nM [^{3}H]-BK. Nonspecific binding determined in the presence of 10 μM unlabelled BK was subtracted from total binding. The points shown are averages of triplicate determinations from one experiment representative of three experiments. The curves are best non-linear least squares, computer generated fits to the data as described under Methods.

[^{3}H]-inositol, stimulated with agonist, and [^{3}H]-IPs were extracted. The aqueous phase of cell extracts was used to determine the amount of [^{3}H]-IPs formation elicited by the agonist. BK-induced IPs accumulation increased rapidly up to 5 min of incubation and reached a maximal value (22600±1870 d.p.m./well, $n=5$) at 60 min in the presence of 10 mM LiCl, 10 μM phosphoramidon, and 2 μM indomethacin (Figure 4). The basal level of IPs was 10500±1750 d.p.m./well. LiCl alone caused no significant accumulation of IPs within 60 min (data not shown). The effects of BK and kallidin on IPs accumulation were dose-dependent over the range of 1 nM to 10 μM with EC_{50} values of 17.6±3.5 nM, and 26.6±5.3 nM, $n=5$, respectively (Figure 5). des-Arg^9-BK at a concentration of 10 μM did not cause any significant increase in IPs accumulation.

Effects of B_2 antagonists on BK-induced IPs accumulation

To determine which type of BK receptor is involved in the activation of PLC in canine TECs, we examined the effects of the B_1-selective antagonist ([Leu^8, des-Arg^9]-BK) and the B_2-selective antagonists, ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK, Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK), on BK-induced IPs accumulation. As shown in Figure 6, preincubation of TECs with these B_2-selective antagonists inhibited the BK-induced IPs accumulation. The concentration-effect relationship of BK was shifted to the right in a concentration-dependent manner, upon addition of Hoe 140 (0.3 and 0.5 μM) and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK (3 and 5 μM). At low concentrations, these two antagonists inhibited the BK-induced PI response in a competitive manner. However, at higher concentrations, both antagonists not only shifted the BK concentration-effect curve to the right, but also markedly suppressed the maximal response. The EC_{50} values of BK and dissociation constants (pK_B) of Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK calculated from the dose-ratios method are listed in Table 2. There was no significant difference in pK_B values between the antagonistic effect of these two antagonists on BK-induced IPs accumulation (Table 2). The pK_B values of Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK were 8.8±0.3 and 7.0±0.3, respectively. In contrast, the B_1-selective antagonist, [Leu^8, D-Arg^9]-BK, did not change the BK-induced

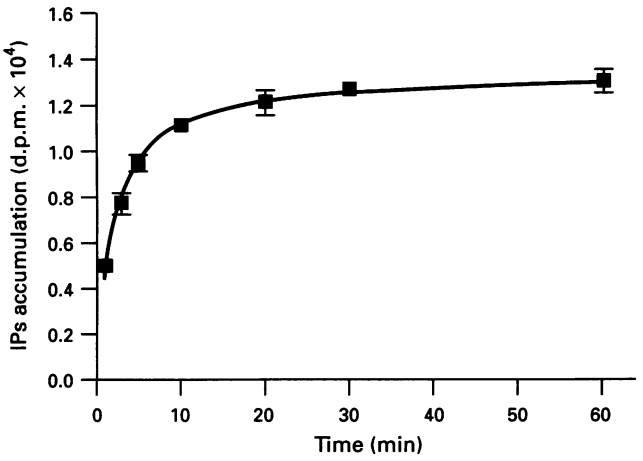

Figure 4 Time course for BK-induced [^{3}H]-IPs accumulation in cultured TECs. BK (10 μM) was added and the reaction was stopped at the time indicated. [^{3}H]-IPs was determined as described under Methods. The basal level of IPs (10500 ± 1750 d.p.m./well) was subtracted from the total IPs induced by BK. Results are expressed as the mean ± s.e.mean of five separate experiments determined in triplicate.

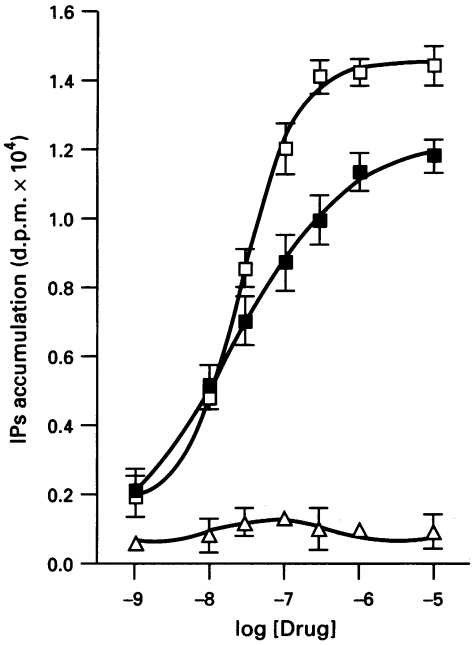

Figure 5 Dose-response curve for BK-, kallidin-, and des-Arg^9-BK-stimulated [^{3}H]-IPs accumulation in cultured TECs. Agonist (1 nM – 10 μM) was added and the reaction was stopped after 60 min incubation. [^{3}H]-IPs was determined as described under Methods. The basal level of IPs (10350 ± 1660 d.p.m./well) was subtracted from the total IPs induced by each agonist. Each point represents the mean ± s.e.mean of five separate experiments determined in triplicate. (□) BK; (■) kallidin; (△) des-Arg^9-BK.

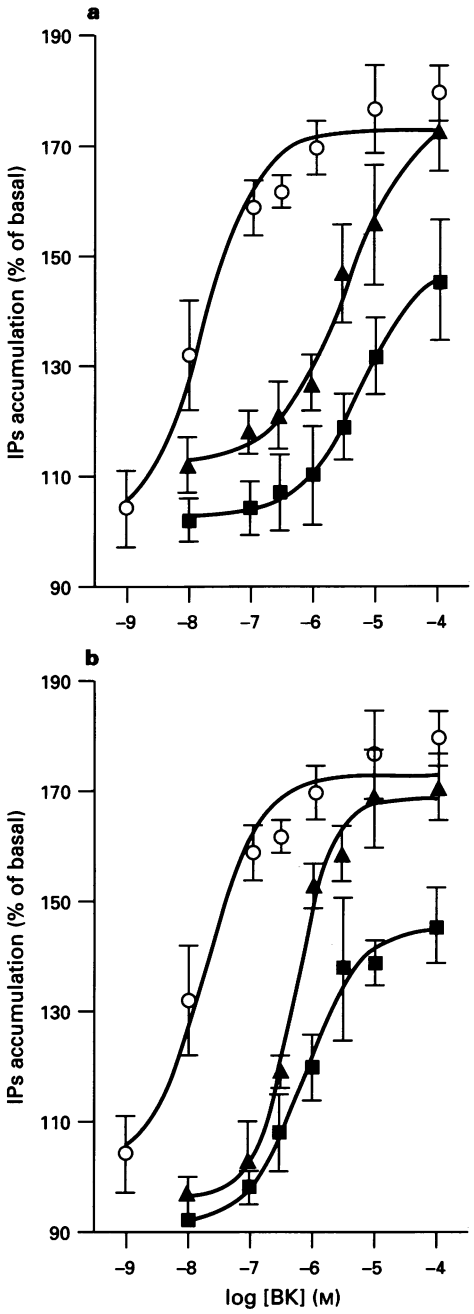

Figure 6 Inhibition of BK-induced [^{3}H]-IPs accumulation in TECs by (a) Hoe 140 and (b) [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK. [^{3}H]-IPs was determined as described under Methods. Each point was normalized to the basal levels of IPs (10050 ± 3500 d.p.m./well) and represents the mean with s.e.mean of nine experiments determined in triplicate. BK stimulated IPs accumulation was measured in the absence (○) and presence of Hoe 140 (▲, 0.3 μM; ■, 0.5 μM) or [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK (▲) 3 μM; (■) 5 μM).

IPs response (data not shown). These results indicate that the BK receptors mediating IPs accumulation in canine TECs have pharmacological properties similar to the BK B_2 receptors (Hock *et al.*, 1991; Eggerickx *et al.*, 1992; Marsh & Hill, 1992; Yang *et al.*, 1994a).

Discussion

Several lines of evidence demonstrate the presence of BK receptors in airway tissues (Leikauf *et al.*, 1985; Farmer *et al.*, 1989; Trifilieff *et al.*, 1991; 1994). It is well established that BK induces an increase in PI hydrolysis and a rise in $[Ca^{2+}]_i$ in canine and bovine cultured tracheal smooth muscle cells which appear to be mediated via the activation of the B_2 receptors (Marsh & Hill, 1992; 1993; Yang *et al.*, 1994a,b). In this study, we have characterized the pharmacological properties of BK receptors in canine cultured TECs by receptor binding assay with [^{3}H]-BK. [^{3}H]-BK has been shown to label BK receptors in guinea-pig lung and trachea, human and canine tracheal smooth muscle cells, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994; Yang *et al.*, 1995). Since [^{3}H]-BK is a peptide which does not penetrate the cell membrane, it allows the determination of cell surface BK

Table 2 The apparent dissosociation constants (K_B) for the BK B_2 receptor anatagonists inhibiting the BK-induced IPs accumulation in TECs

Antagonist	*EC_{50} (μM)*	*Dose-ratio*	*pKb*
[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK			
(3 μM)	0.49±0.09	28±3	7.0±0.2
(5 μM)	0.75±0.05	43±4	6.9±0.3
Hoe 140			
(0.3 μM)	3.29+0.43	187±15	8.8±0.3
(0.5 μM)	5.83±0.65	332±21	8.8±0.4

Values [means±s.e. for nine experiments] were calculated from the log concentration-response curves for BK as shown in Figure 6. The Ec_{50} value of BK-induced IPs accumulation in the absence of antagonist was 17.6±3.5 nM. $pK_B = -\log K_B$.

receptors on intact TECs. It is important to characterize the BK receptors on intact TECs, because the initiation of target cell response is dependent on the number and affinity of the cell surface receptors for its agonists. Therefore, this study helps in the clarification and understanding of the role of these receptors in tracheal functions.

The interaction of [^{3}H]-BK with BK receptors occurs rapidly at room temperature. The half-time for association of [^{3}H]-BK with TECs was 5 min. Dissociation occurred slowly with a half-life of approximately 20 min at room temperature. The equilibrium dissociation constant (K_D) of 1.2±0.3 nM as determined by kinetic experiments was in reasonable agreement with the K_D value of 1.5±0.2 nM derived from saturation isotherms, which indicates that the assumption of a single biomolecular reaction is valid.

Canine TECs were found to be enriched in [^{3}H]-BK-specific, saturable, high-affinity BK receptor-like binding sites. Scatchard analysis of binding data gave a K_D value of 1.5±0.2 nM and a B_{max} value of 53.2±5.2 fmol mg^{-1} protein. The binding sites most probably represent a single class of receptors without significant cooperative interactions, since Scatchard plots were linear and the Hill coefficients were near unity. Furthermore, the BK receptor active drugs competed with [^{3}H]-BK binding in a concentration-related manner. The order of potency was BK = kallidin > > des-Arg^9-BK (Table 1). It has been suggested that BK induces its effects through at least two receptor subtypes which have been characterized as B_1 and B_2 receptors (Regoli *et al.*, 1990). BK and kallidin have a high affinity for B_2 receptors and a low affinity for B_1 receptors (Regoli *et al.*, 1990). In contrast, des-Arg^9-BK has a high affinity for B_1 receptors, but low affinity for B_2 receptors (Regoli *et al.*, 1990). In this study, the B_1 receptor-selective antagonist, [Leu^8, des-Arg^9]-BK and agonist, des-Arg^9-BK, gave no displacement at concentrations up to 10 μM, excluding the presence of B_1 receptors in canine cultured TECs. The binding characteristics are similar to those of the BK receptors described for guinea-pig lung and trachea, canine and human tracheal smooth muscle cells, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994; Yang *et al.*, 1995).

Moreover, the competitive inhibition of specific [^{3}H]-BK binding by two B_2 receptor-selective antagonists, Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK confirmed the existence of a single population of BK binding sites in canine cultured TECs (Table 1). The order of potency of antagonists in this binding assay was Hoe 140 > [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK > > [Leu^8, des-Arg^9]-BK (Table 1). The highest affinity was obtained with Hoe 140, a potent B_2 receptor-selective antagonist (Hock *et al.*, 1991). The binding affinities for Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK were 2.8±0.6 and 83.4±13.9 nM, respectively, corresponding to the high affinity for B_2 receptors described in other tissues (Trifilieff *et al.*, 1991; 1994; Eggerickx *et al.*, 1992; Hess *et al.*, 1994; Yang *et al.*, 1995).

Furthermore, these pharmacological properties are shown by the receptors mediating BK-induced hydrolysis of PI. The discrepancy in the dose-response relationship for the effects of BK, kallidin, and des-Arg^9-BK on the IPs accumulation in canine TECs is consistent with those reported by others using different tissues and different cell preparations (Regoli *et al.*, 1990; Yang *et al.*, 1994a,b). Our data obtained from the IPs accumulation induced by these agonists show that the order of potency was BK = kallidin > des-Arg^9-BK (Figure 5). However, the EC_{50} for the BK-induced PI response is approximately an order of magnitude higher than the K_D values obtained from binding studies. This difference may be due to different experimental conditions. The PI response was conducted at 37°C vs. binding assay at 4°C. The other possibility may reflect that BK receptors could couple to different effector systems which may cross regulate each other. des-Arg^9-BK did not cause a significant accumulation of IPs. Therefore, the receptors involved in this response are not B_1 receptors; our findings reflect the presence of B_2 receptors in canine TECs.

As an alternative approach to define the receptor subtypes, the determination of antagonist affinities can provide more accurate information than the relative potencies of agonists. In these experiments, the effects of discriminating antagonists have been analysed at the level of this specific biochemical response. The results obtained with Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK, differentiated the receptor subtype mediating IPs accumulation (Figure 6). The pK_B values for the B_2 antagonists Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK were 8.8±0.3 and 7.0±0.3, respectively, with high affinity in antagonizing B_2 receptor-mediated IPs accumulation. These results are in good agreement with the K_i values obtained from the binding studies and those reported by others in the rabbit jugular vein (Regoli *et al.*, 1990) and in the bovine (Marsh & Hill, 1992) and canine (Yang *et al.*, 1994a,b,c) tracheal smooth muscle cells. Moreover, BK has been associated with activation of PI-specific phospholipase C as a second messenger pathway in a variety of cell types (Osugi *et al.*, 1987; Balmforth *et al.*, 1992; Marsh & Hill, 1992; Yang *et al.*, 1994a). BK stimulates IPs accumulation and an increase in cytosolic calcium concentration (Marsh & Hill, 1993; Yang *et al.*, 1994b). The raised Ca^{2+} further regulated the Cl^- secretory pathway (Clarke *et al.*, 1994). A Ca^{2+}-dependent Cl^- conductance, thought to be regulated by Ca^{2+}-calmodulin-dependent kinase, is functional in airway epithelium (Wargner *et al.*, 1991).

In conclusion, our experiments demonstrate that radioligand binding assays are effective means of directly measuring parameters of receptor occupancy in canine cultured TECs. Canine TECs possess high-affinity, specific [^{3}H]-BK binding sites. The pharmacological properties of BK receptors could be further differentiated on the basis of competitive inhibition binding profiles of investigated agonists and antagonists. The BK receptors are primarily B_2 receptors which may regulate the tracheal function through the activation of this receptor subtype coupled to PI hydrolysis in canine TECs.

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