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Pharmacological characterization of bradykinin receptors in canine cultured tracheal smooth muscle cells

[1]Chuen Mao Yang, *Shue-Fen Luo & Hui-Chuan Hsia

Department of Pharmacology and *Department of Internal Medicine, Chang Gung College of Medicine and Technology, Kwei-San, Tao-Yuan, Taiwan

1 [^{3}H]-bradykinin was used to characterize the bradykinin receptors associated with canine cultured tracheal smooth muscle cells (TSMCs). Receptor binding assay showed that TSMCs had specific, saturable, high-affinity binding sites for [^{3}H]-bradykinin.

2 The specific [^{3}H]-bradykinin binding increased linearly with increasing cell concentrations. The equilibrium for association of [^{3}H]-bradykinin with the bradykinin receptors was attained within 2 h at 4°C and 1 h at room temperature, respectively.

3 Analysis of binding isotherms yielded an apparent equilibrium dissociation constant (K_D) of 2.5 ± 0.3 nM and a maximum receptor density (B_{max}) of 25.1 ± 0.3 fmol mg^{-1} protein. The Hill coefficient for [^{3}H]-bradykinin binding was 1.00 ± 0.02. The association (K_1) and dissociation (K_{-1}) rate constants were $(8.67 \pm 2.60) \times 10^6$ M^{-1} min^{-1} and 0.024 ± 0.005 min^{-1}, respectively. K_D, calculated from the ratio of K_{-1} and K_1 was 2.8 ± 0.5 nM, a value close to that of K_D calculated from Scatchard plots of binding isotherms.

4 The B_1 receptor selective agonist, (des-Arg^9-bradykinin, 0.1 nM–10 μM) and antagonist ([Leu^8, des-Arg^9]-bradykinin, 0.1 nM–10 μM) did not inhibit the [^{3}H]-bradykinin binding to TSMCs, which excludes the presence of B_1 receptors in canine TSMCs.

5 The specific binding of [^{3}H]-bradykinin to canine TSMCs was inhibited by B_2 receptor selective antagonists ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin, Hoe 140, 0.1 nM–10 μM and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin, 0.1 nM–10 μM) and agonists (bradykinin 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]-bradykinin binding was kallidin = bradykinin = Hoe 140 > [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin.

6 Preincubation of TSMCs with forskolin for 24 h led to an up-regulation of B_2 receptors, increasing in B_{max} from 25.1 ± 0.3 to 218 ± 24 fmol mg^{-1} protein without changing the K_D values. [^{3}H]-bradykinin binding to TSMCs was inhibited by the B_2 receptor selective antagonists and agonists, but not by the B_1 receptor selective reagents. The up-regulation of the B_2 receptor by forskolin was mediated through protein synthesis, since cycloheximide blocked this response.

7 It is concluded that the pharmacological characteristics of the bradykinin receptors in canine cultured TSMCs are primarily of the B_2 receptor subtype.

Keywords: Bradykinin receptor; canine tracheal smooth muscle cells; cyclic AMP elevating agents; kinins

Introduction

Bradykinin is a classic mediator of inflammatory diseases of the airways and is implicated in allergic asthma (Christiansen *et al.*, 1987; Farmer *et al.*, 1991). In the airways, bradykinin causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). It is well established that the kinins, bradykinin, kallidin, and des-Arg^9-bradykinin, interact with two bradykinin 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 sensitivity to bradykinin than to des-Arg^9-bradykinin are considered to express B_2 receptor activity, while preparations having opposite sensitivity are considered to express B_1 receptor activity (Proud & Kaplan, 1988; Regoli *et al.*, 1990). The use of selective antagonists has further strengthened the existence of such receptor subtypes (Regoli *et al.*, 1990). In addition, studies with B_2 receptor selective antagonists have revealed that further heterogeneity exists among bradykinin 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 a 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). In previous studies, we have reported that bradykinin can induce an increase in phosphoinositide hydrolysis and a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in canine cultured TSMCs which appear to be mediated via the activation of B_2 receptors (Yang *et al.*, 1994a,b). However, studies on guinea-pig (Farmer *et al.*, 1991) and bovine (Marsh & Hill, 1993) TSMCs suggested that activation of B_3 and B_2 receptors were responsible for the bradykinin-induced increase in $[Ca^{2+}]_i$, respectively. However, it is possible that differences in affinities of B_2 receptor antagonists are due to species homologues of B_2 receptors (Hall *et al.*, 1993). Consequently, the pharmacological characteristics of the bradykinin receptor subtypes in canine cultured TSMCs need to be defined.

It has been shown that long-term cholera toxin treatment potentiates the bradykinin-stimulated phosphoinositide turnover in canine TSMCs, human foreskin fibroblasts, osteoblast-like cell line MC3T3-E1 and BALB/c/3T3 cells (Banno *et al.*, 1993; Etscheid *et al.*, 1991; Olashaw & Pledger, 1988; Yang *et al.*, 1994c). This potentiating action was inhibited by cycloheximide, suggesting that it involves an increase in protein synthesis following elevation of the con-

[1] Author for correspondence.

centration of adenosine 3':5'-cyclic monophosphate (cyclic AMP), probably through an increase in the density of bradykinin receptors (Etscheid *et al.*, 1991; Banno *et al.*, 1993; Yang *et al.*, 1994c). Although only B_2 receptor density has been shown to increase in osteoblast-like MC3T3-E1 cells treated with cholera toxin (Banno *et al.*, 1993), the changes in pharmacological properties of bradykinin receptor subtypes are not clearly delineated in canine TSMCs treated with cyclic AMP elevating agents.

The purpose of this study was to characterize the bradykinin receptors in canine cultured TSMCs by radioligand [^{3}H]-bradykinin binding assay. To determine whether bradykinin receptor subtypes are present in canine TSMCs, competitive inhibition of [^{3}H]-bradykinin binding was performed using B_1 and B_2 receptor selective agonists and antagonists as competing ligands.

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 temperatures and light cycle conditions and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with ketamine (20 mg kg^{-1}, i.m.) and pentobarbitone (30 mg kg^{-1}, i.v.). The tracheae were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods previously described (Yang *et al.*, 1991). The muscle was dissected, minced and transferred to the dissociation medium containing 0.1% collagenase IV, 0.025% deoxyribonuclease I, 0.025% elastase IV, and antibiotics (100 u ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 250 ng ml^{-1} fungizone) 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 residuum was again digested with fresh enzyme solution for an additional 1 h at 37°C. The released cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F-12 (F-12) medium (1:1, vol/vol). The cells, suspended in DMEM/F-12 containing 10% foetal bovine serum (FBS), were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cells were diluted with DMEM/F-12 to a final concentration of 2×10^5 cells ml^{-1}. The cells (0.5 ml/well) were plated onto 24-well culture plates for receptor binding assay. The medium was changed after 24 h and then every 3 days. After 5 days, the cells were cultured in DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were grown in DMEM/F-12 containing 1% FBS supplemented with insulin-like growth factor I (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 an indirect immunofluorescence method using a monoclonal antibody of light chain myosin (Gown *et al.*, 1985). Under the above culture conditions, over 95% of the cells were smooth muscle cells.

[^{3}H]-bradykinin binding assay

Binding assays were performed with confluent TSMCs in 24-well culture plates, with or without forskolin in DMEM/F-12 containing 1% FBS for 24 h prior to the binding experiments, as described by Yang *et al.* (1994c). Culture medium was removed and 1 ml of binding buffer (composition, mM: HEPES 20, pH 7.4, NaCl 17, KCl 5.4, KH_2PO_4 0.44, $CaCl_2$ 0.63, $MgSO_4$ 0.21, Na_2HPO_4 0.34, N-methylglucamine 110, 0.1% (w/v) BSA and bacitracin 2) 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]-bradykinin in the absence or presence of unlabelled bradykinin (10 μM). After 4 h incubation at 4°C, the binding buffer was removed, cells were washed three times with 2 ml of binding buffer at 4°C, 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 bradykinin. 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).

For cell concentration binding experiments, the cells were grown onto 100 mm culture dishes and released by 0.25% trypsin and 0.5 mM EDTA. In the assay, 50 μl cell suspension in binding buffer (10^3 to 10^6 cells) was added to culture test tubes in triplicate each containing 0.2 ml binding buffer with 3 nM [^{3}H]-bradykinin. Nonspecific binding was determined in the presence of 10 μM unlabelled bradykinin. The mixture was incubated at 4°C for 4 h. The reaction was terminated by rapid filtration under vacuum through Whatman GF/C fibre filters, using a cell harvester manifold, followed by four washes with 5 ml of chilled binding buffer. After the filtration step, each filter was transferred to a vial containing 5 ml scintillation solution, and the radioactivity was determined in a liquid scintillation counter (Beckman LS5000TA).

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]-bradykinin (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]-bradykinin at room temperature and 4°C for 1 h and 2 h, respectively. At this time (time 0), 10 μM unlabelled bradykinin was added and determinations were made at various time interals over a 2 h period.

Analysis of binding data

Equilibrium dissociation constant (K_D) and maximal receptor density (B_{max}) were calculated by 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]-bradykinin with statistical significance established by Fisher's F test (Zar, 1974).

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). [^{3}H]-bradykinin (67 Ci mmol^{-1}) was from Dupont NEN (Boston, MA, U.S.A.). Bradykinin, des-Arg^9-bradykinin, [Leu^8, des-Arg^9]-bradykinin, [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin and Hoe 140 ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) were from Peninsula Laboratories (Belmont, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.). All of the reagents were prepared as 1 mM stock solutions and diluted with deionized water to the appropriate concentration.

Results

Kinetic constants of [^{3}H]-bradykinin binding to TSMCs

Specific [^{3}H]-bradykinin binding increased linearly with increasing cell concentrations (from 10^3 to 10^6 cells), when the cells were incubated with 3 nM [^{3}H]-bradykinin at 4°C for 4 h (Figure 1). Specific [^{3}H]-bradykinin binding to TSMCs was time- and temperature-dependent. Binding of [^{3}H]-bradykinin to TSMCs reached an apparent equilibrium within 2 h at 4°C and 1 h at room temperature (Figure 2a). Half maximal bindings occurred within 21 and 8 min at 4°C and room temperature, respectively. At 4°C, no [^{3}H]-bradykinin dissociation from TSMCs was observed (Figure 2b). After 60 min association at room temperature, dissociation was initiated by addition of 10 μM unlabelled bradykinin which resulted in a reduction of specifically bound [^{3}H]-bradykinin. Half dissociation occurred within 20 min. The observed association rate constant (K_{obs}) was 2.625 ± 0.126 min^{-1} and the dissociation rate constant (K_{-1}) was 0.024 ± 0.005 min^{-1}. The value of the association rate constant (K_1) calculated from the equation $K_1 = (K_{obs} - K_{-1})/[\text{radioligand}]$ was $(8.67 \pm 2.60) \times 10^6$ M^{-1} min^{-1}. A K_D value of 2.8 ± 0.5 nM calculated from the ratio of the rate constants, K_{-1}/K_1, ageed reasonably well with the K_D (2.5 ± 0.3 nM) determined by Scatchard analysis of saturation isotherms, as shown in Figure 3b.

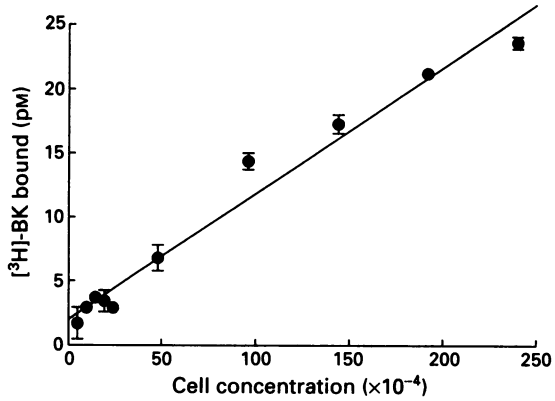

Figure 1 Specific binding of [^{3}H]-bradykinin as a function of cell concentration. Various concentrations of TSMCs were incubated with 3 nM [^{3}H] bradykinin at 4°C for 4 h in 0.25 ml binding buffer. The relationship between cell concentration and specific binding of [^{3}H]-bradykinin is linear in the range from 10^3 to 10^6 cells.

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

The saturability of [^{3}H]-bradykinin binding was measured by incubating the cultured TSMCs with various concentrations of [^{3}H]-bradykinin from 0.1 to 11 nM (Figure 3a). The saturation isotherm is a rectangular hyperbola, suggesting that a single population of saturable high affinity bradykinin binding sites exists. Nonspecific binding, on the other hand, increased linearly with increasing [^{3}H]-bradykinin concentra-

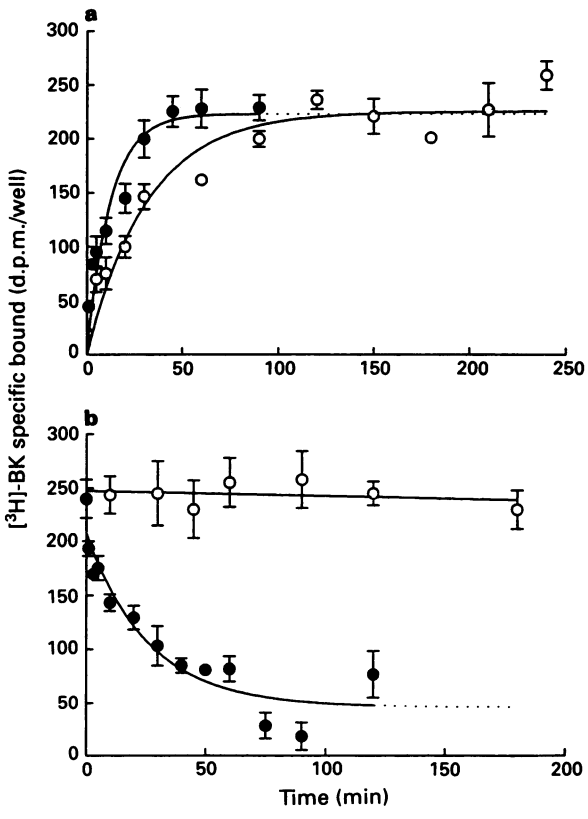

Figure 2 Time course of association and dissociation of specific [^{3}H]-bradykinin binding to cultured TSMCs. For association (a), the cells were incubated with 3 nM [^{3}H]-bradykinin for the various periods of time indicated at room temperature (●) and 4°C (○). For determination of dissociation rate constant (b), unlabelled bradykinin at a final concentration 10 μM was added after incubation with [^{3}H]-bradykinin 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.

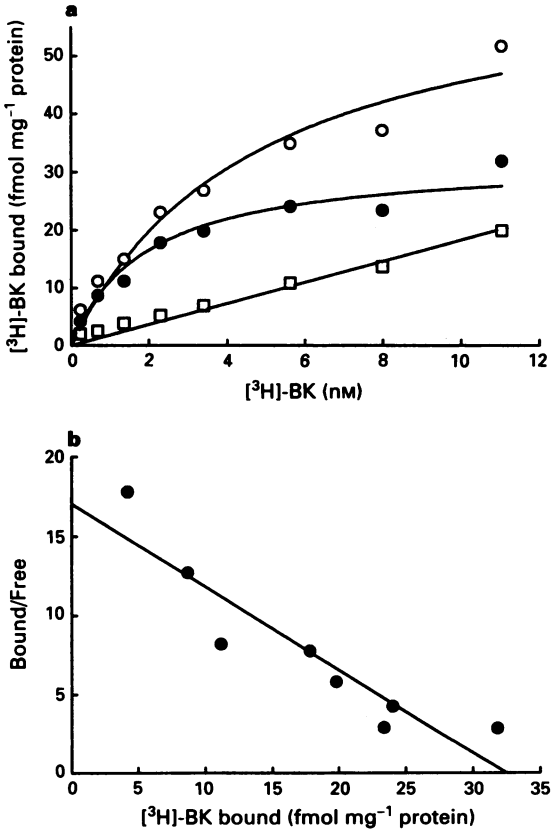

Figure 3 (a) Saturation isotherms of [^{3}H]-bradykinin binding to canine cultured TSMCs incubated in triplicates with 0.1 to 11 nM of [^{3}H]-bradykinin at 4°C for 4 h as described under Methods. Specific binding (●) was determined as the difference between total (○) and nonspecific binding (□) in the absence and presence of 10 μM unlabelled bradykinin. (b) Scatchard plots of specific [^{3}H]-bradykinin binding data in (a). X-intercept of least-squares fit to Scatchard plot is a measure of maximal receptor density (B_{max}); negative reciprocal of the slope is the dissociation constant (K_D). Data are expressed as one of six separate experiments.

tion up to 11 nM. Scatchard plot analysis of specific bound [^{3}H]-bradykinin in the cultured TSMCs gave an apparent dissociation constant (K_D) of 2.5 ± 0.3 nM and a maximal receptor density (B_{max}) of 25.1 ± 0.3 fmol mg^{-1} protein, $n = 6$ (Figure 3b). A plot of binding data for [^{3}H]-bradykinin 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.

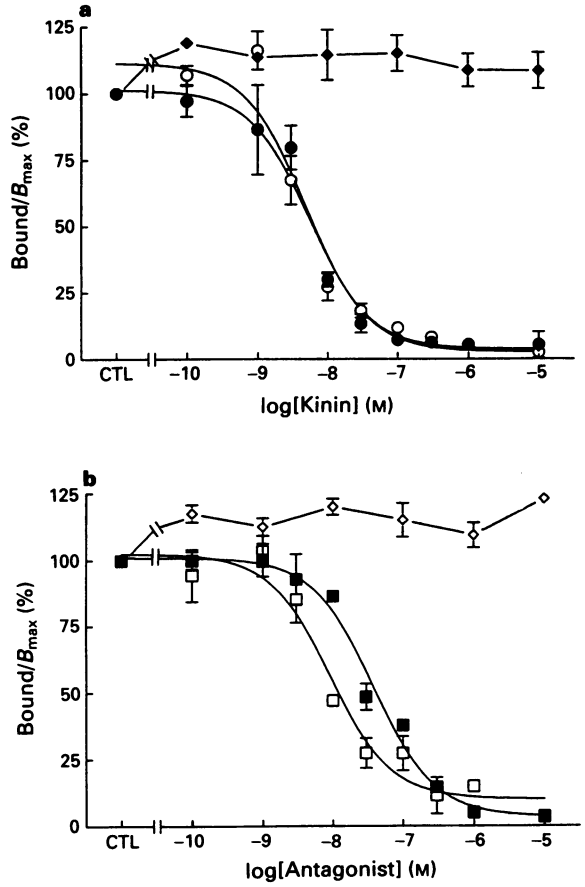

Figure 4 Competition for [^{3}H]-bradykinin binding to canine cultured TSMCs by bradykinin receptor (a) agonists; (◆) des-Arg^9-bradykinin; (○) kallidin; (●) bradykinin and (b) antagonists; (◇) [Leu^8, des-Arg^9]-bradykinin; (■) [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe]-bradykinin; (□) Hoe 140. Cells were incubated with various concentrations of the competing ligand in the presence of 3 nM [^{3}H]-bradykinin. Nonspecific binding, determined in the presence of 10 μM unlablled bradykinin, was subtracted from total binding. The points shown are averages of triplicate determinations from one representative of three experiments. The curves are best non-linear least squares, computer generated fits to the data, as described under Methods.

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

The pharmacological specificity of binding was defined by studying the inhibition of [^{3}H]-bradykinin binding by bradykinin receptor agonists and antagonists. Figure 4 shows that all of the drugs tested displaced [^{3}H]-bradykinin binding at bradykinin receptors in a concentration-related manner except the B_1 receptor-selective agonist des-Arg^9-bradykinin and antagonist [Leu^8, des-Arg^9]-bradykinin, even at the highest concentration tested (10 μM). The concentration of these drugs required to inhibit 50% of the specific binding (IC_{50}) and inhibition constant (K_i) in competition with [^{3}H]-bradykinin are listed in Table 1. The order of potency for the inhibition of [^{3}H]-bradykinin binding was kallidin = bradykinin = Hoe 140 > [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin >> des-Arg^9-bradykinin, [Leu^8, des-Arg^9]-bradykinin. The competition curves were steep and could be adequately described by a one binding site model for these drugs (Figure 4). The pharmacological properties of the bradykinin receptors are consistent with those of B_2 receptors (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994).

Effect of forskolin on [^{3}H]-bradykinin binding

Cyclic AMP elevating agents have been shown to exert their effect by receptor up-regulation (Etscheid *et al.*, 1991; Banno *et al.*, 1993). We previously reported that pretreatment of TSMCs with cholera toxin, forskolin, and dibutyryl cyclic AMP enhanced bradykinin-induced inositol phosphates accumulation and a rise in $[Ca^{2+}]_i$ through an increase in density of the bradykinin receptor (Yang *et al.*, 1994c). To examine further which bradykinin receptor subtype was increased in the cells treated with forskolin for 24 h, we chose des-Arg^9-bradykinin, [Leu^8, des-Arg^9]-bradykinin, bradykinin, Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin to differentiate between the bradykinin binding sites. All tested drugs inhibited [^{3}H]-bradykinin binding with the same affinities as those of the control (Table 1). [^{3}H]-bradykinin binding to TSMCs treated with forskolin was inhibited by the B_2 receptor-selective antagonists and agonist, but was not affected by the B_1 receptor-selective agonist and antagonist. Moreover, preincubation with forskolin led to an increase in B_{max} from 25.1 ± 0.3 to 218 ± 24 fmol mg^{-1} protein without changing the K_D value. These results suggest that the increase in receptor density is primarily due to the B_2 receptor.

Discussion

It is well established that bradykinin-induced tracheal smooth muscle contraction contributes to an increase in phos-

Table 1 Relative potencies of drugs for [^{3}H]-bradykinin binding to canine cultured TSMCs preincubated with forskolin

	Control		*Forskolin*	
Drug	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
Agonists				
des-Arg^9-bradykinin	> 10000	> 10000	> 10000	> 10000
Kallidin	5.6 ± 1.4	2.4 ± 0.8	–	–
Bradykinin	4.6 ± 0.6	1.9 ± 0.2	5.2 ± 0.3	2.2 ± 0.4
Antagonists				
[Leu^8, des-Arg^9]-bradykinin	> 10000	> 10000	> 10000	> 10000
[D-Arg^0, Hyp^3, Thi^{5,8},D-Phe^7]-bradykinin	40 ± 9	21 ± 5	52 ± 9	25 ± 7
Hoe 140	5.3 ± 0.9	2.7 ± 0.4	6.1 ± 1.6	3.0 ± 0.4

Cultured TSMCs were preincubated in the absence or presence of forskolin (10 μM) for 24 h. [^{3}H]-bradykinin (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.

phoinositide hydrolysis and a rise in $[Ca^{2+}]_i$ in cultured canine and bovine TSMCs which appears 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 bradykinin receptors in canine cultured TSMCs by receptor binding assay with [^{3}H]-bradykinin. [^{3}H]-bradykinin has been shown to label bradykinin receptors in guinea-pig lung and trachea, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994). Since [^{3}H]-bradykinin is a peptide which does not penetrate the cell membrane, it allows the determination of cell surface bradykinin receptors on intact TSMCs. It is important to characterize the bradykinin receptors on intact TSMCs, 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 to clarify the role of these receptors in tracheal functions.

The interaction of [^{3}H]-bradykinin with bradykinin receptors occurs rapidly at room temperature. The half-time for association of [^{3}H]-bradykinin with TSMCs was 8 min. Dissociation occurred slowly with a half-life of approximately 20 min at room temperature. The equilibrium dissociation constant (K_D) of 2.8 ± 0.5 nM as determined by kinetic experiments was in reasonable agreement with the K_D value of 2.5 ± 0.3 nM derived from saturation isotherms, which indicates that the assumption of a single biomolecular reaction is valid.

Canine TSMCs were found to be rich in [^{3}H]-bradykinin-specific, saturable, high-affinity bradykinin receptor-like binding sites. Scatchard analysis of binding data indicated a K_D value of 2.5 ± 0.3 nM and a B_{max} value of 25.1 ± 0.3 fmol mg^{-1} protein. The binding sites most likely represent a single population without significant cooperative interactions, since Scatchard plots were linear and the Hill coefficients were near unity. Furthermore, the bradykinin receptor acting drugs competed with [^{3}H]-bradykinin binding in a concentration-related manner. The order of potency was bradykinin = kallidin >> des-Arg^9-bradykinin (Table 1). It has been suggested that bradykinin induces its effects through at least two receptor subtypes which have been characterized as B_1 and B_2 reeptors (Regoli *et al.*, 1990). Bradykinin and kallidin have high affinity for B_2 and low affinity for B_1 receptors (Regoli *et al.*, 1990). In contrast, des-Arg^9-bradykinin has a high affinity for B_1, 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]-bradykinin and agonist des-Arg^9-bradykinin, triggered no displacement at concentrations up to 10 μM, thus excluding the presence of B_1 receptors in canine cultured TSMCs. The binding characteristics are similar to those of the bradykinin receptors described for guinea-pig lung and trachea, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifillieff *et al.*, 1991; 1994).

Moreover, the competitive inhibition of specific [^{3}H]-bradykinin binding by the B_2 receptor-selective antagonists, Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin showed a single population of bradykinin binding sites in canine cultured TSMCs (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]-bradykinin >> [Leu^8, des-Arg^9]-bradykinin (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]-bradykinin were 2.7 ± 0.4 and 21 ± 5 nM, respectively, confirming their high affinity for B_2 receptors described for other tissues (Eggerickx *et al.*, 1992; Hess *et al.*, 1994).

We found that forskolin increased the total number of [^{3}H]-bradykinin binding sites without changing their affinity in canine TSMCs. The increase in [^{3}H]-bradykinin binding was inhibited by B_2 receptor-selective antagonists (Hoe 140 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin) and bradykinin, but not by the B_1 receptor-selective agonist, des-Arg^9-bradykinin and antagonist [Leu^8, des-Arg^9]-bradykinin (Table 1). We speculate that these cyclic AMP elevating agents affect the B_2 receptor number at the gene level, and are associated with the sustained elevation of intracellular cyclic AMP, since TSMCs have to be exposed to cholera toxin, forskolin, or dibutyryl cyclic AMP for several hours before any enhancing effect occurs (Yang *et al.*, 1994c).

In conclusion, our experiments demonstrate that radioligand binding assays are effective means of directly measuring parameters of receptor occupancy in canine cultured TSMCs. Canine TSMCs possess high-affinity, specific [^{3}H]-bradykinin binding sites. The bradykinin receptors in canine TSMCs are primarily B_2 receptors which may regulate the activity of tracheal smooth muscle through activation of this receptor subtype (Yang *et al.*, 1994a,b,c).

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