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Characterization and localization of bradykinin B_2 receptors in the guinea-pig using a radioiodinated HOE140 analogue

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Abstract

The potent bradykinin B_2 receptor antagonist analogue, [^{125}I]HPP-HOE140, ([^{125}I]-3-4-hydroxyphenyl-propionyl-D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin), was used to localize and characterize guinea-pig tissue bradykinin B_2 receptors. Analysis of competition for the radioligand binding, using membrane preparations of lung, ileum and uterus, revealed the presence of a high- and low-affinity binding site: at the high-affinity site, the apparent K_i for the various bradykinin B_2 receptor ligands ranged from 0.26 to 2.13 nM for HPP-HOE140, from 0.25 to 1.45 nM for HOE140, from 129 to 625 nM for D-Arg^0[Hyp^3,Phe^7]bradykinin and from 0.05 to 1.11 nM for bradykinin. At the low-affinity site, the apparent K_i values ranged from 4.90 to 10.5 nM, from 1.23 to 1.90 nM, 4760 nM and from 2.01 to 62.1 nM, respectively. By contrast, the bradykinin B_1 receptor antagonist, des-Arg^9[Leu^8]bradykinin did not compete for [^{125}I]HPP-HOE140 binding from membrane preparations at concentrations up to 1 μM. Using in vitro autoradiography on tissue sections, intense binding was observed in the lamina propria of the villi of ileum and the arteriolar smooth muscle cells in lung. In the uterus, dense binding was found in the inner third of the myometrium and over epithelial cells of the glandular endometrium, while diffuse binding was observed throughout the endometrial stroma. In the brain, intense binding was observed in the nucleus of the solitary tract, spinal trigeminal tract and area postrema of the hindbrain, the middle cerebral arteries, and the choroid plexus of the third and lateral ventricles. Moderate binding was observed in the CA3 region of the hippocampus and posterior and ventroposterior thalamic nuclei. In the spinal cord, high-density binding occurred in the laminae I and II of the dorsal horn. Unlike previous autoradiographic localization studies of the bradykinin B_2 receptor using radiolabeled bradykinin, the radiolabeled antagonist HPP-HOE140 did not bind to bradykinin-degrading peptidases, such as angiotensin-converting enzyme, and displayed subtype specificity. Therefore, binding studies with [^{125}I]HPP-HOE140 offers high sensitivity and specificity for characterization, quantitation and localization of subtypes of bradykinin B_2 receptors in tissues, and offers new information on uterine and brain bradykinin B_2 receptors.

Keywords: Bradykinin B_2 receptor; HOE140; Autoradiography

1. Introduction

Bradykinin (BK) is a nonapeptide implicated in inflammation, pain sensation, regulation of blood pressure, oedema formation and contraction of smooth muscle cells (Marceau et al., 1983; Dray and Perkins, 1993; Regoli and Barabé, 1980; Bhoola et al., 1992).

Two subtypes of the BK receptors have been pharmacologically characterized: the B_1 subtype occurs predominantly in the rabbit vascular smooth muscle and its synthesis is induced by tissue damage or inflammation (Bathon and Proud, 1991). The BK B_1 receptor clone isolated from human embryonic lung fibroblasts (Menke et al., 1994) is characterized by its high affinity for the agonists des-Arg^9-BK and des-Arg^{10}-Lys-BK and the antagonists des-Arg^9[Leu^8]BK and des-Arg^{10}-Lys-[Leu^8]BK (Bathon and Proud, 1991).

The pharmacological properties of the BK B_2 receptor are better known and it is this subtype which mediates most of the biological actions of BK. Activation of the BK B_2 receptors on sensory fibres induces pain in response to inflammation and facilitates the release of noradrenaline from sympathetic nerve terminals (Regoli et al., 1990). BK B_2 receptors also stimulate the release of prostacyclin and

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nitric oxide, thereby mediating relaxation of smooth muscle in peripheral vessels, the final outcome being an increase in blood flow and vascular permeability (Plevin and Owen, 1988).

Recently, a BK B_3 receptor, located in guinea-pig trachea, has been characterized by the inability of BK B_1 or B_2 receptor antagonists to inhibit BK binding in airway smooth muscle or to inhibit BK-induced tracheal contraction (Farmer et al., 1989, 1991). However, other studies have contradicted this finding, suggesting that the new site is more likely to be a subtype of the BK B_2 receptor (Trifilieff et al., 1991).

The development of more potent and specific antagonists of the BK receptors, such as HOE140, which contains several synthetic amino acid substitutions (Rhaleb et al., 1992; Hock et al., 1991) and is a selective and potent BK B_2 receptor antagonist (Bao et al., 1991), has enabled the delineation and characterization of the BK B_2 receptor protein by affinity cross-linking studies (Abd Alla et al., 1993). HOE140 displays higher affinity and specificity than previous BK B_2 receptor antagonists (Regoli et al., 1990; Rhaleb et al., 1991; Togo et al., 1989) which are limited by their partial agonist activity and are subject to degradation by proteolytic enzymes (Regoli et al., 1990; Dray and Perkins, 1993).

Previous radioligand-binding studies using radiolabeled ligands for the BK B_2 receptor have been conducted predominantly on membrane tissue preparations. Consequently, there is little information on the cellular distribution of this BK receptor subtype.

This study employed 3-4-hydroxyphenyl-propionyl-D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK (HPP-HOE140), a new analogue of the potent and specific BK B_2 receptor antagonist HOE140. BK B_2 receptors were characterized by tissue membrane-binding studies and were visualized on tissue sections by in vitro autoradiography.

2. Methods

2.1. Tissue membrane preparations

Adult female guinea-pigs received a lethal dose of sodium pentobarbitone (30–50 mg/kg i.p.) the lung, ileum and uterus removed and processed at 4°C to minimize receptor degradation. Tissues were finely chopped (1 mm^3), suspended in 5 mM sodium phosphate buffer, pH 7.4, containing 10 mM sodium chloride, 0.32 M sucrose, 2 mM $MgCl_2$, 2 mM $CaCl_2$ (buffer A) and homogenized for two bursts of 10 s each at 13 500 rpm (Ultraturrax T-25; Janke and Kunkel, Staufen, Germany). Homogenates were then centrifuged at $1000 \times g$ for 15 min at 4°C (Beckman GPR centrifuge, CA, USA) and the supernatants collected. The pellets were resuspended in buffer and recentrifuged at $1000 \times g$ for 15 min. The supernatants from both low-speed centrifugations were pooled and centrifuged for 30 min at $50\,000 \times g$ at 4°C (Beckman XL-90 Ultracentrifuge, SW41Ti rotor, CA, USA). Following resuspension of the pellets in buffer A, the membrane preparations were manually homogenized using a glass tube and tight-fitting Teflon plunger (10 strokes), dispensed into aliquots and rapidly frozen in liquid nitrogen.

2.2. Radioligand preparation

The BK B_2 receptor antagonist analogue, HPP-HOE140 (Abd Alla et al., 1993), was radioiodinated by the Chloramine T method using 3 μg HPP-HOE140 and 1 mCi ^{125}I (Greenwood and Hunter, 1963). The reaction product was separated on a Sep-pak C_{18} cartridge by elution with 40–100% gradient of methanol in 0.1% trifluoroacetic acid, giving an approximate specific activity of 820 μCi/μg. Aliquots of the radioligand were stored at −20°C and were stable for at least 2 months.

2.3. Radioligand-binding studies

The time course for the association of the radioligand binding to the membranes was determined from 2 min to 8 h at 22°C. The radioligand and tissue membranes were diluted in buffer A containing 0.2% bovine serum albumin, 1 mM benzamidine, 2.5 mM 1,10 phenanthroline, 1 μM leupeptin, 1 μM phosphoramidon, 1 μM lisinopril, 1 μM Plummer's inhibitor (2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid) and 1 mg/ml bacitracin, at pH 7.4. The membrane concentrations were 1.28, 0.14 and 0.30 mg/ml of protein for guinea-pig lung, ileum and uterus, respectively. All assay tubes were set up in duplicate and contained 260 nCi of [^{125}I]HPP-HOE140 (~2 nM) and 100 μl of membrane preparation and were incubated at 22°C. [^{125}I]HPP-HOE140 bound to tissue membranes was separated from free radioligand by rapid filtration (Brandel Cell Harvester, MD, USA) through a GF/B Whatman filter presoaked in 5 mM sodium phosphate buffer, pH 7.4, containing 10 mM sodium chloride, 2 mM calcium chloride, 2 mM magnesium chloride and 0.5% polyethylenimine. Each tube was washed 5× (2.5 ml/wash) with buffer A containing 0.2% bovine serum albumin (4°C). Filter-bound radioactivity was determined in a gamma counter (LKB 1260, Multigamma II counter, Turku, Finland). The amounts of bound radioligand were corrected for protein concentration (Lowry et al., 1951). In subsequent experiments, membrane preparations were incubated for 4 h at which time the binding achieved equilibrium as determined by this association experiment.

The saturation study was carried out in duplicate on lung membrane preparations (1.28 mg/ml protein) using increasing concentrations of [^{125}I]HPP-HOE140 from 0.01 to 20 nM.

Competition studies were performed on guinea-pig lung, ileum and uterus tissue membrane preparations using the following unlabeled BK analogues: the BK B_2 receptor antagonists, HPP-HOE140, D-Arg^0[Hyp^3,D-Phe^7]BK and HOE140; the BK B_1 receptor antagonist, des-Arg^9[Leu^8]BK; and BK at concentrations ranging from 10^{-12} to 10^{-6} M. At least 20 different concentrations of each unlabeled BK analogue were assessed in duplicate for each tissue.

2.4. In vitro autoradiography on tissue sections

Twenty-μm-thick frozen sections of unfixed lung, ileum, uterus and brain were cut in a cryostat (Microm, HM 505E; Walldorf, Germany) at −17°C, thaw-mounted onto gelatin-coated slides, dehydrated at 4°C overnight and stored at −70°C until use. The slide-mounted sections were preincubated for 15 min at room temperature in a 170 mM Tris buffer, pH 7.4, containing 0.2% bovine serum albumin, to remove endogenous ligand and then incubated for 24 h at 4°C in a fresh volume of Tris buffer containing 0.28 μCi/ml (~0.2 nM) of [^{125}I]HPP-HOE140. Non-specific binding was determined in parallel incubations containing 1 μM unlabeled HOE140. After incubation, the sections were rinsed in ice-cold incubation buffer without bovine serum albumin for 2 min in four consecutive washes, dried under a stream of cool air and exposed for 24 h to X-ray film (Kodak-Ektascan, EB-1; Eastman Kodak, USA), and developed in a RPX-OMAT automatic developer.

For higher-resolution localization, the slide-mounted sections obtained after the incubation, washing and drying steps, were fixed in 10% formalin solution in 70 mM phosphate buffer for 30 min at 4°C. The sections were then dehydrated in increasing concentrations of ethanol (50–100%), defated in xylene followed by 100% ethanol and allowed to air-dry. These sections were then coated with liquid emulsion (LM-1, Amersham, UK) and exposed in light tight boxes stored at 4°C for 5–14 days. The emulsion dipped slides were developed in Kodak D19 developer at 20°C for 6 min, and fixed in Ilford rapid fix containing 1 : 40 dilution of hardener for 2 min.

2.5. Data analysis

Binding data was analysed using Equilibrium Binding Data Analysis (EBDA; McPherson Melbourne, Australia) for the association experiments and LIGAND (Munson and Rodbard, 1980) for the competition experiments. GraphPad Prism (San Diego, CA, USA) was used to analyse the hot saturation experiments.

2.6. Materials

Benzamidine, leupeptin, bacitracin, polyethyleneimine D-Arg^0[Hyp^3,D-Phe^7]BK and des-Arg^9[Leu^8]BK were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin was purchased from Commonwealth Serum Laboratories (Parkville, Victoria, Australia) and phosphoramidon and BK were purchased from Peninsula Laboratories (Belmont, CA, USA). Plummer's inhibitor (2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid) was purchased from Calbiochem (La Jolla, CA, USA) and 1,10-phenanthroline from Merck (Darmstadt, Germany). Lisinopril and SQ20,881 were provided as generous gifts from Merck, Sharp and Dohme Research Laboratories (West Point, PN, USA) and Squibb (Princeton, NJ, USA), respectively. HPP-HOE140 and HOE140 were provided as generous gifts from Hoechst (Frankfurt, Germany).

Table 1
Binding characteristics for BK analogues competing for [^{125}I]HPP-HOE140 radioligand from BK B_2 receptors on membrane preparations of guinea-pig tissues

Tissue	Competing ligand	High-affinity site		Low-affinity site	
		K_i (nM)	B_{max} (fmol mg^{-1})	K_i (nM)	B_{max} (fmol mg^{-1})
Guinea-pig lung	HPP-HOE140 [a]	2.13	92	10.5	650
	HOE140 [a]	0.25		1.90	
	D-Arg^0[Hyp^3,D-Phe^7]BK	227		ND	
	BK [c]	1.11		62.1	
Guinea-pig ileum	HPP-HOE140 [c]	0.83	538	4.90	3725
	HOE140 [b]	1.14		1.23	
	D-Arg^0[Hyp^3,D-Phe^7]BK [a]	129		4760	
	BK [c]	0.05		2.01	
Guinea-pig uterus	HPP-HOE140 [c]	0.26	53	9.56	2973
	HOE140	1.45		ND	
	D-Arg^0[Hyp^3,D-Phe^7]BK	625		ND	
	BK [c]	0.17		5.76	

Experiments were performed in duplicate as described in Methods. [a] $0.01 < P < 0.04$, [b] $0.002 < P < 0.006$ and [c] $P < 0.0001$ for 2 vs. 1 site fit. ND, a second binding site was not detected.

3. Results

3.1. Association and saturation experiments

At 22°C, the binding of [^{125}I]HPP-HOE140 to tissue membranes reached equilibrium by 4 h and remained stable for at least 6 h (Fig. 1). An apparent K_D value of 3 nM was calculated for lung tissue using the saturation data and analysis by the GraphPad Prism program (San Diego, CA, USA) (Fig. 2).

3.2. Ligand specificity

The binding properties of BK and its analogues in various tissues are summarized in Table 1. Competition of [^{125}I]HPP-HOE140 binding to membrane preparations of lung, ileum and uterus by HPP-HOE140, HOE140, D-Arg^0[Hyp^3,D-Phe^7]BK and BK are shown in Fig. 3. Non-specific binding was 6–12% of total binding in the uterus membrane preparations, 3–6% in ileum membrane preparations and 16–23% in lung membrane preparations. The BK B_1 receptor antagonist, des-Arg^9[Leu^8]BK, failed to displace [^{125}I]HPP-HOE140 from all tissues at concentrations up to 1 μM. In lung membranes, the BK B_2 receptor antagonists HPP-HOE140, HOE140 and D-Arg^0[Hyp^3,D-Phe^7]BK and BK competed for the radioligand binding with apparent K_i values of 2.13, 0.25, 227 and 1.11 nM, respectively, for the high-affinity site while for the low-affinity site K_i values of 10.5, 1.90 and 62.1 nM were elucidated. Note that a low-affinity site for D-Arg^0[Hyp^3,D-

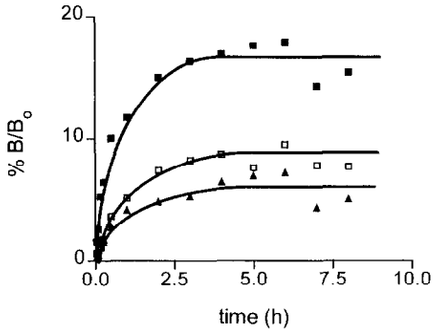


Fig. 1. Time course of [^{125}I]HPP-HOE140 association to lung (□), ileum (■) and uterus (▲) membranes. The points shown are mean values of an assay performed in triplicate.

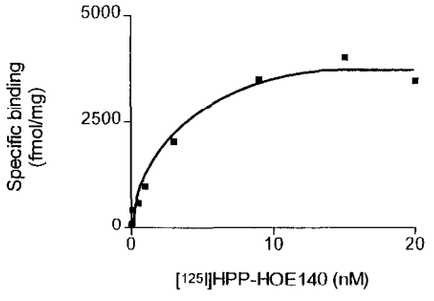


Fig. 2. Saturation binding isotherm for [^{125}I]HPP-HOE140 binding to lung membranes which were incubated in duplicate for 4 h at 22°C with various concentrations of [^{125}I]HPP-HOE140 (apparent K_D value of 3 nM and B_{max} of 92 fmol/mg protein).

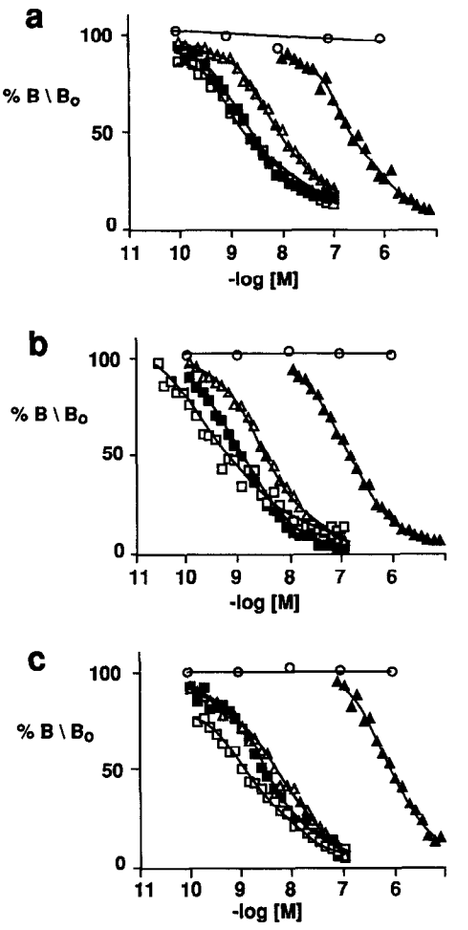
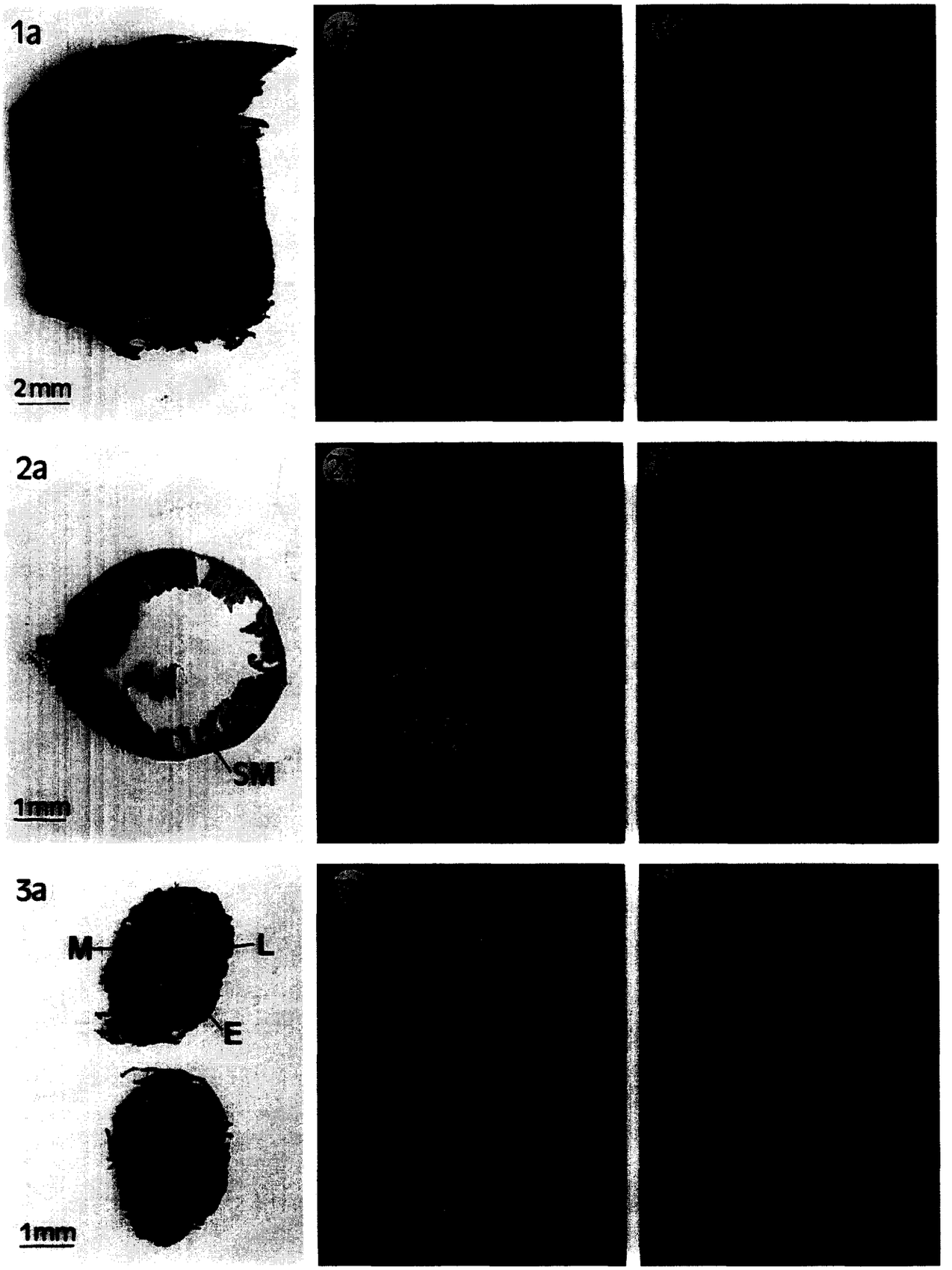
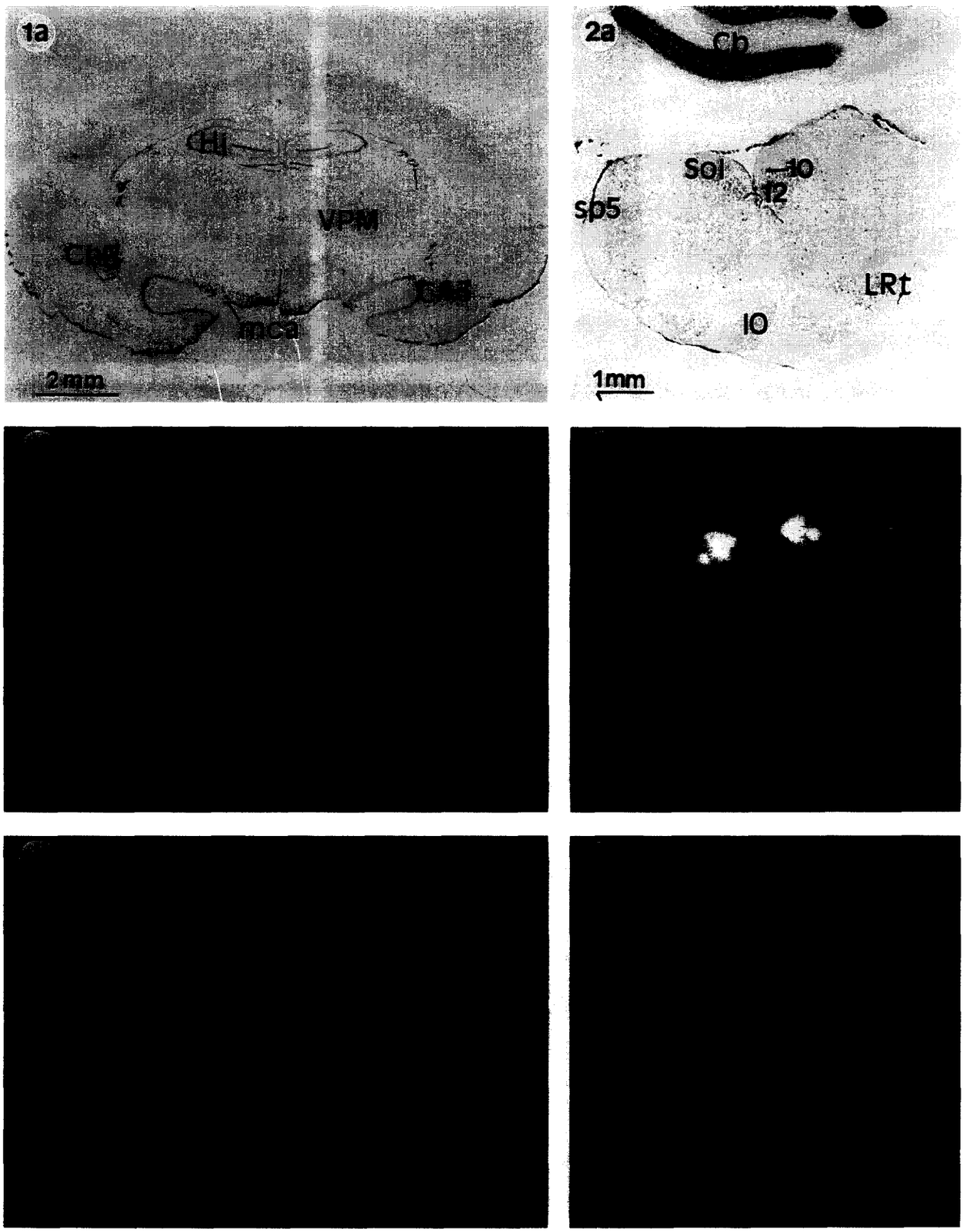


Fig. 3. Competition of [^{125}I]HPP-HOE140 binding to lung (panel a); ileum (panel b) and uterus (panel c) membranes by BK and its analogues: (□) BK; (■) HOE140; (△) HPP-HOE140; (○) des-Arg^9[Leu^8]BK; and (▲) D-Arg^0[Hyp^3,D-Phe^7]BK. At least 20 different concentrations of each unlabeled BK analogue and BK itself were assessed in duplicate for each tissue.

Fig. 4. Distribution of BK B_2-binding sites in guinea-pig lung (1), ileum (2) and uterus (3) demonstrated by in vitro autoradiography employing [^{125}I]HPP-HOE140 as the ligand. Panels 1a–3a show haematoxylin- and eosin-stained sections. Panels 1b–3b show total specific BK B_2 receptor binding. Panels 1c–3c show BK B_2 receptor binding in the presence of 1 μM HOE140 and represent non-specific binding. The white areas in panels 1b–3b represent areas of specific radioligand binding. A, arteriole; B, bronchiole; E, endometrium; L, lumen; M, myometrium; SM, smooth muscle layer; V, villi. Scale bars, 2 mm (1), 1 mm (2) and 1 mm (3).


1a
2mm
2a
SM
1mm
3a
M
L
E
1mm

Phe[7]]BK was not detected (Fig. 3a). For ileum membranes, the corresponding compounds displaced the radioligand with apparent K_i values of 0.83, 1.14, 129 and 0.05 nM, respectively, at the high-affinity site and 4.90, 1.23, 4760 and 2.01 nM at the low-affinity site (Fig. 3b). The uterine membranes showed similar affinities at the high-affinity



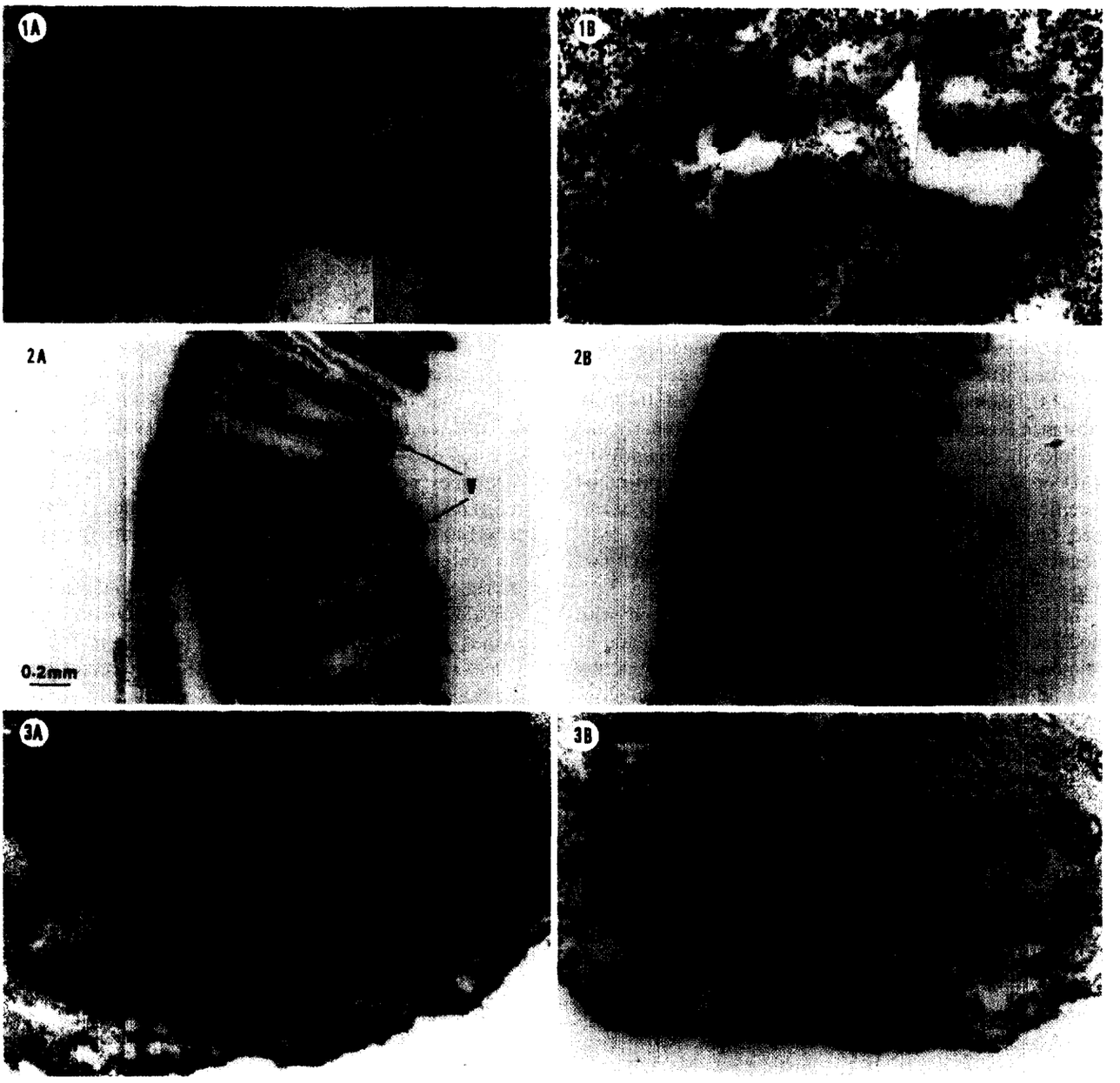


Fig. 6. Localization of BK B_2 receptors in guinea-pig lung (1), ileum (2) and uterus (3). All sections have been stained with haematoxylin and eosin. In panels 1A–3A, the black grains depict the areas of total binding. Panels 1B–3B represent non-specific binding determined in the presence of 1 μM HOE140. A, alveoli; B, bronchiole; G, glands; L, lumen; LP, lamina propria; M, myometrium; SM, smooth muscle; V, villi. Scale bars, 50 μm (1) and 0.2 mm (2, 3).

site as guinea-pig lung and ileum with the respective apparent K_i values: 0.26, 1.45, 625 and 0.17 nM. A low-affinity site was detected only when HPP-HOE140 and BK were used to compete with [^{125}I]HPP-HOE140 binding to uterine membrane preparations with the respective K_i values of 9.56 and 5.76 nM (Fig. 3c).

Fig. 5. Distribution of BK B_2-binding sites in guinea-pig diencephalon (1) and hindbrain (2) demonstrated by in vitro autoradiography employing [^{125}I]HPP-HOE140. Panels 1a and 2a show Nissl-stained sections. White areas in panels 1b and 2b depict total binding. Panels 1c and 2c represent non-specific binding. 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; CA3, CA3 region of Ammon's horn; Cb, cerebellum; ChP, choroid plexus; Hi, hippocampus; IO, inferior olive; LRt, lateral reticular nucleus; mca, middle cerebral artery; Sol, nucleus of the solitary tract; sp5, spinal trigeminal tract. Scale bars, 2 mm (1) and 1 mm (2).

3.3. Autoradiography

Optimal binding conditions for the in vitro autoradiography experiments were obtained using 170 mM Tris buffer, pH 7.4. Total but not non-specific binding was enhanced when incubations were carried out at 4°C as compared to those carried out at 22°C. The addition of the peptidase inhibitors 2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid, phosphoramidon and leupeptin or dithiothreitol or polyethleneimine had no effect on the pattern or intensity of the binding (data not shown). No displacement of specific binding occurred in the presence of an excess of the BK B_1 receptor antagonist des-Arg^9[Leu^8]BK (10 μM) or in a range of unrelated peptides (1 μM), including angiotensin II, angiotensin II-(3–8), substance P or adrenocorticotropic hormone. Similarly, the angiotensin-converting enzyme inhibitors, lisinopril (1 μM) or SQ20,881 (1 μM), had no significant effect on specific binding of [^{125}I]HPP-HOE140 in the tissues examined (Table 2). The presence of 1 μM HOE140 completely abolished specific binding by [^{125}I]HPP-HOE140 (Fig. 4–1c to 4–3c and Fig. 5–1c to 5–2c).

In lung (Fig. 4–1b), [^{125}I]HPP-HOE140 bound with moderate intensity to the endothelium and muscularis of pulmonary arteries and patchy binding occurred in the cells lining alveolar spaces. Emulsion autoradiography revealed a lower but significant density of binding over the smooth muscle cells of bronchioles (Fig. 6–1A).

In ileum, intense binding is observed over the villi (Fig. 4–2b). High-power emulsion autoradiography revealed this binding to be over the lamina propria of the villi with a lower density of receptors being present in the submucosa and the muscularis propria of the intestinal wall (Fig. 6–2A).

In the uterus, diffuse binding was observed in the stratum submucosum of the myometrium and endometrial stroma (Fig. 4–3b). Emulsion autoradiography revealed dense binding over the epithelium of the endometrial glands nearest the myometrial submucosum. No binding was observed on the luminal epithelium (Fig. 6–3A).

In the brain, a high density of BK B_2 receptors occurs in the area postrema, nucleus of the solitary tract, the solitary tract itself, the spinal trigeminal tract, choroid plexus, the middle cerebral arteries (Fig. 5) and laminae I and II of the dorsal horn of the spinal cord (data not shown). A moderate density of receptors was detected in the posterior and ventroposterior thalamic nuclei and the CA3 region of Ammon's horn of the hippocampus. No binding was observed on the pyramidal cells of the hippocampus.

4. Discussion

In this study, a K_D value of 3 nM was calculated using increasing concentrations of [^{125}I]HPP-HOE14 on guinea-pig lung membranes. This was comparable to the K_i value of 2.13 nM for the unlabeled compound, HPP-HOE140 and 0.25 nM for HOE140, its parent compound. This is in agreement with previously published work which reported a K_i value of 2.4 nM for the unlabeled HPP-HOE140 and 0.6 nM for HOE140 (Abd Alla et al., 1993). Hence, the addition of 3-4-hydroxyphenyl-propionyl or radioactive iodine does not alter the binding affinity to the BK B_2 receptors. Consequently, the radioligand, [^{125}I]HPP-HOE140 was found to bind with high affinity to saturable sites in guinea-pig lung, ileum and uterus.

The inability of the BK B_1 receptor antagonist des-Arg^9[Leu^8]BK to compete for [^{125}I]HPP-HOE140 from all tissues at concentrations of either 1 or 10 μM and the potent competition by a range of BK B_2 receptor ligands indicates that this radioligand binds specifically to the BK B_2 receptor. Analysis of saturation experiments using the radioligand alone detected only a high-affinity site, although detailed competition-binding studies using more data demonstrate that [^{125}I]HPP-HOE140 is able to differentiate a high- and a low-affinity site of the BK B_2 receptor.

[^{3}H]BK binding to guinea-pig ileal membranes has been described by several groups: two subclasses of the BK B_2 receptor subtype with K_D values of 13 pM and 910 pM were reported (Manning et al., 1986), but another study (Ransom et al., 1992) detected only a single binding site with a K_D of 16 pM. In contrast, studies using [^{125}I][Tyr^8]BK (Tousignant et al., 1991) described a single binding site with a K_D of 1600 pM. Heterogeneity of binding sites in the guinea-pig ileum was also observed when either [^{3}H]BK or [^{125}I][p-Phe^5]BK were used (Liebmann et al., 1994): [^{3}H]BK was used to detect a high-affinity site of 24 pM and a low-affinity site of 200 pM whereas [^{125}I][p-Phe^5]BK demonstrated a very high-affinity site with a K_D of 3 pM. The K_D of the low-affinity

Table 2
Effect of angiotensin-converting enzyme inhibitors on the binding of [^{125}I]HPP-HOE140 on guinea-pig tissues

	Total (dpm/mm^2)	Lisinopril (1 μM) (dpm/mm^2)	SQ 20881 (1 μM) (dpm/mm^2)	F ratio	P
Guinea-pig lung	55 ± 3.8	50 ± 11	46 ± 3.5	2.04	0.16
Guinea-pig ileum	197 ± 24	194 ± 6.4	207 ± 19	0.84	0.51
Guinea-pig uterus	189 ± 16	182 ± 6.1	184 ± 9.1	0.43	0.66

Mean ± S.D. (n = 5). Data was analysed using one-way ANOVA.

site using [^{125}I][p-Phe^5]BK was comparable to that found with [^{3}H]BK.

There is also evidence for two subclasses of the BK B_2 receptor based on their affinities for BK receptor antagonists and their coupling to G-proteins: the BK B_{2a} subclass is G-protein-coupled, a characteristic not shown for the BK B_{2b} subclass. The BK B_{2b} subclass, which has a larger population of sites, possesses a 20-fold lower affinity for Hyp^3-BK and was found on smooth muscle of guinea-pig ileum (Seguin and Widdowson, 1993). In our study, the density of binding over ileal smooth muscle was distinct, but less striking than the intense binding of [^{125}I]HPP-HOE140 to the lamina propria of the villi. Our competition studies using guinea-pig ileum membranes revealed two populations of BK B_2-binding sites. BK B_2 receptors were proposed to mediate the effects of chloride secretion in the mucosa and contractile actions in smooth muscle (Manning et al., 1986). We clearly demonstrate dense BK B_2 receptor binding in muscle layers which may be responsible for the contractile action of BK.

Studies using [^{3}H]BK also suggest the existence of two pharmacological subclasses of the B_2-binding site in guinea-pig lung, with K_D values of 15 pM and 570 pM (Trifilieff et al., 1991). In contrast, only a high-affinity binding site (K_D of 500 pM) was detected in another study (Mak and Barnes, 1991), probably because a higher concentration of the ligand was employed. Interestingly, in physiological experiments low doses of BK potentiate acetylcholine release (Omini et al., 1989), while higher doses increase thromboxane A_2 levels (Rossoni et al., 1980). These effects could be attributed to the existence of a high- and low-affinity BK B_2 receptor site in the lung.

[^{3}H]BK binding, characterized pharmacologically as being a BK B_2 receptor, was localized in the muscularis of the pulmonary vessels with lighter binding in the adventitia, the smooth muscle of the bronchioles and the alveolar walls (Mak and Barnes, 1991). We confirm the vascular and bronchiolar binding to BK B_2 receptors in this study using [^{125}I]HPP-HOE140.

To date, the distribution of BK B_2 receptors in uterine tissues has not been described. Our data demonstates BK B_2 receptors in very high density within the glandular endometrium and stratum submucosum of the myometrium, with moderate density of more diffuse binding in the endometrial stroma. Saturation studies using [^{3}H]BK binding to rat myometrial membranes show K_D values of 16 pM and 1.01 nM for the high-affinity and low-affinity BK B_2-binding sites, respectively (Liebmann et al., 1991). Our results show K_i values of 170 pM and 5.76 nM for BK in competing for the binding of [^{125}I]HPP-HOE140 to guinea-pig membranes. In rat myometrial membranes, binding of the BK B_2 receptor antagonist [Thi^{5,8},D-Phe^7]BK has been reported to distinguish two BK B_2 receptor subclasses (Liebmann et al., 1991) and it was speculated that the high-affinity binding site could represent presynaptic BK B_2 receptors, whilst the lower-affinity site might mediate BK-induced uterine contraction (Liebmann et al., 1991). However, molecular cloning from rat uterus yielded a single BK B_2 receptor which belongs to the seven-transmembrane G-protein-coupled receptor superfamily (McEachern et al., 1991; Burch and Kyle, 1992). Similarly, a single BK B_2 receptor gene has been found in humans (Ma et al., 1994). Therefore, the structural basis of the pharmacologically well-defined BK B_2 receptor subtypes remains to be established, although differential splicing of mRNA transcripts or posttranslational modifications remains a possible explanation for the receptor heterogeneity.

Our detection of a high density of BK B_2 receptors in the guinea-pig myometrium supports a role for BK in inducing uterine contraction. However, the physiological role for BK in the endometrium is unknown. Both BK and kallidin stimulate release of arachidonic acid and inositol monophosphate from human endometrial stromal cells, with endometrial glandular cells being less responsive (Bonney et al., 1993). Interestingly, the BK B_1 receptor agonist, des-Arg^9-BK also stimulates release of arachidonic acid from stromal cells but does not alter phosphoinositide hydrolysis, suggesting that the two cellular responses were mediated by different receptors, BK B_1 and B_2, respectively (Bonney et al., 1993).

In the guinea-pig brain, in vitro autoradiographic studies using [^{125}I][Tyr^8]BK have previously localized BK B_2 receptors in hindbrain regions, specifically the nucleus of the solitary tract, area postrema, dorsal motor nucleus of the vagus and the spinal trigeminal nucleus (Privitera et al., 1991). Using [^{3}H]BK in membrane-binding assays, specific high-affinity BK B_2-binding sites in the pons, medulla and spinal cord and moderate density binding in the cerebral cortex and hippocampus and low-density binding in other brain regions have been reported (Fujiwara et al., 1989). Our results using [^{125}I]HPP-HOE140 to localize BK B_2 receptors support these hindbrain findings but we believe the localization in the spinal trigeminal is in the tract, rather than the nucleus as previously described (Privitera et al., 1991). The distribution of BK B_2-binding sites in the nucleus of the solitary tract and area postrema, as described in the current study and by others (Privitera et al., 1991; Fujiwara et al., 1989), supports the role of BK in the mediation of blood pressure and cardiovascular control. Similarly, the finding of BK B_2 receptors in the spinal trigeminal tract and substantia gelatinosa of the dorsal horn supports a role for BK involvement in nociception (Steranka et al., 1988; Lopes et al., 1993). In the current study, a moderate density of diffuse binding was detected in the posterior and ventroposterior thalamic nuclei, which has not been described previously. The ventromedial portion of these thalamic nuclei receives projections from the caudal nucleus of the spinal trigeminal and this pathway is involved in the relay of tactile information via the spinal trigeminal tract and the spinal trigeminal nucleus from the facial nerves (Dodd and Kelly, 1991). The involvement of

BK in nociception is well-documented (Dray and Perkins, 1993) and the presence of BK B_2 receptors in these areas of the brain provides further evidence for the role of BK in pain perception.

Our current work also demonstrates a high density of BK B_2 receptors on the choroid plexus, a circumventricular organ composed of highly vascularized connective tissue which is responsible for the production of cerebrospinal fluid (Nilsson et al., 1992). Perfusion of an angiotensin-converting enzyme inhibitor ventriculocisternally results in an increase in cerebrospinal fluid production (Vogh and Godman, 1989), a possible action of BK on the choroid plexus. Furthermore, we demonstrate a high density of BK B_2 receptors in the middle cerebral arteries where BK has a vasodilatory action (Onoue et al., 1994).

In summary, [^{125}I]HPP-HOE140 binds specifically to BK B_2 receptors in a range of tissues, with no detectable binding to BK B_1 receptors. Unlike [^{3}H]BK (De Vries et al., 1989), [^{125}I]HPP-HOE140 does not bind either of the active sites of angiotensin-converting enzyme as shown by the lack of effect of the angiotensin-converting enzyme inhibitors, lisinopril or SQ20,881 on displacing the binding of this radioligand. We conclude that this radioiodinated BK analogue offers a high-affinity, specific radioligand, with low non-specific binding, which is suitable for the pharmacological characterization of BK B_2-binding sites and for in vitro autoradiographic localization of BK B_2 receptors in tissues. Using this radioligand, we have confirmed some earlier reports using [^{3}H]BK and extended them to show distribution of BK B_2 receptors in uterus and the CNS.

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