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Biochimica et Biophysica Acta 1191 (1994) 109–116

BBA Biochimica et Biophysica Acta

Co-solubilization of bradykinin B_2 receptors and angiotensin-converting enzyme from guinea pig lung membranes

Alexandre Trifilieff, Estelle Lach, Mouafak Mousli, El-Bdaoui Haddad, Yves Landry, Jean-Pierre Gies *

Laboratoire de Neuroimmunopharmacologie INSERM CJF 91-05, Université Louis Pasteur – Strasbourg I, BP 24, 67401 Illkirch Cedex, France

(Received 1 July 1993)

Abstract

Bradykinin B_2 receptor-like binding activity was solubilized from guinea pig lung using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (Chaps). The binding of [^{3}H]bradykinin to the soluble fraction was time-dependent and saturable. Scatchard analysis of equilibrium binding data indicated that the soluble extract contained a single class of binding sites with a K_d of 696 pM and a B_{max} of 57 fmol/mg protein. Unlabelled bradykinin and B_2 antagonists inhibited the binding of [^{3}H]bradykinin to Chaps-solubilized extracts with relative potencies similar to those observed with the low-affinity membrane-bound binding sites. Following partial purification of the soluble preparation, using anion exchange (DEAE-Sephacel) and gel filtration (Aca 34) column chromatography steps, two peaks eluted off the column were able to bind [^{3}H]bradykinin and have molecular masses of 168 and 98.5 kDa. The former seems to represent binding of bradykinin to angiotensin converting enzyme (ACE, EC 3.4.15.1) and the latter binding to bradykinin receptor. Using purified commercial ACE, we show that the binding of [^{3}H]bradykinin to ACE can easily be distinguished from that of the bradykinin receptor, since both B_1 and B_2 ligands were able to inhibit bradykinin binding with affinities clearly different from that expected for a bradykinin receptor.

Key words: Bradykinin receptor; Angiotensin-converting enzyme; Solubilization; [^{3}H]Bradykinin binding; Lung; (Guinea pig)

1. Introduction

Kinins, bradykinin (BK) and kallidin are peptide hormones which are known to be involved in a number of physiological events including inflammation and bronchoconstriction. These events are mediated by two classes of receptors, B_1 and B_2 [1,2]. Radioligand binding studies have identified B_2 BK receptors in a variety of tissues including bovine uterus [3,4], guinea pig heart, ileum, kidney and brain [5,6], and more recently rat myometrium [7]. Moreover, the recent cloning and sequencing of the gene coding for the B_2 receptor from rat [8] and human [9] sources indicate that it belongs to the seven transmembrane G protein-coupled superfamilly of receptors. These studies have contributed to the knowledge of the pharmacology of B_2 receptors, but the structural properties of native B_2 receptors are still poorly characterized.

Solubilization of BK binding activity from various tissues known to contain B_2 receptors has been demonstrated [10–12], but evidence has suggested that there exists a novel BK receptor in guinea pig airways, referred to as B_3 [13,14]. On the other hand, other work indicates that this BK airway receptor may belong to the B_2 subtype (see Ref. [15] for review). Moreover, in tissues such as rat mast cells [16] or guinea pig trachea [2] kinins could act via receptor independent mechanisms. In view of this complex situation and in order to clarify the mode of action of BK in the guinea

* Corresponding author. Fax: +33 88 678638.

Abbreviations: BK, bradykinin; DTT, dithiothreitol; BSA, bovine serum albumin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; NPC 17731, D-Arg^0[Hyp^3,HypE(*trans*-propyl)^7, Oic^8]-bradykinin; Hoe 140, D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin; ACE, angiotensin-converting enzyme; DSS, disuccinimidyl suberate; BS_3, bis(sulphosuccinimidyl)suberate; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; GTPγS, guanosine 5'-[γ-thio]triphosphate; NEP, neutral endopeptidase.

SSDI 0005-2736(93)E0384-M

pig airways, the characterization of BK receptor at the biochemical level is required. Solubilization of kinin receptors in guinea-pig airways is the first step necessary in order to understand the mode of action of these peptides. Despite widespread investigation, the molecular and structural properties of the native B_2 BK receptor has been hampered by the lack of a photoaffinity probe able to discriminate between kinin receptor and kininases [17], the aggregation of soluble receptor [12], and the low amount of receptors using immunoprint analysis [18].

We have previously shown the existence of two B_2 binding sites in guinea pig lung [19]. Here we have studied the solubilization of these particulate binding sites and have attempted to partially purify the BK receptor. Some of these findings have been subject of a preliminary communication [20].

2. Materials and methods

Materials

[2,3-*propyl*-3,4(n)-^{3}H]Bradykinin ([^{3}H]BK, spec. act. 103 Ci/mmol) and ^{125}I-[Tyr^8]bradykinin (^{125}I-BK, spec. act. 2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). BK, kallidin, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (Chaps), bovine serum albumin (BSA), EGTA, bacitracin (Zn^{2+} free), dithiothreitol (DTT), 1,10-phenanthroline (a chelating agent which inhibited metalloendopeptidases), $MgSO_4$, DEAE-Sephacel, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (Tes), polyethylenimine, angiotensin-converting enzyme from rabbit lung and molecular mass markers for gel filtration were purchased from Sigma (St Louis, MO). D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, [D-Phe^7]-BK, des-Arg^9-BK and des-Arg^9-[Leu^8]-BK, were obtained from Cambridge Research Biochemicals (Cambridge, UK) and Tris (hydroxymethyl)aminoethane from Merck (Darmstadt, Germany). Captopril (SQ 14225) was a gift from Squibb Pharmaceuticals (Epernon, France). D-Arg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Hoe 140) was a gift from Hoechst AG. (Frankfurt, Germany), D-Arg^0[Hyp^3,HypE(*trans*-propyl)^7,Oic^8]-BK (NPC 17731) was provided by Scios-Nova (Baltimore, USA). Disuccinimidyl suberate (DSS), 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and bis(sulphosuccinimidyl)suberate (BS_3) were obtained from Pierce (Oud-Beijerland, The Netherlands). Guanyl-5′-yl imidodiphosphate (Gpp(NH)p) and guanosine 5′-[γ-thio]triphosphate (GTPγS) were purchased from Boehringer Mannheim (Germany). Ultrogel AcA 34 was from IBF biotecnics (Villeneuve-la-Garenne, France).

Membrane preparation and solubilization

Lung preparation of albino Dunkin-Hartley guinea pigs was as described previously [19]. For the solubilization procedure, final pellets were resuspended in 4–12 vol. of assay buffer (25 mM Tes, 1 mM 1,10-phenanthroline, 140 μg/ml bacitracin, 10 μM captopril, 1 mM DTT (pH 6.8)). These membrane preparations were used immediately for solubilization of BK binding activity. Various concentrations of Chaps were added to the membrane preparations (0–20 mM) in order to determine the optimal conditions for solubilization. Mixing was performed gently in an ice bath for 30 min followed by centrifugation at $100\,000 \times g$ for 1 h. The supernatant obtained was diluted 2-fold with assay buffer. When used, the pellets (detergent-treated membranes) were resuspended in 60 volumes of assay buffer.

Binding assays of soluble receptors

Soluble binding assays were performed in polypropylene tubes with freshly solubilized membranes. To each tube was added 400 μl of supernatant or diluted supernatant, 50 μl of assay buffer or assay buffer containing BK or BK analogues and 50 μl of [^{3}H]BK. The assay tubes were vortexed slowly and incubated in an ice bath. Bound radioactivity was separated from free radioactivity by vacuum filtration through a nitrocellulose filter (BA 85, Schleicher and Schuell) pre-treated with 0.3% aqueous polyethylenimine for 2 h. Filters were then rinsed with 3×3 ml of ice-cold Tris-HCl (25 mM (pH 6.8)) and placed in a vial with 6 ml of scintillation cocktail (Aquasol, New England Nuclear). Vials were shaken for 20 min and counted 12 h later using a Beckman LS1800 counter.

Kinetic experiments were carried out at 4°C. The association reaction was measured at various time intervals after the addition of 0.5 nM [^{3}H]BK, in the presence or absence of unlabelled BK. Dissociation kinetics were studied as follows; after equilibrium had been reached (120 min), tracer dissociation was induced by isotopic dilution with 1 μM unlabelled BK. Specific binding was determined at appropriate time intervals. Saturation experiments were performed using 16 different concentrations of radioligand, ranging from 0.005 to 6 nM. Competition experiments were performed using 0.6 nM [^{3}H]BK. In both cases, incubations were for 3 h at 4°C.

Particulate and detergent-treated membrane binding assays were performed as described previously for high- and low-affinity sites [19] except that incubations were carried out in a total volume of 500 μl.

All binding assays were performed in triplicate. Specific binding was defined as the difference between total binding and the amount bound in the presence of 1 μM unlabelled BK. Binding data were analyzed by use of iterative curve-fitting procedures as described [21] using the LIGAND programme (Elsevier–Biosoft, Cambridge, UK). Protein concentration was determined according to Spector [22].

DEAE-Sephacel column

Soluble fractions of guinea pig lung were loaded onto a DEAE-Sephacel column (12 × 1.5 cm). The column was pre-equilibrated at 4°C with 40 ml 25 mM Tes containing 1 mM EGTA, 1 mM DTT, 3 mM Chaps (pH 6.8) (buffer A). Proteins were eluted at a flow rate of 1 ml/min with a linear gradient of NaCl (0–500 mM) and the column was re-generated with 1 M NaCl. Two ml fractions were collected, diluted twice with binding assay buffer containing two-fold concentrated peptidase inhibitor cocktail, then assayed for specific binding activity. Fractions containing specific binding were pooled and concentrated to 1 ml using the Amicon concentration system with a 10 kDa membrane before loading onto the gel filtration column.

Gel filtration column

An Ultrogel AcA 34 column (90 × 1.6 cm) was equilibrated at 4°C with buffer A containing 1 mM NaCl, at a flow rate of 0.25 ml/min. Purified ACE (25 μg of protein) or the samples concentrated after DEAE-Sephacel column chromatography were loaded onto the Ultrogel column and elution (0.25 ml/min) was performed at 4°C with buffer A containing 1 mM NaCl. Fractions of 2 ml were collected, diluted twice with binding assay buffer (two-fold concentrated peptidase inhibitor cocktail), and assayed for specific binding activity. Competition experiments on purified commercial ACE (2.7 μg/assay) were performed in triplicate using the same buffer. The column was calibrated under the same conditions using the following markers (apparent molecular mass in kDa); Blue dextran (void volume, 2000), β-amylase (200), alcohol dehydrogenase (150), bovine serum albumin (66), carbonic anhydrase (29), cytochrome *c* (12.4).

3. Results

Because the cross-linking of [^{3}H]BK or ^{125}I-BK to membrane bound receptor, using various homobifunctional reagents (DSS, BS_3, DFDNB), were unsuccesful, we have developed a purification procedure of the BK receptor.

Development of optimal solubilization procedure

The yield of solubilized BK binding sites was dependent on the concentration of Chaps used and the amount of protein in the membrane preparation. [^{3}H]BK binding activity (0.5 nM) was optimally solubilized with 10 mM Chaps in a membrane preparation containing 15 mg protein/ml. Under these conditions approximately 30% of total membrane protein was solubilized and specific binding was 4152 ± 34 dpm/tube ($n = 3$, Fig. 1).

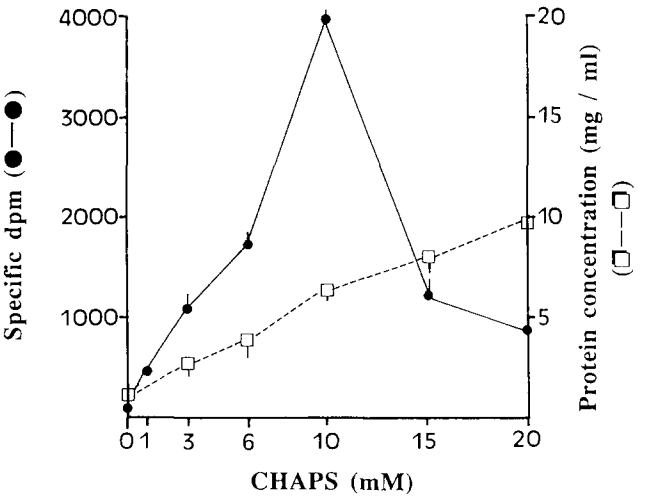

Fig. 1. Effect of Chaps concentration on the solubilization of bradykinin binding activity and proteins from guinea pig lung membrane. A fraction of guinea pig lung membrane (15 mg protein/ml) was extracted using various concentrations of Chaps for 30 min at 4°C. Points are means ± S.E. of three experiments performed in triplicate.

Kinetics of [^{3}H]BK to soluble binding sites

The specific binding of [^{3}H]BK to soluble receptors was time dependent ($n = 2$). Equilibrium was obtained within 120 min, binding remained constant for a further 3 h and after 24 h incubation 60% of binding activity was lost. After 120 min of association, dissociation was initiated by addition of 1 μM unlabelled BK and resulted in an exponential reduction of specifically

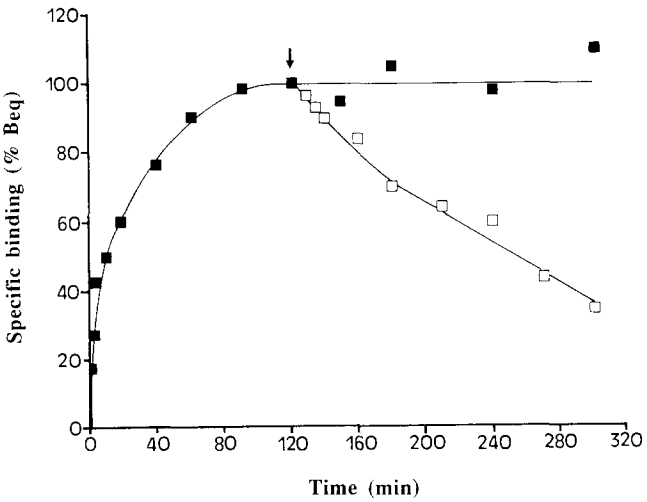

Fig. 2. Kinetics of [^{3}H]bradykinin binding (0.5 nM) to CHAPS-solubilized extracts. Association (■) and dissociation (□) kinetics were examined at 4°C. When equilibrium was reached (120 min), the dissociation was started by addition of 1 μM unlabelled bradykinin (arrow). All data are expressed as percentage of specific [^{3}H]BK binding at equilibrium and represent the average of two experiments performed in triplicate.

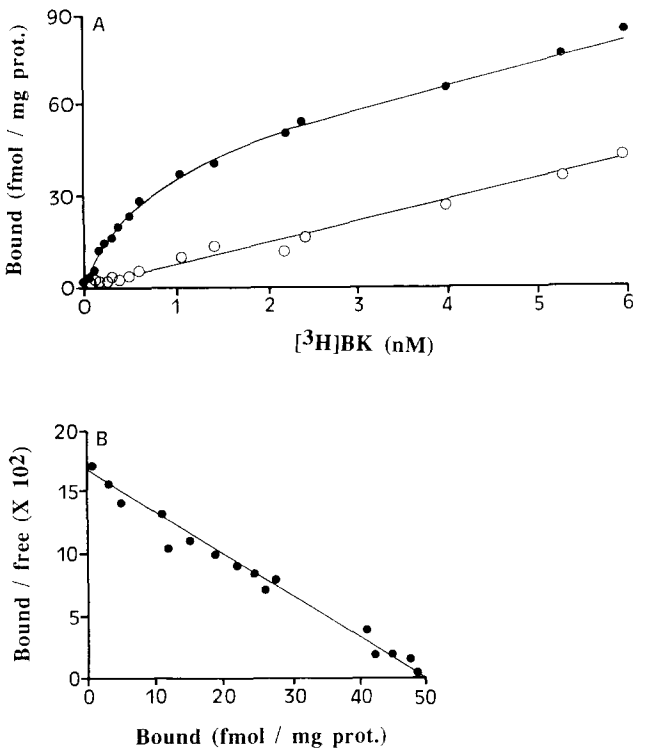

Fig. 3. (A) Saturation binding of [^{3}H]bradykinin to soluble binding sites. After addition of various concentrations of radioligand (0.005–6 nM), total (●) and non-specific (○) binding were measured as described in Materials and methods. (B) Scatchard analysis of saturation isotherms of [^{3}H]bradykinin. Points shown are from one experiment representative of four other independent experiments.

bound [^{3}H]BK (Fig. 2). The association rate constant (k_{obs}) was $2.62 \cdot 10^{-2}$ min^{-1} ($2.1–3.15 \cdot 10^{-2}$ min^{-1}) and the dissociation rate constant (k_{-1}) was $5.01 \cdot 10^{-3}$ min^{-1} ($4.83–5.19 \cdot 10^{-3}$ min^{-1}). The value of the association rate constant (k_{+1}) calculated using the equation $k_{+1} = (k_{obs} - k_{-1})/[^3H]BK$ was $3.99 \cdot 10^7$ min^{-1} M^{-1}. Finally, the equilibrium dissociation constant $K_d = k_{-1}/k_{+1}$ could be calculated from these kinetic parameters, giving value of 126 pM.

Pharmacology and specificity of soluble binding sites compared with membranes sites

The [^{3}H]BK binding to soluble sites was saturable ($n = 4$; Fig. 3A). A linear Scatchard plot was obtained as shown in Fig. 3B. The Hill coefficient was close to one suggesting the presence of a single class of binding sites with a K_d value of 696.3 ± 167.5 pM and a B_{max} value of 57.6 ± 2.5 fmol/mg of protein.

The affinity of B_1 and B_2 ligands for the soluble binding sites was studied using 0.6 nM [^{3}H]BK. Neither the B_1 agonist, des-Arg^9-BK, nor the B_1 antagonist, des-Arg^9-[Leu^8]-BK, displaced the specific binding of [^{3}H]BK. In contrast, all the B_2 antagonists used completely displaced the specific binding of [^{3}H]BK. The Hill coefficient- and K_i values of these BK analogues are shown in Table 1. The new highly potent antagonists Hoe 140 and NPC 17731 [23,24] showed affinities similar to that of BK, whilst classical B_2 antagonists used in the present study and in our previous work [19] show much lower affinities (Table 1). The relative potencies of these analogues for inhibiting [^{3}H]BK binding to soluble and particulate binding sites were calculated from the K_i or IC_{50} ratio as described in the legend of Table 1. There is a good correlation between these relative potency values for the soluble and low-affinity particulate binding site ($R = 0.95$), supporting the selective extraction of the low-affinity particulate binding site. Non-hydrolyzable analogues of guanosine triphosphate, GTPγS and Gpp(NH)p, have no effect on [^{3}H]BK specific binding to intact or soluble binding sites.

Table 1
Affinity and relative potencies of bradykinin analogues for the binding of [^{3}H]BK to soluble and particulate binding sites

	Soluble binding sites				Particulate binding sites			
	nH	K_i (nM)	K_i analogue /K_i BK	(n)	nH	IC_{50} (nM)	IC_{50} analogue /IC_{50} BK	(n)
Bradykinin	0.97 ± 0.05	0.72 ± 0.16	1.0	(6)	0.76 ± 0.07	2.07 ± 0.33	1.0	(9) [a]
NPC 17731	1.24 ± 0.14	2.23 ± 0.39	3.23 ± 0.18	(3)	0.89 ± 0.04	2.09 ± 0.46	1.10 ± 0.16	(3)
Hoe 140	1.19 ± 0.03	2.16 ± 0.67	3.47 ± 0.86	(4)	0.83 ± 0.02	2.11 ± 0.01	1.05 ± 0.00	(3)
Kallidin	0.96 ± 0.09	2.57 ± 0.48	3.02 ± 0.19	(4)	0.74 ± 0.06	11.27 ± 2.29	4.39 ± 0.20	(3) [a]
[Thi^{5,8},D-Phe^7]-BK	0.94 ± 0.04	61.34 ± 5.51	87.57 ± 6.06	(4)	0.81 ± 0.07	242.86 ± 31.72	115.79 ± 7.78	(5) [a]
D-Arg^0-[Hyp^3,Thi^{5,8},D-Phe^7]-BK	0.90 ± 0.09	64.54 ± 1.84	93.57 ± 2.95	(3)	0.78 ± 0.04	278.75 ± 18.43	138.66 ± 8.41	(4)
[D-Phe^7]-BK	1.05 ± 0.09	80.62 ± 8.92	116.4 ± 17.22	(4)	0.83 ± 0.05	259.53 ± 25.58	115.98 ± 15.11	(3) [a]
des-Arg^9-BK	–	> 100,000	–	(3)	–	inactive	–	(6) [a]
des-Arg^9-[Leu^8]-BK	–	> 100,000	–	(3)	–	inactive	–	(4) [a]

The relative potencies (analogue affinity/BK affinity) were calculated from each IC_{50} or K_i and not from the mean IC_{50} or K_i, which accounts for the apparent discrepancy between the average IC_{50} or K_i and the IC_{50} or K_i ratio. The K_i values for soluble binding sites were calculated as described previously [31]. For particulate binding sites experiments were performed under conditions which label both high- and low-affinity particulate binding sites ([^{3}H]BK = 0.6 nM).

[a] Data from Ref. [19].

Table 2
Purification steps of the bradykinin receptor from guinea pig lung membranes

Step	Specific binding (fmol)	Total protein (mg)	Specific activity (fmol/mg protein)
Membranes	16416	833.3	19.7
Solubilization	4795	237.4	20.2
DEAE-Sephacel	3396	146.4	23.2
Aca 34	184	0.6	207

The data are from a single preparation which is representative of three similar experiments performed in triplicate. Specific binding was determined using 0.5 nM of [^{3}H]BK.

To improve the possible selective extraction of the low-affinity sites we also studied the competitive inhibition of [^{3}H]BK (0.6 nM) by unlabelled BK on detergent treated membranes ($n = 4$). Unlabelled BK gave a Hill coefficient of 1.06 ± 0.05 and a K_i of 0.80 ± 0.16 nM.

Partial purification of the bradykinin receptor

Three techniques were used for partial purification of the bradykinin receptor-like binding activity, the data from a representative preparation are summarized in Table 2. Crude membranes were solubilized with 10 mM Chaps. The soluble extract was chromatographed on a DEAE-Sephacel column. The elution with a linear gradient of NaCl (0–500 mM) produced a large peak of protein which generally included over 80% of the total binding applied to the column (Fig. 4). The fractions containing [^{3}H]BK binding activity (230–380 mM of NaCl) were pooled, concentrated

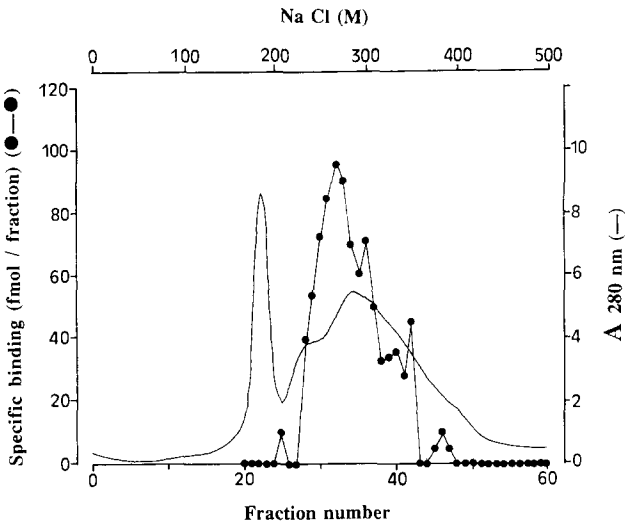

Fig. 4. Anion-exchange column chromatography of Chaps-solubilized bradykinin binding activity. Solubilized membranes were applied to a DEAE-Sephacel column. After washing with buffer as described, the sample was eluted with a linear gradient of NaCl (0–500 mM). The elution was followed by recording the absorbance at 280 nm (—). Specific [^{3}H]bradykinin binding activity (●———●) of 2 ml fractions was determined in triplicate.

to 1 ml using an ultrafiltration system, and loaded onto an AcA 34 gel filtration column (Fig. 5). Application of DEAE-Sephacel-purified binding activity to the AcA 34 column resulted in the recovery of about 75% of the total binding activity loaded onto the column. A small portion of [^{3}H]BK binding activity was eluted in the

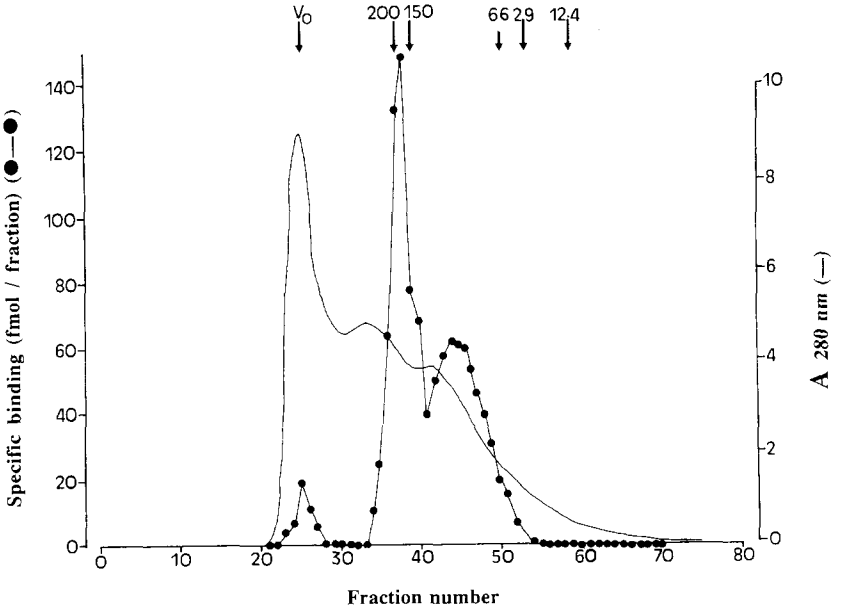

Fig. 5. Gel filtration column chromatography of purified Chaps-soluble bradykinin binding activity. Following DEAE-Sephacel column chromatography, the fractions containing specific [^{3}H]bradykinin binding (28–50) were pooled, concentrated to 1 ml on an Amicon concentration system and loaded onto a AcA 34 column. Elution was followed by recording the absorbance at 280 nm (—). Specific binding activity (●———●) of 2 ml fractions was determined in triplicate. The column was calibrated with; blue dextran (void volume, 2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa).

void volume, probably reflecting aggregation of the soluble receptor or co-migration with other proteins. Two major peaks appeared with apparent molecular masses of 168 kDa (189–156) and 95.8 kDa (110–80). The specific activities of these two peaks were 303.5 and 207 fmol/mg of protein, respectively. Angiotensin-converting enzyme (ACE), with a molecular mass of 166 kDa has been reported to bind kinin ligands [17]. Since lung is a rich source of this enzyme, we examined the possibility that one of the two peaks observed was ACE. Omission of captopril in the binding medium led to an increase by 50% of the specific binding present in the 168 kDa peak, whereas the 98.5 kDa peak was unaffected (data not show). Following gel filtration column chromatography of purified commercial (Sigma) ACE in the presence of the peptidase inhibitor cocktail described in materials and methods, a [^{3}H]BK binding peak with a mobility identical to that of the 168 kDa band (166 kDa) was recovered. Similarly, omission of captopril in the binding medium led to an increase of the signal observed (data not show). This result suggest that the high molecular mass peak (168 kDa) represent binding of bradykinin to ACE.

Ligand specificity of [^{3}H]BK binding to purified commercial ACE

Because [^{3}H]BK seems to be able to bind to ACE in our purified preparation, we tested the specificity of the [^{3}H]BK binding to ACE by studying, in competition experiments ([^{3}H]BK = 0.6 nM; $n = 3$), the binding of various BK analogues to the purified commercial ACE. All the compounds used displaced totally the [^{3}H]BK binding to ACE with the following affinity (IC_{50} in nM): BK (3.00 ± 0.79), Hoe 140 (46.81 ± 9.07), des-Arg^9-[Leu^8]-BK (558.37 ± 11.18) and [Thi^{5,8},D-Phe^7]-BK (5332.94 ± 456.75). The specificity of ACE binding was clearly different from that expected for a BK receptor since both the B_1 (des-Arg^9-[Leu^8]-BK) and B_2 (Hoe 140, [Thi^{5,8},D-Phe^7]-BK) ligands displaced the [^{3}H]BK binding.

4. Discussion

The bradykinin receptor-like binding activity was solubilized from guinea pig lung in an active form using Chaps. This zwitterionic detergent has already been successfully employed for the solubilization of bradykinin receptors from bovine uterine myometrium [10], human fibroblasts [12], and guinea pig ileum [25]. As with most receptors, the binding activity is very sensitive to the concentration of detergent used. Bradykinin receptors have been optimally solubilized with 3 mM Chaps in bovine uterine myometrium [10] or 4 mM for human fibroblasts [12], whereas a combination of Chaps and digitonin was employed for rat uterus and NG 108–15 cells [11]. This apparent discrepancy might be due to species variation in the lipid composition of the membrane or in the molecular structure of the receptor, since the solubilization of bradykinin receptor from guinea pig tissues requires 10 mM of Chaps ([25] and present study).

Scatchard analysis of [^{3}H]BK binding to solublized sites revealed a single class of sites having a K_d of 696 pM. This is comparable with the kinetically determined K_d (126 pM) and the K_i of 720 pM estimated from competitive inhibition studies of [^{3}H]BK to solubilized binding sites (Table 1), which corresponds well with the K_d of low-affinity particulate binding sites reported previously (570 pM) [19]. From our binding data on the solubilized membrane preparation, there was no indication that more than one class of binding site was involved. However, gel filtration column chromatography revealed that BK can label it's receptor (98.5 kDa) and also ACE (168 kDa). Therefore, our binding data on soluble (present study) and particulate membrane preparations [19] might include binding to non-receptor sites (i.e., ACE). Indeed, some workers have reported the binding of BK to ACE [17,26]. Therefore, the membrane-bound low-affinity binding site revealed in our previous study [19] may, in fact, represent binding to ACE. However, increasing concentrations of captopril (10–600 μM) do not affect [^{3}H]BK binding (1.2 nM) to the particulate membrane preparation (data not show). This supports the idea that this low-affinity binding site is not, in fact, related to binding of [^{3}H]BK to ACE. In the soluble preparation, we failed to reveal binding to ACE despite using a range of [^{3}H]BK concentrations in saturation experiments (up to 6 nM). This could be explained by the absence of NaCl in our medium, since Lanzillo and Fanburg [27] have shown that ACE from guinea pig lung requires NaCl for activity. Nevertheless, our binding study when performed on purified commercial ACE showed that the binding specificity of the enzyme can be easily distinguished from the BK receptor binding in respect to the affinity of BK analogues. Indeed, both B_1 and B_2 antagonists are able to displace [^{3}H]BK binding to ACE. Moreover, the affinities of BK antagonists for ACE are clearly different from those reported for the BK receptors. Therefore, the pattern of binding obtained with B_1 and B_2 ligands on soluble (present study) or particulate [19] membrane preparations clearly shows that under our experimental conditions the BK binding activity in the guinea pig lung does not represent ACE binding.

To ensure that the binding activity we solubilized represents the low-affinity receptor for BK in the membrane preparation, we compared the binding properties of soluble and particulate binding sites. For a range of BK analogues there is a good correlation between the ratio K_i analogues/K_i BK for the soluble

binding sites and the ratio IC_{50} analogues/IC_{50} BK for the particulate binding sites, under conditions where the two sites are labelled. The B_1 ligands were unable to displace completely the [^{3}H]BK soluble binding activity (approximatelly 15% at a dose of 10^{-4} M), demonstrating that the soluble activity was not of the B_1 type. All the B_2 antagonists used displaced BK binding and the nanomolar affinities for the BK receptor of the new potent B_2 antagonists (Hoe 140 and NPC 17731) were recovered in both particulate and soluble fractions. Therefore, the soluble BK binding activity appears to be identical to the particulate one and could be classified as a BK B_2 receptor.

Statistical analysis of saturation curves demonstrated the existence of two states having different affinities for BK in guinea pig lung membranes [19]. Similar high- and low-affinity states have been described in different tissues and cell lines [4,5,7,28]. The finding that the soluble extract from guinea pig lung membranes contains only one (low-affinity) population is not a new concept. Similar observations have been made for the A_2 adenosine receptor in rat brain [29] and also for BK in various tissues. In crude membranes of rat [7] or bovine myometrium [4] and NG 108–15 cells [28] binding studies indicated the presence of two classes of binding sites. When these binding sites were solubilized, only one was detected by binding studies [10,11], but no explanation was offered to explain this phenomenon. This can be interpreted in three different ways; 1) Chaps allows the extraction of only the low-affinity state, (2) both kinds of populations are solubilized but the high-affinity state is inactivated when solubilized and (3) both kinds of populations are solubilized but are converted into a single low-affinity class. The fact that the receptor remaining in the detergent-treated membranes displays only low-affinity binding sites eliminates the first possibility. The second hypothesis would implicate a loss of binding sites which does not occur by solubilization. Instead, the values of B_{max} obtained are in agreement with a transition of the high-affinity site into low-affinity sites in the solubilized receptors. A conversion of high- to low-affinity sites in the soluble state might be due to interaction of BK receptors with a G-protein. In this case it could be suggested that during the solubilization procedure the interaction between G-protein and BK receptor is lost. However, we can exclude this hypothesis since non-hydrolyzable analogues of GTP have no effect on [^{3}H]BK binding in the membrane preparation. Taken together, our results seem to indicate that the micro-environment of the intact membrane is responsible for the existence of two states of binding for BK, since when this micro-environment is lost, only one population of binding site is detected.

Cross-linking of the membrane receptors to [^{3}H]BK or ^{125}I-BK with several homobifunctional reagents were unsuccessful, therefore we attempted to determine the molecular mass of the BK receptor in a partially purified soluble preparation. After solubilization, anion-exchange and gel filtration column chromatography steps, [^{3}H]BK labels two peaks with apparent molecular masses of 168 and 98.5 kDa. We examined the possibility that one of these two peaks represents a peptidase since; (1) two types of peptidases have been reported to mimick kinin receptor binding, ACE [26] and neutral metalloendopeptidase (NEP, EC 3.4.24.11) [30], (2) guinea pig lung is known to contain these enzymes [27] and (3) the apparent molecular mass of 168 kDa obtained by gel filtration is similar to that reported for ACE [17].

ACE and NEP are metallopeptidases which are inactivated by chelating agents. In this inactivated state NEP is unable to bind bradykinin [30]. In contrast, ACE is able to bind bradykinin when it is catalytically inactive [26]. The presence of 1 mM EGTA, a chelating agent, allowed us to exclude the possibility of a non-receptor binding site to NEP, but binding to ACE has to be considered since guinea pig lung is a rich source of this enzyme [27]. The presence of 10 μM captopril in our binding medium might prevent binding to ACE, but captopril and its derivatives (enalaprilat) have limited ability to block the labelling of [^{3}H]BK to ACE [17]. Indeed, as observed by DeVries et al. [17] on NG 108–15 cells, omission of captopril in our soluble binding assays led to heavy labelling of the 168 kDa peak. When purified commercial ACE was loaded onto the gel filtration column it yielded an apparent molecular mass (166 kDa) identical to that of the 168 kDa soluble peak. Moreover, as observed for the 168 kDa peak, BK binding activity to purified ACE is enhanced in the absence of captopril. These observations suggest that the 168 kDa peak represents binding to ACE and therefore the 98.5 kDa peak might represent the BK receptor.

In conclusion, we have reported the solubilization of an active B_2 receptor from guinea pig lung. Following partial purification of this soluble preparation and gel filtration column chromatography, we were able to determine an apparent molecular mass of 98.5 kDa for BK receptor. This study is the first step in the purification of the bradykinin receptor. Purified bradykinin receptors should prove useful in studies to unravel the molecular and biochemical events which occur when bradykinin binds to its receptors in vivo.

5. Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche Médicale (grants CRE 900504 and CJF 91-05) and by the Ligue Nationale Contre le Cancer (Fédération Nationale et Départe-

mentale des Centres de Lutte contre le Cancer). We are grateful to Hoechst AG (Frankfurt) for providing Hoe 140 and to Scios-Nova Inc. (Baltimore, MD) for the gift of NPC 17731.

6. References

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