Bradykinin binding sites in healthy and carcinomatous human lung

Alexandre Trifilieff, Estelle Lach, *Pascal Dumont & ¹Jean-Pierre Gies

Laboratoire de Neuroimmunopharmacologie-Pulmonaire INSERM CJF-9105, Université Louis Pasteur-Strasbourg I, B.P. 24, 67401 Illkirch Cedex, France and *Service de Chirurgie Thoracique, Hospices civils de Strasbourg, B.P. 426, 67091 Strasbourg Cedex, France

1 Direct ligand binding techniques have been used to compare bradykinin receptors in squamous- or adeno-carcinoma and healthy lung membranes removed from patients during operations.

2 The binding of [³H]-bradykinin to healthy lung membrane is time-dependent and saturable with a K_D value of 1.08 ± 0.18 nM and a B_{max} value of 46.1 ± 3.1 fmol mg⁻¹ protein ($n = 10$). In squamous-carcinoma tissue ($n = 8$) the same amount of receptors are present, $B_{max} = 52.2 \pm 3.3$ fmol mg⁻¹ protein ($P = 0.22$) but the K_D value is significantly higher 2.57 ± 0.40 nM ($P = 0.004$). Similar measurements were obtained for adeno-carcinoma tissue ($n = 3$), $K_D = 2.80 \pm 0.29$ nM ($P = 0.001$) and $B_{max} = 49.8 \pm 2.1$ fmol mg⁻¹ protein ($P = 0.56$).

3 In both healthy and squamous-carcinoma preparations, bradykinin analogues displace [³H]-bradykinin binding with the following relative order of potency: Hoe 140 > bradykinin > kallidin > D-Arg⁹[Hyp³,D-Phe⁷]bradykinin >>> des-Arg⁹-bradykinin. Of the analogues used, bradykinin and D-Arg⁹[Hyp³,D-Phe⁷]bradykinin appear to be able to differentiate the bradykinin receptors present in both preparations.

4 It is concluded that bradykinin receptors present in healthy and carcinomatous human lung are of the B₂ type.

Keywords: Bradykinin; bradykinin binding sites; human lung; tumours; B₂ receptor

Introduction

Evidence that bradykinin (BK) and related kinins are involved in various pulmonary diseases has accumulated during the past 30 years (for review see Trifilieff *et al.*, 1993). In human lung, kinin interacts with specific receptors which have been mapped by autoradiography techniques (Mak & Barnes, 1991), but the lack of effect by BK on healthy human lung has hampered the characterization of the subtype of BK receptors present in this tissue. On the other hand, multiple neuropeptides, including kinins, have been proposed as acting as autocrine and paracrine growth factors for lung cancer, in particular in the case of neuroendocrine bronchial cancer as an example of small cell lung cancer (SCLC) (Woll & Rozengurt, 1989; Sethi & Rozengurt, 1991). The possible role of kinins in cancer remains speculative but some results obtained to date are of great interest in defining a role for kinin in this pathology. BK stimulates DNA synthesis in human fibroblasts (Owen & Villereal, 1983) and human endometrial stroma cells (Endo *et al.*, 1991). A mitogenic effect of kinins has also been reported on human embryonic lung fibroblasts (Goldstein & Wall, 1984), and SCLC cells (Sethi & Rozengurt, 1991). In human tumour ascites (Maeda *et al.*, 1988) and in plasma from patients with various cancers (Matsumura *et al.*, 1991), the kinin level has been reported to be significantly increased. Moreover, exposure of human SCLC to BK and other neuropeptides has been shown to lead to an increase in intracellular calcium (Woll & Rozengurt, 1989) and proliferation of these cells (Sethi & Rozengurt, 1991). Recently, BK has been reported to be the most potent peptide in increasing intracellular calcium on a number of SCLC or non-SCLC (Bunn *et al.*, 1992). From these observations, it has been proposed that BK may function as a paracrine growth factor for SCLC or large cell lung cancer (Bunn *et al.*, 1990; 1992; Sethi & Rozengurt, 1991).

Two classes of kinin receptors, B₁ and B₂, have been proposed on the basis of the bioassay potencies of BK

analogues (Regoli & Barabé, 1980). Des-Arg⁹-BK, a natural metabolite of BK, has high affinity for B₁ receptors, whilst BK has a low affinity. B₁ receptors are found mainly in vascular tissues and are specifically inhibited by des-Arg⁹-[Leu⁸]BK. B₂ receptors have a high affinity for BK whilst des-Arg⁹-BK and des-Arg⁹-[Leu⁸]BK are mainly inactive. B₂ receptors are widely distributed in mammals and are strongly inhibited by the new antagonist Hoe 140 (D-Arg⁹[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]BK) (Hock *et al.*, 1991). Moreover, the recent cloning and sequencing of the gene coding for a B₂ receptor from rat (Mc Eachern *et al.*, 1991) and from man (Hess *et al.*, 1992) has led to the prediction of a seven transmembrane G-protein coupled receptor. Nothing is known about binding properties of BK in healthy human lung and since alterations of binding sites have been reported for other physiological receptors in human lung tumours (Morin *et al.*, 1987), we decided to investigate the binding of BK in membrane preparations of healthy and carcinomatous human lung.

Methods

Lung preparation

Human lung and tumour samples were obtained after thoracotomy of patients undergoing surgery for lung carcinoma, and were transported, on ice, to the laboratory within 20 to 40 min. All patients had received droperidol (20 mg), thiopentone (250 mg), fentanyl (0.5 mg), atropine (0.5 mg) and curare (10 mg). The histological studies showed that eight of the tumours were well differentiated squamous-carcinoma, the three other were adenocarcinomas. For membrane preparations the following buffer was used: 25 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 6.8 containing 1 mM 1,10-phenanthroline (buffer A). All membranes were prepared as described previously (Trifilieff *et al.*, 1992) with minor modifications. Briefly, tissues were minced and homogenized in 0.25 mM ice-cold sucrose. After

¹ Author for correspondence.

centrifugation at 2500 *g* for 10 min at 4°C, the pellets were resuspended, homogenized and resedimented at the same speed. These two supernatants were pooled and diluted with three volumes of buffer A. The mixtures were then centrifuged twice at 50,000 *g* for 10 min at 4°C. The resulting pellets were resuspended in 10 ml of assay buffer per g of tissue for BK binding studies. Assay buffer is buffer A with 140 µg ml⁻¹ bacitracin, 10 µM captopril, 1 mM dithiothreitol and 0.1% bovine serum albumin added.

Radioligand binding assay

[³H]-BK binding was carried out as described previously (Trifilieff *et al.*, 1992). Competition and kinetic experiments were performed with 0.5 to 0.7 nM and 0.4 nM [³H]-BK, respectively. Saturation experiments were performed with increasing concentrations of radioligand (0.06 to 3.5 nM). Each experiment was performed for 90 min at 25°C in triplicate. All the data were analysed as described previously (Gies *et al.*, 1989). Student's *t* test (two-tailed) for unpaired data was used to determine the significance of differences in K_D and B_{max} values between the healthy and carcinomatous preparations.

Materials

[³H]-BK (74.7 to 110 Ci mmol⁻¹) was obtained from New England Nuclear (Boston, MA, U.S.A.). Des-Arg⁹-BK was purchased from Bachem (Bubendorf, Switzerland). Captopril was a gift from Squibb Pharmaceuticals (Epernon, France), Hoe 140 (D-Arg⁰[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]BK) was kindly provided by Hoechst AG (Frankfurt, Germany). Unlabelled BK, kallidin, D-Arg⁰[Hyp³,D-Phe⁷]BK and all other reagents were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Results

For both tissue sources (healthy and carcinomatous), we verified that binding of [³H]-BK was linear over a broad range of membrane particulates concentration (89 to 785 µg of protein per assay). Binding assays were therefore conducted with 240 to 270 µg of protein for healthy tissue and 650 to 700 µg of protein for cancer tissue. In these conditions, non-specific binding of [³H]-BK (0.6 nM) was 50 and 60% in healthy and carcinomatous tissues, respectively.

The [³H]-BK binding to healthy lung membranes was observed to be time-dependent ($n = 2$, Figure 1). Binding reached a maximum after 20 to 30 min and remained steady up to 150 min. After 90 min of association, dissociation was initiated by addition of 1 µM unlabelled BK and resulted in a rapid reduction of specifically bound [³H]-BK: 50% of dissociation occurred in 4 min (4.3–4.1). The association rate constant (K_{obs}) was 0.28 min⁻¹ (0.31–0.25) and the dissociation rate constant (K_{-1}) was 0.16 min⁻¹ (0.16–0.17). The value of the association rate constant (K_{+1}) calculated from the equation $K_{+1} = (K_{obs} - K_{-1})/[radioligand]$ was $2.98 \cdot 10^8$ min⁻¹ M⁻¹ ($3.49 \cdot 10^8$ – $2.47 \cdot 10^8$). Finally, the equilibrium dissociation constant $K_D = K_{-1}/K_{+1}$ could be calculated from these kinetic parameters, giving value of 0.57 nM (0.46–0.68). Scatchard analysis of saturation studies (Figure 2) indicated the presence of a homogeneous class of binding sites with a dissociation constant (K_D) of 0.73 ± 0.18 nM and a density of binding sites (B_{max}) of 37.7 ± 6.2 fmol mg⁻¹ protein ($n = 3$). The Hill coefficient was determined to be 0.90 ± 0.05 and confirms the existence of one population of binding sites.

Due to the limited amount of human tissue, we used the method described by De Blasi *et al.* (1989) to determine the binding parameters for the tumour membranes and seven of the healthy membrane preparations (Table 1). In order to improve this method, we have compared the results obtained by Scatchard analysis of saturation studies (parameters above) and those obtained by analysis of competition studies

proposed by De Blasi *et al.* (1989) ($K_D = 1.23 \pm 0.23$ nM, $B_{max} = 49.6 \pm 2.4$ fmol mg⁻¹ protein, $n = 7$). As shown, similar values were obtained with both methods. This result allows us to calculate binding parameters for tumour samples using competition experiments. The mean values for all the healthy membrane preparations were $K_D = 1.08 \pm 0.18$ nM and $B_{max} = 46.1 \pm 3.1$ fmol mg⁻¹ protein ($n = 10$).

Figure 1 Kinetics of [³H]-bradykinin binding to healthy lung membranes. Association (■) and dissociation (□) kinetics were examined at 25°C. When equilibrium was reached (90 min), the dissociation was started by addition of 1 µM unlabelled bradykinin (arrow). Points are the mean of triplicate measurements in one representative experiment.

Figure 2 (a) Saturation binding of [³H]-bradykinin to healthy human lung membranes. Specific binding (●) was calculated by subtracting non-specific binding (○) determined in the presence of 1 µM of unlabelled bradykinin from total binding (■). (b) Scatchard plot of the binding data. Points are the mean of triplicate measurements in one representative experiment.

For squamous-carcinoma membranes (Table 1, $n = 8$) no significant change in the $B_{\max}$ value was observed when compared with healthy membranes (52.2 ± 3.3 fmol mg^{-1} protein, $P = 0.22$), but the K_D value is significantly higher (2.57 ± 0.40 nM, $P = 0.004$). Similarly, the K_D value for adeno-carcinoma membranes ($n = 3$) was significantly higher when compared with healthy membranes (2.80 ± 0.29 nM, $P = 0.001$), but the $B_{\max}$ value remains in the same range (49.8 ± 2.1 fmol mg^{-1} protein, $P = 0.56$).

In order to define the pharmacological subtype of the BK binding sites present on healthy and tumour preparations, the affinities of B_1 and B_2 ligands were studied in competition experiments (Figure 3). In both healthy and squamous-carcinoma preparations, the natural B_1 agonist des-Arg⁹-BK did not displace the specific [³H]-BK binding even at the highest dose tested (10^{-5} M). For the other analogues tested, we found for both preparations the same relative order of potency in displacing [³H]-BK binding: Hoe 140 > BK > kallidin > D-Arg⁰[Hyp³,D-Phe⁷]BK. The affinities in healthy and squamous-carcinoma preparations are compared in Table 2. Based on their affinities, Hoe 140 and kallidin did not differentiate the BK binding sites present in both preparations, whereas BK and D-Arg⁰[Hyp³,D-Phe⁷]BK are able to distinguish binding sites in both preparations. Because of the small amount of tissue available for adeno-carcinoma tumour membrane preparations, we could not analyse all the bradykinin analogues tested for healthy and squamous-carcinoma membranes. We chose the B_1 agonist, (des-Arg⁹-BK), and the new more potent B_2 antagonist, Hoe 140. As observed with squamous-carcinoma membranes, des-Arg⁹-BK did not displace [³H]-BK binding, whereas Hoe 140 displaced completely the [³H]-BK binding with an affinity similar to that of bradykinin (Table 2).

Table 1 Binding parameters of [³H]-bradykinin sites in healthy and carcinomatous lung membranes

Subjects	K_D (nM)		$B_{\max}$ (fmol mg^{-1} protein)	
	Healthy	Tumour	Healthy	Tumour
Squamous 1	0.87*	3.00	52.5*	36.7
Squamous 2	1.02*	3.81	33.2*	44.4
Squamous 3	0.76	1.83	55.4	46.7
Squamous 4	0.56	3.03	52.9	56.9
Squamous 5	2.26	0.84	54.1	69.2
Squamous 6	1.93	3.11	52.9	50.2
Squamous 7	—	3.94	—	58.3
Squamous 8	—	0.99	—	55.1
Adeno 1	0.88	2.10	36.7	44.9
Adeno 2	0.82	3.00	43.4	51.1
Adeno 3	—	3.29	—	53.3
Healthy 1	0.29*	—	27.4*	—
Healthy 2	1.40	—	52.1	—

Parameters were determined using competition experiments by the method of De Blasi *et al.* (1989) or saturation experiments (*).

Table 2 Comparison of the potency of kinin analogues for competition in [³H]-bradykinin binding to healthy and carcinomatous lung membranes

	Healthy		Squamous-carcinoma		Adeno-carcinoma	
	K_i (nM)	(n)	K_i (nM)	(n)	K_i (nM)	(n)
Hoe 140	0.73 ± 0.21	(4)	1.23 ± 0.43	(5)	0.56 ± 0.14	(3)
Bradykinin (BK)	1.03 ± 0.17	(7)	$2.39 \pm 0.43^*$	(8)	$2.42 \pm 0.22^*$	(3)
Kallidin	18.37 ± 1.03	(3)	15.41 ± 1.19	(3)	—	—
D-Arg ⁰ [Hyp ³ ,D-Phe ⁷]BK	265.82 ± 12.67	(3)	$82.65 \pm 4.15^*$	(3)	—	—
Des-Arg ⁹ -BK	Inactive	(3)	Inactive	(3)	Inactive	(3)

* $P < 0.005$ when compared with healthy membranes (Student's *t* test for unpaired data, two tailed).

Again, when compared with healthy membranes, Hoe 140 did not differentiate the bradykinin binding sites present in either preparation. The specific [³H]-BK-binding was unaffected by various unrelated peptides such as substance P, bombesin or calcitonin gene-related peptide (each at 10^{-6} M) with the three membrane preparations (data not shown).

Figure 3 Competitive inhibition of specific binding of [³H]-bradykinin in healthy (a), squamous-carcinoma (b) and adeno-carcinoma membranes (c). [³H]-bradykinin (0.5 to 0.7 nM) was incubated for 90 min at 25°C with increasing concentrations of (■) Hoe 140, (□) unlabelled bradykinin, (▲) kallidin, (○) D-Arg⁰[Hyp³,D-Phe⁷]bradykinin or (◆) des-Arg⁹-bradykinin. Each point represents the mean $\pm$ s.e. mean of the number of experiments indicated in Table 2.

Discussion

Here we describe for the first time the determination of [3 H]-BK-binding constants in healthy and carcinomatous human lung. In a previous study (Trifilieff *et al.*, 1991) we showed the presence of two [3 H]-BK binding sites in the guinea-pig lung, such a situation was not observed in the human lung. This could suggest a species variation or may be due to the relative high level of non-specific binding observed in human lung, as has been reported elsewhere (Mak & Barnes, 1991), which prevented the detection of a second binding site. In squamous- and adeno-carcinoma membranes, the same amount of BK receptors are present, whereas the equilibrium dissociation constant of [3 H]-BK is significantly higher compared with the value for healthy membranes. Among the BK analogues used in competition experiments, only the B_2 receptor antagonist, D-Arg 0 [Hyp 3 ,D-Phe 7]BK was able to differentiate the BK binding sites present in healthy and squamous-carcinoma membranes. The two others, Hoe 140 and kallidin, possessed similar affinities in both tissue sources. In the same way, Hoe 140 did not differentiate the BK receptor present in adeno-carcinoma membranes when compared with healthy membranes. Only for two of the eleven samples studied (squamous 5 and probably squamous 8), did BK appear to have a greater affinity for its receptor on carcinomatous tissue rather than its receptor on healthy tissue. However, when taking all the K_D values together, BK had a significantly higher affinity for the healthy receptor than for the tumour receptor. Moreover when other compounds were used (B_2 antagonists and kallidin), in squamous 5 and 8, the affinity values for the BK receptor were in a similar order to the other samples.

We observed a change in affinity values for BK and D-Arg 0 [Hyp 3 ,D-Phe 7]BK between healthy and tumour tissue. A similar magnitude of alteration (two fold) was also observed for [3 H]-quinuclidinyl benzilate ([3 H]-QNB) between healthy carcinomatous human lung (Morin *et al.*, 1987) and the authors suggested that it might be related to a heterogeneity of muscarinic receptors in both tissues. In a previous study, we reported such a heterogeneity of BK receptors in the guinea-pig lung using saturation and kinetic experiments (Trifilieff *et al.*, 1991). However, in the human lung, neither saturation nor kinetic studies were able to reveal two binding sites, but, as mentioned above, this could be due to the relatively high level of non-specific binding in this tissue. It is of importance to note that BK and D-Arg 0 [Hyp 3 ,D-Phe 7]BK were able to differentiate the two binding sites in guinea-pig lung whereas kallidin was not (Trifilieff *et al.*, 1991). A similar behaviour for these compounds was observed between healthy and carcinomatous human lung; therefore, we cannot exclude the possibility of a heterogeneity of BK receptors in these tissues. However, the differences observed between healthy and carcinomatous lung remain rather small. The biological significance of such a difference could be related to an increase in the kinin concentration in tumour cells inducing a desensitization of the BK receptors. In asthmatic subjects,

tachyphylaxis of the kinin receptor has been reported following repeated inhalations of BK (Polosa *et al.*, 1990). As an increase in kinin levels has been observed in various cancers (Maeda *et al.*, 1988; Matsumara *et al.*, 1991), the change in the K_D values of BK could be related to a desensitization of the kinin receptor in tumour samples. Alternatively, a structural modification of the BK receptor could also be involved. Whatever the case, if the change in affinity values observed in the present study had a biological significance, we believe that this might reflect a modification of the membrane environment or a difference in the coupling efficiency of the receptors rather than a structural difference in the BK receptors. Indeed, this hypothesis fits well with the work of Eggericks *et al.* (1992) who have shown that a human BK B_2 receptor gene, when expressed in different eukaryotic systems, encodes a receptor which can be differentiated by BK analogues. In fact, it may be of interest to continue this work with other tumour samples of different histological types to ascertain whether or not there are differences in [3 H]-BK binding.

The failure of the B_1 agonist, des-Arg 9 -BK to displace [3 H]-BK binding in healthy and tumour membrane preparations demonstrates the absence of B_1 receptors in these preparations. On the other hand, the affinity sequence for the other BK analogues allowed us to classify these binding sites as B_2 (Figure 3). These results are in accordance with the presence of B_2 receptors in healthy human lung as suggested by Hess *et al.* (1992) who found the highest level of B_2 receptor mRNA in kidney, uterus and lung.

Since only minor bronchoconstrictor effects for BK have been reported in healthy human subjects, one can speculate as to the physiological role of the B_2 receptors described in our study. Besides its bronchocontractile properties, BK is a well known vasoactive peptide, and has been shown to increase vascular permeability in guinea-pig airways (Saria *et al.*, 1983; Rogers *et al.*, 1990). Moreover, an autoradiographic study of human lung has revealed a high density of BK binding sites on bronchial and pulmonary blood vessels (Mak & Barnes, 1991). As in the guinea-pig, BK could also increase vascular leakage in human airways.

In conclusion, we have shown the presence of a high affinity BK B_2 receptor in healthy and carcinomatous human lung. The physiological role of these receptors remains to be determined, but the recent development of potent BK receptor antagonists, such as Hoe 140, may be useful for its elucidation.

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