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Identification of selective, high affinity [^{125}I]-angiotensin and [^{125}I]-bradykinin binding sites in rat intestinal epithelia

Helen M. Cox[1], Kenneth A. Munday & Judith A. Poat

Department of Physiology and Pharmacology, Medical and Biological Sciences Building, University of Southampton, Bassett Crescent East, Southampton SO9 3TU

1 Specific [^{125}I]-angiotensin II (AII) and [^{125}I]-bradykinin (Bk) binding sites have been identified within epithelial membranes from rat jejunum and descending colon.

2 These high affinity intestinal sites exhibited K_D values of 0.64 ± 0.16 nM for [^{125}I]-AII and 0.69 ± 0.13 nM for [^{125}I]-Bk, which were similar to those for [^{125}I]-AII (0.85 nM) and [^{125}I]-Bk binding sites (1.03 nM) previously identified in renal cortex epithelia.

3 Specific [^{125}I]-AII binding capacity was only 19.77 ± 2.74 fmol mg^{-1} in small intestine and 11.31 ± 2.66 fmol mg^{-1} in descending colon epithelia while a larger population, 332.0 ± 72.9fmol-mg^{-1} of specific [^{125}I]-Bk sites were identified in epithelial membranes from small intestine.

4 Significant hydrolysis of both free [^{125}I]-AII and [^{125}I]-Bk was observed while membrane bound peptides remained relatively resistant to degradation. Whilst no corrections have been made to the observed values of K_D and B_{max} quoted above, one may assume that the calculated reductions in the free hormone concentration will result in a decrease of the K_D value for both peptides. Loss of membrane bound peptide, particularly of [^{125}I]-AII, may indicate that the calculated B_{max} value is an underestimation.

5 Despite the rapid degradation of unbound [^{125}I]-AII and [^{125}I]-Bk during incubations the kinetics of specific peptide binding were reversible and highly selective. The order of potency for specific [^{125}I]-AII binding was [Sar^1, Leu^8]-AII $\geq$ [Sar^1, Thr^8]-AII $\geq$ AII $>$ [Sar^1, Ile^8]-AII $\geq$ [Des, Asp^1, Ile^8] AII $>$ AIII. Specific [^{125}I]-Bk binding was also highly selective, the order of potency being Phe^8-Bk $\geq$ Tyr^8-Bk $\geq$ Lys-Bk $>>$ Des, Arg^1-Bk. AII exhibited an IC_{50} of $>$ 1mM for specific [^{125}I]-Bk binding and likewise Phe^8-Bk for specific [^{125}I]-AII binding.

Introduction

The peptides angiotensin II (AII) and bradykinin (Bk) are both intimately involved in the control of electrolyte transport across intestinal epithelia. Kallidin and Bk stimulate Cl secretion across rat isolated voltage clamped colon and ileum by a direct, prostaglandin-mediated pathway (Cuthbert & Margolius, 1982; Musch *et al.*, 1983; Cuthbert *et al.*, 1984) initiated by the interation of Bk with specific receptors present within the epithelial membranes (Manning *et al.*, 1982). In contrast, AII produces antinatriuretic and antidiuretic effects in transporting epithelia. In isolated sacs of rat intestine the stimulation of an electroneutral NaCl cotransport system by AII (Davies *et al.*, 1970) has been blocked by α-adrenoceptor antagonists (Levens *et al.*, 1981a) indicating the mediation of noradrenaline (NA) in this response in which the transmitter may be released from sympathetic nerves innervating intestinal villi (Schultzberg *et al.*, 1980). Angiotensin also stimulates NaCl and water secretion at higher concentrations and this direct action is thought to involve prostaglandins (Levens *et al.*, 1981b). It is interesting to note that the peptide systems of which Bk and AII are the active components are linked by a common enzyme. A carboxypeptidase termed angiotensin converting enzyme is responsible for the production of AII from AI whilst it cleaves the C terminal dipeptide from Bk rendering it inactive.

The mechanisms of Bk and AII action in renal epithelia appear to be similar to their respective actions in intestinal epithelia (Schuster *et al.*, 1984; Brunton *et al.*, 1978). Specific Bk binding sites in renal

[1] Present address: Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD.

epithelia have been identified (Tomita & Pisano, 1984; Cox *et al.*, 1984b) and, despite the postulated indirect mechanism of AII action, specific binding sites for this octapeptide have also been located in renal cortex epithelia (Cox *et al.*, 1983; Brown & Douglas, 1983) predominantly on basolateral epithelial membranes (Cox *et al.*, 1984a). The identification of these renal [^{125}I]-AII binding sites may indicate a direct mechanism of action for AII independent of NA, whereby blood borne or intrarenally generated AII acts at specific receptor sites in the epithelial basolateral membranes. It has been postulated that these AII receptors may mediate the secondary secretory responses seen with higher doses of AII. Since the mechanism of action of AII and of Bk on ion transport in renal and intestinal epithelia appear to be similar it was pertinent to investigate the presence of AII binding sites in membrane preparations of the latter. The present study describes such an investigation and the results are compared with the binding characteristics of [^{125}I]-Bk in epithelial membranes prepared from rat jejunum.

Methods

Preparation of intestinal membranes

Crude homogenates of jejunum and descending colon epithelia were obtained according to Manning *et al.* (1982). Male Wistar rats weighing 250–300 g were killed by cervical dislocation and lengths of intestine removed as required. These were flushed with 0.9% saline and the mucosal surface scraped gently with a glass slide. Mucosal cells were then homogenized in 20 volumes of 250 mM sucrose containing 10 mM triethanolamine HCl and 0.1 mM phenyl-methylsulphonylflouride (PMSF) pH 7.6 in a tight fitting homogenizer (clearance 0.18 mm). Homogenates were centrifuged twice at 50,000 *g* for 10 min (Beckman J2–21 at 2–4°C) with an intermediate rehomogenization in sucrose buffer. Final membrane pellets were resuspended in hypotonic solution consisting of 10 mM triethanolamine HCl, and 0.1 mM PMSF pH 7.6.

Thin layer chromatography (t.l.c.)

The extent of labelled peptide degradation during binding assays was assessed by t.l.c. as described by Innis *et al.* (1981) for [^{125}I]-Bk and by Goodfriend & Simpson (1981) for [^{125}I]-AII. To examine the degradation of bound and free hormone, incubations were terminated by centrifugation in a microfuge. The supernatant was collected and the resulting pellet washed and resuspended in 50% glacial acetic acid. Aliquots of this suspension (representing bound peptide), the supernatant (representing free peptide) and reference labelled peptide were then spotted onto activated plastic backed cellulose t.l.c. plates (Polygramm Cel 300, Camlab, Cambridge). When assessing [^{125}I]-AII degradation, plates were developed in 3% NH_4OH:butan-2-ol (35:105) while a solvent system of butanol:acetic acid:water (25:4:10) was used to assess [^{125}I]-Bk hydrolysis. Developed, dried plates were first scanned (Camlab-Dunnschicht Scanner, Cambridge) and then cut into 0.5 cm strips and counted in a LKB 1272 Clinigamma counter.

Receptor binding assays

[^{125}I]-Tyr^8-Bk ([^{125}I]-Bk) binding assays were performed according to Manning *et al.* (1982). Membrane protein (50–350 μg per tube) was incubated with 0.6–0.8 nM [^{125}I]-Bk for 90 min on ice in 25 mM N-Tris - (hydroxymethyl) - methyl - 2 - amino-ethanesulphonic acid (TES) buffer, pH 6.8, containing 1 mM 1,10 phenanthroline, 0.1 mM bacitracin, 1 mM dithiothreitol (DTT), 1 μM captopril and 0.2% BSA (final volume, 250 μl). Nonspecific binding was defined by the addition of 1 μM unlabelled Bk. This concentration of Tyr^8-Bk consistently displaced all specifically bound [^{125}I]-Bk. The resulting levels of nonspecific [^{125}I]-Bk binding were not significantly different (Student's *t* test) from those obtained in the presence of 10 μM, 100 nM or 10 nM Tyr^8-Bk. Incubations were terminated by dilution with 10 ml of ice cold 25 mM TES buffer containing 0.2% BSA, pH 6.8 followed by immediate filtration under vacuum, through GF/B filters presoaked in 0.1% aqueous polyethylenimine. Membrane blanks were routinely determined and bound radioactivity counted in a γ-counter. [^{125}I]-AII binding assays were performed in 20 mM Tris-HCl buffer pH 7.4, containing 120 mM NaCl, 5 mM sodium ethylenediaminetetra-acetate (Na_2EDTA) 0.1 mM phenylmethylsulphonylfluoride (PMSF) and 0.2% BSA (final volume 250 μl). Incubations with 30–250 μg of membrane protein were normally for 5 min at 22°C (unless otherwise indicated) and these were terminated by dilution with ice cold 20 mM Tris-HCl, 120 mM NaCl, pH 7.4, and filtration over GF/B filters under vacuum. Nonspecific [^{125}I]-AII binding was assessed by the addition of 1 μM unlabelled AII a concentration which, as described above for Bk, gave consistent displacements which were not significantly different from those obtained in the presence of 20 nM, 100 nM or 10 μM unlabelled peptide. This is in keeping with previously published data on specific AII binding in other tissues (Cox *et al.*, 1984a; Glossmann *et al.*, 1985). Radioactivity bound to the filters was counted in a γ-counter. Any alterations to this procedure are described in the figure and table legends. Protein levels were determined using Coomassie brilliant blue dye (Bradford, 1976).

Data analysis

Analysis of saturable [^{125}I]-Bk and [^{125}I]-AII binding was performed using a computer assisted curve fitting programme which applied unweighted, untransformed binding data directly to the Michaelis-Menten equation (Wilkinson, 1961). This yielded estimations of affinity (K_D) and maximal binding capacity (B_{max}). Association (k_1) and dissociation (k_{-1}) rates were calculated as described by Bennett (1984). Since only 2–5% of the total [^{125}I]-AII and [^{125}I]-Bk added to the incubation media was bound specifically to membranes the equation; $k_1 = k_{obs} - k_{-1}/L$ was used, where k_{obs} is the observed association rate and L the ligand concentration. This equation takes the dissociation constant (k_{-1}) into account and the ratio of the k_{-1} over the resulting k_1 yielded an equilibrium constant (K_{eq}). IC_{50} values were obtained from monophasic, steep, displacement curves (constructed from at least 8 points in quadruplicate) as the concentration of peptide that displaced 50% of the ^{125}I-peptide specific binding. Hill coefficients for the more potent displacers of peptide binding were calculated according to Cornish-Bowden & Koshland (1975) and the best fitting straight lines were obtained by linear regression analysis.

Materials

Monoiodinated [^{125}I]-AII (specific activity 1880 μCi μg^{-1}) and [^{125}I]-Tyr^8-Bk (specific activity 1830 μCi μg^{-1}) were purchased from N.E.N., Boston, U.S.A. Unlabelled AII and Bk as well as a range of peptide analogues were obtained from Sigma, Poole. Angiotensin converting enzyme inhibitor was purchased from C.R.B., Cambridge. All other compounds were of analytical grade.

Results

Both specific [^{125}I]-Bk and [^{125}I]-AII binding to rat jejunum epithelial membranes were sensitive to changes in the pH of the incubating buffer. Bk binding was increased at lower pH. In agreement with the observations of Innis *et al.* (1981) the highest levels of specific [^{125}I]-Bk binding (0.72 nM [^{125}I]-Bk was used) were obtained at pH 6.8 (99.0 ± 4.8 fmol mg^{-1}). Similar levels of Bk binding (93.2 ± 6.9 fmol mg^{-1}) were obtained at pH 6.4 but at pH 7.4 binding was markedly reduced (26.1 ± 7.4 fmol mg^{-1}). In contrast, specific [^{125}I]-AII binding was decreased by reducing the pH. No specific [^{125}I]-AII binding was obtained at pH 6.4, moderate levels were obtained at pH 6.8 whilst 7.3 ± 1.2 fmol mg^{-1} was obtained at pH 7.4 with 0.78 nM [^{125}I]-AII. All further incubations were therefore performed at pH 7.4 for [^{125}I]-AII assays and pH 6.8 for [^{125}I]-Bk assays. As the levels of specific Bk binding were considerably higher than those for AII in crude jejunal membranes, alterations were made to the latter incubation buffer in an attempt to enhance specific [^{125}I]-AII binding. A wide range of protease inhibitors were tested as well as altering the ionic composition of the incubation buffer. However, none of the changes (summarised in Table 1), significantly enhanced specific [^{125}I]-AII binding so the original incubation buffer was adopted for all further studies.

Increases in membrane protein concentration produced corresponding increases in specific binding of both labelled peptides. Specific [^{125}I]-Bk and [^{125}I]-AII binding was linear between 50 and 130 μg of jejunum membrane protein. Specific [^{125}I]-AII binding to mucosal membranes from rat descending colon exhibited a similar linear increase in [^{125}I]-AII binding as seen in jejunal membranes. Absolute levels of [^{125}I]-AII binding were markedly lower than for [^{125}I]-Bk binding in jejunal membranes. The kinetics of specific [^{125}I]-Bk and [^{125}I]-AII binding in jejunal membranes also showed marked differences. Specific [^{125}I]-Bk binding reached equilibrium after 90 min incubations on ice while lower and erratic levels of binding were achieved following incubations at 22°C (Figure 1a). Specific [^{125}I]-AII binding was temperature-sensitive but in contrast with Bk binding, equilibrium was achieved after only 5 min at 22°C. No [^{125}I]-AII binding was observed after assays had been performed on ice (Figure 1b). In addition specific [^{125}I]-AII binding to jejunal epithelial membranes became unstable after 10 min incubation at 22°C and was only 10% of the equilibrium value after 40 min. Application of association rate data to the pseudo-first order rate

Table 1 Effect of varying buffer composition on specific [^{125}I]-angiotensin II binding in rat jejunum epithelial membranes

Incubation buffer	*Specific binding* (fmol mg^{-1})
Control Buffer	4.8 ± 1.2
60 mM NaCl, + 5 mM $MgCl_2$	4.0 ± 0.7
No PMSF, + 0.1 mM 1,10-phenanthroline	ND
+ 100 μM bacitracin	3.7 ± 0.3
+ 5 mM DTT	0.4 ± 0.1
+ 5 mM benzathonium chloride	2.7 ± 0.5
+ 10 μM leupeptin	4.0 ± 0.8
+ 40 μg ml^{-1} chymostatin	4.3 ± 0.3

Control buffer consisted of 20 mM Tris-HCl, 120 mM NaCl, 5 mM Na_2EDTA, 0.1 mM PMSF, 0.2% BSA, pH 7.4 to which the above alterations were made. Values are the means ± 1 s.e.mean from single incubations with 0.3–0.4 nM [^{125}I]-AII performed in triplicate. ND means not detectable.

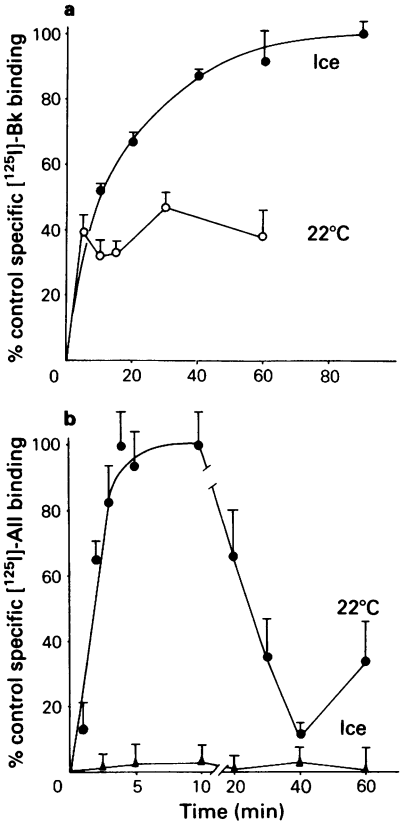

Figure 1 Rates of association of specific [^{125}I]-bradykinin ([^{125}I]-Bk) binding (0.71 nM) (a) and of specific [^{125}I]-angiotensin II ([^{125}I]-AII) binding (0.21 nM) (b) in jejunum epithelial membranes. Incubations were allowed to proceed for the period shown before termination by cooling and filtration. Values are the mean (with s.e.mean shown by vertical line) for triplicate incubations in a single representative experiment.

equation (as described by Bennett, 1984) yielded an observed association rate constant (k_{obs}) of $9.72 \pm 1.36 \times 10^{-3}s^{-1}$ ($n = 3$) for specific [^{125}I]-AII binding at 22°C and $0.34 \pm 0.06 \times 10^{-3}s^{-1}$ ($n = 2$) for specific [^{125}I]-Bk binding on ice.

The rates of ligand dissociation induced either by addition of excess cold peptide or a 40 fold dilution were also investigated for Bk and AII binding (Figure 2a and b). Incubations were allowed to proceed to equilibrium before one of the two methods was implemented to initiate dissociation. Addition of excess unlabelled Tyr^8-Bk resulted in a time-dependent reduction of specific [^{125}I]-Bk binding at 2–4°C with a dissociation rate, k_{-1} of $0.61 \times 10^{-3}s^{-1}$. Specific [^{125}I]-AII binding exhibited a mean dissociation rate of $1.62 \pm 0.2 \times 10^{-3}s^{-1}$ ($n = 3$) after addition of 1 μM AII and this was not significantly different (using an unpaired Student's t test) from the dissociation rate obtained after dilution ($1.56 \pm 0.36 \times 10^{-3}s^{-1}$; $n = 2$) thus indicating the absence of any cooperative interactions between intestinal [^{125}I]-AII binding sites. Estimations of the equilibrium binding constant (K_{eq}) were obtained from the ratio of dissociation (k_{-1}) over the association rate constant (k_1) and yielded values of 0.45 nM for specific

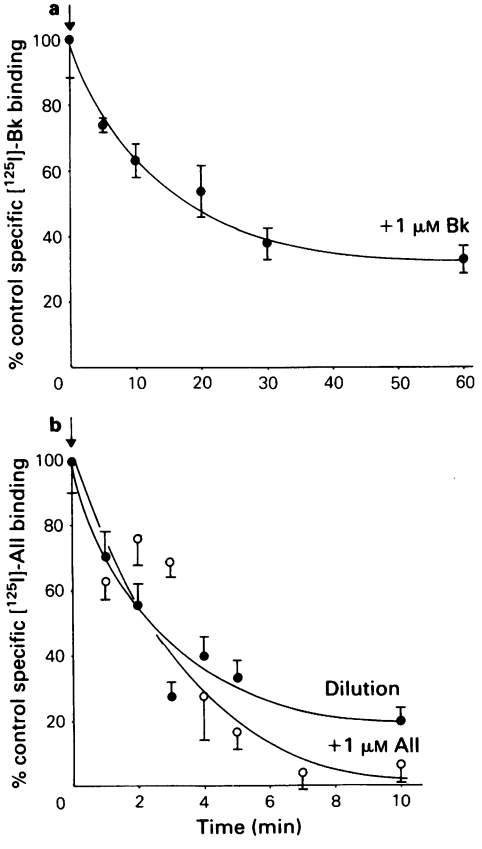

Figure 2 Rates of dissociation of specific [^{125}I]-bradykinin ([^{125}I]-Bk) binding on ice (a) and specific [^{125}I]-angiotensin II ([^{125}I]-AII) binding at 22°C (b). After 90 min incubation of jejunal membranes with 0.7 nM [^{125}I]-Bk, 1 μM unlabelled Bk was added and tubes were cooled and filtered at increasing intervals after this. (b) Incubations at 22°C with 0.3 nM [^{125}I]-AII lasted for 5 min before either addition of excess unlabelled AII (○) or 40 fold dilution (●). Tube contents were filtered at increasing time intervals afterwards. Values are the mean (with s.e.mean shown by vertical line) from single representative experiment performed in triplicate.

[^{125}I]-Bk binding (at 2–4°C) and a K_{eq} of 0.03 nM for specific [^{125}I]-AII binding at 22°C.

The saturable specific binding profiles obtained for [^{125}I]-Bk and [^{125}I]-AII in jejunum epithelial membranes are shown in Figure 3a and b. The equilibrium dissociation constants (K_D) obtained from saturation data were similar but the maximal number of binding sites (B_{max}) was markedly lower for [^{125}I]-AII compared with specific [^{125}I]-Bk binding. The latter labelled peptide exhibited a K_D of 0.69 ± 0.13 nM and maximal binding capacity (B_{max}) of 332.8 ± 72.9 fmol mg^{-1} ($n = 4$) compared with [^{125}I]-AII saturable binding which appeared to be to a single site with a K_D of 0.64 ± 0.16 nM and B_{max} of 19.77 ± 2.74 fmol mg^{-1} ($n = 6$). Very similar binding characteristics were obtained for [^{125}I]-AII binding to descending colon epithelial membranes where the K_D was 0.48 ± 0.06 nM and the B_{max} was 11.31 ± 2.66 fmol mg^{-1} ($n = 3$).

One possible explanation for the differences in the number of specific [^{125}I]-AII and [^{125}I]-Bk binding sites may be a difference in the susceptibility of the bound peptide to hydrolysis by endogenous proteases. In addition enzymatic degradation of free labelled peptides during incubation assays will also lead to significant reductions in free ligand concentration and thus change the observed estimates of k_1, k_{-1} and K_D. The hydrolysis of both bound and free labelled peptides was therefore checked using t.l.c. techniques (Figure 4).

Analysis of the supernatant containing free [^{125}I]-Bk after 90 min incubation on ice (0.65 to 0.08 nM [^{125}I]-Bk) identified 41.5 ± 1.0% hydrolysis of the [^{125}I]-Bk applied to the t.l.c. plate to a less polar product ($R_F = 0.63$; $n = 2$). Membrane bound [^{125}I]-Bk appeared to be relatively resistant to degradation, 85.0 ± 5.5% of the radioactivity running with an R_F of 0.46, exactly the same as reference [^{125}I]-Bk.

Authentic [^{125}I]-AII ran with an R_F of 0.32 in a solvent system of 3% NH_4OH:butan-2-ol (35:105). As seen in Figure 4 both free and membrane bound [^{125}I]-AII showed signs of hydrolysis to a more polar product. Of the acid elutable (membrane bound) radioactivity applied to the t.l.c. plate, 55.0 ± 4.5% ran with an R_F of 0.32 ($n = 3$). As seen with [^{125}I]-Bk, free [^{125}I]-AII was more susceptible to enzymatic degradation. After only 5 min incubation with 0.5 to 0.7 nM [^{125}I]-AII at 22°C 70.0 ± 4.0% of the free hormone had been hydrolysed to a more polar product ($n = 3$).

Despite the rapid degradation of free peptides, both [^{125}I]-Bk and [^{125}I]-AII specific binding were highly selective and the orders of potency obtained with a range of AII and Bk analogues are shown in Table 2. Phe^8-Bk, the naturally occurring sequence of Bk, Tyr^8-Bk and Lys-Bk (kallidin) were most potent at displacing [^{125}I]-Tyr^8-Bk from its binding site. Loss of arginine

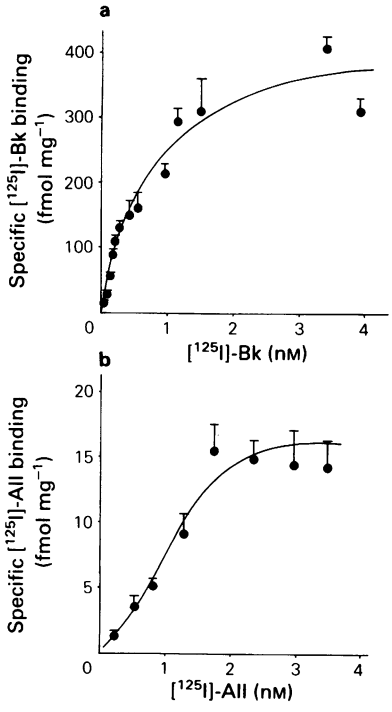

Figure 3 Saturable specific [^{125}I]-bradykinin ([^{125}I]-Bk) and [^{125}I]-angiotensin II ([^{125}I]-AII) binding in jejunum epithelial membranes. Incubations were performed either for 90 min on ice with increasing concentrations of [^{125}I]-Bk (a) or for 5 min at 22°C with increasing concentrations of [^{125}I]-AII (b). Each graph represents values which are mean (with s.e.mean shown by vertical line) from a single representative experiment performed in quadruplicate (n values in the text).

from position 1, Des, Arg^1-Bk, resulted in a far less potent peptide with an IC_{50} value of 1 μM and shorter fragments of Bk, i.e. 1–6 Bk and 2–7 Bk were virtually inactive. Addition of the nonapeptide converting enzyme inhibitor did not attenuate specific [^{125}I]-Bk binding, instead at 0.1 μM and 1 μM the nonapeptide enhanced binding (126.5 ± 13.4% and 163.3 ± 15.6% respectively) compared with controls (100.0 ± 7.2%) in the absence of any inhibitor. Similar increases in specific [^{125}I]-Bk binding were obtained with captopril (SQ14225) which at 1 μM (the concentration normally present in the incubation buffer) increased binding to 165.2 ± 14.5% of control levels. It is important to note that the displacement profiles of both Bk and AII analogues with IC_{50} values < 1 μM exhibited steep, monophasic characteristics indicative of displacement from a single class of high affinity binding sites. The use of 1 μM unlabelled peptide to define nonspecific ^{125}I-peptide binding is justified since it produced

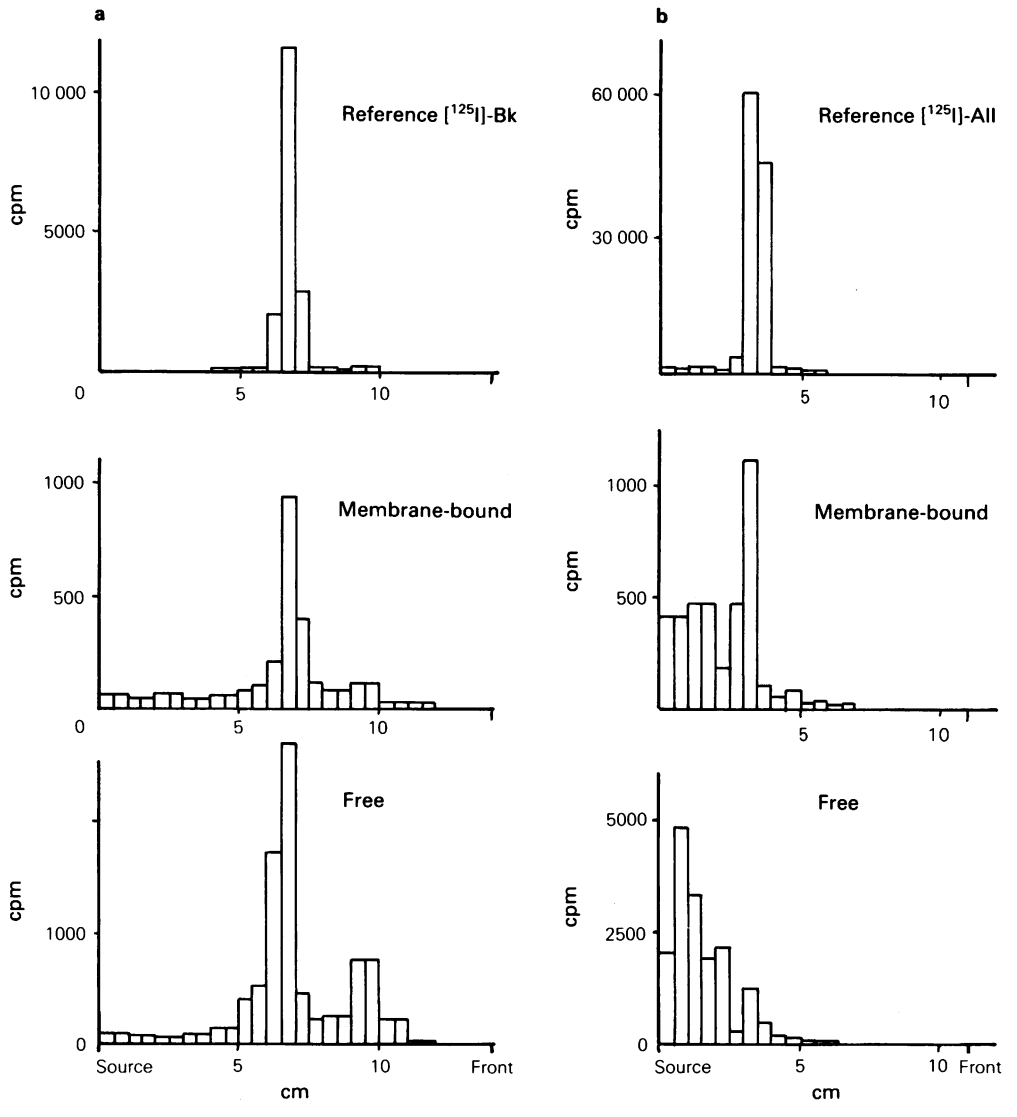

Figure 4 Chromatographs of reference, membrane-bound and free [^{125}I]-bradykinin ([^{125}I]-Bk) (a) and [^{125}I]-angiotensin II ([^{125}I]-AII) (b). Bk (0.65–0.8 nM [^{125}I]-Bk) incubations with epithelial membranes from rat jejunum proceeded for 90 min on ice. Incubations were terminated by microcentrifugation, the supernatant was retained and the remaining pellet washed and resuspended in 50% glacial acetic acid. Aliquots of each were spotted onto activated plastic backed cellulose t.l.c. plates and developed in butanol:acetic acid:water (25:4:10) with reference nonincubated [^{125}I]-Bk. Developed dried plates were cut and counted for radioactivity. Results shown are from one representative experiment. Authentic [^{125}I]-Bk ran with an R_F of 0.5 in this solvent system. (b) [^{125}I]-AII (0.5–0.7 nM) incubations for 5 min at 22°C were performed with jejunum epithelial membranes. Incubations were terminated as above and aliquots of acid elutable [^{125}I]-AII and free [^{125}I]-AII were co-spotted with authentic [^{125}I]-AII onto t.l.c. plates. After development in 3% NH_4OH butan-2-ol (35:105) plates were dried, cut and counted. Results shown are from one representative experiment where authentic [^{125}I]-AII ran with an R_F of 0.3.

Table 2 Displacement of specific [^{125}I]-bradykinin ([^{125}I]-Bk) (A) and [^{125}I]-angiotensin II ([^{125}I]-AII) binding (B) in jejunal epithelial membranes by a range of peptides

Displacing analogue	IC_{50}(nM)	*Hill coefficient* (n)
A *Bradykinin*		
Phe^8-Bk	1.2 ± 0.80	0.94 ± 0.04
Tyr^8-Bk	2.0 ± 0.72	0.96 ± 0.10
Lys-Bk	3.3 ± 1.40	0.97 ± 0.16
Des Arg^1-Bk	800.0 ± 141.40	0.83 ± 0.05
1-6 Bk	> 10 μM	–
2-7 Bk	> 10 μM	–
Ile^5-AII	> 1000 μM	
B *Angiotensin*		
[Sar^1, Leu^8]-AII	1.5 ± 0.40	0.93 ± 0.11
[Sar^1-Thr^8]-AII	2.0 ± 0.71	0.94 ± 0.01
Ile^5-AII	3.5 ± 0.86	0.91 ± 0.06
[Sar^1, Ile^8]-AII	10.0 ± 0.75	0.92 ± 0.15
[Des, Asp^1, Ile^8]-AII	16.5 ± 1.70	0.95 ± 0.05
[Des, Asp^1, Phe^8]-AII (AIII)	330.0 ± 176.8	0.92 ± 0.40
AI	1000.0 ± 91.9	0.90 ± 0.10
Renin substrate	10 μM	0.86
L-Asp, L-Arg	> 1000 μM	–
Phe^8-Bk	> 1000 μM	–

Incubations were performed as described in the Methods with increasing concentrations of displacing analogue. The concentration of unlabelled peptide which reduced specific [^{125}I]-Bk or [^{125}I]-AII binding in intestinal membranes by 50% is denoted as the IC_{50}. Values are the mean from 2–5 experiments and displacement curves were obtained with at least 8 different concentrations (in quadruplicate) for the potent peptide analogues and at least 3 concentrations of the less potent peptides.

reductions in ^{125}I-peptide binding not significantly different from either 100 nM or 10 nM unlabelled AII or Bk. Unlabelled AII or Bk both at 10 μM did not reduce specific [^{125}I]-AII or [^{125}I]-Bk binding further.

Similarly, [^{125}I]-AII binding to rat jejunal epithelial membranes was highly selective. The antagonist analogues [Sar^1, Leu^8]-AII and [Sar^1, Thr^8] AII were the most potent displacers followed by AII and a third antagonist [Sar^1, Ile^8]-AII. AIII (Des, Asp^1-AII) was far less potent as a competitor for [^{125}I]-AII binding sites yet the antagonist analogue [Des, Asp^1, Ile^8]-AII was approximately 20 times more potent than the agonist. The larger peptides, AI and renin substrate were relatively ineffective and the dipeptide fragment, L-Asp, L-Arg and also Phe^8-Bk were totally ineffective at displacing [^{125}I]-AII from its binding site. The Hill coefficients for the more potent Bk and AII displacing analogues were all close to 1.0 indicating the lack of cooperative interactions between these peptides and their binding sites.

Discussion

Specific high affinity binding sites for [^{125}I]-Bk and [^{125}I]-AII have been characterized in a crude preparation of rat jejunum epithelial membranes. Specific [^{125}I]-Bk binding sites exhibited a similar pH profile, specificity (as determined by displacement studies) and affinity as [^{3}H]-Bk binding sites identified in homogenates of guinea-pig ileal mucosa (Innis *et al.*, 1981; Manning *et al.*, 1982). The characteristics of specific [^{125}I]-Bk binding indicate peptide binding to a specific receptor protein as opposed to angiotensin converting enzyme or to a nonspecific protease since addition of increasing concentrations of the nonapeptide converting enzyme inhibitors was necessary in the incubation buffer for maximal specific [^{125}I]-Bk binding. As suggested by Manning *et al.* (1982) these intestinal sites may represent Bk receptors involved in the initiation of chloride secretion across the rat mucosa. The distribution of [^{125}I]-Bk binding sites described here in rat tissue differs from that described in guinea-pig tissue perhaps indicating a species difference. In epithelial membranes from rat renal cortex we identified three times the number of specific [^{125}I]-Bk binding sites seen in jejunum membranes while Innis *et al.* (1981) observed the reverse, more Bk receptors being located in the intestine rather than kidney tissue of the guinea-pig.

The identification of specific [^{125}I]-AII binding sites in rat jejunum and descending colon epithelial membranes is the first such documented study. The uncorrected affinities of these [^{125}I]-AII sites (K_D of 0.64 ± 0.16 nM in jejunum and 0.48 ± 0.06 nM in descending colon epithelia) were very similar to the affinity exhibited by specific [^{125}I]-AII binding sites in renal cortex basolateral and brushborder membranes (K_D of 0.85 nM) and also to [^{125}I]-Bk binding sites in both renal and intestinal epithelial membranes (1.03 nM and 0.69 nM respectively). The B_{max} for both labelled peptides was much larger in renal epithelia compared with intestinal tissue. Some 321 fmol mg^{-1} specific [^{125}I]-AII binding sites were located within basolateral and brushborder membranes from renal cortex (Cox *et al.*, 1984a) whereas only 20 fmol mg^{-1} were observed in jejunal membranes and 11 fmol mg^{-1} in descending colon epithelia. In a similar fashion, 8450 fmol mg^{-1} specific [^{125}I]-Bk binding sites were identified in renal membranes (Cox *et al.*, 1984b) compared with only 333 fmol mg^{-1} in jejunal membranes. The larger B_{max} values in renal compared with intestinal epithelia may either indicate real differences in receptor population or may be a result of more extensive degradation of labelled peptides bound to

the receptors in intestinal membranes. Even in the presence of protease inhibitors and nonspecific proteins (such as bovine serum albumin and bacitracin) substantial hydrolysis of [^{125}I]-AII, and to a lesser extent [^{125}I]-Bk, was identified by t.l.c. Such extensive enzymatic hydrolysis, in a tissue notoriously rich in proteases, may account for the apparent lack of other receptor types in intestinal tissue (Gaginella *et al.*, 1983).

Whilst no corrections to the observed binding parameters have been made, the loss of 70.0 ± 4.0% of free [^{125}I]-AII and 41.5% ± 1.0% of free [^{125}I]-Bk will result in changes in the estimates of K_D, k_1 and k_{-1}. If one assumes that the same percentage of each concentration of labelled peptides is hydrolysed then a shift to the left of each saturation curve could be expected. This assumption appears to be justified in the light of current knowledge regarding the enzymatic hydrolysis of neuropeptides. Most, if not all, peptidases exhibit K_m values in the micromolar to millimolar concentration range (Turner *et al.*, 1985). Corrected K_D values might therefore be approximately 0.2 nM for specific [^{125}I]-AII and 0.34 nM for [^{125}I]-Bk binding sites. A decrease in free ligand concentration will also result in increased values for k_1 and k_{-1} however K_{eq} values will remain unchanged. Degradation of specifically bound peptide will result in underestimations of the maximal binding capacities. Given that 45.0 ± 4.5% of [^{125}I]-AII and 15.0 ± 5.0% of [^{125}I]-Bk bound to epithelial sites from the small intestine are hydrolysed then the corrected B_{max} values may be expected to be 28.67 ± 2.74 fmol mg^{-1} for [^{125}I]-AII and 381.8 ± 72.9 fmol mg^{-1} for [^{125}I]-Bk. The changes summarised above serve as a reminder to the reader that extensive hydrolysis of labelled peptides may result in either an overestimation of the K_D, an underestimation of the B_{max}, or perhaps both. However, this does not detract from the main emphasis of the study, given the high specificity of each population of binding sites and their close similarity with sites previously identified in renal cortex epithelia where peptide hydrolysis is less pronounced.

The estimation of equilibrium binding constants (K_{eq}) from uncorrected rate constants yielded interesting results. The K_{eq} value of 0.45 nM correlated well with the K_D of 0.69 ± 0.13 nM obtained for saturable specific [^{125}I]-Bk binding in epithelial membranes from rat jejunum. Kinetic data for specific [^{125}I]-AII binding however resulted in a K_{eq} value of 0.03 nM, one order of magnitude lower than the uncorrected K_D obtained from saturation studies. This may indicate the presence of an additional class of higher affinity specific [^{125}I]-AII binding sites in jejunal epithelia, a possibility that is currently under investigation.

Intestinal [^{125}I]-Bk and [^{125}I]-AII specific binding were saturable, reversible, protein-dependent and highly selective. Only closely related analogues of the respective labelled peptides exhibited high affinities for the binding sites. Peptide fragments and enzyme inhibitors did not reduce specific binding and neither peptide had any affinity for the other labelled binding sites. The presence of high affinity, specific [^{125}I]-AII binding sites, albeit in low numbers, with characteristics similar to those found in epithelial membranes from renal cortex suggest a direct role for AII in the regulation of intestinal ion transport in addition to the indirect hormonal action mediated by noradrenaline. Thus these specific [^{125}I]-AII sites may represent the epithelial AII receptors involved in eliciting the prostaglandin-mediated secretory responses to AII (Levens *et al.*, 1981b). This study has also highlighted the problem of ligand, and possibly receptor protein, degradation during incubations with intestinal membrane preparations. An enzyme-resistant AII analogue with the capacity to label the specific AII binding sites with high affinity would be a useful tool in the further characterization of these intestinal sites.

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