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Adrenomedullin receptor binding sites in rat brain and peripheral tissues

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Abstract

The existence of specific adrenomedullin receptor binding sites was investigated using the agonist peptide fragment [^{125}I]human adrenomedullin-(13–52) in rat brain, lung and vas deferens homogenates. Saturation-binding experiments suggest that [^{125}I]human adrenomedullin-(13–52) binds to an apparent single population of sites with similar affinities (K_D of 0.3 to 0.6 nM) but with different maximal binding capacity in the rat brain, lung and vas deferens homogenates (B_{max} of 73, 1760 and 144 fmol/mg protein, respectively). Competition-binding experiments using various analogues and fragments of calcitonin gene-related peptide (CGRP) and adrenomedullin were also performed using this radioligand. Competition-binding profiles suggest the possible existence of heterogeneous populations of adrenomedullin receptor binding sites. For example, in rat brain, human adrenomedullin-(1–52) and human adrenomedullin-(13–52) competed against specific [^{125}I]human adrenomedullin-(13–52) sites with competition curves best fitted to a two-site model. Additionally, human calcitonin gene-related peptide α (hCGRPα), [Cys(Et)^{2,7}]hCGRPα and [[*R*-(*R*,(*R**,*S**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-Pi-peridinecarboxamide] (BIBN4096BS) competed against specific [^{125}I]human adrenomedullin-(13–52) binding with profiles that were also best fitted to a two-site model. Furthermore, binding assays performed in the presence of GTPγS (100 μM) revealed that this compound inhibited 20% of specific [^{125}I]human adrenomedullin-(13–52) sites in rat brain homogenates and competition curves of human adrenomedullin-(1–52) and [Cys(Et)^{2,7}]hCGRPα against specific [^{125}I]human adrenomedullin-(13–52) sites remained best fitted to a two-site model. Moreover, the existence of specific [^{125}I]human adrenomedullin-(13–52) binding sites that are resistant to human adrenomedullin-(22–52) and human CGRP-(8–37) is suggested in the rat brain and vas deferens. Taken together, these data provide evidence for the possible existence of heterogeneous populations of adrenomedullin binding sites in rat brain and peripheral tissues.

Keywords: Adrenomedullin; CGRP (calcitonin gene-related peptide); Binding, Receptor; Brain; Lung; Vas deferen; BIBN4096BS; [Cys(Et)^{2,7}]hCGRPα; Adrenomedullin-(22–52), human; (Rat)

1. Introduction

Human adrenomedullin is a 52-amino acid peptide isolated from human adrenal pheochromocytoma (Kitamura et al., 1993). Both human and rat adrenomedullin are derived from a 185-amino acid preproadrenomedullin; however, the mature form of rat adrenomedullin is a 50-amino acid peptide (Sakata et al., 1993).

Adrenomedullin-like immunoreactivity and its corresponding mRNA have been reported to be expressed in various tissues including the adrenal gland, heart, lungs and kidneys (Ichiki et al., 1995; Kitamura et al., 1994; Sakata et al., 1994) as well as in the hypothalami of the human and rat (Satoh et al., 1996; Ueta et al., 1995). As reported for calcitonin gene-related peptide (CGRP) and to a lesser extent amylin, intracerebroventricular injections of adrenomedullin increased blood pressure (Takahashi et al., 1994) inhibited water intake (Murphy and Samson, 1995) and produced anorexia (Taylor et al., 1996), while inducing a potent vasodilatation in peripheral tissues (Nuki et al., 1993). On the basis of the comparative biological effects induced by adrenomedullin and CGRP, early studies suggested that both peptides were acting via a common CGRP-like receptor (Eguchi et al., 1994a; Taylor et al., 1996). For example, it has been shown that the potent vasodilator action of adrenomedullin was antagonized by a CGRP receptor antagonist, CGRP-(8–37), suggesting the involvement of

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the $CGRP_1$-like receptor subtype (Baskaya et al., 1995; Berthiaume et al., 1995; Eguchi et al., 1994b; Entzeroth et al., 1995; Hall et al., 1995). Similarly, the anorexic property and hypertensive effects of brain administered adrenomedullin were proposed to be mediated via $CGRP_1$ receptors (Taylor et al., 1996).

However, various in vitro and in vivo bioassays studies supported the existence of a specific population of adrenomedullin receptors on the basis of the very low to low potencies of CGRP-(8–37) to antagonize the effects of adrenomedullin in rat systemic vascular bed (Nandha et al., 1996), guinea pig pulmonary artery (Pinto et al., 1996), mouse astrocytes or renal glomeruli (Hjelmqvist et al., 1997; Yeung et al., 1996) and aldosterone secretion (Hinson et al., 1998;Kapas et al., 1998). Additionally, receptor binding assays have shown that hCGRPα is 100 to 1000 times less potent than adrenomedullin to compete for specific [^{125}I]human or rat adrenomedullin-binding sites in the rat spinal cord (Owji et al., 1995), rat systemic vascular bed (Nandha et al., 1996) and in a rat skeletal muscle cell line (L6) (Coppock et al., 1996) as well as in several other tissues (Hinson et al., 2000). Moreover, structure–activity studies revealed that removing the first 21 N-terminal amino acid residues of human adrenomedullin, just next to the disulfide bridge (human adrenomedullin-(22–52)), generated a fragment with antagonistic properties (Coppock et al., 1996). In most cases, human adrenomedullin-(22–52) was able to block the effects of adrenomedullin without affecting those of CGRP (Fujioka et al., 1999; Gardiner et al., 1999; Nishikimi et al., 1998). Taken together, these results suggested that at least some of biological effects of adrenomedullin were mediated by specific adrenomedullin receptors.

One of the most convincing evidence for the existence of a distinct adrenomedullin receptors was provided by the cloning of single transmembrane proteins known as the receptor-activity-modifying proteins (RAMP) which confer to the calcitonin-receptor like receptor (CRL receptor) either a CGRP or an adrenomedullin-like ligand selectivity profile depending on the associated RAMP (Poyner et al., 2002). When CRL receptor is co-transfected with either RAMP2 or RAMP3, an adrenomedullin receptor was generated while the co-transfection of CRL receptor with RAMP1 resulted in a functional CGRP receptor in human embryonic kidney (HEK) 293 cells (McLatchie et al., 1998).

Good correlation between the distribution of CGRP binding sites and CRL receptor/RAMP1, as well as adrenomedullin receptors and CRL receptor/RAMP2 has been shown in several tissues (Chakravarty et al., 2000), However, this may not account for the totality of [^{125}I]adrenomedullin and [^{125}I]CGRP receptor-binding sites. For example, the expression of the CRL receptor mRNA failed to be detected in the spinal cord and cerebellum (Fluhmann et al., 1997; Oliver et al., 1998), two areas highly enriched with specific [^{125}I]adrenomedullin and [^{125}I]hCGRPα-binding sites (Jacques et al., 2000; Juaneda et al., 2000, 2001; Owji et al., 1995; Van Rossum et al., 1997). Moreover, in rat aortic vascular smooth muscle cells, a prototypical adrenomedullin bioassay, CRL receptor mRNA is not expressed (Autelitano and Tang, 1999), suggesting the possible existence of other genes coding for additional adrenomedullin receptors.

We studied here the respective binding profile of [^{125}I]human adrenomedullin-(13–52) (a potent agonist) in rat brain, lung and vas deferens membrane homogenates. Our results suggest that [^{125}I]human adrenomedullin-(13–52) may recognize heterogeneous populations of receptor-binding sites in these tissues.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (250–300 g) were obtained from Charles River (St-Constant, QC, Canada) and kept on a 12-h light, 12-h dark cycle (lights on at 07:00) in temperature- and humidity-controlled rooms. Animals were fed with standard laboratory chow and had access to tap water ad libitum. Animal care was according to the protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

Human calcitonin gene-related peptide alpha (hCGRPα), hCGRP-(8–37), [$Cys(ACM)^{2,7}$]hCGRPα and [$Cys(Et)^{2,7}$]hCGRPα were synthesized as previously described in details (Mimeault et al., 1992). [[*R*-(*R*,(*R**,*S**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-Piperidinecarboxamide] code name BIBN4096BS was generously provided by Boehringer Ingelheim (Germany). Human adrenomedullin was purchased from Bachem California (Torrance, CA, USA). Human adrenomedullin-(13–52) and human adrenomedullin-(22–52) were generously provided by Dr. Jaw-Kang Chang (Phoenix Pharmaceuticals, Mountain View, CA, USA). [^{125}I]human adrenomedullin-(13–52) was kindly provided by New England Nuclear (Boston, MA). Trisma base, NaCl, $MgCl_2$, bovine serum albumin, bacitracin, leupeptin and chymostatin were obtained from Sigma (St-Louis, MO, USA). GTPγS was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Schleicher and Schuell glass-fiber filters No. 32 were obtained from Zymotech (Montreal, QC, Canada). All other chemicals of analytical grade were obtained from Fischer Scientific (Montreal, QC, Canada).

2.2. Receptor-binding assays

Animals were decapitated and their brains, lungs and vasa deferentia were rapidly removed and placed in 15

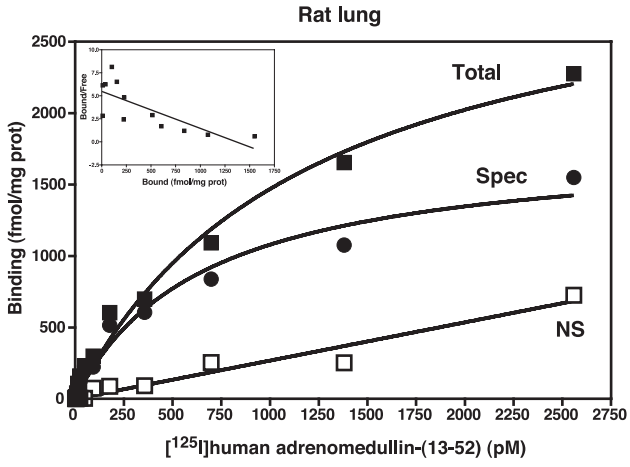

Fig. 1. Typical profile of saturation-binding isotherms of [^{125}I]human adrenomedullin-(13–52) in rat lung membrane homogenates in the presence (NS: nonspecific binding) or absence (total binding) of 1 μM human adrenomedullin-(13–52). Specific binding was established as the difference between total and nonspecific bindings. Inserts are the mathematical transformation as Rosenthal plots of the binding isotherms of [^{125}I]human adrenomedullin-(13–52).

volumes of 25 mM Tris, 50 mM NaCl and 2 mM $MgCl_2$ at pH 7.4. Tissues were then homogenized using a Brinkman polytron (20 s, setting 6) and centrifuged at 49,000 × *g* for 20 min at 4 °C. Pellets were washed and resuspended in the original volume of buffer and recentrifuged. This step was repeated twice. The crude membranes were resuspended in 50 mM Tris, 100 mM NaCl and 4 mM $MgCl_2$ at pH 7.4 at final protein concentration of 4 to 6 mg/ml for binding performed in rat brain and vas deferens, and 0.1 to 0.2 mg/ml for rat lung-binding experiments (final protein concentration being five times lower in binding test tubes). Protein concentration was determined using bovine serum albumin as the standard (Bradford, 1976). All binding assays were performed at room temperature and initiated by adding 100 μl of membrane preparations in a final volume (0.5 ml) of 0.5 mM Tris, pH 7.4, containing 100 mM NaCl, 4 mM $MgCl_2$, 0.1% bovine serum albumin, 0.05% bacitracin, 10 μg/ml leupeptin, 5 μg/ml chymostatin, the iodinated peptide and competitor as required. Saturation-binding experiments were performed in the presence of increasing concentrations of [^{125}I]human adrenomedullin-(13–52), whereas competition binding studies were performed in the presence of 35 pM of [^{125}I]human adrenomedullin-(13–52) and various competitors at concentrations ranging from 10^{-13} to 10^{-5} M. Competition-binding experiments were also performed in the presence and absence of a stable GTP analogue, GTPγS. Nonspecific binding was determined in the presence of 1 μM human adrenomedullin-(13–52). After 90 min (time to reach equilibrium), the reaction was stopped by rapid filtration through glass fiber filters (previously soaked in 0.1% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instrument, Gaithersburg, MD). Filters were rinsed three times with 3 ml of cold 50 mM Tris, 100 mM NaCl and 4 mM $MgCl_2$ at pH 7.4 and the radioactivity remaining on the filters was quantified using gamma counter (Packard Instruments, Cobra II; Mississauga, ON, Canada).

2.3. Data analysis

All binding experiments were performed in triplicate and repeated 3 to 10 times. All binding parameters were determined using GraphPad Prism program (version 3.0). The affinity (K_D) and maximal binding capacity (B_{max}) of the saturation isotherms were estimated by a nonlinear regression curves and were fitted to a one-site and two-site model using hyperbola equations. All saturation isotherms curves were best fitted to a one-site model ($P < 0.05$). The concentration of unlabeled peptide required to compete for 50% of specific binding of the radioligand of various peptides were calculated from the competition binding assays and the results were expressed as the percentage of specific binding representing the mean ± S.E.M. of 3 to 10 individual determinations each in triplicate. Competition curves were fitted to a one-site and a two-site models using GraphPad Prism program (version 3.0). Khigh (K_H) and Klow (K_L) values represent the high and low affinity sites for competition curves that were best fitted to a two-site model with $P < 0.05$ using the Fisher test.

3. Results

Prototypical isotherm saturation binding experiments revealed that [^{125}I]human adrenomedullin-(13–52) binds to an apparent single population of sites in the rat lung (Fig. 1) as well as in rat brain and vas deferens (data not shown). Binding parameters derived from saturation-binding experiments demonstrate that [^{125}I]human adrenomedullin-(13–52) binds with high affinity ($K_D = 0.32 \pm 0.05$, 0. 66 ± 0.16 and 0.33 ± 0.11 nM) and saturable amount ($B_{max} = 73 \pm 4$, 1760 ± 170 and 144 ± 10) of sites in rat brain, lung and vas deferens, respectively (Table 1). It is important to mention here that based on signal-to-noise ratio, [^{125}I]human adrenomedullin-(13–52) is a much better

Table 1
Binding data derived from saturation isotherms of [^{125}I]human adrenomedullin-(13–52) in rat brain, lung and vas deferens membrane preparations

Tissue	[^{125}I]human adrenomedullin-(13–52)	
	K_D (nM)	B_{max} (fmol/mg protein)
Brain	0.32 ± 0.05	73 ± 4
Lung	0.66 ± 0.16	1760 ± 170
Vas deferens	0.33 ± 0.11	144 ± 10

Data represent the mean ± S.E.M. of three to four individual determinations, each performed in triplicate.

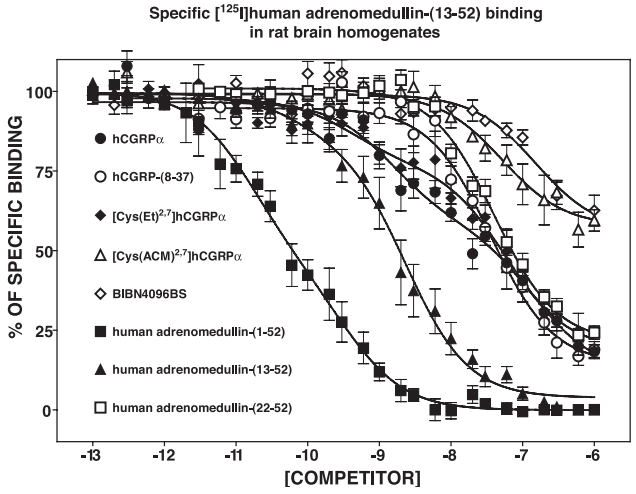

Fig. 2. Competition-binding profiles of human adrenomedullin-(1–52), human adrenomedullin-(13–52), human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37), BIBN4096BS, [Cys(Et)^{2,7}]hCGRPα and [Cys(Acm)^{2,7}]hCGRPα against specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat brain membrane preparations. Each point represents the mean ± S.E.M. of data obtained from 3 to 10 determinations, each performed in triplicate and expressed as percentage of specific binding.

radioligand than [^{125}I]human adrenomedullin-(1–52) used in previous studies (Belloni et al., 1998; Sone et al., 1997; Zimmermann et al., 1996). In fact, at 35 pM, specific [^{125}I]human adrenomedullin-(13–52) binding represented 60% and 50% of totally bound ligand in rat brain and vas deferens homogenates, respectively, while specific [^{125}I]human adrenomedullin-(1–52) binding accounted for only 25% or less of total binding (Sone et al., 1997). Similar results were obtained in rat lung homogenates, but much lower nonspecific [^{125}I]human adrenomedullin-(13–52) binding was observed in this tissue (Fig. 1).

The ligand selectivity profile of binding sites targeted by [^{125}I]human adrenomedullin-(13–52) was investigated next. As shown in Fig. 2, human adrenomedullin-(1–52) and human adrenomedullin-(13–52) competed for specific [^{125}I]human adrenomedullin-(13–52) binding with affinities in the low nM range in rat brain homogenates (Table 2). The competition curves of human adrenomedullin-(1–52) and human adrenomedullin-(13–52) for specific [^{125}I]human adrenomedullin-(13–52) binding revealed that these two peptides may recognize more than one class of sites or different affinity states of the same site as Hill coefficients were significantly lower than unity (n_H of 0.5 to 0.7). Furthermore, as shown in Table 2, the competition curves of these two adrenomedullin agonists were best fitted to a two-site model. In fact, human adrenomedullin-(1–52) competed for specific [^{125}I]human adrenomedullin-(13–52) with high (K_H 0.01 ± 0.005 nM) and low (K_L 0.4 ± 0.1 nM) affinities whereas human adrenomedullin-(13–52) was around 10 times less potent (K_H 0.06 ± 0.2 and K_L 2.2 ± 0.4 nM) in rat brain homogenates. As observed in the rat brain, human adrenomedullin-(1–52) and human adrenomedullin-(13–52) were the most potent competitors against specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat lung (Fig. 3) and vas deferens (Fig. 4) membrane homogenates (Table 2). Furthermore, the purported adrenomedullin receptor antagonist, human adrenomedullin-(22–52), was able to compete against specific [^{125}I]human adrenomedullin-(13–52) binding sites with affinities of 12 to 30 nM in all these preparations (Table 2). However, and most interestingly, human adrenomedullin-(22–52) competed for only up to 80% of specific [^{125}I]human adrenomedullin-(13–52) binding sites in the rat brain (Fig. 2) and vas deferens (Fig. 4) while all specific binding was competed by human adrenomedullin-(22–52) in the lung (Fig. 3). Hence, approximately 20% of specific [^{125}I]human adrenomedullin-(13–52) sites in the rat brain and vas deferens appears to be rather insensitive to the antagonist human adrenomedullin-(22–52). Human CGRPα, the fragment 8–37 (CGRP-(8–37)) and the linear CGRP analogue, [Cys(Et)^{2,7}]hCGRPα demonstrated similar properties in rat brain (Fig. 2; Table 2). On the other hand, in the rat lung, as seen for human adrenomedullin-(22–52), CGRP-related peptides competed for all specific [^{125}I]human adrenomedullin-(13–52) binding sites (Fig. 3).

A highly complex competition profile for specific [^{125}I]human adrenomedullin-(13–52) sites was observed

Table 2
Comparative affinities of AM, CGRP and their analogues for specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat brain, lung and vas deferens membrane homogenates

	Rat brain		Rat lung		Rat vas deferens	
	K_H (nM)	K_L (nM)	K_H (nM)	K_L (nM)	K_H (nM)	K_L (nM)
Human adrenomedullin-(1–52)	0.01 ± 0.005 (44 ± 6%)	0.4 ± 0.1	0.003 ± 0.001 (24 ± 4%)	0.021 ± 0.01	0.05 ± 0.01 (80 ± 6%)	3.4 ± 1.2
Human adrenomedullin-(13–52)	0.06 ± 0.02 (15 ± 4%)	2.2 ± 0.4	4.3 ± 1.5		3 ± 1.5	
Human adrenomedullin-(22–52)	20 ± 6 (78 ± 4%)	>1000	12 ± 4 (45 ± 7%)	150 ± 29	28 ± 4 (77 ± 4%)	>1000
hCGRPα	1.4 ± 0.5 (40 ± 7%)	142 ± 35	2.4 ± 0.8 (11 ± 3%)	120 ± 40	1.8 ± 0.6 (48 ± 7%)	230 ± 60
hCGRP-(8–37)	42 ± 10 (85 ± 13%)	>1000	20 ± 7 (34 ± 5%)	450 ± 110	57 ± 11 (83 ± 5%)	>1000
BIBN4096BS	150 ± 45 (45 ± 8%)	>1000	>1000		>1000	
[Cys(ACM)^{2,7}]hCGRPα	52 ± 12 (46 ± 5%)	>1000	ND		>1000	
[Cys(Et)^{2,7}]hCGRPα	0.4 ± 0.29 (27 ± 5%)	60 ± 8	0.5 ± 0.2	180 ± 35	4.4 ± 1.08 (68 ± 9%)	310 ± 50

Data represent the mean ± S.E.M. of 3 to 10 determinations. All binding curves were fitted to a one-site or two-site model using GraphPad Prism Software competition data analysis. The goodness of fit between the two models was tested by F-test ($P < 0.05$). K_H and K_L represent the affinity of competitors for the high- and low-affinity component, respectively. Values in parenthesis represent the percentage sites for the high-affinity component. ND means not determined.

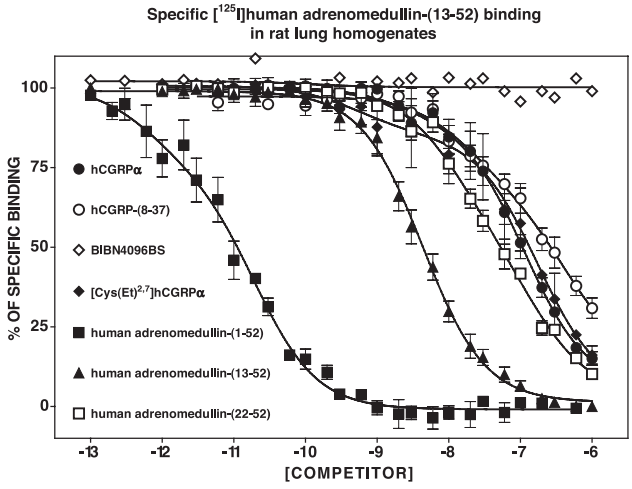

Fig. 3. Competition-binding profiles of human adrenomedullin-(1–52), human adrenomedullin-(13–52), human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37), BIBN4096BS, [Cys(Et)^{2,7}]hCGRPα and [Cys(Acm)^{2,7}]hCGRPα against specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat lung membrane preparations. Each point represents the mean ± S.E.M. of data obtained from three to five determinations, each performed in triplicate and expressed as percentage of specific binding.

in rat brain and vas deferens homogenates. As shown in Fig. 2, the binding curve of hCGRPα is shallow and best fitted to a two-site model ($P < 0.05$) with high ($K_H = 1.4 \pm 0.5$ nM) and low ($K_L = 142 \pm 35$ nM) affinity components (Table 2). Similarly, the two linear analogues of CGRP, [Cys(ACM)^{2,7}]hCGRPα and [Cys(Et)^{2,7}]hCGRPα competed for specific[^{125}I]human adrenomedullin-(13–52) sites with binding curves that were also best fitted to a two-site model ($K_H = 52$ and 0.4 nM; $K_L > 1000$ and 60 nM, respectively) in

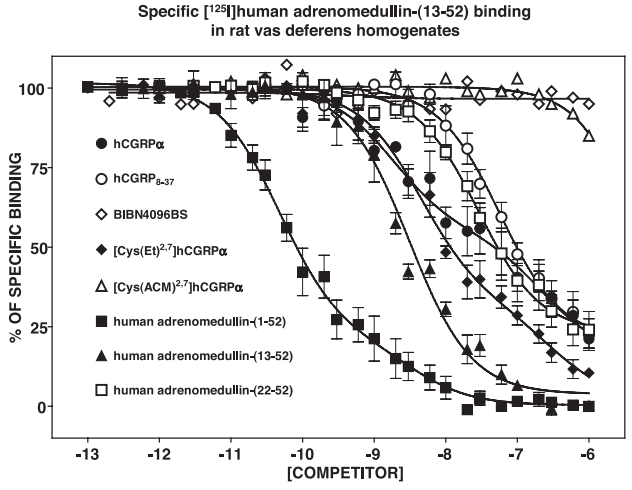

Fig. 4. Competition-binding profiles of human adrenomedullin-(1–52), human adrenomedullin-(13–52), human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37), BIBN4096BS, [Cys(Et)^{2,7}]hCGRPα and [Cys(Acm)^{2,7}]hCGRPα against specific [^{125}I]human adrenomedullin-(13–52) binding sites in vas deferens membrane preparations. Each point represents the mean ± S.E.M. of data obtained from three to five determinations, each performed in triplicate and expressed as percentage of specific binding.

rat brain homogenates (Fig. 2, Table 2). Furthermore, as shown in Table 2, comparable results were obtained for hCGRPα and [Cys(Et)^{2,7}]hCGRPα in the rat vas deferens homogenates (K_H of 1.8 and 4.4 nM; K_L of 230 and 310 nM, respectively).

The non-peptide CGRP receptor antagonist, BIBN 4096BS was inactive against specific [^{125}I]human adrenomedullin-(13–52) sites in rat lung and vas deferens ($IC_{50} \gg 1000$ nM) (Figs. 3 and 4; Table 2). However, in rat brain homogenates, BIBN4096BS was able to compete for a significant proportion of specific [^{125}I]human adrenomedullin-(13–52) binding sites (Fig. 2).

Experiments were also conducted in order to determine the effect of GTPγS on specific [^{125}I]human adrenomedullin-(13–52) binding in rat brain membrane homogenates. As shown in Fig. 5, increasing concentrations of GTPγS resulted in a decrease in specific [^{125}I]human adrenomedullin-(13–52) binding with an affinity of 460 nM. Additionally, rat brain membrane preparations incubated with 100 μM GTPγS resulted in an inhibition of only 20% of specific [^{125}I]human adrenomedullin-(13–52) binding. Competition-binding experiments performed in the presence of GTPγS (100 μM) have shown that human adrenomedullin-(1–52) and [Cys(Et)^{2,7}]hCGRP were able to compete against specific [^{125}I]human adrenomedullin-(13–52) binding with competition curves best fitted to a two-site model (Fig. 6) with affinities similar to that observed in the absence of GTPγS (Table 2). In fact, human adrenomedullin competed against specific [^{125}I]human adrenomedullin-(13–52) binding with affinities of 0.01 and 0.4 nM while in the presence of GTPγS, (100 μM) human adrenomedullin competed for those sites with affinities of 0.02 and 0.5 nM. Similar results were obtained with [Cys(Et)^{2,7}]CGRP. This analogue competed against specific [^{125}I]human adrenomedullin-(13–52) binding with affinities of 0.54 and 92 nM in

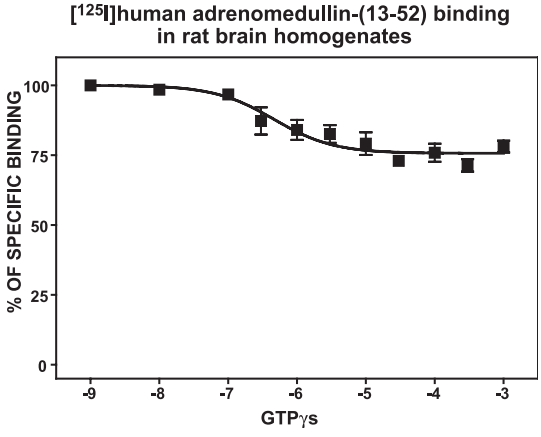

Fig. 5. Competition-binding profiles of GTPγS against specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat brain membrane preparations. Each point represents the mean ± S.E.M. of data obtained from three determinations, each performed in triplicate and expressed as percentage of specific binding.

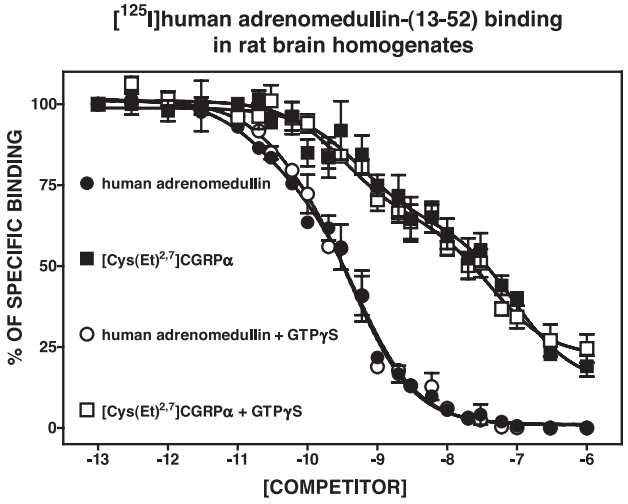

Fig. 6. Competition-binding profiles of human adrenomedullin-(1–52) [Cys(Et)^{2,7}]hCGRPα in the presence and absence of 100 μM GTPγS against specific [^{125}I]human adrenomedullin-(13–52) binding sites in rat brain membrane preparations. Each point represents the mean ± S.E.M. of data obtained from three determinations, each performed in triplicate and expressed as percentage of specific binding.

the absence of GTPγS and of 0.34 and 40 nM in the presence of 100 μM GTPγS.

4. Discussion

This study provides evidence for the existence of the possible heterogeneity of adrenomedullin receptors in rat tissues. This hypothesis is based on the differential affinity and ability of some competitors such as human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37), [Cys(Et)^{2,7}]hCGRPα and BIBN4096BS to inhibit only a fraction of specific [^{125}I]human adrenomedullin-(13–52) binding sites. Furthermore, the existence of an heterogeneous population of [^{125}I]human adrenomedullin-(13–52) sites in the rat vas deferens is also suggested by the complex competition binding curves of human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37) and [Cys(Et)^{2,7}]hCGRPα. Binding data suggest that one site recognized by [^{125}I]human adrenomedullin-(13–52) is rather insensitive to human adrenomedullin-(22–52), hCGRPα, hCGRP-(8–37) and [Cys(Et)^{2,7}]hCGRPα while the second displays high affinity for human adrenomedullin-(22–52) and two affinity components for hCGRPα and [Cys(Et)^{2,7}]hCGRPα (K_H nM range and K_L high nM range) and BIBN4096BS (150 vs. ≫1000 nM). Additionally, in rat brain membrane homogenates, experiments performed in the presence of GTPγS revealed that this compound was able to inhibit only 20% of specific [^{125}I]human adrenomedullin-(13–52) binding. Most importantly, in this same tissue, competition binding curves performed in the presence of 100 μM GTPγS demonstrated that human adrenomedullin and [Cys(Et)^{2,7}]CGRP competed against specific [^{125}I]human adrenomedullin-(13–52) binding with a best fit to a two-site model, suggesting that those sites most likely do not represent a single population of sites in different affinity states, but rather distinct population of specific binding sites.

Early studies suggested the existence of at least two CGRP receptor subtypes (for recent reviews, see Juaneda et al., 2000; Poyner et al., 2002). The $CGRP_1$ subtype is particularly sensitive (pA_2 value of 7 to 7.5) to CGRP receptor antagonists such as CGRP-(8–37) and more recently BIBN4096BS (Doods et al., 2000; Wu et al., 2000) while the $CGRP_2$ subtype is usually less sensitive to these antagonists (Dennis et al., 1989, 1990; Doods et al., 2000; Wu et al., 2000). Additionally, it has been established that adrenomedullin could mediate some of its effects through the activation of the $CGRP_1$ receptor (Baskaya et al., 1995; Berthiaume et al., 1995; Eguchi et al., 1994b; Entzeroth et al., 1995; Hall et al., 1995; Taylor et al., 1996). However, limited information is currently available on the effect of adrenomedullin on the $CGRP_2$ receptor subtype. In prototypical $CGRP_2$ in vitro bioassays such as the guinea pig (Poyner et al., 1999; Quirion et al., 1992) and rat (Dennis et al., 1989, 1990; Quirion et al., 1992; Wu et al., 2000) vasa deferentia, CGRP-(8–37) and BIBN4096BS were able to antagonize the effects of hCGRPα with pA_2 values in the 6.0–7.0 range. In the guinea pig vas deferens, it has also been reported that CGRP-(8–37) was a weak antagonist against adrenomedullin-induced responses (Poyner et al., 1999), suggesting that adrenomedullin can possibly act on $CGRP_2$ receptor subtypes in this tissue. However, in the rat vas deferens, Wu et al. (2000) reported that adrenomedullin and [Cys(Et)^{2,7}]hCGRPα inhibited electrically induced-twitch responses that were antagonized by CGRP-(8–37) and BIBN4096BS with pA_2 values of 7.0 and 8.0, respectively. These authors proposed the existence of an atypical adrenomedullin/[Cys(Et)^{2,7}]hCGRPα preferring receptor that was sensitive to these two CGRP receptor antagonists. Taken together, these functional assays support the existence of multiple CGRP/adrenomedullin receptor subtypes.

It is now well accepted that the $CGRP_1$ receptor subtype represents a protein complex that includes CRL receptor and RAMP1 while the adrenomedullin AM_1 receptor is an heterodimer composed of CRL receptor and RAMP2 (Poyner et al., 2002). The respective pharmacological profile of CRL receptor/RAMP1 (CGRP, BIBN4096BS>CGRP-(8–37) ≥ adrenomedullin ≫ adrenomedullin-(22–52)) and CRL receptor/RAMP2 (adrenomedullin ≥ adrenomedullin-(22–52) ≫ CGRP, CGRP8–37>BIBN4096BS) is now well documented and has been replicated in several laboratories (Autelitano and Ridings, 2001; Born et al., 2002; Buhlmann et al., 1999; Choksi et al., 2002; Drake et al., 1999; Husmann et al., 2000; Kamitani et al., 1999; Moreno et al., 2002). On the other hand, the profile of the adrenomedullin AM_2 receptor (CRL receptor/RAMP3) has been much less studied but may possess high affinity for both adrenomedullin and CGRP (Born et al., 2002; Poyner et al., 2000). It is not clear at this time if the adrenomedullin AM_2

receptor (CRL receptor/RAMP3) may in fact represent the so-called $CGRP_2$ subtype or the atypical adrenomedullin receptor reported by Wu et al. (2000).

Receptor-binding assays and receptor autoradiographic studies have demonstrated that [^{125}I]hCGRPα and [^{125}I] adrenomedullin recognized different populations of binding sites in the rat brain (Juaneda et al., 2001; Van Rossum et al., 1995), rat lung (Dang et al., 1999) and rat adrenal cortex (Kapas et al., 2001). The fact that competition-binding studies using rat or human [^{125}I]adrenomedullin failed to provide evidence for the heterogeneity of adrenomedullin-binding sites (Belloni et al., 1999; Coppock et al., 1999; Dang et al., 1999; Kennedy et al., 1998; Owji et al., 1995; Renshaw et al., 2000; Sone et al., 1997; Taylor et al., 1996; Yeung et al., 1996) is most likely related to technical issues associated to the use of these radioligands. For example, [^{125}I] human adrenomedullin-(1–52) is known to generate a very poor signal-to-noise ratio limiting its usefulness to detect the possible existence of subtypes expressed in small proportions or to distinguish between affinity states of a given receptor population.

In that regard, one of the main advantages of using [^{125}I]human adrenomedullin-(13–52) as radioligand is the much higher percentage of specific binding (up to 60% vs. 25% for human adrenomedullin-(1–52)). Moreover, the overall ligand selectivity profile of various competitors to inhibit [^{125}I]human adrenomedullin-(13–52) binding is in accordance with that expected for adrenomedullin receptors with adrenomedullin ≈ long C-terminal fragments of adrenomedullin>short C-terminal fragments>CGRP related molecules>BIBN4096BS. Furthermore, and in contrast to data obtained in rat lung, human adrenomedullin-(22–52) was only able to compete for a fraction of total specific [^{125}I]human adrenomedullin-(13–52) binding sites in the rat brain and vas deferens. These data can be taken as evidence for the existence of two populations of adrenomedullin-binding sites; one that is sensitive to the purported antagonist, human adrenomedullin-(22–52) while the other is not. Rather surprisingly, the linear analogue of hCGRPα, [$Cys(Et)^{2,7}$]hCGRPα, as well as hCGRPα and hCGRP-(8–37), were also only able to compete for a proportion of specific [^{125}I]human adrenomedullin-(13–52) binding sites in the rat brain. It is tempting to speculate that adrenomedullin-(22–52) and [$Cys(Et)^{2,7}$]hCGRPα- insensitive [^{125}I]human adrenomedullin-(13–52) sites may represent a unique population of adrenomedullin receptors. Additional molecular and biochemical studies will be required to establish more precisely the nature of these unique [^{125}I]human adrenomedullin-(13–52) binding sites.

Already, however, we observed that both CGRP linear analogues, [$Cys(Et)^{2,7}$]hCGRPα and [$Cys(ACM)^{2,7}$] hCGRPα, as well as hCGRPα itself competed in a clearly biphasic manner against specific [^{125}I]human adrenomedullin-(13–52) binding in rat brain homogenates. In fact, their respective competition binding curve was best fitted to a two-site model with high (K_H of 0.4, 52 and 1.4 nM, respectively) and low (K_L of 60, >1000 and 142 nM, respectively) affinity components. Moreover, in the presence of 100 μM GTPγS, [$Cys(Et)^{2,7}$]CGRP competed against [^{125}I]human adrenomedullin-(13–52) binding with competition curves best fitted to a two-site model with high and low affinity components (0.32 and 40 nM, respectively), suggesting the existence of an heterogeneous population of [^{125}I]human adrenomedullin-(13–52) sites in the rat brain. These complex competition profiles could relate the different ligand selectivity pattern reported in various bioassays (Hinson et al., 2000; Poyner, 1995, 2002; Quirion and Dumont, 2000). A receptor site could possibly represent the $CGRP_2$ receptor subtype, explaining the high affinity/potency of the linear analogues of hCGRPα. Alternatively, it could relate to the adrenomedullin AM_2 subtype. Further characterization of the CRL RECEPTOR/RAMP3 complex and adrenomedullin AM2 subtype will be required to clarify this issue.

Most recently, BIBN4096BS was used as a radioligand and competition binding experiments revealed that adrenomedullin was almost inactive to compete against specific [^{3}H]BIBN4096BS-binding sites (Schindler and Doods, 2002). These results are in agreement with the poor affinity of BIBN4096BS to compete for specific [^{125}I]human adrenomedullin-(13–52) binding sites. Interestingly however, BIBN4096BS was able to compete for a certain proportion of specific [^{125}I]human adrenomedullin-(13–52) sites in the rat brain while being ineffective in the lung and vas deferens. It has recently been shown that BIBN4096BS could antagonize adrenomedullin and [$Cys(Et)^{2,7}$]hCGRPα-induced inhibitory twitch responses in the rat vas deferens (Wu et al., 2000). Taken together, these results could indicate that one of the [^{125}I]human adrenomedullin-(13–52) binding sites that is sensitive to [$Cys(Et)^{2,7}$]hCGRPα in the rat brain may represent the adrenomedullin/[$Cys(Et)^{2,7}$]hCGRPα-preferring receptor proposed by Wu et al. (2000). Unfortunately, we failed to observe a significant inhibition of specific [^{125}I]human adrenomedullin-(13–52) binding by BIBN4096BS in rat vas deferens homogenates. This could relate to the fact that specific [^{125}I]human adrenomedullin-(13–52)/[$Cys(Et)^{2,7}$]CGRP-sensitive and BIBN4096BS-sensitive sites may represent only a small fraction of the total population of specific [^{125}I]human adrenomedullin-(13–52) sites detected in the rat vas deferens in comparison to a higher proportion in the rat brain.

In summary, our results suggest the possible heterogeneity of adrenomedullin receptors, particularly in the rat brain and vas deferens. This hypothesis is supported by the findings that a significant proportion of specific [^{125}I]human adrenomedullin-(13–52) sites in the rat brain are resistant to analogues such as human adrenomedullin-(22–52) and hCGRP-(8–37), and on the basis of the complexity of binding curves of various competitors, even in the presence GTPγS (100 μM). Molecular studies and a better characterization of the adrenomedullin AM_2 receptor are now

required to characterize the unique features of these populations of [^{125}I]human adrenomedullin-(13–52) receptors sites expressed in the rat brain and vas deferens.

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