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Characterization of [^{125}I]-endothelin-1 and [^{125}I]-BQ3020 binding to rat cerebellar endothelin receptors

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1 We performed radioligand binding experiments on rat cerebellar homogenates using [^{125}I]-endothelin-1 ([^{125}I]-ET-1) and [^{125}I]-BQ3020 to examine the pharmacology of endothelin receptors in rat brain. Saturation experiments demonstrated a single population of binding sites with high affinity for both radioligands ([^{125}I]-ET-1, $pK_d = 8.94 \pm 0.17$; [^{125}I]-BQ3020, $pK_d = 9.18 \pm 0.14$ nM; mean ± s.e.mean). However, [^{125}I]-BQ3020 only recognised approximately one third the number of endothelin receptors measured with [^{125}I]-ET-1.

2 Saturation binding experiments with [^{125}I]-PD151242 revealed high affinity binding to a single population of ET_A receptors in the cerebellar homogenates ($pK_d = 9.95 \pm 0.14$; $B_{max} = 30 \pm 15$ fmol mg^{-1} protein).

3 Competition experiments were performed with ligands that are either non-selective or selective for endothelin receptor subtypes. The rat cerebellar endothelin receptor displayed a high affinity for endothelin-1 (ET-1), endothelin-3 (ET-3) and sarafotoxin-S6c (STX-6c) although the affinity for ET-3 was slightly higher than the affinity for ET-1 using both radioligands. The selective ET_A antagonists, BQ123, BMS-182,874 and JKC-301 all displayed low affinities at the endothelin receptors. In contrast the selective ET_B agonists, IRL1620 and [Ala^{1,3,11,15}]ET-1 and the selective ET_B antagonist, BQ-788 had moderate affinities at the endothelin receptor, in the low nanomolar range. The ET_B agonist, BQ3020, had approximately 10 fold higher affinity than IRL1620 and [Ala^{1,3,11,15}]ET-1 at the rat cerebellar endothelin receptors. The non-selective antagonists, Ro-46,2005, Ro-47,0203 and PD-142,893 displayed moderate affinities at the cerebellar receptor.

4 Since [^{125}I]-BQ3020 recognises only a fraction of the [^{125}I]-ET-1 binding sites, the majority of the endothelin receptors in the cerebellum cannot be classed as ET_B. Although [^{125}I]-PD151242 was able to detect ET_A receptors in the rat cerebellar homogenates, the small population of ET_A receptors (2% of the total endothelin population as measured with [^{125}I]-ET-1) could not account for the non-ET_B receptor population. We conclude that the rat brain cerebellar receptor has a profile similar to the ET_{B1} receptor as it has a high affinity for ET-1, ET-3, STX-6c and was moderately sensitive to PD-142,893. However, as the ET_B ligands BQ-788, IRL1620 and [Ala^{1,3,11,15}]ET-1 have only a moderate affinity for the rat cerebellar endothelin receptor and since ET-3 has a higher affinity as compared to ET-1, our findings suggest that the rat cerebellum contains predominately ET_C receptors.

Keywords: Endothelin receptor subtypes; [^{125}I]-endothelin-1; [^{125}I]-BQ3020; [^{125}I]-PD151242; rat cerebellum

Introduction

Endothelins are a family of potent vasoactive peptides (Yanagisawa *et al.*, 1988; Yanagisawa & Masaki, 1989; Inoue *et al.*, 1989) that produce potent and sustained vasoconstriction. It has also been suggested that endothelins play a role in neurotransmission (Supattapone *et al.*, 1989) and in the processes accompanying neuronal cell injury (MacCumber *et al.*, 1990; Widdowson *et al.*, 1995). Endothelin receptors are found in moderate densities throughout the brain with the highest in the cerebellum (Nambi *et al.*, 1990). The endothelin family, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) (Yanagisawa & Masaki, 1989) which also includes the structurally related sarafotoxins, sarafotoxin-6b (STX-6b) and sarafotoxin-6c (STX-6c), interact with at least two classes of receptors (Schvartz *et al.*, 1991; Masaki *et al.*, 1994) called ET_A and ET_B receptors. ET_A and ET_B receptors have now been isolated, sequenced and cloned in a number of mammalian species, including man (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Lin *et al.*, 1991). In pharmacological assays, including isolated organs and radiolabelled binding techniques, ET_A receptors demonstrate an approximately 100 fold higher affinity for ET-1, ET-2 and STX-6b over ET-3 and STX-6c (Williams *et al.*, 1991). In contrast, all the endothelins and STX-6b and STX-6c have approximately the same potency at ET_B receptors. A number of peptide and nonpeptide ligands that display selectivity for the two endothelin receptors have now been synthesized. BQ123, JKC-301 and BMS-182,874 act as selective ET_A antagonists with more than 100 fold selectively over the ET_B receptors (Bax & Saxena, 1994). In contrast, the synthesis of high affinity, selective ET_B antagonists has proved more elusive and there are currently only two compounds reported as displaying selectivity for ET_B receptors over ET_A receptors, namely BQ-788 (Ishikawa *et al.*, 1994) and RES-701-1 (Tanaka *et al.*, 1994). However, a number of peptide ET_B agonists are now available in addition to ET-3 and STX-6c, such as BQ-3020, IRL-1620 (Nambi *et al.*, 1994) and [Ala^{1,3,11,15}]ET-1 (Saeki *et al.*, 1991). There are no reports of selective ET_A agonists. The synthesis of non-peptide antagonists, for example Ro-46,2005, Ro-47-0203, SB 209670 (Clozel *et al.*, 1993; 1994; Douglas *et al.*, 1995) that show oral activity (Breu *et al.*, 1993), has further aided the understanding of the role of endothelins in modulating cardiovascular function in addition to that obtained with peptide ligands such as TAK-044, PD 142,893 and FR 139317 (Warner *et al.*, 1993b; Yamamoto *et al.*, 1994; Bax & Saxena, 1994).

A number of recent studies on isolated organs have suggested that the action of the endothelins cannot be explained

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based on the interaction at only two classes of receptors (Warner *et al.*, 1993a; b; Bax & Saxena, 1994). For example, it has been demonstrated that the ET_B receptor located on the endothelium mediating vasodilatation is different from the ET_B receptor located on smooth muscle and which produces vasoconstriction, based on the sensitivity to the non-selective antagonist, PD142,893 (Warner *et al.*, 1993b). The ET_B receptor subclasses have been tentatively classified as ET_{B1} (located on the endothelium and PD142,893-sensitive) and ET_{B2} (located on smooth muscle and PD142,893-insensitive) (Warner *et al.*, 1993b). Recently another endothelin receptor has been proposed, displaying a preference for ET-3 over ET-1, as in the bovine carotid artery (Emori *et al.*, 1990) and called the ET_C receptor. The endothelin receptors in bovine carotid artery may be related to receptors cloned from *Xenopus laevis* melanophores (Karne *et al.*, 1993) that also have a higher affinity for ET-3 over ET-1. Although the endothelin receptor in rat brain has been tentatively classified as an ET_B type, the higher affinity of ET-3 over ET-1 in binding assays suggests that the rat brain receptor may be an ET_C type (Nambi *et al.*, 1990).

We have characterized the cerebellar endothelin receptors using the non-selective ligand [^{125}I]-ET-1 and the selective ET_B ligand [^{125}I]-BQ3020 and using selective ET_A and ET_B ligands to examine whether the endothelin receptors in rat brain fall into an ET_A, ET_{B1}, ET_{B2} or ET_C classification.

Methods

The cerebellum was removed from male and female Alderley Park rats and homogenized in 50 mM Tris-HCl buffer (pH 7.4; 4°C) with a Teflon/glass motorised homogenizer. The homogenates were centrifuged for 10 min at 1,000 *g* (4°C) to remove cellular debris and nuclei. The supernatant was subsequently centrifuged for 30 min at 20,000 *g* (4°c) to pellet the synaptosomal/mitochondrial fraction. The pellet was resuspended in buffer at between 5 and 10 mg protein ml^{-1}, divided into 1 ml aliquots and frozen. The protein suspensions were stored at −70°C until used.

Receptor binding was performed in 96-well microplates. [^{125}I]-ET-1, [^{125}I]-BQ3020 and [^{125}I]-PD151242 (all 2000 Ci $mmol^{-1}$) binding was performed in 50 mM Tris buffer (pH 7.4) containing 1 mM $CaCl_2$, 0.1% BSA (w/v), 0.002% sodium azide, 5 $\mu g\ ml^{-1}$ soybean trypsin inhibitor and 100 $\mu g\ ml^{-1}$ bacitracin in a total volume of 200 μl for 3 h at 22°C. Saturation experiments were performed with concentration ranges of 10 pM to 5 nM for all the radioligands, whilst 20 pM of [^{125}I]-ET-1 and [^{125}I]-BQ3020 were used for competition experiments. Non-specific binding for [^{125}I]-ET-1, [^{125}I]-BQ3020 and [^{125}I]-PD151242 was measured using either 100 nM ET-1, 100 nM BQ3020 or 5 μM JKC-301, respectively. Non-specific binding for [^{125}I]-ET-1 was typically around 10% of the total binding whilst the non-specific binding for both [^{125}I]-BQ3020 and [^{125}I]-PD151242 was approximately 25%. Bound radioactivity was separated from free by rapid filtration through Packard Unifilter GF/B filters, using a Packard Topcount cell harvester. The protein was washed three times with ice-cold 50 mM Tris buffer (pH 7.4) and the radioactivity on the filters estimated by liquid scintillation counting using Packard Scint-O scintillation cocktail. Data were analysed using the iterative nonlinear least square curve-fitting programme, LIGAND.

Peptides and drugs

[^{125}I]-ET-1, [^{125}I]-BQ3020 and [^{125}I]-PD151242 (N-[Chexahydro-1-azepinyl)carbonyl]L-Leu(1-Me)D-Trp-D-Tyr) were obtained from Amersham International, Bucks, U.K. ET-1, ET-3, STX-S6c, IRL1620 (N-succinyl-[Glu^9,Ala^{11,15}]ET-1(8-21)), BQ3020 (N-acetyl-[Ala^{11,15}]ET-1(6-21)), [Ala^{1,3,11,15}]ET-1, JCK-301 (cyclo[D-Asp-Pro-D-Ile-Leu-D-Trp]) and BQ123 (cyclo[D-Asp-Pro-D-Val-Leu-D-Trp]) were from BACHEM (UK) Ltd, Saffron Walden, Essex. Ro-46,2005 (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5(3-methoxy-phenoxy)-4-pyrimidinyl]-benzenesulphonamide), Ro-47,0203 (4-tert-butyl-N-[6-(2-hydroxy-eth-oxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulphonamide) and BMS-182,874 (5-[dimethy-amino]-N-[3,4-dimethyl-5-isoxazolyl]-1-naphthalene-sulphonamide) were synthesized at ZENECA Pharmaceuticals, Alderley Park, Macclesfield, BQ-788 (N-*cis*-2,6-dimethyl-piperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) was purchased from Penninsula Laboratories, Cheshire, U.K. PD142,893 (Ac-[β-phenyl]-D-Phe-Leu-Asp-Ile-Ile-Trp) was a gift from Dr A Doherty, Parke Davis Pharmaceuticals, Ann Arbor, MI, U.S.A. Peptides were dissolved in dimethylsulphoxide (DMSO) and diluted in water so that the final concentration of DMSO did not exceed 1% of the total volume. All other reagents were purchased from the Sigma-Aldrich Chemical Company, Poole Dorset and were of the highest quality commercially available.

Results

All three radioligands, namely [^{125}I]-ET-1, [^{125}I]-BQ3020 and [^{125}I]-PD151242 bound to a single population of sites in rat cerebellum homogenates with high affinity (Table 1; Figure 1) and with Hill coefficients that were not significantly different from unity. The number of sites labelled with [^{125}I]-BQ3020 was approximately half that measured with [^{125}I]-ET-1. The selective ET_A antagonist, [^{125}I]-PD151242 was able to recognise only a small population of ET_A receptors in the cerebellar homogenate preparation (approximately 2% of the total number of binding sites measured by [^{125}I]-ET-1).

Competition experiments using a variety of selective and non-selective endothelin agonists and antagonists confirmed that [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding was to endothelin receptors since ET-3 and STX-6c exhibited a high affinity with both ligands (Table 2; Figure 2). The affinity of ET-3 was higher (between 2 and 20 fold) at the [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites as compared with ET-1 but the difference between the affinity of ET-3 versus ET-1 was 10 fold greater when the radioligand was [^{125}I]-BQ3020. In contrast, the affinities of STX-6c at [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites was approximately the same as with ET-1. The selective ET_B agonist, BQ3020, had a similar, high affinity at both [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites. However the reported selective ET_B agonists, IRL1620 and [Ala^{1,3,11,15}]ET-1 and the ET_B antagonist, BQ-788 had between 10 and 20 fold lower affinities than BQ3020 at [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites. As with ET-3 the affinities of BQ-788 and PD142,893 were slightly higher with [^{125}I]-BQ3020 than with [^{125}I]-ET-1 (Table 2). The selective ET_A antagonist, BQ123 had a low affinity at [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites in rat cerebellar homogenates whilst neither of the ET_A antagonists, BMS-182,874 and JKC-301 competed with [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding, up to 1 mM. The non-selective, non-peptide endothelin antagonists, Ro-46,2005 and Ro-47,0205 displayed a moderate affinity at both [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites as did the peptide antagonist, PD142,893. An analysis of the values for

Table 1 Saturation experiments using [^{125}I]-ET-1 and [^{125}I]-BQ3020 and rat cerebellar homogenates

	[^{125}I]-ET-1	*[^{125}I]-BQ3020*	*[^{125}I]-PD151242*
pK_D (nM)	8.94±0.17	9.18±0.14	9.95±0.14
B_{max} (fmol mg^{-1} protein)	2207±150	787±46	31±15
Hill coefficient	1.01±0.06	0.96±0.03	0.93±0.10

Data shown as mean±s.e.mean for separate experiments performed on three to four membrane preparations in duplicate.

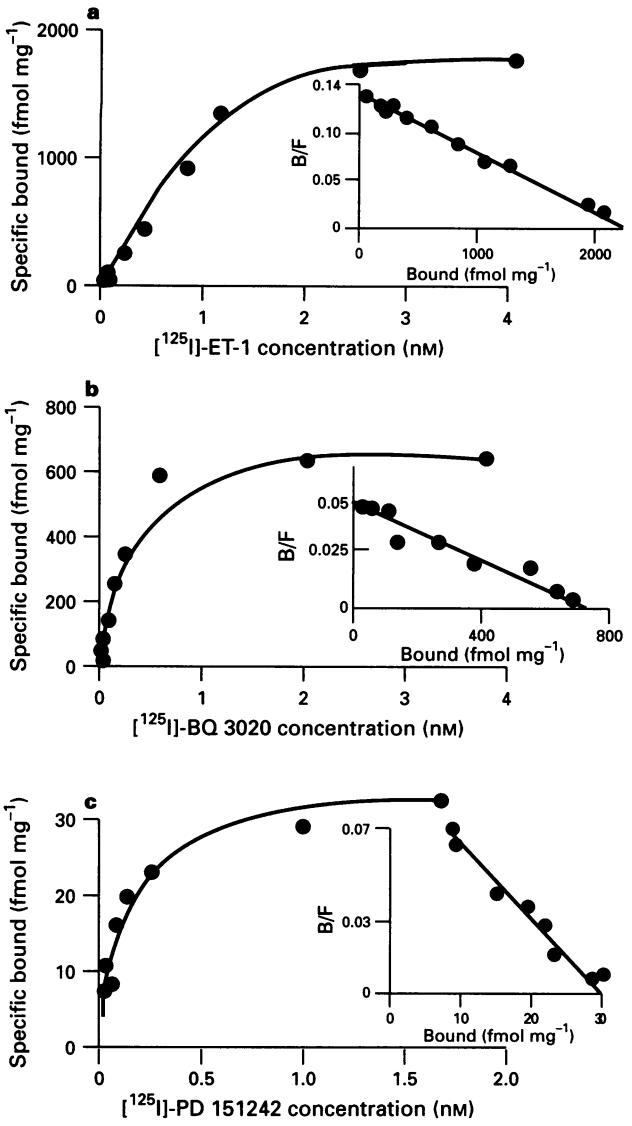

Figure 1 Representative saturation experiments and their respective Scatchard transformations for (a) [^{125}I]-ET-1 binding, (b) [^{125}I]-BQ3020 binding and (c) [^{125}I]-PD151242 binding to cerebellar homogenates.

pK_i obtained from the endothelin ligands against [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites resulted in a highly significant correlation between the two ligands ($r=0.970$; $P<0.0001$).

Discussion

Both [^{125}I]-ET-1 and [^{125}I]-BQ3020 bound with high affinities to endothelin receptors in rat cerebellar homogenates with affinities for [^{125}I]-ET-1 similar to previous studies (Schvatz *et al.*, 1991; Bousso-Mittler *et al.*, 1991; Gulati & Rebello, 1992). The affinity of the selective ET_A antagonist, [^{125}I]-PD151242 was also similar to previous experiments on preparations containing ET_A receptors (Yu *et al.*, 1995). Competition experiments with both [^{125}I]-ET-1 and [^{125}I]-BQ3020 resulted in high affinities for ET-1, ET-3 and STX-S6c in the rat cerebellum. Also the high correlation between the affinities of the various endothelin ligands at [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites suggests that both radioligands recognise an identical endothelin receptor. Based on the current nomenclature, this would suggest that the rat cerebellar endothelin receptor fits into the ET_B classification where all endothelins and sarafotoxins display equal affinities (Sakurai *et al.*, 1990; Masaki *et al.*, 1994). However, [^{125}I]-BQ3020 recognised approximately only one third of the total number of endothelin receptors measured by [^{125}I]-ET-1. The endothelin receptors that were not recognised by [^{125}I]-BQ3020 could not have been ET_A receptors since saturation binding using the selective ET_A antagonist, [^{125}I]-PD151242 (Yu *et al.*, 1995) recognised only a

Table 2 Competition experiments using 20 pM [^{125}I]-ET-1 or 20 pM [^{125}I]-BQ3020 on rat cerebellar homogenates

	pK_i (M)	
	[^{125}I]-ET-1	*[^{125}I]-BQ3020*
ET-1	8.36±0.09	8.39±0.09
ET-3	8.71±0.08	9.38±0.41
STX-6c	8.24±0.07	8.34±0.05
BQ3020	7.67±0.01	7.46±0.06
IRL1620	6.39±0.16	6.29±0.24
Ro-46,2005	6.07±0.03	6.82±0.21
Ro-47,0203	6.21±0.11	6.50±0.06
[Ala^{1,3,11,15}]ET-1	6.70±0.16	7.01±0.13
BQ-788	6.87±0.14	7.65±0.22
PD-142,893	6.48±0.04	7.53±0.04
BQ123	5.03±0.00	5.01±0.00
BMS-182,874	<3	<3
JKC-301	<3	<3

Results expressed as mean±s.e.mean for between 3 and 4 experiments performed in duplicate.

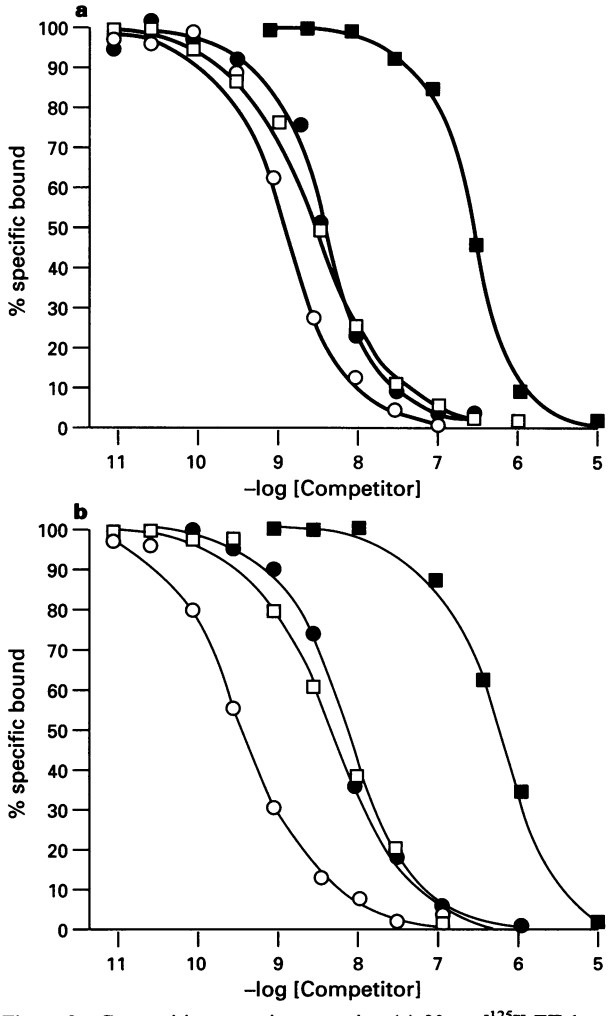

Figure 2 Competition experiments using (a) 20 nM [^{125}I]-ET-1 or (b) 20 nM [^{125}I]-BQ3020 in cerebellar homogenates. Data shown as the mean of between 3 and 4 experiments: (●) ET-1; (○) ET-3; (□) STX-S6c; (■) IRL1620.

very small amount of ET_A receptors in the cerebellar homogenate (i.e. less than 2% of the receptors recognised by [^{125}I]-ET-1). Moreover the affinity of [^{125}I]-BQ3020 in our study was approximately 100 fold lower than reported by Jarvis *et al.* (1994) who also used rat cerebellar homogenates. One possible reason for the lower affinity for IRL1620 and BQ3020 in our studies against ET_B receptors as compared to previous studies (Jarvis *et al.*, 1994; Nambi *et al.*, 1994) may be the species and/or strain differences in rat of the ET_B receptor pharmacology. However, Jarvis *et al.* (1994) reported equal potency for ET-1 and ET-3 using [^{125}I]-BQ3020 whereas in our study, ET-3 displayed a higher affinity than ET-1. The slightly higher affinity of ET-3 over ET-1 may suggest that in our study the [^{125}I]-ET-1 and [^{125}I]-BQ3020 may be binding to an ET_C subtype in rat cerebellum (Karne *et al.*, 1993; Masaki *et al.*, 1994). Our affinity of ET-3 at the cerebellar site compares favourably with the low affinity ET-3 sensitive/ET-1 insensitive site reported in bovine endothelial cells (Emori *et al.*, 1990). The difference in affinity towards ET-3 and ET-1 in our study compares favourably with the data obtained in *Xenopus* dermal melanophores where ET-3 was approximately 2 fold higher than ET-1 at the putative ET_C receptors using receptor binding assays (Karne *et al.*, 1993).

The low affinity of the selective ET_B agonists, IRL1620 and [Ala^{1,3,11,15}]ET-1 and of the selective ET_B antagonist, BQ-788 at both [^{125}I]-ET-1 and [^{125}I]-BQ3020 binding sites suggests that the radioligands recognise a site that is similar to, but not identical to the classical ET_B site. Both IRL 1620 and [Ala^{1,3,11,15}]ET-1 exhibit activities in the low nanomolar range towards ET_B receptors (Saeki *et al.*, 1991; Buchan *et al.*, 1994) whereas in our rat brain preparation, the affinities were approximately 100 fold lower. In addition, the affinity of BQ-788 at ET_B receptors in porcine cerebellar membranes and human Girardi heart cells (Ishikawa *et al.*, 1994) has been reported to be around 1 nM, yet again in our rat brain preparation, we calculate the affinity to be approximately 100 fold lower at the cerebellar endothelin receptor. Furthermore, the affinities of BQ-788 and PD-142,893 were slightly higher when using [^{125}I]-BQ3020 than [^{125}I]-ET-1 suggesting that both radioligands might either recognise slightly different binding sites on the same receptor or that they may be binding to different receptors. Although it has been reported that the rat brain contains a small population of ET_A receptors (Nandasoma & Davenport, 1994) as measured by the binding of the selective ET_A antagonist, [^{125}I]-PD151242, we failed to demonstrate any binding of [^{125}I]-ET-1 or [^{125}I]-BQ3020 to ET_A receptors as demonstrated by the lack of displacement by BMS-182,874 or JKC-301 and the low affinity of BQ123 at the binding sites. Further evidence that the receptor located in the cerebellum may not be the ET_B subtype comes from immunocytochemical studies (Hagiwara *et al.*, 1993). Using a specific antibody directed against purified bovine ET_B receptors, Hagiwara *et al.* (1993) reported that most of the staining for the ET_B receptors was located in the molecular layer of the cerebellum. These data conflict with autoradiographic studies using [^{125}I]-ET-1 that demonstrate higher endothelin receptor densities in the granular layer of the cerebellum (Widdowson *et al.*, 1995). Thus it suggests that the majority of endothelin receptors located in the cerebellum and concentrated in the granular layer may not ET_B receptors. Recently a similar phenomenon of a difference in receptor density recognised by two radioligands supposedly binding to the same receptor was reported by Patel *et al.* (1996). Using both [^{3}H]-nicotine and [^{3}H]-epibatidine to label neuronal nicotinic receptors, [^{3}H]-epibatidine was demonstrated to recognise approximately twice as many receptors as [^{3}H]-nicotine (Patel *et al.*, 1996). Competition curves showed similar pharmacological profiles for both radioligands, but there were some notable differences for some nicotinic agonists (Patel *et al.*, 1996). As with our binding studies using [^{125}I]-ET-1 and [^{125}I]-BQ3020, the reasons for the differences in the number of binding sites using [^{3}H]-nicotine and [^{3}H]-epibatidine is not known.

In conclusion, the binding of [^{125}I]-ET-1 and [^{125}I]-BQ3020 to rat cerebellar homogenates resembles the binding to ET_{B1} receptors. However, the lower affinity of selective ET_B receptor ligands, such as IRL1620, [Ala^{1,3,11,15}]ET-1 and BQ-788 and the higher affinity of ET-3 as compared to ET-1 may suggest that the binding of both radioligands is to the putative ET_C receptor.

References

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BAX, W.A. & SAXENA, P.R. (1994). The current endothelin receptor classification: time for reconsideration? *Trends Pharmacol. Sci.*, **15**, 379–386.

BOUSSO-MITTLER, D., GALRON, R. & SOKOLOVSKY, M. (1991). Endothelin/sarafotoxin receptor heterogeneity: evidence for different glycosylation in receptors from different tissues. *Biochem. Biophys. Res. Commun.*, **178**, 921–926.

BREU, V., LOFFLER, B-M. & CLOZEL, M. (1993). In vitro characterization of Ro 46-2005, a novel synthetic non-peptide endothelin antagonist of ET_A and ET_B receptors. *FEBS Lett.*, **334**, 210–214.

BUCHAN, K.W., ALLDUS, C., CHRISTODOULOU, C., CLARK, K.L., DYKES, C.W., SUMMER, M.J., WALLACE, D.M., WHITE, D.G. & WATTS, I.S. (1994). Characterization of three non-peptide endothelin receptor ligands using human cloned ET_A and ET_B receptors. *Br. J. Pharmacol.*, **112**, 1251–1257.

CLOZEL, M., BREU, V., BURRI, K., CASSAL, J-M., FISCLI, W., GREY, G.A., HIRTH, G., LOFFLER, B-M., MULLER, M., NEIDHART, W. & RAMUZ, H. (1993). Pathological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature*, **365**, 759–761.

CLOZEL, M., BREU, V., GREY, G.A., KALINA, B., LOFFLER, B-M., BURRI, K., CASSAL, J-M., HIRTH, G., MULLER, M., NEIDHART, W. & RAMUZ, H. (1994). Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J. Pharmacol. Exp. Ther.*, **270**, 228–235.

DOUGLAS, S.A., EDWARDS, R.M., ELLIOTT, J.D. & OHLSTEIN, E.H. (1995). *In vivo* pharmacological characterization of the non-peptide endothelin receptor antagonist SB 209670. *Br. J. Pharmacol.*, **114**, 405–413.

EMORI, T., HIRATA, Y. & MARUMO, F. (1990). Specific receptors for endothelin-3 in cultured bovine endothelin cells and its mechanism of action. *FEBS Lett.*, **263**, 261–264.

GULATI, A. & REBELLO, S. (1992). Characteristics of endothelin receptors in the central nervous system of spontaneously hypertensive rats. *Neuropharmacology*, **31**, 243–250.

HAGIWARA, H., NAGASAWA, T., YAMAMOTO, T., LODHI, K.M., ITO, T., TAEMURA, N. & HIROSE, S. (1993). Immunochemical characterization and localization of endothelin ET_B receptor. *Am. J. Physiol.*, **264**, R777–R783.

INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2863–2867.

ISHIKAWA, K., IHARA, M., NOGUCHI, K., MASE, T., MINO, N., SAEKI, T., FUKURODA, T., OZAKI, S., NAGASE, T., NISHIKIBE, M. & YANO, M. (1994). Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc. Natl Acad. Sci. U.S.A.*, **91**, 4892–4896.

JARVIS, M.F., ASSAL, A.A. & GESSNER, G. (1994). Pharmacological characterization of the rat cerebellar endothelin$_B$ (ET_B) receptor using the novel agonist radioligand [^{125}I]BQ3020. *Brain Res.*, **665**, 33–38.

KARNE, S., JAYAWICKREME, C.K. & LERNER, M.R. (1993). Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus Laevis* dermal melanophores. *J. Biol. Chem.*, **268,** 19126–19133.

LIN, H.Y., KAJI, E.H., WINKEL, G.K., IVES, H.E. & LODISH, H.F. (1991). Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 3185–3189.

MACCUMBER, M.W., ROSS, C.A. & SNYDER, S.H. (1990). Endothelin in brain: receptors, mitogenesis and biosynthesis in glial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2359–2363.

MASAKI, T., VANE, J.R. & VANHOUTE, P.M. (1994). International union of pharmacology nomenclature of endothelin receptors. *Pharmacol. Rev.*, **46,** 137–139.

NAMBI, P., PULLEN, M. & FEUERSTEIN, G. (1990). Identification of endothelin receptors in various regions of rat brain. (1990). *Neuropeptides*, **16,** 195–199.

NAMBI, P., PULLEN, M. & SPIELMAN, W. (1994). Species differences in the binding characteristics of [^{125}I]IRL-1620, a potent agonist specific for endothelin-B receptors. *J. Pharmacol. Exp. Ther.*, **268,** 202–207.

NANDASOMA, U. & DAVENPORT, A.P. (1994). Localisation of endothelin ET_A receptors in rat brain using [^{125}I]PD151242. *Br. J. Pharmacol.*, **113,** 156P.

PATEL, S., FREEDMAN, S.B. & PATEL, S. (1996). [^{3}H]epibatidine: a novel high affinity ligand at neuronal nicotinic acetylcholine receptors. *Br. J. Pharmacol.*, (in press).

SAEKI, T., IHARA, M., FUKURODA, T., YANAGIWA, M. & YANO, M. (1991). [Ala^{1,3,11,15}]Endothelin-1 analogs with ET_B agonistic activity. *Biochem. Biophys. Res. Commun.*, **179,** 286–292.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348,** 732–735.

SCHVARTZ, I., ITTOOP, O. & HAZUM, E. (1991). Direct evidence for multiple endothelin receptors. *Biochemistry*, **30,** 5325–5327.

SUPPATTAPONE, S., SIMPSON, A.W.M. & ASHLEY, C.C. (1989). Free calcium rise and mitogenesis in glial cells caused by endothelin. *Biochem. Biophys. Res. Commun.*, **3,** 1115–1122.

TANAKA, T., TSUKUDA, E., NOZAWA, M., OHNO, T., KASE, H., YAMADA, K. & MATSUDA, Y. (1994). RES-701-1, a novel, potent, endothelin type B receptor selective antagonist of microbial origin. *Mol. Pharmacol.*, **45,** 724–730.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993a). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and release of EDRF. *Br. J. Pharmacol.*, **110,** 777–782.

WARNER, T.D., ALLCOCK, G.H., MICKLEY, E.J., CORDER, R. & VANE, J.R. (1993b). Comparative studies with the endothelin receptor antagonists BQ-123 and PD142893 indicate at least three endothelin receptors. *J. Cardiovasc. Pharmacol.*, **22,** suppl. 8, S117–S120.

WIDDOWSON, P.S., SIMPSON, M.G., WYATT, I. & LOCK, E.A. (1995). [^{125}I]endothelin binding in rat cerebellum is increased following L-2-chloropropionic acid-induced granule cell necrosis. *Peptides*, **16,** 897–902.

WILLIAMS, D.L., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESCHMIDT, B.V. (1991). Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.*, **175,** 556–561.

YAMAMOTO, T., SHIMOYAMA, N., ASANO, H. & MIZUGUCHI, T. (1994). Analysis of the role of endothelin-A and endothelin-B receptors on nociceptive information transmission in the spinal cord with FR139317, an endothelin-A receptor antagonist and sarafotoxin S6c, an endothelin-B receptor agonist. *J. Pharmacol. Exp. Ther.*, **27,** 156–163.

YANAGISAWA, M., INOUE, A., ISHIKAWA, T., KASUYA, Y., KIMURA, S., KUMAGAYE, S-I., NAKAJIMA, K., WATANABE, T.X., SAKIBARA, S., GOTO, K. & MASAKI, T. (1988). Primary structure, synthesis and biological activity of rat endothelin, an endothelin-derived vasoconstrictor peptide. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 6964–6967.

YANAGISAWA, M. & MASAKI, T. (1989). Molecular biology and biochemistry of the endothelins. *Trends Pharmacol. Sci.*, **10,** 374–378.

YU, J.C.M., PICKARD, J.D. & DAVENPORT, A.P. (1995). Characterisation of endothelin receptors in cultured human cerebral vascular smooth muscle cells. *Br. J. Pharmacol.*, **114,** 195P.

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