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Rat striatum contains pure population of ET_B receptors

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Abstract

In most organs of the body, endothelin acts on endothelin ET_A and ET_B receptors that co-exist (albeit often on different cell types). Although virtually pure endothelin ET_A receptors have been identified in some tissues (e.g., lung), no essentially pure endothelin ET_B receptor tissue has been reported to date. [^{125}I]Endothelin-1 bound to striatal membrane preparations with a K_d of 19.4 ± 0.2 pM and B_{max} of 496 ± 8 fmol/mg protein. Endothelin-1 displaced [^{125}I]endothelin-1 receptor binding with an IC_{50} of 23 pM. The endothelin ET_B-selective antagonist BQ788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-leucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) and agonist sarafotoxin 6C displaced [^{125}I]endothelin-1 monophasically with IC_{50} values of 25 nM and 110 pM, respectively, whereas that of the endothelin ET_A-selective antagonist BQ123 (cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)) was 24 μM, values agreeing with cloned human endothelin ET_B but not ET_A receptors. Receptor autoradiography confirmed that rat striatum (but not white matter) contains essentially exclusively endothelin ET_B receptors.

Keywords: Endothelin; Endothelin receptor; BQ123; BQ788; Autoradiography

1. Introduction

Since its discovery in 1988 (Yanagisawa et al., 1988), the endothelin peptide family has been investigated extensively. The 21 amino acid peptide with two disulfide bonds is the most potent and long-lasting (Rubanyi and Polokoff, 1994) vasoconstrictor known to date. Three isoforms, namely endothelin-1, endothelin-2, and endothelin-3 have been discovered (Inoue et al., 1995). Two receptor subtypes, endothelin ET_A and ET_B receptors have been identified and cloned (Arai et al., 1990; Ogawa et al., 1991; Rubanyi and Polokoff, 1994), stably expressed, and pharmacologically characterized as members of the G protein hepta-helical superfamily, with 63% amino acid identity between the two subtypes (Sakurai et al., 1992).

In the central nervous system, endothelin has not been as extensively and vigorously studied as in other systems such as the cardiovascular, renal, and gastrointestinal systems. Previous receptor autoradiography binding studies of the rat brain revealed highly concentrated specific [^{125}I]endothelin-1 binding sites in the choroid plexus, subfornical organ, lacunosum molecular layer of the hippocampus and granular layer of the cerebellum (Niwa et al., 1991; Jones et al., 1989; Koseki et al., 1989). However, the endothelin receptor subtypes in these tissues have not been elucidated. With the advent of selective and potent antagonists (Warner, 1994), the endothelin receptors in the brain can be characterized specifically.

Evidence suggests clinically significant roles for endothelins in the brain. For instance, a number of studies indicate endothelin levels increase dramatically days after various types of acute brain injury. Six days after subarachnoid hemorrhage endothelin-1-like immunoreactivity has increased close to a factor of ten in cerebrospinal fluid from patients with subarachnoid hemorrhage, correlating identically in time with the onset of vasospasm (Suzuki et al., 1990).

The present study utilizes the well-characterized compounds BQ123, an endothelin ET_A receptor antagonist, and BQ788, an endothelin ET_B receptor antagonist, in in vitro receptor binding and autoradiography to characterize fully the specific distribution of the known endothelin receptors. Studies were performed in the striatum, which is a neurochemically well-characterized region that is relatively ho-

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mogeneous and of known neuronal cell types (Kotter, 1994; Gerfen, 1993).

2. Materials and methods

2.1. Preparation of striatal membranes

Adult male Wistar rats ([CRrl:(Wwo)BR]; 250–400 g; Charles River, Wilmington, MA, USA) were killed by decapitation while under halothane anesthesia. The striatum was dissected immediately, frozen on dry ice, and stored at −70°C. The rat striatal tissue, pooled from 5 rats, was homogenized in 50 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), pH 7.0, at a ratio of 1 g tissue/5 ml buffer. The tissue debris was removed by brief centrifugation, and the supernatant was centrifuged again at 100 000 × g for 1 h. The resulting pellet was resuspended in 50 mM TES, pH 7.0, at a protein concentration of 15 mg/ml and stored in aliquots at −80°C.

2.2. Production of recombinant receptors

Chinese hamster ovary cells (CHO-K1, ATCC CCL 61) were transfected with pME eukaryotic expression vector containing cDNAs encoding for human endothelin ET_A or endothelin ET_B receptors (Sakamoto et al., 1993). These CHO-ET_A and CHO-ET_B cells were cultured in D-MEM/F12 medium (GibcoBRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 1 × antibiotic-antimycotic (GibcoBRL, Grand Island, NY), and 0.3 mg/ml geneticin. Cells were harvested by scraping, pelleted by centrifugation, and homogenized in a buffer containing 1 mM EDTA and 10 mM Tris, pH 8.0. The cell debris was removed by brief centrifugation, and the supernatant was centrifuged again at 50 000 × g for 10 min. The resulting pellet was resuspended in Hanks' balanced salt solution at a protein concentration of 0.2 mg/ml and stored in aliquots at −80°C.

2.3. Ligand binding assays

Competition binding was carried out by incubating 0.2–1.0 μg of membrane protein from CHO-ET_A or CHO-ET_B cells or 5 μg of membrane protein from rat striatum with 12 000 cpm [^{125}I]endothelin-1 (specific activity 2200 Ci/mmol; New England Nuclear, Boston, MA, USA) in the presence of varying concentrations of the competing ligand for 2 h at 37°C in a buffer containing 0.2 mg/ml bovine serum albumin, 0.002% Triton X-100, 0.02% NaN_3, and 50 mM Tris, pH 7.0. The reaction was terminated by filtration through a cell harvester (Brandel, Gaithersburg, MD, USA) using GF/C filters. The radioactivity on the filters was counted in a gamma counter. Duplicate samples were carried out in each experiment, and the full dose-response curve for each competing ligand was repeated 2–4 times. The ALLFIT program was used to fit data to a one-site model for the determination of IC_{50} values.

The saturation binding of [^{125}I]endothelin-1 to the striatal membrane preparations was carried out in a manner similar to the competition binding described above except that various concentrations of the radioligand were used. In general, a concentration range of the radioligand from 1 pM to 1 nM was used, incubated with 20 μg of protein for 1 h at 37°C. The bound and free ligands were separated by centrifugation. The K_d and B_{max} values were calculated according to a published method (Scatchard, 1949).

2.4. In vitro receptor autoradiography of brain

Frozen whole brains from adult male Wistar rats ($n = 5$) were sectioned (20 μm thickness) at the striatal level on a cryostat (Microm, Germany), thaw-mounted onto gelatin-covered slides, and stored overnight at −70°C. Each brain section was incubated for 2 h at room temperature with 30–40 pM [^{125}I]endothelin-1. The incubation buffer was identical to that used above. After incubation, the sections were washed 3 times in water, kept for 2 h at room temperature in the incubation buffer, dried overnight at room temperature under vacuum, and exposed to X-OMAT AR films (Eastman Kodak Co., Rochester, NY, USA) for 48 h. Specific and non-specific binding sites for [^{125}I]endothelin-1 and the effects of different selective endothelin receptor ligands on [^{125}I]endothelin-1 binding were compared. Autoradiographs were scanned by CCD camera under identical darkness and contrast settings, and printed on a dye sublimation printer. Frozen human brain cortical tissue was obtained and sectioned (20 μm thickness) on a cryostat (Microm, Germany), thaw-mounted onto gelatin-covered slides, and stored overnight at −70°C. The sections were incubated with the same radioactive ligands under the same conditions as above.

2.5. Compounds

Endothelin-1, BQ123 (cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)), BQ 788 (*N*-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-leucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine), and sarafotoxin 6C (Cys-Thr-Cys-Asp-Met-Thr-Asp-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp) were obtained from American Peptide Company (Sunnyvale, CA, USA).

3. Results

Experiments with rat striatal tissue established that [^{125}I]endothelin-1 binding was linear with respect to protein and came to equilibrium within 1 h of incubation at 37°C (data not shown). The K_d and B_{max} values for [^{125}I]endothelin-1 binding were 19.4 ± 0.2 pM and 496 ± 8

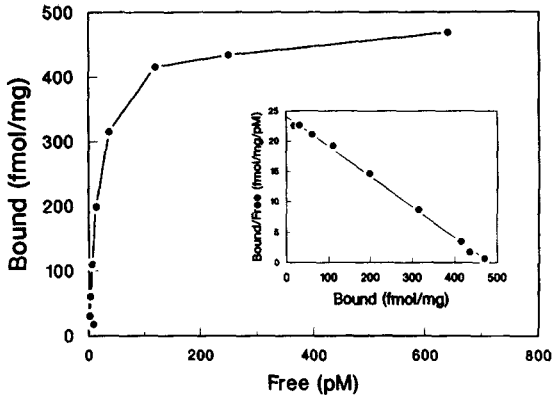

Fig. 1. Saturation binding of [^{125}I]endothelin-1 in rat striatal membranes. Concentration of free [^{125}I]endothelin-1 is shown on the abscissa, and amount of specific binding to striatal membranes is shown on the ordinate. Inset shows the same data plotted by the method of Scatchard, which yielded values of $K_d = 19.4 \pm 0.2$ pM and $B_{max} = 496 \pm 8$ fmol/mg protein (means ± S.E.M. of three separate determinations). The non-specific binding (defined by 1 μM ET-1) represented less than 5% of total binding at the concentrations tested.

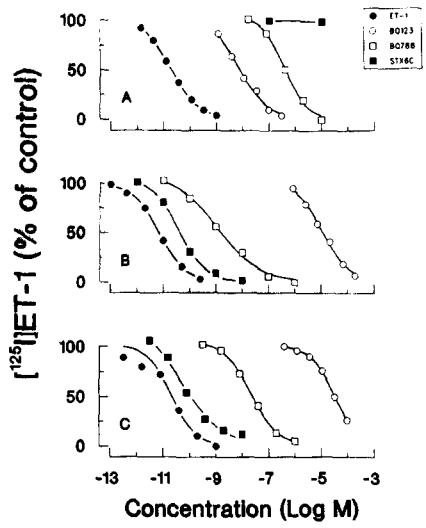

Fig. 2. Concentration-dependent displacement curves of subtype-specific ligands in [^{125}I]endothelin-1 binding. The four characteristic compounds were tested in: (A) recombinant human endothelin ET_A receptors expressed in CHO cells; (B) recombinant human endothelin ET_B receptors expressed in CHO cells; (C) rat brain striatal membranes, prepared by polytron homogenization.

fmol/mg protein (mean ± S.E.M., $n = 3$; Fig. 1). The Scatchard plot indicated there was only a single class of binding site.

CHO cells expressing human endothelin ET_A receptors (Fig. 2A) showed the classical profile of this receptor, i.e., endothelin-1 ≫ BQ123 ≫ BQ788, with sarafotoxin 6C inactive. CHO cells expressing human endothelin ET_B receptors (Fig. 2B) showed this receptor's characteristic profile, endothelin-1 > sarafotoxin 6C ≫ BQ788 ≫ BQ123. The profile of the rat striatum (Fig. 2C) matched identically that of human endothelin ET_B receptors, i.e., endothelin-1 > sarafotoxin 6C ≫ BQ788 ≫ BQ123. None of the curves for these inhibitors in the striatum suggested biphasic nature or had Hill coefficients substantially different from unity. Table 1 shows the IC_{50} values determined for these compounds.

Studies were performed using receptor autoradiography in the region of the striatum. [^{125}I]Endothelin-1-labeled sections showed intense labeling in the brain (Fig. 3A), all of which was displaced by 1 μM endothelin-1 (Fig. 3B). At this coronal level, very little inhibition by BQ123 (1 μM) of [^{125}I]endothelin-1 binding throughout the brain

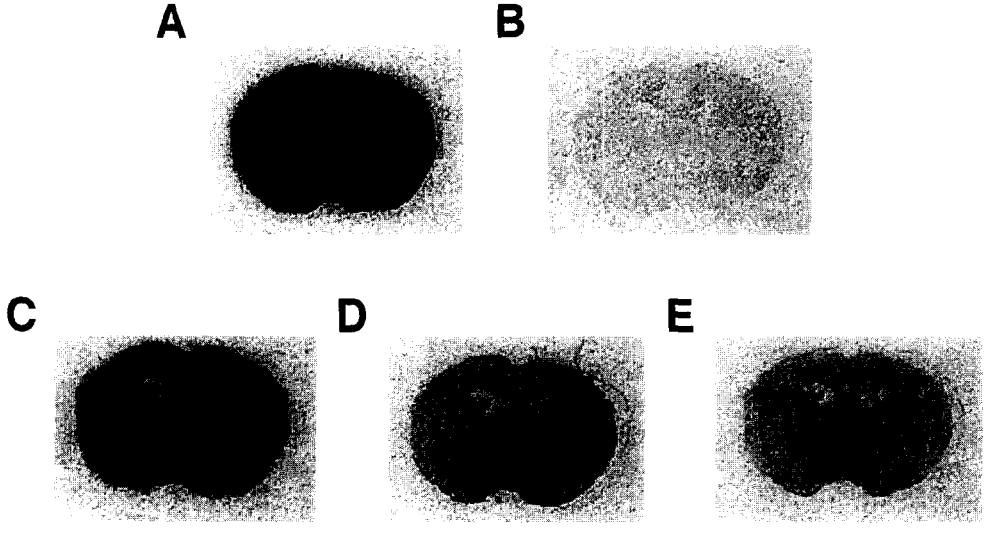

Fig. 3. In vitro receptor autoradiographic localization of [^{125}I]endothelin-1 binding sites in the rat striatum. Consecutive 20 μm thick tissue sections at the level of the striatum were labeled with 35 pM [^{125}I]endothelin-1. (A) Total binding, obtained in the absence of any other ligands. (B) Non-specific binding, i.e., background, non-receptor bound radioactivity (determined by adding unlabeled 1 μM endothelin-1 to the medium during the incubation). (C) Endothelin ET_B receptors revealed by the presence of the selective endothelin ET_A antagonist BQ123 (1 μM). (D) Endothelin ET_A receptors revealed by the presence of the selective endothelin ET_B antagonist BQ788 (1 μM). (E) The possible existence of endothelin receptors different from the characterized ET_A and ET_B receptor subtypes was tested by adding both BQ123 (1 μM) and BQ788 (1 μM).

Table 1
Relative potencies of compounds examined in rat striatum and recombinant endothelin receptors

Ligand	IC_{50}, nM		
	CHO-ET_A	CHO-ET_B	Striatum
ET-1	0.014 ± 0.002 [a] (4)	0.0064 ± 0.0004 [a] (4)	0.023 ± 0.003 (3)
BQ123	6.7 ± 2.2 [a] (3)	16000 ± 4400 [a] (3)	24000 ± 7300 (3)
BQ788	420 ± 24 (4)	1.3 ± 0.2 (4)	25 ± 1 (3)
STX6C	ND (4)	0.025 ± 0.006 (4)	0.11 ± 0.02 (3)

Each IC_{50} value represents the mean ± S.E.M. of three to four independent determinations carried out in duplicate. ND: No displacement at 10 μM. [a] From (Balwierczak et al., 1995). Abbreviations: ET-1, endothelin-1; STX6C, sarafotoxin 6C.

was observed (Fig. 3C). In contrast, BQ788 (1 μM) effectively inhibited [^{125}I]endothelin-1 binding to the majority of brain regions at this coronal level, particularly in the striatum (Fig. 3D). Noteworthy exceptions are the ependymal cell layer on the dorsomedial ventricular surface of the striatum, the choroid plexus, and pial-meningial surfaces in general. Regionally within the striatum, the myelinated corticofugal fiber bundles and the anterior commissure showed a lack of endothelin ET_B receptors, but the latter could be identified as having endothelin ET_A receptors. Simultaneous incubation in the presence of both BQ123 (1 μM) and BQ788 (1 μM) almost entirely abolished specific binding of [^{125}I]endothelin-1 (Fig. 3E). At this level, neocortex was also predominantly of endothelin ET_B receptor subtype as well (Fig. 3). Preliminary studies on human cortex identified that at least some cortical regions also consist of exclusively endothelin ET_B receptors (data not shown).

4. Discussion

Previously endothelin receptors were localized autoradiographically in the brain using [^{125}I]endothelin-1 as ligand (Koseki et al., 1989), but their localization using the highly selective endothelin receptor antagonists BQ123 and BQ788 has not been previously reported to our knowledge. The highest densities of binding sites included cerebellum, choroid plexus, and median eminence. Only low binding was reported for the striatum, although curiously, this tissue seems to contain among the highest levels in the brain of mRNA encoding endothelin-1 (Lee et al., 1990).

Based upon the use of the most characteristic ligands for differentiating endothelin ET_A and ET_B receptors, the subtype of endothelin receptor predominating in the striatum is identified as the ET_B subtype. Detailed mapping of the rest of the rat brain suggests that, although the striatum is not the only region which is enriched in endothelin ET_B receptors, other parts of the brain contain a more complex population of endothelin receptors (manuscript in preparation). Tissues such as lung containing virtually pure populations of endothelin ET_A receptors have been characterized, but until now no tissue has been identified that contains virtually only endothelin ET_B receptors (for recent reviews, see Rubanyi and Polokoff, 1994; Battistini and Botting, 1995).

Since reactive astrocytes and some neurons synthesize endothelins (MacCumber et al., 1990), it is important to elucidate the localization, role, and pharmacological properties of endothelin receptors in the brain. It has been shown that endothelin-1 causes lesions in the brain (Fuxe et al., 1989), and that endothelin-1-like immunoreactivity increases during focal cerebral ischemia (Rubanyi and Polokoff, 1994). Notably, exogenous endothelin-1 also causes breakdown in the blood-brain barrier (Miller et al., 1996), suggesting that under pathophysiological conditions such as stroke and traumatic brain injury, endogenous endothelin may cause similar breakdown in the blood-brain barrier. However, the respective roles of endothelin ET_A and ET_B receptors in mediating this neurodegeneration is not yet established. The identification of the striatum as an essentially pure source of endothelin ET_B receptors is useful information in understanding the involvement of this subtype of endothelin receptor in mediating the physiology and pathophysiology of endothelin in the brain. Notably, natural transduction mechanisms for endothelin ET_B receptors may be conveniently studied in vitro and in vivo in the striatum, due to its high abundance of endothelin ET_B receptors compared to other tissues. Previously, interaction with endothelin receptors in fibroblasts has been shown to increase intracellular Ca^{2+} levels (probably via L-type channels), and by a Ca^{2+}-independent mechanism, increased diacylglycerol and inositol trisphosphate (Rodland et al., 1991), presumably via activation of phospholipase C.

The function of endothelin ET_B receptors in the brain has not been studied extensively. However, recently it was shown that ET_B receptors apparently mediate the effects of endothelins on release of dopamine (Kataoka et al., 1995). For instance, the 20-min exposure of hypoglycemic/hypoxic striatal slices to ET-3 (4 μM) causes a 50% reduction in subsequent KCl-evoked dopamine release, an effect that is reduced by co-administration of the NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (1 μM), or removal of Ca^{2+}, during the hypoglycemic/hypoxic period. Although endothelin-3 presumably stimulates release of glutamate, the second messengers underlying this effect remain to be identified. A number of other studies have also noted the enrichment of primarily endothelin ET_B receptors in some brain tissues, based upon mRNA localization (Hori et al., 1992). Other studies have suggested the primary role of endothelin ET_B receptors in hypothalamic responses (Yamamoto et al., 1992) and astrocytes in culture (Hama et al., 1992). Because of the high purity of endothelin ET_B receptors in the striatum, this is an ideal tissue for studying its associated transduction mechanisms and second messengers.

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