ELSEVIER

Biochemical Pharmacology 62 (2001) 537–545

Biochemical Pharmacology

Regulation of agonist binding to rat ET_B receptors by cations and GTPγS

Francesca Ceccarelli, Laura Giusti, Gianni Bigini, Barbara Costa, Dante Grillotti, Erika Fiumalbi, Antonio Lucacchini, Maria Rosa Mazzoni*

Department of Psychiatry, Neurobiology, Pharmacology and Biotechnology, Laboratory of Neurobiology and Pharmacology, University of Pisa, Via Bonanno 6, I-56126 Pisa, Italy

Received 24 November 2000; accepted 26 April 2001

Abstract

Endothelins exert their physiological effects through interaction with cell surface receptors that are members of the G-protein-coupled receptor family. The endothelin receptor subtype B (ET_B receptor) is abundantly expressed in rat cerebellum. Since agonist binding to G-protein-coupled receptors may be modulated by cations and guanine nucleotides, we investigated the effects of cations and guanosine 5′-*O*-(2-thiotriphosphate) (GTPγS) on ^{125}I-endothelin-1 (^{125}I-ET-1) binding to rat cerebellar membranes. Both Na^+ and Mg^{2+}-stimulated ^{125}I-ET-1 binding causing an increase in receptor affinity for the agonist. While the effect of the divalent cation was evident at relatively low concentrations (5–10 mM), the stimulatory activity of the monovalent cation appeared at relatively high concentrations (50 mM). Additive activities of 25–50 mM NaCl and 1 mM $MgCl_2$ suggested that monovalent and divalent cations increased receptor affinity for ET-1 by different mechanisms. In the presence of 5 mM $MgCl_2$, 50 mM NaCl caused an additional modest reduction of the K_d value. Whereas 5 mM $MgCl_2$ affected the displacement curves of both ET-3 and suc-[Glu^9, Ala^{11,15}]-endothelin-1 (8–21) (IRL 1620), the influence of 50 mM NaCl on these curves was less substantial. All together, these results suggest that modulation of receptor affinity by NaCl depends on the nature of the displacing agonist. In the presence of 5 mM $MgCl_2$ or 50 mM NaCl, a partial regulation of ^{125}I-ET-1 binding by GTPγS was detectable, while in the absence of cations no GTPγS-dependent inhibition was evident.

Keywords: Endothelins; Endothelin receptors; ET_B receptor; G-protein-coupled receptors; Heterotrimeric G-proteins; Cations

1. Introduction

Endothelins (ETs), a family of three closely related peptide hormones (ET-1, -2, -3), are potent modulators of vascular functions. They all consist of 21 amino acid residues with two intramolecular disulfide bonds. Endothelins are produced in a wide variety of tissues where they exert diverse physiological effects on cellular development, differentiation, vasoconstriction, and mitogenesis [for reviews see: 1,2]. Their actions are mediated by specific cell surface receptors that belong to the superfamily of heptahelical G-protein-coupled receptors. Two ET receptor (ETR) subtypes, ET_A and ET_B, have been cloned, sequenced, and expressed [3–7]. They can be pharmacologically distinguished by different rank orders of affinity toward ET isopeptides. The ET_A receptor is ET-1 selective, whereas the ET_B receptor exhibits similar affinities for all three isopeptides [1,2].

Binding of ligands to a number of G-protein-coupled receptors and subsequent regulation of cell function is influenced by cations such as Mg^{2+}, Ca^{2+}, and Na^+ [8–12]. The ionic requirement of certain receptors allows to differentiate between distinct subpopulations [13,14]. This occurs for somatostatin receptors, which consist of a Mg^{2+}-dependent as well as a Mg^{2+}-independent receptor population in rat brain [15]. Two distinct pharmacological populations of somatostatin receptors characterized in several tissues correspond to two receptor subtypes which have been cloned [16]. In some instances, such as the glucagon receptor [17],

* Corresponding author. Tel.: +39-050-240-92; fax: +39-050-503-534.
E-mail address: mariarm@farm.unipi.it (M.R. Mazzoni).

Abbreviations: ETs, endothelins; ETR, endothelin receptors; GTPγS, guanosine 5′-*O*-(2-thiotriphosphate); BQ 788, *N*-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa; IRL 1620, suc-[Glu^9, Ala^{11,15}]-endothelin-1 (8-21); and PMSF, phenylmethanesulphonyl fluoride.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00706-7

different patterns of Mg^{2+} regulation of ligand–receptor interaction have been observed depending on the animal species. In most systems, divalent cations enhance agonist binding to receptors such as A_1 [18] and A_{2a} adenosine [11,19], α_2- [20,21] and β-adrenergic [22], opiate [23], and H_1 histamine [24] receptors. The important role of Mg^{2+} in activation of G-proteins and formation of the high-affinity receptor–G-protein complex is well documented [8].

For a number of G-protein-coupled receptors, including the α_2- [25,26] and β-adrenergic [27], D_2 dopamine [28], μ- and δ-opiate [29,30], and muscarinic [31] receptors, Na^+ has been reported to reduce the affinity for agonists. Several of these receptors contain a conserved Asp residue in their second transmembrane-spanning region [26,32]. Site-directed mutagenesis of the Asp to an Asn results in mutant receptors that are insensitive to the effects of Na^+ and to modulation of agonist binding by GTP analogs [33,34]. Thus, this conserved Asp site is essential for Na^+ regulation of agonist binding and its mutation may perturb receptor–G-protein coupling [26,33,34]. Similar mutagenesis studies of the somatostatin receptor subtype 2 have shown that this same residue participates in the allosteric regulation of agonist binding by the monovalent cation but that its mutation does not affect receptor interaction with G-protein [35]. In addition, agonist binding to some types of G-protein-coupled receptors is either unmodified (somatostatin receptor subtype 1) [35] or increased (angiotensin II and α_1-adrenergic receptors) [36,37] by Na^+ ions.

So far, cation modulation of agonist binding to ETRs has not been extensively investigated. Agonist binding to ET_B receptors has been shown to be positively regulated by Mn^{2+} [38]. However, ET-1 binds with high affinity to ETRs even in the absence of divalent cations, such as Mg^{2+} [38,39]. This lack of Mg^{2+} requirement for agonist binding seems to be associated with the absence of modulation by GTP analogs [39].

In order to elucidate whether cations and GTP analogs modulate agonist binding to ET_B receptors in rat cerebellar membranes, we analyzed ^{125}I-ET-1 binding in the presence and absence of monovalent (Na^+, K^+, or Li^+), divalent (Mg^{2+}, Ca^{2+}, or Mn^{2+}) cations and/or guanosine 5′-*O*-(2-thiotriphosphate) (GTPγS). Our investigation was particularly focused to understand the role of Na^+ and Mg^{2+} on agonist binding regulation. Both Na^+ and Mg^{2+} increased receptor affinity for ET-1. Their effects were additive but a Na^+-modulation was less evident in competition experiments with either ET-3 or IRL 1620. In addition, both cations influenced the sensitivity of ^{125}I-ET-1 binding to GTPγS.

2. Materials and methods

2.1. Materials

^{125}I-ET-1 (2000 Ci/mmol) was purchased from Amersham Corp. ET-1, ET-3, *N*-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa (BQ 788) and suc-[Glu^9, Ala^{11,15}]-endothelin-1 (8–21) (IRL 1620) were obtained from Alexis. GTPγS and benzamidine were purchased from Sigma Chemical Co. Bacitracin and phenylmethanesulphonyl fluoride (PMSF) were products of Fluka Chemie AG. Aprotinin and leupeptin were purchased from Boehringer-Mannheim (Roche Diagnostic, F. Hoffmann-La Roche Ltd.). All other agents and reagents were from standard commercial sources.

2.2. Membrane preparation

Male Sprague–Dawley rats (150 g) were killed by cervical dislocation, rapidly bled, and cerebellum was immediately removed. Fresh tissue was homogenised using a Polytron homogenizer in 10 vols. of ice-cold 20 mM HEPES–Tris pH 7.4 (buffer A) containing 0.32 M sucrose, 5 mM EDTA, 1 μg/mL of leupeptin, 0.1 mM bacitracin, 0.1 mM benzamidine, and 0.1 mM PMSF [40]. The homogenate was centrifuged at 1000 X *g* for 5 min at 4°. The supernatant was centrifuged at 48,000 X *g* for 15 min at 4°. The pellet was resuspended in 10 vols. of buffer A containing 5 mM EDTA and protease inhibitors (as above) and centrifuged at 48,000 X *g* for 15 min at 4°. The membrane pellet was resuspended in buffer A (0.1 g/mL), divided into Eppendorf tubes (1 mL), and centrifuged at 13,000 X *g* for 10 min at 4°. The resulting pellets were stored at −80° until the time of assay. Protein content was determined according to the method of Lowry *et al*. [41], using BSA as a standard.

2.3. Binding assay

^{125}I-ET-1 binding assays were performed as described by Cody *et al*. [42] with some modifications [40]. Briefly, cerebellar membranes (~3 μg of proteins) were incubated with ^{125}I-ET-1 (15 pM) in 0.25 mL of 20 mM Tris–HCl buffer, pH 7.4 at 37°, containing 2 mM EDTA, 0.1 mM bacitracin, 0.1 mM PMSF, 1 μg/mL of leupeptin, 5 μg/mL of aprotinin (buffer B), and 0.08 mg BSA for 2 hr at 37° [40]. After incubation, reactions were stopped with 3 mL of ice-cold 50 mM Tris–HCl, pH 7.3 at 4°, containing 0.1 mM bacitracin (buffer C). Membrane-bound radioactivity was separated from free ligand by filtration through Whatman GF/C filters that had been presoaked in buffer C containing 2 mg/mL of BSA. Filters were then washed three times with 3 mL of buffer C and then counted in a γ-counter. Nonspecific binding was defined in the presence of 100 nM ET-1. In order to study the effects of cations, binding assays were performed in the presence and absence of various concentrations of the following salts: $MgCl_2$ (0.01 to 50 mM), $MnCl_2$ (0.01 to 50 mM), $CaCl_2$ (0.01 to 50 mM), LiCl (0.1 to 200 mM), NaCl (0.1 to 200 mM), KCl (0.1 to 200 mM), Na_2SO_4 (1 to 200 mM), and $C_5H_{14}NOCl$ (choline) (0.01 to 250 mM). Dilution and competition binding experiments were performed incubating membranes with ^{125}I-ET-1 (15 pM) in the presence and absence of various con-

centrations of the following compounds: ET-1 (0.003 to 1 nM), ET-3 (0.001 to 1 nM), BQ 788 (0.250 to 1000 nM), and IRL 1620 (0.003 to 50 nM). Stock solutions of ET-1 and ET-3 were prepared in buffer B, while BQ 788 and IRL 1620 were dissolved in dimethyl sulfoxide and then diluted in buffer B to the proper concentration. In the assay, the final concentration of dimethyl sulfoxide never exceeded 0.2%. In order to study the nucleoside triphosphate effect, membranes were incubated with ^{125}I-ET-1 (as above) in the presence and absence of six different concentrations of GTPγS, ranging from 0.1 to 500 μM, with and without 5 mM $MgCl_2$ or 50 mM NaCl.

2.4. Analysis of data

A non-linear multipurpose curve-fitting computer program (EBDA/LIGAND, Elsevier-Biosoft) [43] was used to analyze and transform dilution experiments of ^{125}I-ET-1 with unlabelled ET-1. Displacement and dilution curves were analyzed and fitted using the GraphPad Prism (Version 3.0) computer program (GraphPad Software). Transformed saturation data were also fitted with the GraphPad Prism (Version 3.0) program. Single- and multiple-site models were statistically compared to determine the best fit, and differences between models were tested by comparing the residual variance using a partial F test and a significance level of $P < 0.05$. Derived IC_{50} values obtained from displacement and dilution curves were converted to K_i values by the Cheng and Prusoff equation [44]. Values represent the means ± SEM of at least three independent experiments except when otherwise stated. Statistical analysis was performed by means of unpaired Student's *t*-test and ANOVA using GraphPad Prism Version 3.0.

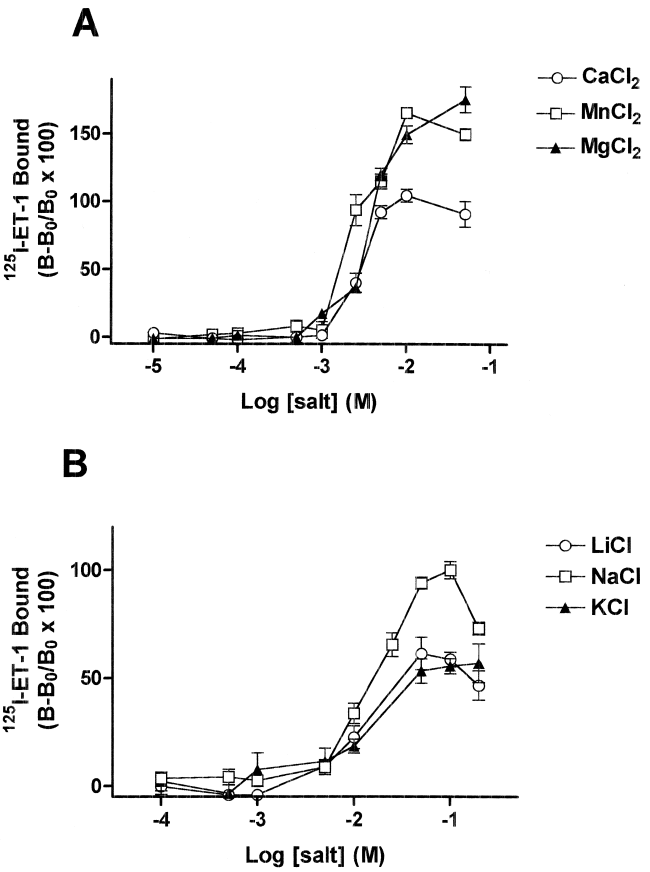

Fig. 1. Effects of cations on ^{125}I-ET-1 binding to rat cerebellar membranes. Membranes were incubated with 15 pM ^{125}I-ET-1 in the presence (Bound) and absence ($Bound_0$) of various concentrations of $CaCl_2$, $MnCl_2$, and $MgCl_2$ (0.01 to 50 mM) (*panel A*) or LiCl, NaCl, and KCl (0.1 to 200 mM) (*panel B*) for 2 hr at 37°, as described in "Methods." Data points represent means ± SEM of three or four independent experiments. B (Bound), specifically ^{125}I-ET-1 bound in the presence of the salt; B_0 ($Bound_0$), specifically ^{125}I-ET-1 bound in the absence of salts. $Bound_0$ (control) was 127 ± 7 fmol/mg protein (N = 12). Abscissa: total salt concentrations.

3. Results

Previous studies [40] have shown that under our standard binding conditions, specific ^{125}I-ET-1 binding to rat cerebellar membranes is linearly related to protein concentration up to 50 μg of proteins and reaches equilibrium by 2 hr at 37°. Saturability of specific binding has also been demonstrated [40]. Under these experimental conditions, both divalent and monovalent cations enhanced specific ^{125}I-ET-1 binding to rat cerebellar membranes (Fig. 1). Divalent cations were substantially more potent and effective than monovalent cations. Among divalent cations, Mg^{2+} and Mn^{2+} were more effective than Ca^{2+} at concentrations of their chloride salts ranging from 5 to 50 mM. Whereas Mg^{2+} stimulation of ^{125}I-ET-1 binding increased up to a $MgCl_2$ concentration of 50 mM, both Ca^{2+} and Mn^{2+} reached their maximal efficacy at salt concentrations of 10 mM (Fig. 1, *panel A*). The three monovalent cations examined showed similar potency. However, Na^+ was the most effective with a maximal efficacy at a NaCl concentration of 100 mM (Fig. 1, *panel B*). Both Li^+ and K^+ were less active stimulators of ^{125}I-ET-1 binding to ET_B receptors in rat cerebellar membranes. To ensure that the striking effect of the Na^+ salt was not due to Cl^- or changes in the ionic strength, we compared the activity of a range of NaCl and Na_2SO_4 concentrations. Both 100 mM NaCl and 50 mM Na_2SO_4 produced a similar stimulation of ^{125}I-ET-1 binding, independent from the ionic strength of the solutions (0.1 and 0.15 for NaCl and Na_2SO_4, respectively) (data not shown). In addition, the positive modulation of agonist binding was not produced by the Cl^- salt of a bulky monovalent cation such as choline. Within a concentration range of 0.01–250 mM, choline did not cause any increase in binding but only a modest inhibition at 250 mM (data not shown).

In order to investigate the general nature of cation effects, we studied Mg^{2+} and Na^+ activities in more detail. To test whether they acted at the same or different sites, we examined the effects of three NaCl concentrations (10, 25, and 50 mM) at three $MgCl_2$ concentrations (1, 5, and 10 mM) in the assays (Fig. 2). At a $MgCl_2$ concentration of 1

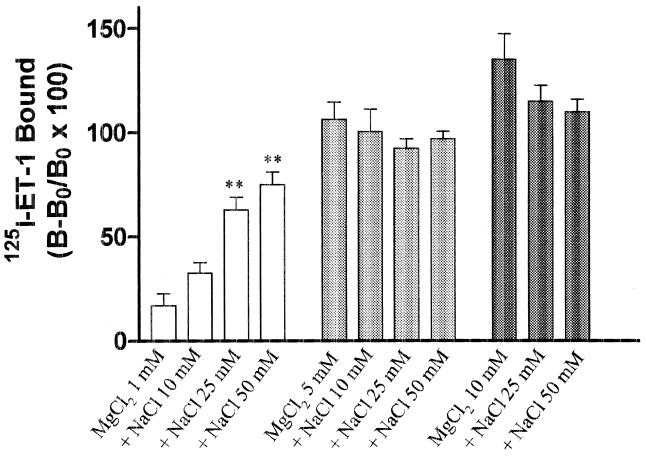

Fig. 2. Influence of NaCl on ^{125}I-ET-1 binding to rat cerebellar membranes in the presence of three concentrations of $MgCl_2$. Membranes were incubated with 15 pM ^{125}I-ET-1 in the presence (Bound) and absence ($Bound_0$) of three concentrations of $MgCl_2$ (1, 5, and 10 mM) plus or minus NaCl (10, 25, and 50 mM) for 2 hr at 37°, as described in "Methods." Data points represent means ± SEM of four independent experiments. B (Bound), specifically ^{125}I-ET-1 bound in the presence of $MgCl_2$ or $MgCl_2$ plus NaCl; B_0 ($Bound_0$), specifically ^{125}I-ET-1 bound in the absence of salts. $Bound_0$ (control) was 161 ± 44 fmol/mg protein (N = 4). **, $P < 0.01$.

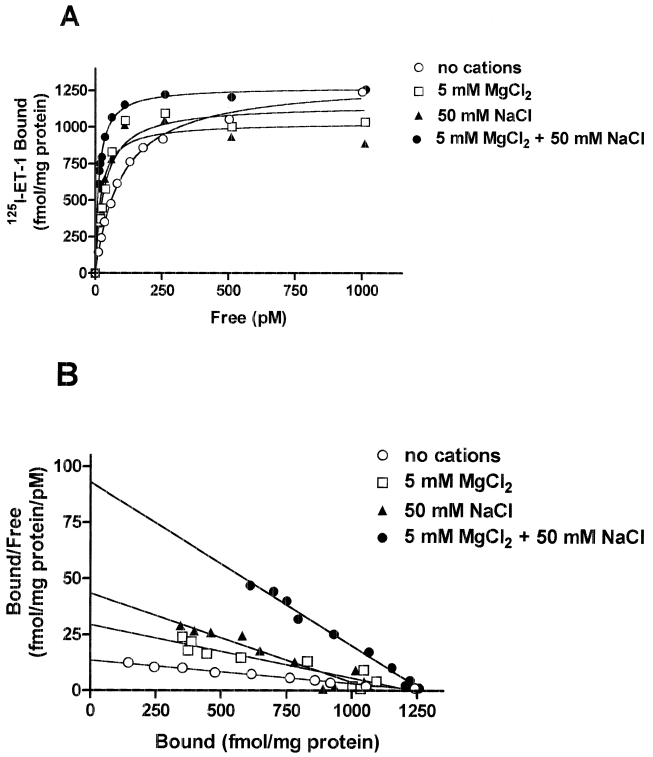

Fig. 3. Equilibrium binding of ^{125}I-ET-1 to rat cerebellar membranes in the presence and absence of 5 mM $MgCl_2$, 50 mM NaCl, or 5 mM $MgCl_2$ plus 50 mM NaCl. Saturation isotherms (*panel A*) were obtained by transformation of dilution binding data. Saturation data were further transformed using Scatchard's analysis (*panel B*). Membranes (3 μg of proteins) were incubated with ^{125}I-ET-1 (15 pM) in the presence and absence of increasing concentrations of unlabeled ET-1 (0.003 to 1 nM) with and without salts for 2 hr at 37°, as described in "Methods." Values shown are means from a representative experiment performed in duplicate and repeated two or three additional times with similar results. For these experiments, in the absence of salts the K_d and B_{max} values were 98.3 pM and 1315 fmol/mg proteins while in the presence of 5 mM $MgCl_2$, 50 mM NaCl or both salts, K_d and B_{max} values were 33.5 pM and 1152 fmol/mg protein, 20.0 pM and 1030 fmol/mg protein, and 13.4 pM and 1272 fmol/mg proteins, respectively. In *panel A*, curves are based on results of computer analysis with GraphPad Prism (Version 3.0) program.

mM that induced a modest increase in specific ^{125}I-ET-1 binding by itself (Fig. 1, *panel A*), all three concentrations of NaCl stimulated specific binding (Fig. 2). The induced increase was significant at 25 and 50 mM NaCl as compared to the control ($P < 0.01$). At 5 and 10 mM $MgCl_2$, 25 and 50 mM NaCl caused a modest decrease in specific binding (Fig. 2).

To determine whether these cations modified receptor affinity for ET-1 and/or the maximal number of binding sites (B_{max}), we performed dilution experiments of ^{125}I-ET-1 with unlabelled ET-1, in the presence and absence of 5 mM $MgCl_2$, 50 mM NaCl or both salts. Data of the dilution curves were transformed as saturation data using the EBDA software [43]. Under each condition, specific binding was saturable (Fig. 3, *panel A*). In the presence of Mg^{2+} or Na^+, saturation was reached at lower concentrations of the agonist ligand than in the absence of either cations. This effect was particularly conspicuous in the presence of both salts (Fig. 3, *panel A*). Analysis of saturation data using the non-linear curve fitting techniques of EBDA/LIGAND and GraphPad Prism computer programs revealed that the best fits observed were for one-site models. Transformation of data according to Scatchard resulted in linear plots (Fig. 3, *panel B*), confirming the existence of a single class of binding sites under all experimental conditions. The maximal number of binding sites was not influenced by either one of the cations or their combination. In the absence of cations, the derived B_{max} value was 1367 ± 45 fmol/mg protein (N = 3), while in the presence of 5 mM $MgCl_2$, 50 mM NaCl or both salts B_{max} values were 1124 ± 166 (N = 4), 1165 ± 95 (N = 3), and 1653 ± 269 (N = 3) fmol/mg protein, respectively. In contrast, receptor affinity for ET-1 significantly increased in the presence of salts. In the absence of cations, the derived K_d value was 88.9 ± 7.8 pM while in the presence of 5 mM $MgCl_2$, 50 mM NaCl or both salts K_d values were 30.2 ± 3.6 ($P < 0.001$), 22.5 ± 1.7 ($P < 0.01$), and 13.3 ± 0.1 ($P < 0.001$) pM, respectively. Thus, the K_d values decreased, respectively, 3-, 4-, and 7-fold in the presence of 5 mM $MgCl_2$, 50 mM NaCl, or both salts. Representation of ^{125}I-ET-1 dilution data as competitions showed monophasic curves which were fitted by one-site models (Fig. 4, *panel A*). The addition of cations caused a left-shift of the curves. In the presence of 5 mM $MgCl_2$ or 50 mM NaCl, a 4- and 3-fold decrease of K_i values was detectable (Table 1), while in the presence of both salts the K_i value was 19.4 ± 3.3 pM (N = 3).

The Mg^{2+}-induced increase in ET_B receptor affinity for agonist ligands was also supported by competition binding experiments with ET-3 (Fig. 4, *panel B*) and IRL 1620 (Fig.

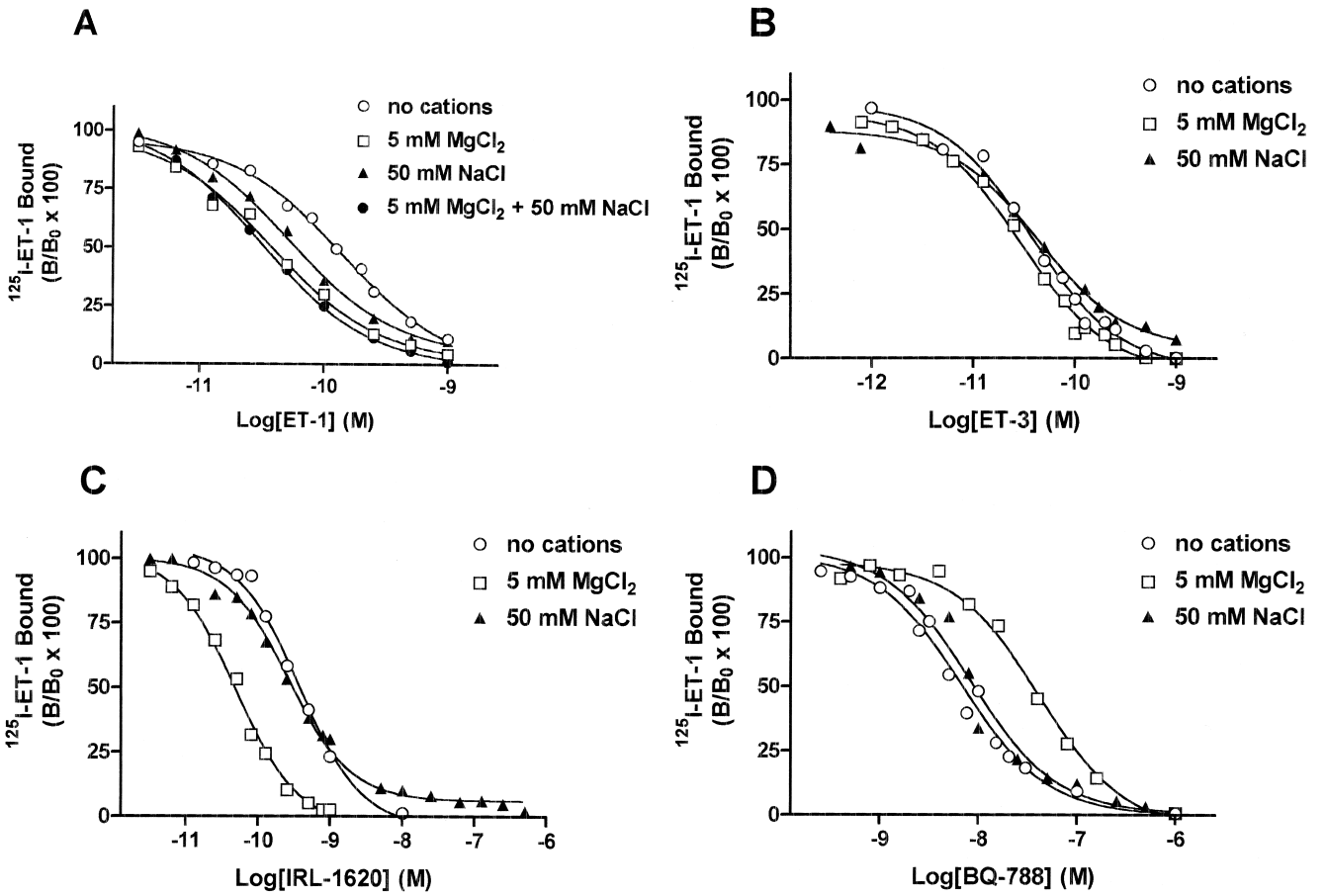

Fig. 4. Competition of ^{125}I-ET-1 binding to rat cerebellar membranes by agonist and antagonist ligands in the presence and absence of 5 mM $MgCl_2$, 50 mM NaCl, or both salts. Membranes (3 μg of proteins) were incubated with ^{125}I-ET-1 (15 pM) in the presence and absence of increasing concentrations of ET-1 (0.003 to 1 nM) (*panel A*), ET-3 (0.001 to 1 nM) (*panel B*), IRL 1620 (0.003 to 50 nM) (*panel C*), or BQ 788 (0.25 to 1000 nM) (*panel D*) with and without salts for 2 hr at 37°, as described in "Methods." A non-linear regression analysis of the GraphPad Prism (Version 3.0) computer program was used to fit the dose–response curves and derive IC_{50} values. Values shown are means from a representative experiment performed in duplicate and repeated two or three additional times with similar results. B (Bound), specifically ^{125}I-ET-1 bound at various concentrations of the displacement ligand; B_0 ($Bound_0$), specifically ^{125}I-ET-1 bound in the absence of the displacement ligand.

4, *panel C*) performed in the presence and absence of 5 mM $MgCl_2$. Under both conditions, ET-3 and IRL 1620 displacement curves were monophasic and represented by one-site models. However, they were left-shifted in the presence of the divalent cation (Fig. 4, *panel B* and *C*). This shift was particularly evident for the IRL 1620 competition curve. Whereas in the presence of 5 mM $MgCl_2$, the K_i value for IRL 1620 decreased 7-fold, that for ET-3 was reduced 2-fold (Table 1). We also investigated the Mg^{2+} effect on displacement of ^{125}I-ET-1 binding by the ET_B receptor antagonist, BQ 788. Both in the presence and absence of the divalent cation the curves were monophasic and fitted by one-site models (Fig. 4, *panel D*). However, in the presence of 5 mM $MgCl_2$, the BQ 788 competition curve was right-

Table 1
Influence of cations on inhibition of ^{125}I-ET-1 binding to rat cerebellar membranes by agonist and antagonist ligands

K_i (pM)			
Ligand	No cation (control)	5 mM $MgCl_2$	50 mM NaCl
ET-1	118.0 ± 8.6 (3)	26.7 ± 4.8 (4)***	33.3 ± 0.4 (3)***
ET-3	36.8 ± 2.6 (3)	17.4 ± 1.3 (3)**	30.7 ± 2.7 (3)
IRL1620	262.3 ± 40.0 (3)	40.2 ± 2.6 (3)**	107.3 ± 31.1 (3)*
BQ788	5,800.0 ± 20.0 (3)	27,300.0 ± 140.0 (3)***	5,740.0 ± 120.0 (3)

Values are means ± SEM; the number of experiments is reported in parentheses.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (as compared to corresponding controls).

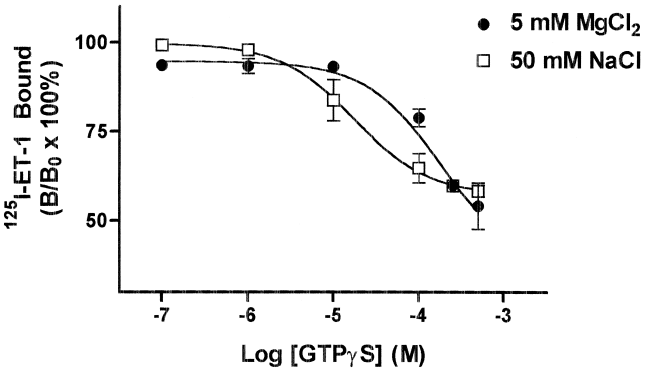

Fig. 5. Effect of various concentrations of GTPγS on ^{125}I-ET-1 binding to rat cerebellar membranes. Membranes and ^{125}I-ET-1 (15 pM) were incubated with (Bound) and without (Bound$_0$) GTPγS (0.1 to 500 μM) in the presence of 5 mM $MgCl_2$ or 50 mM NaCl for 2 hr at 37°, as described in "Methods." Data points represent means ± SEM of three or four independent experiments. B (Bound), specifically ^{125}I-ET-1 bound in the presence of the various concentrations of GTPγS; B_0 (Bound$_0$), specifically ^{125}I-ET-1 bound in the absence of the guanine nucleotide. In the presence of $MgCl_2$ and NaCl, bound$_0$ (control) was 420 ± 74 and 332 ± 30 fmol/mg protein, respectively.

shifted as compared to the control with a 5-fold increase in the K_i value (Table 1).

The influence of Na^+ on competition of ET-3, IRL 1620, and BQ 788 for ^{125}I-ET-1 binding was also examined (Fig. 4). All competition curves were monophasic and fitted by one-site models (Fig. 4). In the presence of 50 mM NaCl, competition curves of ET-3 (Fig. 4, *panel B*), IRL 1620 (Fig. 4, *panel C*), and BQ 788 (Fig. 4, *panel D*) showed modest changes. The K_i values for ET-3 and BQ 788 were similar to those of corresponding control experiments (Table 1). However, the addition of NaCl produced a 2-fold decrease in the K_i value for IRL 1620 (Table 1).

We also examined the effect of a non-hydrolizable GTP analog, GTPγS, on ^{125}I-ET-1 binding to rat cerebellar membranes. In the presence of 5 mM $MgCl_2$ or 50 mM NaCl, GTPγS caused a concentration-dependent inhibition of specific binding (Fig. 5). The inhibitory effect was not complete and reached 40–45% at a nucleotide concentration of 500 μM. Thus, apparently 60–55% of total ET_B receptor sites were not sensitive to modulation of agonist binding by GTPγS. However, in the absence of cations no inhibition was detectable even at the highest concentration (500 μM) of the guanine nucleotide (data not shown). To determine whether GTPγS was able to modify ET-1 binding parameters, we performed dilution experiments of ^{125}I-ET-1 with unlabelled ET-1 in the presence of 5 mM $MgCl_2$ and 500 μM GTPγS. Data were transformed and analyzed as saturation. Specific binding was saturable and the curve was represented by a one-site model indicating the existence of a single class of binding sites (data not shown). The derived K_d and B_{max} values were 52.0 ± 10.8 pM and 1284 ± 151 fmol/mg protein (N = 3), respectively.

4. Discussion

The major findings of the present paper are that both monovalent and divalent cations modulate agonist binding to ET_B receptors in rat cerebellar membranes. Divalent cations are more effective in increasing ^{125}I-ET-1 binding than monovalent cations. Among the former, Mg^{2+} and Mn^{2+} are more effective than Ca^{2+} whereas among the latter, Na^+ shows the highest efficacy.

A positive effect of Mg^{2+} ions on ^{125}I-ET-1 binding to ETRs has not been described previously. ET-1 binds with high affinity to ET_B receptors even in the absence of divalent cations. However, the addition of Mg^{2+} ions (5–10 mM $MgCl_2$) causes a considerable increase in agonist binding that is similar to that observed for several other G-protein-coupled receptors including α_2- and β-adrenergic [20–22], A_1 and A_{2a} adenosine [11,18,19], H_1 histamine [24], opiate [23], glucagon [12], somatostatin [15], and formyl-peptide [45] receptors. Stimulation of ^{125}I-ET-1 binding is the consequence of an increased receptor affinity for the agonist ligand, as indicated by the significant reduction in the K_d value without any change in the number of binding sites. This result is in agreement with previous data reported by us [11] for Mg^{2+} modulation of [^{3}H]CGS21680 binding to rat A_{2a} adenosine receptors. Indeed, several reports indicate that Mg^{2+} ions cause an increase in G-protein-coupled receptor affinity for their agonist ligands without affecting antagonist affinity [15,17,18,22]. For some receptors, a partial or selective effect of the cation on B_{max} values has also been described [18,46]. The addition of $MgCl_2$ increases ET_B receptor affinity for other agonists as shown by competition of ^{125}I-ET-1 binding with ET-3 or IRL 1620. The receptor affinity for the ET_B-selective synthetic agonist, IRL 1620, is particularly sensitive to regulation by the divalent cation. Thus, the receptor sensitivity to modulation of its affinity is dependent on the type of agonist. On the other hand, the receptor affinity for an antagonist, BQ 788, decreases as shown with ^{125}I-ET-1 competition assays. This effect of Mg^{2+} ions is likely the consequence of the increased receptor affinity for the radiolabelled agonist. A positive influence of Mn^{2+} ions on the affinity of human placental endothelin receptors [38] and ET_A receptors in neuroblastoma cells [47] has been described. This modulation appears to affect a cross-linking reaction [38] and is maintained after receptor solubilization [38,47]. Overall, these data and our present findings suggest that the presence of divalent cations is required for the high-affinity state of endothelin receptors while in the absence of these cations a low-affinity state is detected. Divalent cations may enhance receptor–G-protein coupling acting at the level of receptor and/or its interaction with G-proteins. Since in the millimolar range Mg^{2+} and Mn^{2+} share a similar activity, their influence on ^{125}I-ET-1 binding cannot be related to an action on the guanine nucleotide site of Gα-subunits.

The monovalent cation, Na^+, stimulates ^{125}I-ET-1 binding to rat cerebellar membranes. This effect is also mim-

icked by other monovalent cations but is not supported by a bulky monovalent cation, suggesting the specificity of small cation activity. Na^+ causes a significant decrease in the K_d value while the B_{max} value does not show any change. The addition of NaCl induces an increase in receptor affinity for ET-1 but that for other agonists is either unmodified (ET-3) or slightly changed (IRL 1620). Thus, this effect seems selective for ET-1. In addition, Na^+ stimulation of specific binding is additive with that induced by 1 mM $MgCl_2$. At higher concentrations of the divalent cation (5–10 mM $MgCl_2$), the addition of Na^+ causes modest effects. However, ^{125}I-ET-1 dilution experiments shows that the combination of 5 mM $MgCl_2$ and 50 mM NaCl induces a further increase in receptor affinity for ET-1, suggesting cation cooperation in modulating the receptor affinity state. A similar stimulatory effect of Na^+ ions on agonist binding has been reported for angiotensin II [36], α_1-adrenergic [37], and dopamine receptors [46]. For other G-protein-coupled receptors, this cation has been shown to reduce the affinity for agonists [25–31].

A conserved Asp residue in the second membrane-spanning region appears to be involved in supporting the Na^+-mediated inhibition of agonist binding [33–35]. The recent resolution of the crystal structure of rhodopsin [48] has shown that this conserved Asp residue can form a hydrogen bond with the peptide carbonyl of an Ala residue (Ala^{299} of rhodopsin) in the seventh membrane-spanning region and be connected via a water molecule to the peptide carbonyl of a Gly residue (Gly^{120} of rhodopsin) in the third membrane-spanning region. Thus, the Asp residue participates in those intramolecular interactions required to maintain the tridimensional arrangement of receptor transmembrane domains. Monovalent cations may perturb these interactions between amino acid side chains and water molecules. Two Asp residues are present in the second membrane-spanning region of the rat ET_B receptor [49]. The effect of Na^+ on ^{125}I-ET-1 binding to ET_B receptors may be caused by a perturbation of receptor intramolecular interactions which stabilizes the intrinsic receptor affinity for ET-1. Modifications of these interactions may also produce an indirect enhancement of receptor-G-protein coupling. In fact, the addition of Na^+ as well as Mg^{2+} ions reveals a GTPγS-dependent modulation of ^{125}I-ET-1 binding to rat cerebellar membranes. However, Na^+ and Mg^{2+} appear to influence the receptor–G-protein system at different levels.

Na^+ ions may exert the stimulatory effect through stabilization of an ET-1 conformation that is the most appropriate for binding to the ET_B receptor. In fact, this stimulation is observed only for ET-1 while ET-3 or IRL 1620 binding is modestly affected by the addition of the monovalent cation. NMR analysis of ET-1 [50] and ET-3 [51] structures has shown that both isopeptides assume a helical conformation between Lys^9 and Cys^{15}. Their C-terminus, which lies in close opposition to the helical region, makes contacts with it through hydrophobic interactions [51]. Minimal variations in isopeptide structure may influence its response to the increase of salt concentration.

Sokolovsky *et al.* [39] have reported that rat ET_B receptors are not sensitive to modulation of the affinity state by a guanine nucleotide triphosphate analog. We have found that in the presence of 5 mM $MgCl_2$ or 50 mM NaCl, GTPγS inhibits ^{125}I-ET-1 binding to rat cerebellar membranes while in the absence of salts no regulation of agonist binding is detectable. However, only 40–45% of receptor sites are sensitive to modulation by the guanine nucleotide. A G_s-coupled receptor, the A_{2a} adenosine receptor, also shows Mg^{2+} requirement for modulation of the affinity state by GTPγS together with an incomplete inhibition [11]. Under our experimental conditions, the addition of GTPγS (500 μM) does not induce a complete shift to receptor low-affinity state. This is either the consequence of a reduced sensitivity of the ET_B receptor–G-protein system to modulation by guanine nucleotides or due to intrinsic pharmacological properties of ET-1. As mentioned before, our data also support an indirect positive effect of Na^+ on receptor–G-protein coupling.

In conclusion, we have found that in rat cerebellar membranes the ET_B receptor affinity state is positively regulated by Mg^{2+} and Na^+ ions, while guanine nucleoside triphosphate exerts an inhibitory effect. Both mono- and divalent cations facilitate the high-affinity state of receptors acting at different levels of the ET_B receptor–G-protein system.

Acknowledgment

This work was supported by M.U.R.S.T. grants to A. L. and M. R. M.

References

[1] Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. Pharmacol Rev 1994;46:325–415.
[2] Webb DJ, Monge JC, Rabelink TJ, Yanagisawa M. Endothelin: new discoveries and rapid progress in the clinic. Trends Pharmacol Sci 1998;19:5–8.
[3] Adachi M, Yang YY, Furuichi Y, Miamoto C. Cloning and characterization of cDNA encoding human A-type endothelin receptor. Biochem Biophys Res Commun 1991;180:1265–72.
[4] Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 1990; 348:730–2.
[5] Lin HY, Kaji EH, Winkel GK, Ives HE, Lodish HF. Cloning and functional expression of a vascular smooth muscle endothelin-1 receptor. Proc Natl Acad Sci USA 1991;88:3185–9.
[6] Sakamoto A, Yanagisawa M, Sakurai T, Takuwa Y, Yanagisawa H, Masaki T. Cloning and functional expression of human cDNA for the ET_B endothelin receptor. Biochem Biophys Res Commun 1991;178: 656–63.
[7] Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. Cloning of a non-isopeptide selective subtype of the endothelin receptor. Nature 1990;348:732–5.

[8] Birnbaumer L, Abramowitz J, Brown AM. Receptor-effector coupling by G-proteins. Biochim Biophys Acta 1990;1031:163–224.
[9] Blume AJ. Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. Proc Natl Acad Sci USA 1978;75:1713–7.
[10] Iyengar R, Abramowitz J, Bordelon-Riser M, Blume AJ, Birnbaumer L. Regulation of hormone-receptor coupling to adenylyl cyclase. Effects of GTP and GDP. J Biol Chem 1980;255:10312–21.
[11] Mazzoni MR, Martini C, Lucacchini A. Regulation of agonist binding to A_{2A} adenosine receptors: effects of guanine nucleotides (GDP[S] and GTP[S]) and Mg^{2+} ion. Biochim Biophys Acta 1993;1220:76–84.
[12] Rojas FJ, Birnbaumer L. Regulation of glucagon receptor binding. Lack of effect of Mg and preferential role for GDP. J Biol Chem 1985;260:7829–35.
[13] Fagg GE, Riederer B, Matus A. Sodium ions regulate a specific population of acidic amino acid receptors in synaptic membranes. Life Sci 1984;34:1739–45.
[14] Rouot BM, U'Prichard DC, Snyder SH. Multiple α_2-noradrenergic receptor sites in rat brain: selective regulation of high affinity [^{3}H]clonidine binding by guanine nucleotides and divalent cations. J Neurochem 1980;34:374–84.
[15] Reubi JC, Maurer R. Different ionic requirements for somatostatin receptor subpopulations in the brain. Regul Peptides 1986;14:301–11.
[16] Rens-Damiano S, Law SF, Yamada Y, Seino S, Bell GI, Reisine T. Pharmacological properties of two cloned somatostatin receptors. Mol Pharmacol 1992;42:28–34.
[17] Padrell E, Herberg JT, Monsatirsky B, Floyd G, Premont RT, Iyengar R. The hepatic glucagon receptor: a comparative study of the regulatory and structural properties. Endocrinology 1987;120:2316–25.
[18] Goodman RR, Cooper MJ, Gavish M, Snyder SH. Guanine nucleotide and cation regulation of the binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A_1 receptors in brain membranes. Mol Pharmacol 1982;21:329–35.
[19] Johansson B, Parkinson FE, Fredholm BB. Effects of mono- and divalent ions on the binding of the adenosine analogue CGS21680 to adenosine A_2 receptors in rat striatum. Biochem Pharmacol 1992;44:2365–70.
[20] Glossmann H, Presek P. Alpha noradrenergic receptors in brain membranes: sodium, magnesium and guanyl nucleotides modulate agonist binding. Naunyn-Schmiedeberg's Arch Pharmacol 1979;306:67–73.
[21] U'Prichard DC, Snyder SH. Interactions of divalent cations and guanine nucleotides at α_2-noradrenergic receptor binding sites in bovine brain membranes. J Neurochem 1980;34:385–94.
[22] Williams LT, Mullikin D, Lefkowitz RJ. Magnesium dependence of agonist binding to adenylate cyclase-coupled hormone receptors. J Biol Chem 1978;253:2984–9.
[23] Pasternak GW, Snowman AM, Snyder SH. Selective enhancement of [^{3}H]opiate agonist binding by divalent cations. Mol Pharmacol 1975;11:735–44.
[24] Chang RS, Snyder SH. Histamine H_1-receptor binding sites in guinea pig brain membranes: regulation of agonist interactions by guanine nucleotides and cations. J Neurochem 1980;34:916–22.
[25] Limbird LE, Speck JL, Smith SK. Sodium ion modulates agonist and antagonist interactions with the human platelet α_2-adrenergic receptor in membrane and solubilized preparations. Mol Pharmacol 1982;21:609–17.
[26] Horstman DA, Brandon S, Wilson AL, Guyer CA, Cragoe EJ, Limbird LE. An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. J Biol Chem 1990;265:21590–5.
[27] Minuth M, Jakobs KH. Sodium regulation of agonist and antagonist binding to beta-adrenoceptors in intact and N_s-deficient membranes. Naunyn-Schmeidebergs Arch Pharmacol 1986;333:124–9.
[28] Neve KA, Henningsen RA, Kinzie JM, DePaulis T, Schmidt DE, Kessler RM, Janowsky A. Sodium-dependent isomerization of dopamine D-2 receptors characterized using ^{125}I-epidepride, a high affinity substituted benzamide ligand. J Pharmacol Exp Ther 1990;252:1108–16.
[29] Pert CB, Snyder SH. Opiate receptor binding of agonists and antagonists affected differentially by sodium. Mol Pharmacol 1974;10:868–79.
[30] Kosterlitz HW, Paterson SJ, Robson LE. Modulation of binding at opioid receptors by mono- and divalent cations and by cholinergic compounds. J Recept Res 1988;8:363–73.
[31] Jakobs K. Inhibition of adenylate cyclase by hormones and neurotransmitters. Mol Cell Endocrinol 1979;16:147–56.
[32] Strader CD, Sigal IS, Register RB, Candelore MR, Rands E, Dixon RA. Identification of residues required for ligand binding to the β-adrenergic receptor. Proc Natl Acad Sci USA 1987;84:4384–8.
[33] Chung FZ, Wang CD, Potter PC, Venter JC, Fraser CM. Site-directed mutagenesis and continuous expression of human β-adrenergic receptors. Identification of a conserved aspartate residue involved in agonist binding and receptor activation. J Biol Chem 1988;263:4052–5.
[34] Surprenant A, Horstman DH, Akbarali H, Limbird LE. A point mutation of the α_2-adrenoreceptor that blocks coupling to potassium but not calcium currents. Science 1992;257:977–80.
[35] Kong H, Raynor K, Yasuda K, Bell GI, Reisine T. Mutation of an aspartate at residue 89 in somatostatin receptor subtype 2 prevents Na^+ regulation of agonist binding but does not alter receptor-G protein association. Mol Pharmacol 1993;44:380–4.
[36] Glossman H, Baukal A, Catt KJ. Cation dependence of high-affinity angiotensin II binding to adrenal cortex receptors. Science 1974;185:281–3.
[37] Glossman H, Hornung R. Sodium ions increase the affinity of clonidine for α_1-adrenoceptors in rat brain. Naunyn-Schmeidebergs Arch Pharmacol 1980;312:105–6.
[38] Kalina B, Loffler BM. Crosslinking analysis of an endothelin receptor protein from human placenta. Biochem Int 1992;27:735–44.
[39] Sokolovsky M, Ambar I, Galron R. A novel subtype of endothelin receptors. J Biol Chem 1992;267:20551–4.
[40] Macchia M, Barontini S, Ceccarelli F, Galoppini C, Giusti L, Hamdan M, Lucacchini A, Martinelli A, Menchini E, Mazzoni MR, Revoltella RP, Romagnoli F, Rovero P. Toward the rational development of peptidomimetic analogs of the C-terminal endothelin hexapeptide: development of a theoretical model. Il Farmaco 1998;53:545–56.
[41] Lowry OH, Rosebrough NJ, Farr A, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951;193:265–75.
[42] Cody WL, He JX, Depue PL, Waite LA, Leonard DM, Sefler AM, Kaltembroun JS, Haleen SJ, Walker DM, Flynn MA, Welch KM, Reynolds EE, Doherty AM. Structure–activity relationships of the potent combined endothelin-A/endothelin-B receptor antagonist Ac-Ddip16-Leu-Asp-Ile-Ile-Trp21: development of endothelin-B receptor selective antagonists. J Med Chem 1995;38:2809–19.
[43] Macchia M, Barontini S, Ceccarelli F, Galoppini C, Giusti L, Hamdan M, Lucacchini A, Martinelli A, Menchini E, Mazzoni MR, Revoltella RP, Romagnoli F, Rovero P. Toward the rational development of peptidomimetic analogs of the C-terminal endothelin hexapeptide: development of a theoretical model. Il Farmaco 1998;53:545–56.
[44] McPherson GA. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J Pharmacol Methods 1985;14:213–28.
[45] Cheng YC, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of the inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.
[46] Gierschik P, Stisslinger M, Sidiropoulos D, Herrmann E, Jackobs KH. Dual Mg^{2+} control of formyl-peptide-receptor–G-protein interaction in HL60 cells. Evidence that the low-agonist-affinity receptor interacts with and activates G-protein. Eur J Biochem 1989;183:97–105.

[47] Usdin TB, Creese I, Snyder SH. Regulation by cations of [^{3}H]spiroperidol binding associated with dopamine receptors of rat brain. J Neurochem 1980;34:669–76.
[48] Huggins JP, Pelton JT, van Giersbergen PL. The receptors for endothelins and their analogues in SK-N-MC neuroblastoma cells. Peptides 1994;15:529–36.
[49] Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: a G protein-coupled receptor. Science 2000;289:739–45.
[50] Cheng HF, Suh YM, Yeh JR, Chang KJ. Alternative transcript of the nonselective-type endothelin receptor from rat brain. Mol Pharmacol 1993;44:533–8.
[51] Andersen NH, Chen CP, Marschner TM, Krystek SR Jr, Bassolino DA. Conformational isomerism of endothelin in acid aqueous media: a quantitative NOESY analysis. Biochemistry 1992;31:1280–95.
[52] Mills RG, O'Donoghue SI, Smith R, King GF. Solution structure of endothelin-3 determined using NMR spectroscopy. Biochemistry 1992;31:5640–5.