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Molecular Cloning and Characterization of the Major Endothelin Receptor Subtype in Porcine Cerebellum

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SUMMARY

Endothelin receptors (ETRs) display subtype heterogeneity and are widely distributed throughout the tissues of the periphery and central nervous system. In order to gain further insight into the potential molecular differences of ETRs, we initiated molecular cloning of ETR genes by screening for the appearance of ^{125}I-ET-1 binding activity in COS cells transfected with pools of a porcine cerebellum cDNA expression library. Two independent clones (pPCETR 1.1 and pPCETR 5.6) were identified and isolated by repeated rounds of pool enrichment and COS cell expression. DNA sequence analysis of pPCET 1.1 and pPCET 5.6 indicated that both clones have the same nucleotide sequence; the deduced amino acid sequence indicated that the porcine cerebellum ETR is 443 residues in length and consists of seven potential transmembrane domains, with homology to members of the GTP-binding protein-coupled receptor superfamily. Northern analysis indicated a single mRNA species of about 5 kilobases, which is expressed significantly in cerebellum, lung, kidney, and pituitary. Expression of functional receptor was demonstrated by endothelin-1 (ET-1)-mediated Ca^{2+} mobilization in COS cells transfected with pPCETR 1.1 (COS/ETR 1.1) and ET-1-mediated electrophysiological responses in *Xenopus* oocytes injected with RNA derived from pPCETR 1.1. Quantitative comparison of saturation binding of ^{125}I-ET-1 to either porcine cerebellum or COS/ETR 1.1 membranes indicated an identical apparent dissociation constant. The relative efficacy of ET-related peptides to compete for binding of ^{125}I-ET-1 to receptor from porcine cerebellum and COS/ETR 1.1 indicated that both preparations encode a nonselective or ET_BR subtype. Chemical cross-linking of ^{125}I-ET-1 to receptor derived from cerebellum or COS/ETR 1 revealed two bands, with apparent molecular masses of 47 and 35 kDa. These data demonstrate that the pPCETR 1.1 encodes the major ETR subtype in the porcine cerebellum.

ETs are a family of 21-residue peptide hormones with profound vasoactive, mitogenic, and potential neuroregulatory functions. In mammals, the ET peptide family is composed of three members, ET-1, ET-2, and ET-3, that are encoded by three separate genes, which are differentially expressed in the tissues of the periphery and CNS (for reviews see Refs. 1 and 2). Mammalian ETs share high sequence homology and structural similarity with a family of 21-residue peptide toxins from the snake *Atractaspis engaddensis*, the SRTs (3). Receptors for the ET-related peptides are known to occur in at least two major subtypes, denoted ET_A and ET_B (4), as defined by rank order binding of ET-1, ET-2, ET-3, and SRTC (5, 6). Thus, ET_AR are defined by high and equal affinity for ET-1 and ET-2, approximately 70–100-fold lower affinity for ET-3, and >1000-fold lower affinity for SRTC. In contrast, the ET_BR subtype displays high and similar affinity for all ET-related peptides. Like the ET-related peptides, receptors for ET and SRT are differentially expressed in a wide variety of tissues and cell types of the periphery and CNS (5–10). ETs and SRTs bind to a common receptor and initiate a common signal-transduction pathway, principally a G protein-mediated activation of phospholipase C and subsequent inositol triphosphate-mediated increase in intracellular Ca^{2+} levels (1, 11), although alternate signal-transduction effectors, such as Ca^{2+}-independent activation of Na^+/H^+ exchange (12) and tyrosine phosphorylation of unidentified proteins (13), have been observed. The physiological effects of ET are also diverse and include potent vasoconstrictive effects (14), vasodialative effects (15), induction of c-*fos* transcription (16), activation of DNA synthesis and mitogenesis in a variety of cells (17, 18), stimulation of PI hydrolysis in granular cell neurons (18, 19), depolarization of spinal neurons (20), and stimulation of the release of substance P (21), vasopressin (21a), prolactin (22), aldosterone (23), and the glycoprotein hormones follicle-stim-

ABBREVIATIONS: ET, endothelin; CNS, central nervous system; COS, African green monkey kidney cells; COS/ETR, COS cells transfected with endothelin receptor cDNA; DMEM, Dulbecco's modified Eagle's medium; ETR, endothelin receptors; PI, phosphatidylinositol; pPCETR, plasmid containing the endothelin receptor cDNA: PTX, pertussis toxin; G protein; GTP-binding protein; SRT, sarafotoxin; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; KRH, Krebs-Ringer-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; kb, kilobases; AM, acetoxy methyl ester; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TSH, thyrotropin.

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ulating hormone, luteinizing hormone, and TSH, (24, 25). These diverse and complex physiological effects mediated by ET, in conjunction with the molecular heterogeneity and differential tissue distribution of the ET-related peptides and their receptors, underscore the importance of using a molecular biological approach to dissect the components of ET physiology. For these reasons, we have undertaken the molecular cloning of ETR subtypes from porcine tissues of the periphery and CNS. We report here the *de novo* cloning and functional characterization of the predominant ETR subtype of porcine cerebellum, the first ETR cDNA isolated from a tissue of the CNS. The availability of cDNA clones for ETR will allow investigation of the neurophysiological relevance of ETR heterogeneity and tissue distribution.

Experimental Procedures

Construction and screening of the porcine cDNA expression library. Initial screening of different porcine tissues for ^{125}I-ET-1 (New England Nuclear) binding indicated that the porcine cerebellum and heart tissues had the highest receptor density (500 and 750 fmol/mg of protein, respectively). Total RNA was isolated from porcine cerebellum tissue as described earlier (26). Poly(A)$^+$ RNA was then isolated by two cycles of chromatography on oligo(dT)-cellulose (Collaborative Research) (27). The ability of the mRNA to serve as a template for the synthesis of ETR was determined by injection of mRNA into *Xenopus laevis* oocytes (28), as described below. The preparation of mRNA that produced the highest receptor activity was used to construct an oriented, size-selected (>1.5-kb), cDNA library in pcDNAI vector (Invitrogen). Plasmid DNA was prepared from pools of 1500–2000 recombinant clones, by the alkaline lysis method (29), and purified using Quaigen columns. DNA (10 μg) was transfected into COS cells in 60-mm plates, using the DEAE-dextran/chloroquine method (30), incubated in DMEM supplemented with 10% fetal bovine serum for 24 hr, shifted into serum-free DMEM containing 0.1% BSA and 0.1 nM ^{125}I-ET-1 (2200 Ci/mmol), and incubated for 12 hr. The cells were then washed with ice-cold PBS, containing 10 mM $MgCl_2$ and 0.2% powdered milk, and were fixed in 2.5% glutaraldehyde/PBS for 30 min at room temperature. Plates were dried, the vertical edges were removed, and plates were exposed to X-ray film (Kodak) for 3–5 days at −70°. Positive pools were enriched by pool subdivision and assayed by repeated rounds of expressions screening until two single clones were obtained (pPCETR 1.1 and pPCETR 5.6).

Nucleotide sequence analysis. The inserts of pPCETR 1.1 and pPCETR 5.6 were subcloned into the Bluescript vector (Stratagene), and a series of *Exo*III deletions were made. Nucleotide sequence on both strands was determined by a modification of the dideoxy chain termination method (31), using the Sequenase II kit (US Biochemicals). The Wisconsin Genetics Computer Group Software package (32) was used to assemble composite sequences from the various fragments and for further sequence analysis.

Northern and Southern analysis. For Northern analysis, total RNA was isolated from various porcine tissues using the guanidinium-thiocyanate-acid-phenol method (33). Fifteen micrograms of each RNA were fractionated on 1% agarose-formaldehyde gels (34) and transferred to nitrocellulose membranes. Northern hybridization reactions were performed at 42° in 50% formamide, 5× SSPE (0.75 M sodium chloride, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 5× Denhardt's reagent, 0.1% SDS, 100 μg/ml yeast tRNA. The blots were washed with 0.5× SSC (75 mM sodium chloride, 7.5 mM sodium citrate, pH 7.0), 0.1% SDS, at 55° and autoradiographed for 48 hr at −70°. For Southern analysis, genomic DNA was isolated from porcine cerebellum tissue as described (35). Samples of genomic DNA were digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nitrocellulose membranes. Southern hybridization reactions were performed in 45% formamide, 4× SSC, 1× Denhardt's reagent, 0.1% SDS, 50 μg/ml salmon sperm DNA. The blots were washed with 2× SSC at 55° and autoradiographed for 24 hr at −70°. The ETR cDNA probe (pPCETR 1.1) was labeled to a specific activity of 1 × 10^9 cpm/μg of DNA with [α-^{32}P]dCTP (ICN), by the random priming method (36).

Preparation of membranes. Frozen cerebellums were disrupted and homogenized in ice-cold 250 mM sucrose, 50 mM HEPES, pH 7.5, in the presence of 0.5 mM phenyl-methylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin (protease cocktail). Unbroken cells and tissue debris were removed by centrifugation (15 min at 2500 × *g* at 4°), and crude membranes were harvested from the supernatant by centrifugation (30 min at 70,000 × *g* at 4°). The crude membrane pellet was resuspended and washed with 250 mM sucrose, 50 mM HEPES, pH 7.5, rapidly frozen, and stored at −70° until use. COS cells were scaled proportionately for growth in 245-mm × 245-mm tissue culture plates, transfected with 30 μg of pPCETR 1.1 DNA, and grown in DMEM supplemented with 10% fetal bovine serum. After 2 days, the cells were washed with PBS and released by scraping into PBS containing 5 mM EDTA and protease cocktail. Cells were harvested by centrifugation, resuspended in PBS-protease cocktail, and lysed by rapid dilution into ice-cold 20 mM HEPES buffer, pH 7.5, containing protease cocktail. Crude membranes were collected by centrifugation (30,000 × *g* for 30 min at 4°), resuspended in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$ (Mg-HEPES), divided into aliquots, and rapidly frozen. Both membrane preparations were stable at −70° for up to 3 months. Protein was determined by the bicinchoninic acid method (Pierce), using BSA as a standard.

Radioligand binding assays. Saturation binding assays were initiated by addition of either porcine cerebellum (12 μg/ml) or COS/ETR 1.1 (0.67 μg/ml) membranes to an assay cocktail composed of 0.1% BSA and the indicated concentration of ^{125}I-ET-1 (2200 Ci/mmol) in Mg-HEPES. Reactions were incubated for 2 hr at 37° with constant agitation, terminated by dilution with cold Mg-HEPES, filtered onto GF/C filters (Whatman), rinsed with cold Mg-HEPES, and counted in a Beckman 8500 γ counter (counting efficiency, 70%). Filters were pretreated with 0.1% BSA in Mg-HEPES. Nonspecific binding was determined in the presence of 100 nM ET-1; total ^{125}I-ET-1 binding was ≤10% of added isotope. Duplicates of total and nonspecific binding determinations varied by <10%. The concentration of ^{125}I-ET-1 was calculated directly by utilizing a constant specific activity, due to the catastrophic decay of labeled ligand (New England Nuclear). The dissociation constant (K_d) and maximum ligand binding capacity (B_{max}) were determined by nonlinear regression using the LIGAND program (37) and by the method of Scatchard (38) after linear regression. Equilibrium binding data were plotted using Kaleidagraph (Abelbeck Software).

Competition binding was initiated by addition of either porcine cerebellum of COS/ETR membranes to an assay reaction composed of 0.1% BSA, 0.1 nM ^{125}I-ET-1 (2200 Ci/mmol), and unlabeled ET-related peptides (American Peptides), in Mg-HEPES. Binding reactions were incubated for 1 hr at 37° with constant agitation, terminated by dilution with cold Mg-HEPES, and filtered as described above. The concentration of non-labeled ET-related peptides was determined by quantitative amino acid analysis; the concentration of ^{125}I-ET-1 was determined as described above. First the homologous displacement data were analyzed by LIGAND (37), and then the calculated K_d was used to determine the K_i, B_{max}, and n, the pseudo-Hill coefficient, for the ET-related peptides, by analysis of the heterologous displacement data with EBDA, a nonlinear regression program in the KELL data analysis package (Bisoft).

Cross-linking of ^{125}I-ET-1 to receptor. Porcine or COS/ETR membranes (22 nM in ET binding sites) were incubated with 50 pM ^{125}I-ET-1 (2200 Ci/mmol), in the presence or absence of 100 nM unlabeled ET-1 in Mg-HEPES, for 1 hr at 37°. Membranes were harvested by centrifugation, resuspended in Mg-HEPES, made 0.5 mM in BS3 (Pierce), and incubated at room temperature for 30 min before addition of 100 mM glycine to quench the cross-linking reaction. The

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membranes were harvested by centrifugation, dissolved in 5% SDS, 100 mM dithiothreitol, at 37°, diluted with sample buffer, and subjected to SDS-PAGE (39). The gel was fixed in 40% methanol/10% acetic acid, dried, and autoradiographed overnight at −70°.

***In vitro* transcription, microinjection into *X. laevis* oocytes, and electrophysiology.** RNA transcripts were synthesized (40) from linearized pPCETR 1.1 with T7 RNA polymerase (Stratagene) and were digested with DNase I (10 units/μg of DNA) to remove template DNA. The reaction mixture was extracted with phenol/$CHCl_3$/isoamyl alcohol (50:49:1) and precipitated with ethanol after addition of 3.75 M ammonium acetate. For microinjection, large *X. laevis* females (NASCO) were anesthetized by hypothermia, and the ovaries were surgically removed. Follicle cells were dispersed, and individual oocytes were released by incubation with 2 mg/ml collagenase (Worthington) in modified Barth's medium, as described (28). After collagenase treatment and washing, the oocytes were allowed to recover overnight at 18° in Barth's medium containing gentamycin (100 μg/ml). Stage V–VI oocytes were selected and the follicular membranes were manually removed. For each experimental group, 30–40 defolliculated oocytes were injected with 50 nl of solution containing 5 ng of *in vitro* transcribed RNA derived from pPCETR 1.1 (Drummond injection apparatus) and were maintained in modified Barth's medium containing 100 μg/ml gentamycin, at 18°, until electrophysiological measurements were made.Where indicated, oocytes were treated with pertussis toxin (2 μg/ml) for 20 hr (41). Electrophysiology was performed using the voltage-clamp technique, with an oocyte voltage-clamp apparatus (Warner Instruments). Oocytes were clamped at −60 mV and the Ca^{2+}-activated Cl^- channel activity was recorded in Barth's medium at room temperature, as described (42).

Ca^{2+} mobilization in COS cells. COS/ETR 1.1 cells were incubated with 5 μM indo-1 AM (43) in KRH buffer (118 mM NaCl, 4.6 mM KCl, 25 mM $NaHCO_3$, 1 mM KH_2PO_4, 11 mM glucose, 0.1% BSA, 1.1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) at 37° in the dark. After 1 hr, the indo-1 AM solution was removed and the cells were rinsed with KRH buffer and incubated in KRH buffer at 37°, to hydrolyze intracellular indo-1 AM. After 45 min, the incubation medium was removed, the plates were washed with KRH buffer, and the cells were gently suspended in PBS containing 3 mM EDTA and protease cocktail. The indo-loaded cells were harvested by centrifugation and resuspended in KRH buffer. Relative intracellular Ca^{2+} levels were measured by monitoring the fluorescence of Ca^{2+}-bound indo-1 (excitation wavelength, 340 nm; emission wavelength, 510 nm) at 37°, in an SLM-8000 fluorescence spectrophotometer equipped with a stirred and temperature-regulated sample compartment. At the indicated time, 15 μM ET-1 was added to the cell suspension and the relative changes in intracellular Ca^{2+} levels were monitored fluorometrically.

Results and Discussion

Construction and screening of a porcine cerebellum cDNA expression library. In addition to its potent vasoactive effects, ET appears to play multiple roles in the CNS. ET-1 and ET-3 are differentially synthesized in various regions of the rat and human brains (44) and have important physiological effects in the CNS (17–25, 45, 46). In order to gain insight into the molecular mechanisms of ET action in the CNS and to compare these mechanisms with those in the periphery and cardiovasculature, we undertook the expression cloning of ETR from porcine cerebellum.

Poly(A)$^+$ RNA was isolated from porcine cerebellum and injected into *X. laevis* oocytes. Two to 3 days after injection, binding assays were carried out directly on the intact oocyte membranes, using ^{125}I-ET-1 (data not shown). The preparation of mRNA inducing the highest ET receptor activity was used to construct a undirectional cDNA library in the high efficiency COS cell expression vector pcDNA1 (Invitrogen). Plasmid DNA from approximately 1500–2000 clones was transfected into COS cells and screened for the expression of ET binding sites by incubation with ^{125}I-ET-1, followed by autoradiography (Fig. 1) (47). Positive pools were identified, subdivided for plasmid preparation, retransfected into COS cells, and assayed for ^{125}I-ET-1 binding activity (Fig. 1). After four rounds of pool subdivision two positive clones of 2×10^5 total clones conferred ET-1 binding to COS cells.

Nucleotide sequence analysis of pPCETR1.1. The DNA sequence analysis of pPCETR 1.1 revealed that there is only one open reading frame, beginning at a methionine codon ATG (nucleotide 1) and ending at a stop codon TGA (nucleotide 1329) (Fig. 2). The second positive clone pPCETR 5.6 has an identical nucleotide sequence but differs in the length of the 5′ untranslated region. The deduced polypeptide consists of 443 amino acid residues, with an expected molecular mass of approximately 49 kDa. Analysis of the ETR hydropathy profile (48) indicates the presence of seven hydrophobic regions, putative transmembrane domains. Potential extracellular *N*-linked glycosylation sites and intracellular Ser/Thr phosphorylation sites are indicated in Fig. 2. The presence of several conserved cysteine and proline residues, in addition to sequence similarity in putative transmembrane domains, indicates that porcine ETR is a member of the G protein-coupled receptor superfamily (49–51).

The deduced amino acid sequence of the porcine cerebellum ETR (pPCETR 1.1) is 86% and 87% identical to that of rat (4) and human (52) ET_BR and 58% and 57% identical to that of the bovine (53) and smooth muscle (54) ET_AR (Fig. 3), respectively. Thus, pPCETR 1.1 encodes an ET_BR subtype (Fig. 2). Comparison of the primary structure of the ET_A- and ET_BR subtypes indicates a high degree of homology in the region of the transmembrane domains but, interestingly, a much more

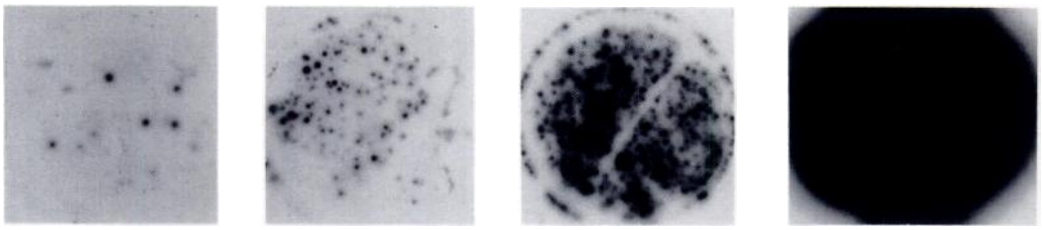

Fig. 1. Identification and enrichment of a porcine ETR cDNA clone by COS expression. Autoradiogram of ^{125}I-ET-1 bound to COS cells transfected with a positive pool of 1500–2000 independent cDNA clones (round 1), a positive pool of 250 cDNA clones (round 2), a positive pool of 20 cDNA clones (round 3), and a single cDNA clone pPCETR 1.1 (round 4).

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 -87  aagcttggtaccgagctcggatccactagtaacggccgccagtgtgctctaaagcagccaccaacgtccaccggagcaggcagcaac atg cag ccg ctg cgc agc cta tgc gga cgc gcc
                                                                                            Met Gln Pro Leu Arg Ser Leu Cys Gly Arg Ala    11

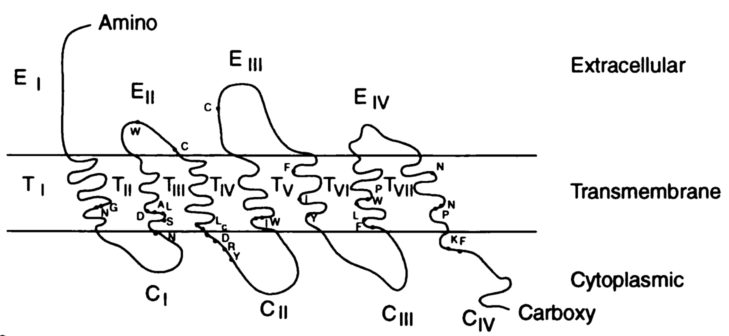
  34  ctg gtg gcg ctg atc ttt gcc tgt ggc gtg gcc ggg gtc cag tct gag gag agg gga ttc ccg ccg gcc ggg gcc act cca cca gcc ctg agg acc gga gag ata gtg gcg ccc cct act
      Leu Val Ala Leu Ile Phe Ala Cys Gly Val Ala Gly Val Gln Ser Glu Glu Arg Gly Phe Pro Pro Ala Gly Ala Thr Pro Pro Ala Leu Arg Thr Gly Glu Ile Val Ala Pro Pro Thr    51
                                          G
 154  aag acc ttc tgg ccc agg ggg tcc aac gcc agc ctg cct cgg tcg tcc tcc ccc ccg cag atg cct aaa gga ggg agg atg gcg gga ccc cca gca cgc acg ctc acc cct cct ccg tgc
      Lys Thr Phe Trp Pro Arg Gly Ser Asn Ala Ser Leu Pro Arg Ser Ser Ser Pro Pro Gln Met Pro Lys Gly Gly Arg Met Ala Gly Pro Pro Ala Arg Thr Leu Thr Pro Pro Pro Cys    91

 274  gaa gga ccc atc gag atc aag gac act ttc aag tac atc aac act gtg gtg tcc tgc cta gtg ttc gtg ctg ggc atc atc gga aac tcc aca ctg ctg cga atc att tac aag aac aag
      Glu Gly Pro Ile Glu Ile Lys Asp Thr Phe Lys Tyr Ile Asn Thr Val Val Ser Cys Leu Val Phe Val Leu Gly Ile Ile Gly Asn Ser Thr Leu Leu Arg Ile Ile Tyr Lys Asn Lys   131
                                                                                    I
 394  tgc atg cga aac ggc cct aac atc ttg ata gcc agc ctg gct ctg gga gac ctg ctt cac atc atc att gat atc ccc atc aac gtc tac aag ctg ctc gcc gag gac tgg ccc ttt gga
      Cys Met Arg Asn Gly Pro Asn Ile Leu Ile Ala Ser Leu Ala Leu Gly Asp Leu Leu His Ile Ile Ile Asp Ile Pro Ile Asn Val Tyr Lys Leu Leu Ala Glu Asp Trp Pro Phe Gly   171
                                                                     II
 514  gtt gag atg tgt aag ctg gtg cct ttc ata cag aag gcc tcc gtg gga atc act gtg ctg agt cta tgt gct ctc agt att gac aga tat cga gcc gtt gct tct tgg agt cga att aaa
      Val Glu Met Cys Lys Leu Val Pro Phe Ile Gln Lys Ala Ser Val Gly Ile Thr Val Leu Ser Leu Cys Ala Leu Ser Ile Asp Arg Tyr Arg Ala Val Ala Ser Trp Ser Arg Ile Lys   211
                                                              III
 634  gga atc ggg gtt cca aaa tgg aca gca gtg gaa att gtt tta att tgg gtg gtc tcc gtg gtc ctg gcc gtc ccg gaa gcc ttg ggt ttt gac atg att acc act gac tat aaa gga aat
      Gly Ile Gly Val Pro Lys Trp Thr Ala Val Glu Ile Val Leu Ile Trp Val Val Ser Val Val Leu Ala Val Pro Glu Ala Leu Gly Phe Asp Met Ile Thr Thr Asp Tyr Lys Gly Asn   251
                                                                         IV
 754  cgc ctg cga atc tgc ttg ctc cat cct act cag aaa aca gcc ttc atg cag ttt tac aag aca gct aaa gat tgg tgg cta ttc agt ttc tat ttc tgc ttg cca cta gcc atc act gca
      Arg Leu Arg Ile Cys Leu Leu His Pro Thr Gln Lys Thr Ala Phe Met Gln Phe Tyr Lys Thr Ala Lys Asp Trp Trp Leu Phe Ser Phe Tyr Phe Cys Leu Pro Leu Ala Ile Thr Ala   291
                                                          *                                                        V
 874  ttt ttt tat acc ctg atg acc tgt gaa atg ctg aga aag aag agt ggc atg caa att gct tta aat gat cac tta aag cag aga cgg gaa gtg gcc aaa acc gta ttt tgc ctg gtc ctt
      Phe Phe Tyr Thr Leu Met Thr Cys Glu Met Leu Arg Lys Lys Ser Gly Met Gln Ile Ala Leu Asn Asp His Leu Lys Gln Arg Arg Glu Val Ala Lys Thr Val Phe Cys Leu Val Leu   331

 994  gtc ttt gcc ctg tgt tgg ctt ccc ctt cat ctc agc agg att ttg aag ctc act ctg tat gat caa aat gat tcg aat aga tgt gaa ctt ttg agc ttt ttg ttg gta ttg gat tac att
      Val Phe Ala Leu Cys Trp Leu Pro Leu His Leu Ser Arg Ile Leu Lys Leu Thr Leu Tyr Asp Gln Asn Asp Ser Asn Arg Cys Glu Leu Leu Ser Phe Leu Leu Val Leu Asp Tyr Ile   371
                                  VI
1114  ggc atc aac atg gcg tcc ctg aat tcc tgt att aat cca ata gct ctg tat ttg gtg agc aaa aga ttc aaa aac tgc ttt aag tca tgc tta tgc tgc tgg tgc cag tca ttt gaa gaa
      Gly Ile Asn Met Ala Ser Leu Asn Ser Cys Ile Asn Pro Ile Ala Leu Tyr Leu Val Ser Lys Arg Phe Lys Asn Cys Phe Lys Ser Cys Leu Cys Cys Trp Cys Gln Ser Phe Glu Glu   411
                                      VII
1234  aaa cag tcc ttg gag gaa aag cag tca tgc tta aag ttc aaa gct aat gat cac gga tat gac aac ttc cgt tcc agt aat aaa tac agc tca tct tga aaggaggaaaaaaaaaaaaaaaacggg
      Lys Gln Ser Leu Glu Glu Lys Gln Ser Cys Leu Lys Phe Lys Ala Asn Asp His Gly Tyr Asp Asn Phe Arg Ser Ser Asn Lys Tyr Ser Ser Ser End                                443

1360  ccgctcgagcatgcatctaga  1380
```

Fig. 2. Nucleotide and deduced amino acid sequences of porcine cerebellum ETR cDNA clone (pPCETR 1.1). Nucleotides are numbered (*left*); deduced amino acids are numbered (*right*) beginning with the initiation methionine. Regions corresponding to putative transmembrane domains are *underlined* (I–VII). Potential sites for phosphorylation (*) and *N*-glycosylation (*G*) are indicated.



% Identity to pPCETR1.1	T I	T II	T III	T IV	T V	T VI	T VII	E I	E II	E III	E IV	C I	C II	C III	C IV
ET_A Bovine Lung	68	65	81	60	76	80	77	28	47	42	75	90	76	68	43
ET_A A10	68	73	77	60	76	73	78	14	47	42	75	91	76	69	40
ET_B Rat Lung	96	96	100	92	96	100	100	59	83	75	66	100	100	100	98
ET_B Human	100	96	100	92	100	100	100	64	91	85	83	100	100	100	100

(Column group heading: Domain)

Fig. 3. Membrane topology and amino acid identity of ETR subtypes. The folding of the polypeptide was predicted by the method of Kyte and Doolittle (48) and is similar to models established for other G protein-coupled receptors. Regions corresponding to putative transmembrane (*TI–TVII*), cytoplasmic (*CI–CIV*), and extracellular (*EI–EIV*) domains are indicated. The conserved amino acid substitutions of porcine ETR with other G protein-coupled receptor superfamily members (*β*-adrenergic, *α*-adrenergic, serotin, muscarinic, dopamingergic, and substance K) are indicated. The percentage of identity of pPCETR 1.1, in transmembrane, cytoplasmic, and extracellular domains, with other ETRs (4, 52–54) is indicated in the table.

divergent sequence in the putative extracellular domains (Fig. 2). Further studies will delineate the importance of these domains to ligand binding, signal transduction, and receptor regulation.

ETRs have several unusual features with respect to other members of the superfamily. ETRs are predicted to have a signal sequence and a relatively large amino-terminal domain, structural features shared by TSH and lutropin-choriogonadotropin receptors (55–57). In addition, the predicted third cytoplasmic domain (C_{III}) of ETRs is only approximately 30 residues, a feature shared with all G protein-coupled receptors that have peptide or protein ligands (58–65), in dramatic contrast to the 100–150-residue length of the C_{III} loop in the receptors for bioactive amines (66).

Southern and Northern analysis. In order to determine the genetic complexity of ETR, Southern blot analysis was performed (Fig. 4A). Porcine and human genomic DNAs were digested with several restriction enzymes and hybridized with the pPCETR 1.1 receptor cDNA probe (Fig. 4A). For each digest, two or three bands hybridized to the pPCETR 1.1 receptor probe under low stringency conditions. This indicates that ETR belongs to a relatively simple gene family, consistent with either the presence of single-copy gene encoding both the ET_A- and ET_BR subtypes (67) or the presence of introns within ETR genes.[1] Availability of the cDNA clone will allow the isolation of the chromosomal genes for the ETR, in order to understand the complexity of the ETR gene family.

Northern hybridization analysis of RNA from various porcine tissues with the porcine cerebellum cDNA clone (pPCETR 1.1) (Fig. 4B) indicates the presence of a single major band of

[1] Y. Furuichi, personal communication.

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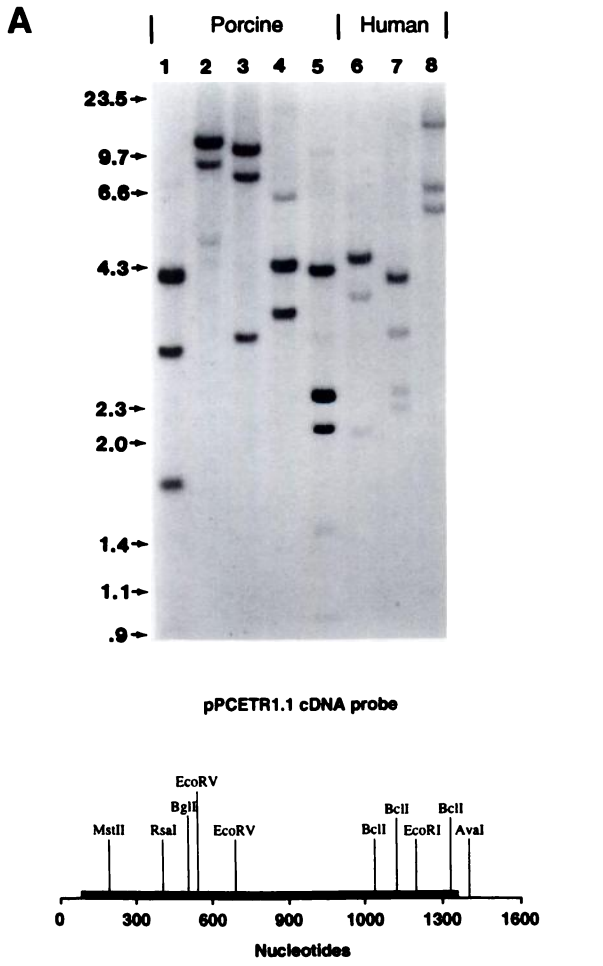

Fig. 4. A, Southern blot analysis of porcine and human genomic DNA. Genomic DNA was digested to completion with restriction endonucleases, fractionated on an 0.8% agarose gel, blotted, and hybridized with ^{32}P-labeled pPCETR 1.1 (see Experimental Procedures). *Lanes 1–5*, porcine genomic DNA digested with restriction endonucleases *Pvu*II, *Hind*III, *Eco*RI, *Bgl*II, and *Bam*HI, respectively. *Lanes 6–8*, human genomic DNA digested with *Pvu*II, *Hind*III, and *Eco*RI, respectively. Positions of the *Hind*III fragments of λDNA are indicated. *Bottom bar*, restriction map of pPCETR1.1 cDNA. The protein-coding region (*thick line*) and untranslated regions (*thin lines*) are indicated. B, Northern blot analysis of RNA isolated from various porcine tissues. Total RNA was prepared from the designated porcine tissues, fractionated on an 1% agarose-formaldehyde gel, blotted, and hybridized with ^{32}P-labeled pPCETR 1.1. The intensity of the 28 S and 18 S rRNAs was visualized by ethidium bromide staining, in order to obtain an internal standard for the amount of RNA loaded (data not shown). The position of a 4.4-kb band of a RNA ladder is indicated.

about 5 kb in most of the tissues examined. Tissues rich in ET_AR, such as porcine heart, contain a small population of ET_BRs[2] and do not appreciably hybridize to the ET_B probe under the high stringency conditions used (Fig. 4B). The intensity of the hybridization signals indicates that cerebellum, lung, and kidney have the highest density of ET_B mRNA. Interestingly, significant amounts of ET_BR mRNA are detected in porcine pituitary (Fig. 4B). These data, in conjunction with previous studies with rat pituitary (21), suggest that both ET_A- and ET_BR subtypes may have important neuroregulatory roles.

Ligand binding properties. Incubation of ^{125}I-ET-1 with membranes derived from porcine cerebellum and COS/ETR 1.1 cells reveals high affinity and saturable binding of ligand (Fig. 5). Analysis of both data sets by nonlinear regression or Scatchard transformation (Fig. 5, *insets*) indicates the presence of a single high affinity site for ET-1, without a statistically significant preference for a two-site receptor model. Nonlinear regression and Scatchard analysis yield apparent K_d values of 74–79 pM, with a B_{max} of 0.58–0.59 pmol/mg, for cerebellum membranes. The K_d determined with COS/ETR 1.1 membranes is 33 pM, in good agreement with the value determined for the native cerebellum membranes, with a B_{max} of 5.7 pmol/mg.

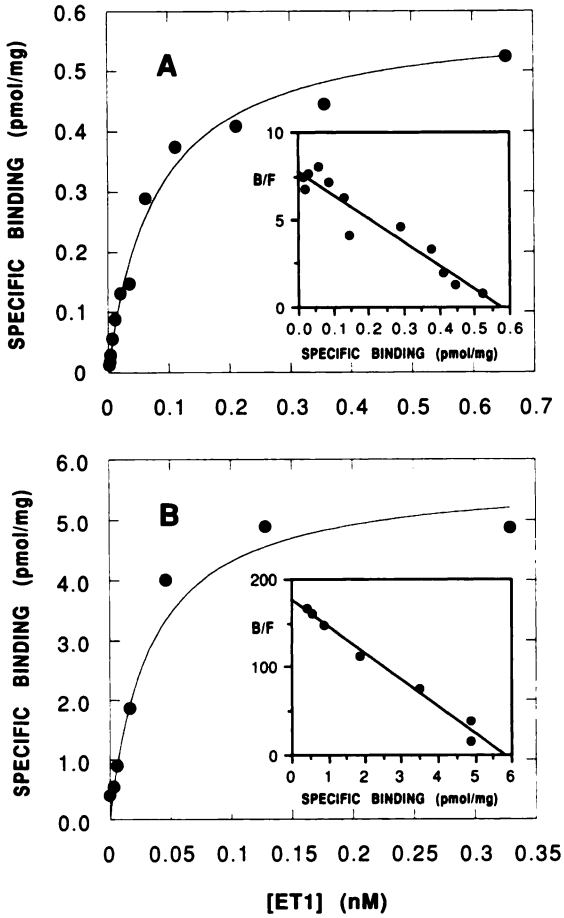

Fig. 5. Saturation binding of ^{125}I-ET-1 to membranes of porcine cerebellum (A) or COS cells transfected with pPCETR 1.1 (B). Assays were conducted in duplicate at 37°, as detailed in Experimental Procedures. Values for K_d and B_{max} were determined by nonlinear regression and used to generate the binding isotherm. *Insets*, linear regressions of Scatchard transformed data.

[2] J. A. Sutiphong and J. A. Lee, unpublished observations.

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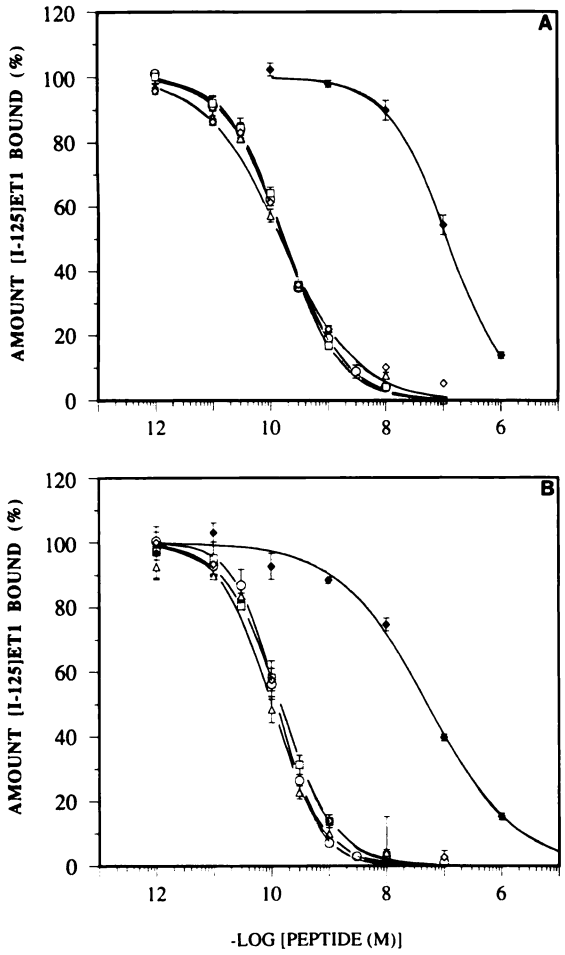

Fig. 6. Competition binding of ET-related peptides to membranes of porcine cerebellum (A) or COS cells transfected with pPCETR 1.1 (B). The indicated concentrations of unlabeled ET-1 (○), ET-2 (□), ET-3 (△), SRTB (◇), and big ET (◆) were mixed with 0.1 nM ^{125}I-ET-1 before addition of membranes, as detailed in Experimental Procedures. Analysis of the homologous competition data was used to determine an apparent K_d and subsequently utilized to determine by nonlinear regression, B_{max}, an apparent K_i, and n for the heterologous competition data, which were used to generate the competition curves.

The ability of ET-1, ET-2, ET-3, big ET, and SRTX6B to compete for ^{125}I-ET-1 binding to membranes derived from porcine cerebellum and COS/ETR 1.1 cells is shown in Fig. 6. Nonlinear regression analysis of the homologous competition data indicates apparent K_d values of 63 pM and 24 pM for ET-1 binding to cerebellum and COS/ETR 1.1 membranes, respectively, in good agreement with the values determined from saturation binding (Fig. 5). The apparent K_i values for ET-1, ET-2, ET-3, and SRTX6B range between 57 and 63 pM and between 19 and 27 pM in studies with porcine cerebellum and COS/ETR 1.1 membranes, respectively, whereas the K_i for big ET is 45.3 nM and 9.13 nM for the respective preparations. In both cerebellum and COS/ETR 1.1 membranes, ET-1, ET-2, ET-3, and SRTX6B are equipotent in competing with ^{125}I-ET-1 binding, whereas big ET is 400–800 times less potent (Fig. 6). Arginine vasopressin, calcitonin gene-related peptide, bradykinin, and angiotensin II (at concentrations of 10 μM) do not compete for ^{125}I-ET-1 binding to either ETR preparation (data not shown).

The competition binding studies indicate that pPCETR 1.1 encodes an ETR that binds ET-1, ET-2, ET-3, and SRTX6B with approximately the same apparent affinity, the identical binding profile observed with membranes from porcine cerebellum (Fig. 6). Therefore, pPCETR 1.1 encodes an ET_B (nonselective) ETR subtype, the predominant ETR subtype in porcine cerebellum.

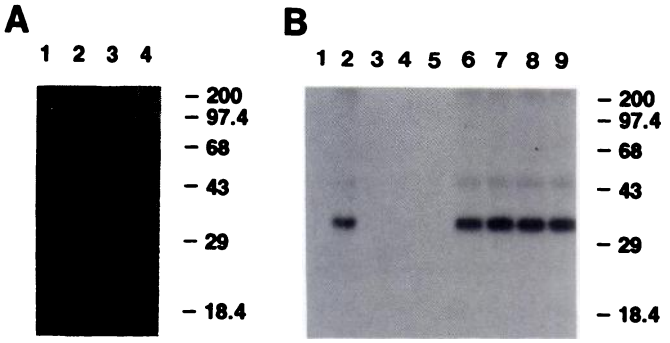

Fig. 7. Autoradiograph of ^{125}I-ET-1 cross-linking reactions. A, Comparison of ETR encoded by pETR1.1 and ETR of porcine cerebellum. ^{125}I-ET-1 was incubated with membranes from COS/ETR 1.1 (*lanes 1* and *3*) or porcine cerebellum (*lanes 2* and *4*), in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of 100 nM unlabeled ET-1. B, Specificity of ^{125}I-ET-1 cross-linking. ^{125}I-ET-1 was incubated with membranes derived from untransfected COS cells (*lane 1*) or COS/ETR 1.1 (*lanes 2–9*), in the absence of unlabeled peptide (*lanes 1* and *2*) or the presence of 100 nM unlabeled peptide (*lane 3*, ET-1; *lane 4*, ET-2; *lane 5*, ET-3; *lane 6*, angiotensin II; *lane 7*, arginine vasopressin; *lane 8*, substance P; *lane 9*, bradykinin). Each reaction was treated with BS3 as described in Experimental Procedures, resolved by SDS-PAGE (12%), and exposed overnight. Position of the prestained high range molecular weight markers (BRL) is indicated.

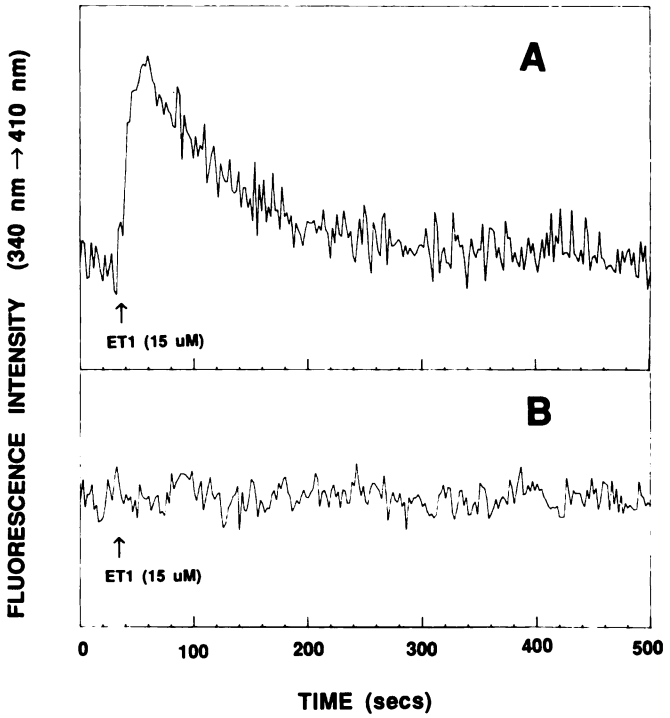

Fig. 8. ET-mediated mobilization of intracellular Ca^{2+} in COS cells. COS cells transfected with pPCETR 1.1 (A) or transfected with pcDNAI alone (B) were loaded with indo-1, as described in Experimental Procedures. At the indicated time, ET-1 (15 μM) was added to a cell suspension and the relative intracellular Ca^{2+} levels were monitored by the increase in fluorescence intensity of the Ca^{2+}-indo-1 complex.

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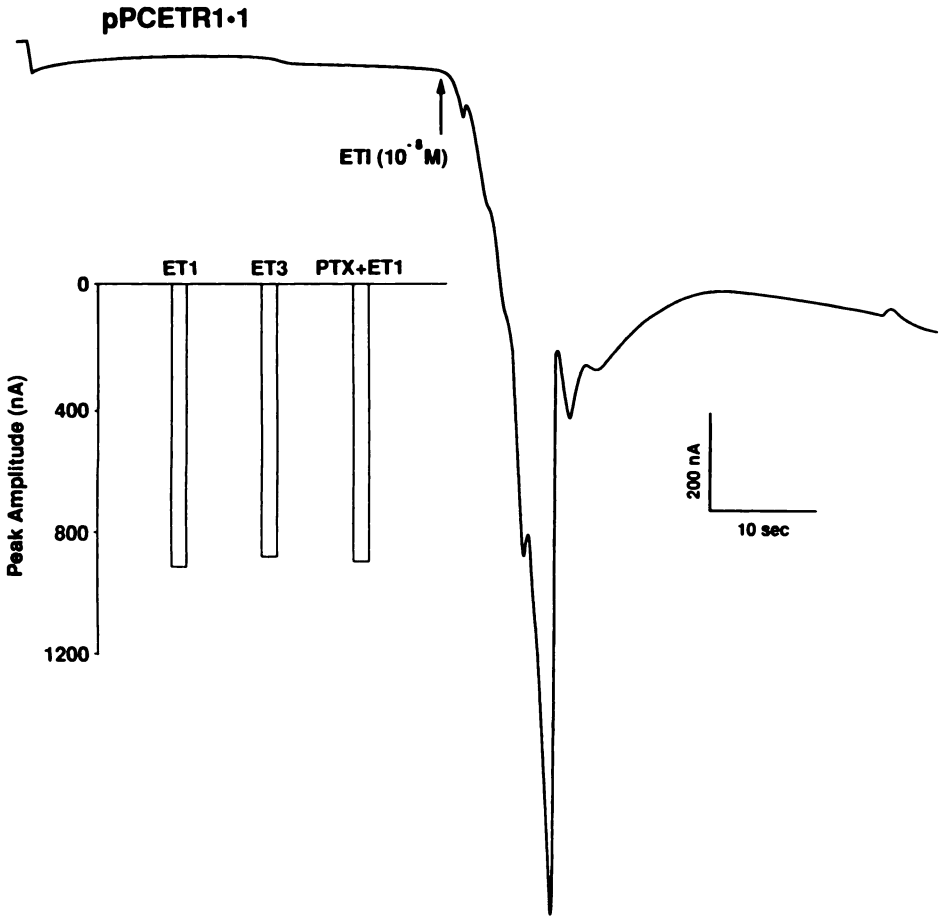

Fig. 9. ET-1- and ET-3-induced currents in oocytes injected with pPCETR 1.1-derived RNA transcripts. The *trace* shows ET-1-mediated current (namp) induced 2 days after injection with pPCETR 1.1 transcripts (5 ng/oocyte). *Inset*, mean peak responses to ET-1, ET-3, and PTX plus ET-1 treatments. The mean ± standard error peak current response to ET-1 is 912 ± 269 (n=30), to ET3 is 880 ± 193 (n=30), and to PTX plus ET-1 is 880 ± 322 (n=30).

Chemical Cross-linking of ^{125}I-ET-1. In order to examine possible molecular differences in the ETR populations from the two preparations, ^{125}I-ET-1 was bound to membranes from porcine cerebellum and COS/ETR 1.1 cells and cross-linked to ETR by addition of BS3. Autoradiography of the reaction mixtures resolved on SDS-PAGE reveals ^{125}I-ET-1 cross-linking to predominant proteins of apparent molecular mass of 47 and 35 kDa in porcine cerebellum membranes and COS/ETR 1.1 cells (Fig. 7A, *lanes 1* and *2*). ET specificity of the cross-linking is indicated by 1) the absence of ^{125}I-ET-1 labeling in untransfected COS cells (Fig. 7B), 2) the elimination of ^{125}I-ET-1 labeling by inclusion of unlabeled ET-1, ET-2, and ET-3, and 3) the persistence of ^{125}I-ET-1 cross-linking in the presence of angiotensin II, arginine vasopressin, substance P, and bradykinin. Because pPCETR 1.1 encodes only a single open reading frame (Fig. 2), the ET-specific cross-linking of two molecular species (47 and 35 kDa) (Fig. 7) is consistent with previous suggestions that the 35-kDa band is a proteolytic degradation product of the 47-kDa band and not a separate subunit or receptor (68, 69) and with the variation in the ratio of ^{125}I-ET-1-labeled 35- and 47-kDa bands between preparations (compare Fig. 7, A and B). Therefore, chemical cross-linking of ^{125}I-ET-1 and limited proteolysis of the receptor indicates that the predominant ETR of porcine cerebellum is identical to the ETR subtype encoded by pPCETR 1.1

Functional studies. The ability of pPCETR 1.1 to trigger a functional response was demonstrated by ET-1-mediated Ca^{2+} mobilization in COS/ETR 1.1 cells and ET-1-mediated electrophysiological responses in *Xenopus* oocytes injected with RNA derived from pPCETR 1.1. Addition of ET-1 to COS/ETR 1.1 leads to a transient increase in intracellular Ca^{2+} levels, as detected by an increase in the fluorescence intensity of a Ca^{2+} indicator dye, indo-1 (Fig. 8A). COS cells transfected with vector alone are nonresponsive to ET-1 (Fig. 8B).

Oocytes injected with RNA derived from pPCETR 1.1 display strong and rapid activation of Ca^{2+}-activated Cl^- currents upon addition of ET-1 and ET-3 (Fig. 9). In order to determine the class of G proteins with which the ETR interacts, oocytes injected with pPCETR 1.1-derived RNA were pretreated with PTX, which is known to catalyze the ADP-ribosylation of certain G proteins and abolish their interaction with the receptor. The results presented in Fig. 9 indicate that the ET-1- and ET-3-stimulated Cl^- currents are resistant to PTX pretreatment, suggesting that in *X. laevis* oocytes the ET responses are mediated by coupling to PTX-insensitive G proteins. These data suggest that pPCETR 1.1 encodes a functional receptor capable of coupling to PI production and intracellular Ca^{2+} mobilization.

This study describes the *de novo* cloning of the first ETR from a tissue of the CNS. Rank order of binding of ET-related peptides and a comparison of the derived primary sequence with recently published sequences (4, 52) indicates that pPCETR 1.1 encodes an ET_B or nonselective ETR subtype. The ETR encoded by pPCETR 1.1 and the predominant ETR of porcine cerebellum are similar in terms of their ligand-binding properties (Figs. 5 and 6), apparent molecular mass,

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and limited proteolysis pattern (Fig. 7). These data suggest that pPCETR 1.1 encodes the predominant ETR (ET_B) in the porcine cerebellum.

ET has been implicated as having multiple physiological effects in the CNS, such as stimulation of DNA synthesis in astroglial cells (17), Ca^{2+} mobilization and release of TSH, follicle-stimulating hormone, luteinizing hormone, and growth hormone from pituitary cells, release of substance P and arginine vasopressin from rat hypothalamus (19–21, 24, 25). PI hydrolysis in cultures of cerebellar neurons (19), and Ca^{2+} mobilization and K^+ channel activation in glial cells (46). In short, ETs are mediators of neural and glial cell physiology, are modulators of hormone release, and may be involved in K^+-buffering and, therefore, important in neural injury (46). However, the role of ET_BR in these processes is not clear. The availability of this clone will facilitate investigation of the potential neuromodulator roles of ET and its possible participation in cerebral pathophysiological processes.

Acknowledgments

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