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Identification and Characterization of Type A Endothelin Receptors in MMQ Cells

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SUMMARY

Recently the identification of endothelin (ET) receptors and ET in the pituitary gland has induced much interest in studying the potential role of ET in neuroendocrine regulation. MMQ, isolated from rat pituitary, is a prolactin-secreting cell line. Similar to primary pituitary cells, the secretory response in MMQ cells is regulated by calcium and cAMP. In this report, by combining radioligand binding, cross-linking, and reverse transcription-polymerase chain reaction (RT-PCR) techniques, we characterized the properties of ET receptors in MMQ cells. ^{125}I-ET-1 bound to membranes prepared from MMQ cells in a time-dependent manner, reaching a plateau at 150 min at 25°. ^{125}I-ET-1 binding was inhibited by ET-1 with an IC_{50} value of 0.17 nM but was only partially (~60%) inhibited by 1 μM ET-3. BQ123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]) and FR139317 (cC6N-L-Leu-D-Trp-Me-D-2Pya-OH), two antagonists that are selective for the ET_A receptor, inhibited ^{125}I-ET-1 binding with IC_{50} values of 5 nM and 0.9 nM, respectively. RT-PCR detected mRNA for the ET_A receptor but not the ET_B receptor. RT-PCR detected mRNA for both ET_A and ET_B receptors in control experiments using rat kidney RNA. ^{125}I-ET-1 binding was saturable, reaching a plateau at 0.1 nM. Scatchard analysis of the data from saturation studies yielded a straight line, with B_{max} and K_d values of 0.11 pmol/mg and 0.038 nM, respectively. The number of receptors was 6.6×10^{10} sites/mg of protein or 13,200 sites/cell. Cross-linking studies using bis(sulfosuccinimidyl)suberate revealed an apparent molecular mass of 65 kDa for the ET receptor. Labeling of the 65-kDa protein was abolished by ET-1, BQ123, or FR139317 at 0.1 μM. ET-1 stimulated the formation of total inositol phosphates in a dose-dependent manner, with an EC_{50} of 0.1 nM. The phosphatidylinositol hydrolysis response was also inhibited by BQ123 and FR139317. We conclude that MMQ cells express the ET_A receptor, which is coupled to phosphatidylinositol hydrolysis. MMQ cells may be useful for elucidating the mechanisms through which ET exerts its regulatory effects on pituitary cells.

ET, originally isolated from cultured porcine aortic endothelial cells, is a highly potent vasoconstricting peptide with 21 amino acid residues (1). Three distinct members of the ET family, namely, ET-1, ET-2, and ET-3, have been identified through cloning (2). Although the roles of ET *in vivo* are still not fully understood, pharmacological characterization of the ET peptides has led to the suggestion that ETs, especially ET-1, may be involved in a wide range of pathological conditions (3, 4).

The effects of ETs on mammalian organs and cells are initiated by their binding to membrane receptors. High affinity receptors specific for ET are found in various tissues and cells, such as brain, lung, and mesangial cells (5–7). Binding of ET to its receptor triggers a variety of intracellular signaling pathways, including phosphatidylinositol hydrolysis (8–10). Two types of ET receptors, ET_A and ET_B, have been characterized and purified (6, 11) and their cDNA genes have been cloned (12–14). ET_A receptors are selective for ET-1 and ET-2, whereas ET_B receptors bind ET-1, ET-2, and ET-3 with equal potencies.

ET receptors and ET have been identified in the brain (15) and neurohypophysis (16). The effect of ET on Ca^{2+} mobilization and prolactin release in cells prepared from the pituitary has also been actively studied (18–21). The observation that ET modulates the release of prolactin, luteinizing hormone, and follicle-stimulating hormone from pituitary cells *in vitro* suggests that ET may be involved in neuroendocrine regulation *in vivo* (17, 18). In this report, we have identified and characterized ET receptors in MMQ cells, an established cell line cloned from the 7315a transplantable rat pituitary tumor (22). The cell line is similar to primary cells isolated from pituitary with respect to the effect of regulatory agents on cAMP formation, calcium flux, and inositol phosphate formation. Our results indicate that MMQ cells express the ET_A receptor. The receptor is a 65-kDa protein and is coupled to phosphatidylinositol hydrolysis.

Experimental Procedures

Materials. *myo*-[^{3}H]Inositol (12.3 Ci/mmol) was purchased from New England Nuclear Corp. ^{125}I-ET-1 (2000 Ci/mmol) was obtained

ABBREVIATIONS: ET, endothelin; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

from Amersham Corp. ET-1 and ET-3 were purchased from American Peptide Company (Sunnyvale, CA). BQ123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]) and FR139317 (cC6N-L-Leu-D-Trp-Me-D-2Pya-OH) were synthesized in-house. Other reagents were of analytical grade.

Cell culture. MMQ cells were licensed from Dr. Robert M. MacLeod of the University of Virginia and were cultured as described previously (22). Cell viability was examined by the trypan blue exclusion method.

Preparation of membranes from cells. MMQ cells from 150-ml culture flasks were collected by centrifugation (1000 × *g* for 10 min) and were then homogenized in 25 ml of 10 mM HEPES, pH 7.4, containing 0.25 M sucrose and protease inhibitors (3 mM EDTA, 0.1 mM PMSF, and 5 μg/ml pepstatin A), using a micro-ultrasonic cell disruptor (Kontes). The mixture was centrifuged at 1000 × *g* for 10 min. The supernatant was collected and centrifuged at 60,000 × *g* for 60 min. The precipitate was resuspended in buffer A (Earle's solution: 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, buffered with 25 mM HEPES, pH 7.4) containing the aforementioned protease inhibitors and was centrifuged again. The final pellet was resuspended in buffer A containing protease inhibitors and was stored at −80° until used. Protein content was determined by the Bio-Rad dye-binding protein assay.

^{125}I-ET-1 binding to membranes. Binding assays were performed in 96-well microtiter plates that had been pretreated with 0.1% bovine serum albumin. Membranes prepared from cells were diluted ~100-fold in buffer B (20 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4, with 0.2% bovine serum albumin, 0.1 mM PMSF, 5 μg/ml pepstatin A, 0.025% bacitracin, and 3 mM EDTA) to a final concentration of 0.2 or 0.4 mg/ml protein. In saturation studies, membranes (0.04 mg of protein) were incubated for 4 hr at 25° with increasing concentrations of ^{125}I-ET-1 in buffer B (final volume, 0.2 ml). In time course studies, membranes (0.04 mg of protein) were incubated for different periods of time with 0.1 nM ^{125}I-ET-1 in buffer B (final volume, 0.2 ml). In competition studies, membranes (0.02 mg) were incubated for 4 hr at 25° with 0.1 nM ^{125}I-ET-1 in buffer B (final volume, 0.2 ml) in the presence of increasing concentrations of unlabeled ET-1, ET-3, BQ123, or FR139317. After incubation, unbound ligand was separated from bound ligand by vacuum filtration using glass fiber filter strips in PHD cell harvesters (Cambridge Technology, Inc.), followed by three washes of the filter strips with saline (1 ml). Nonspecific binding was determined in the presence of 1 μM ET-1.

^{125}I-ET-1 cross-linking studies. Membranes (0.7 mg) in 0.1 ml of buffer A with protease inhibitors were incubated with 1 nM ^{125}I-ET-1 for 4 hr at 25°. Incubation was terminated by addition of 1 ml of ice-cold PBS containing protease inhibitors (3 mM EDTA, 0.1 mM PMSF, and 5 μg/ml pepstatin A), and the mixture was centrifuged at 20,000 × *g* for 15 min. ^{125}I-ET-1 was cross-linked to the receptor by resuspending the pellet in 0.5 ml of PBS containing 0.2 mM bis(sulfosuccinimidyl)suberate, followed by incubation for 20 min at 25°. The reaction was terminated by the addition of 0.5 ml of 1 M Tris, pH 6.8, containing 0.4 M EDTA. Membranes were collected by centrifugation and analyzed by SDS-PAGE. Gels were sliced into 2-mm pieces and radioactivity in each piece was determined.

Measurement of phosphatidylinositol hydrolysis. MMQ cells (0.4×10^6 cells/ml) were labeled for 16 hr with 10 μCi/ml *myo*-[^{3}H] inositol in RPMI 1640 medium. Cells were washed with PBS and then incubated with buffer A containing protease inhibitors (as described above) and 10 mM LiCl for 60 min before challenge with ET-1. ET-1 challenge was terminated by the addition of 1.5 ml of chloroform/methanol (1:2, v/v). Total inositol phosphates were extracted after addition of chloroform and water to give final proportions of chloroform/methanol/water of 1:1:0.9 (v/v/v), as described by Berridge *et al.* (23). The upper aqueous phase (1 ml) was retained and a small portion (100 μl) was counted. The rest of the aqueous sample was analyzed by batch chromatography using the anion exchange resin AG1-X8 (Bio-Rad).

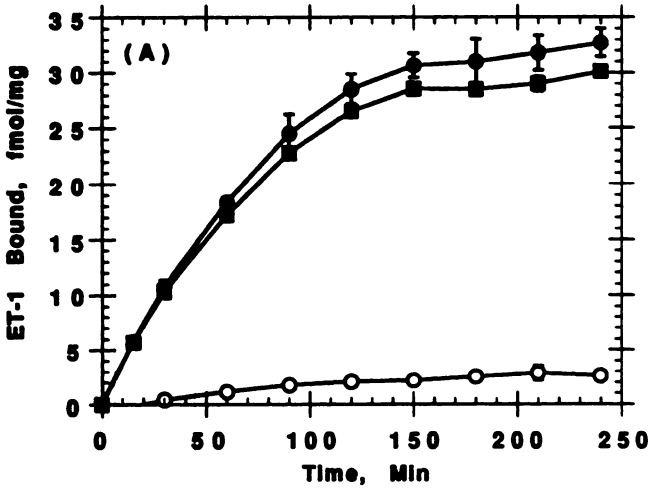
RNA isolation. Total RNA was isolated from MMQ cells by a modification of the LiCl-urea method (24). Briefly, ~70×10^6 cells were dissolved in 6.0 ml of RNA lysis buffer (3 M LiCl, 6 M urea, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.1 M β-mercaptoethanol, 0.1% sarkosyl). The sample was centrifuged at 5000 × *g* for 90 min. The pellet was resuspended in 5.0 ml of 3 M LiCl and centrifuged again. The pellet was resuspended in 3.0 ml of RNA-proteinase K solution (10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% SDS, 200 μg/ml proteinase K), extracted twice with equal volumes of phenol/$CHCl_3$ (1:1, v/v), and then extracted twice with $CHCl_3$. The aqueous layer, containing the RNA, was precipitated with ethanol.

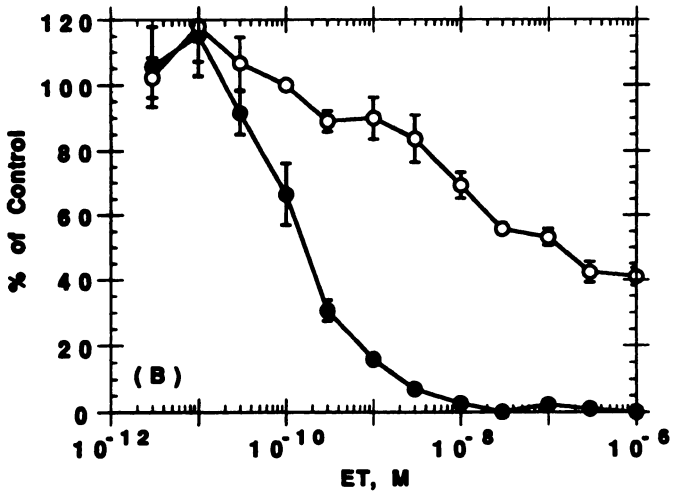
RT-PCR. The RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer Cetus) according to the manufacturer's instructions. Briefly, 1.0 μg of total RNA from MMQ or rat kidney was reverse transcribed using random hexamer primers. Each PCR cycle consisted of 1 min at 94° (denaturation), 2 min at 60° (annealing), and 3 min at 72° (extension). The cDNA was amplified for 30 cycles using one of the following sets of primers. Four primer sets, based on published sequences of rat ET_A and ET_B receptors (13, 14), were synthesized in-house [set 1 (ET_A), 5′-TCCTTTTGGCTGGCCCTGGTG-3′ and 5′-CAATCCGCAAGCTCCCATTCCT-3′; set 2 (ET_B), 5′-GACGCGCCTTGGTGGCGCTG-3′ and 5′-AACTGCATGAAGGCTGTTTTCTG-3′; set 3 (ET_B), 5′-AGCTGGTGCCCTTCATACAGAAGGC-3′ and 5′-TGCACACCTTTCCGCAAGCACG-3′ (25); set 4 (ET_B), 5′-CGAGCTGTTGCTTCTTGGAGTCGA-3′ and 5′-AGGACCAGGCAGAATACTGTCTTGG-3′ (26)]. The RT-PCR samples were analyzed by standard agarose gel electrophoresis.

Results

To characterize ET receptors in MMQ cells, radioligand binding studies using ^{125}I-ET were performed. In intact cells, ET-1 has been shown to bind to receptors initially and then be rapidly internalized (19, 27). To avoid the complication of receptor internalization and to determine binding parameters under steady state conditions, membranes prepared from cells were used in the present study. Fig. 1A shows that ^{125}I-ET-1 bound to membranes from MMQ cells in a time-dependent manner, reaching a steady state at 150 min at 25°. Addition of 1 μM ET-1 at time 0 blocked >90% of ^{125}I-ET-1 binding. Fig. 1B shows the results from competition studies in which unlabeled ET-1 and ET-3 at various concentrations were used to compete against ^{125}I-ET-1 binding. ET-1 at 10 nM completely abolished specific ^{125}I-ET-1 binding. The IC_{50} value for ET-1 was 0.17 nM. ^{125}I-ET-1 binding was partially inhibited by ET-3; only 60% of specific binding was inhibited by 1 μM ET-3. To further characterize the receptor, binding studies using ^{125}I-ET-3 were performed. No specific binding could be detected when membranes were incubated with ^{125}I-ET-3 (ranging from 68 pM to 570 pM) for 6 hr at 25° (data not shown). Specific binding was defined as the difference in membrane-associated radioactivity between the presence and absence of high concentrations of ET-3 (1–10 μM). Furthermore, BQ123 (28) and FR139317 (29), two selective antagonists for the ET_A receptor, were used to study the subtype of the ET receptors in MMQ cells. As shown in Fig. 1C, BQ123 and FR139317 inhibited ^{125}I-ET-1 binding in a dose-dependent manner, with IC_{50} values of 5 and 0.9 nM, respectively. ^{125}I-ET-1 binding was completely inhibited by either antagonist at 0.1 μM. These results suggest that ET receptors identified in MMQ cells are likely to be the ET_A type.

The number of ET binding sites per cell was determined in saturation studies. Fig. 2A shows that ^{125}I-ET-1 binding reached a plateau at 0.1 nM ET-1. The results were fitted by the radioligand binding analysis programs EBDA and LIGAND (Biosoft). Scatchard analysis (Fig. 2B) yielded one straight line,





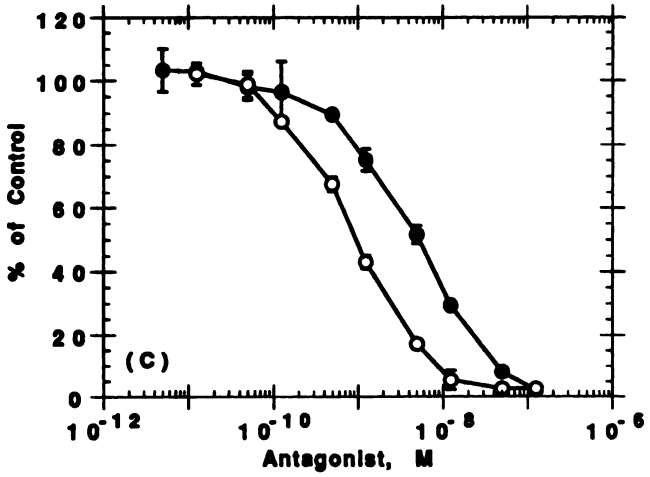
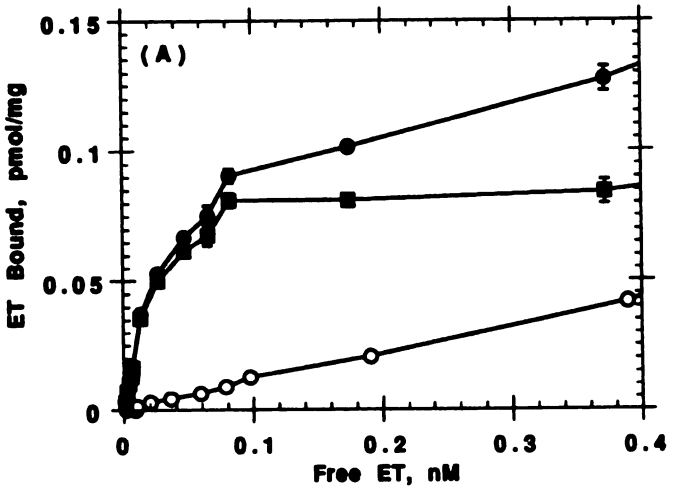


Fig. 1. Binding of ^{125}I-ET-1 to MMQ cell membranes. A, Time course study. Membranes (0.04 mg) were incubated for different periods of time at 25° with ^{125}I-ET-1 (0.05 nM) in the absence (●) or presence (○) of 1 μM unlabeled ET-1. Specific binding (■) was determined by subtraction of nonspecific binding (○) from total binding (●). B, ET-1 and ET-3 competition studies. Membranes (0.02 mg) were incubated for 4 hr at 25° with ^{125}I-ET-1 (0.05 nM) in the presence of increasing concentrations of unlabeled ET-1 (●) or ET-3 (○). C, BQ123 and FR139317 competition studies. MMQ membranes (0.02 mg) were incubated for 4 hr at 25° with ^{125}I-ET-1 (0.05 nM) in the presence of increasing concentrations of BQ123 (●) or FR139317 (○). Results are expressed as a percentage of control (specific binding in the absence of unlabeled ET-1). Nonspecific binding, determined in the presence of 1 μM ET-1, was subtracted from total binding to give specific binding. Each value represents the mean ± standard deviation of three determinations. Specific ^{125}I-ET-1 binding in the absence of unlabeled ligand was 0.151 ± 0.009 pmol/mg in B and 0.139 ± 0.006 pmol/mg in C.



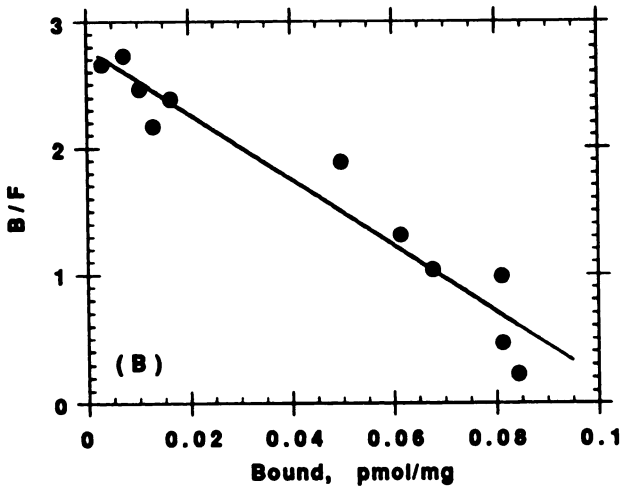


Fig. 2. Saturation studies. A, Membranes (0.04 mg) were incubated for 4 hr at 25° with increasing concentrations of ^{125}I-ET-1 in the absence (●) or presence (○) of 1 μM unlabeled ET-1. Specific binding (■) was determined by subtraction of nonspecific binding (○) from total binding (●). Each value represents the mean ± standard deviation of three determinations. B, Scatchard analysis of the data in A.

with B_{max} and K_d values of 0.11 pmol/mg and 0.038 nM, respectively. The Hill coefficient was calculated to be 0.921. The number of receptors was calculated to be 6.6×10^{10} sites/mg of protein. The protein content in membranes prepared from 1×10^6 cells was approximately 0.2 mg. Based on this number, the calculated number of receptor sites was 13,200 sites/cell.

RT-PCR experiments were performed to further examine whether MMQ cells indeed express ET_A receptors. Fig. 3 shows the result of these RT-PCR experiments. When primers for the ET_A receptor (set 1) were used with MMQ RNA, a specific DNA band migrating at the expected size of 852 bp was observed (Fig. 3, *lane 2*). This fragment was subsequently isolated, cloned, and sequenced, verifying that the PCR product was derived from the rat ET_A receptor (data not shown). No DNA band could be detected when the primers were omitted or when

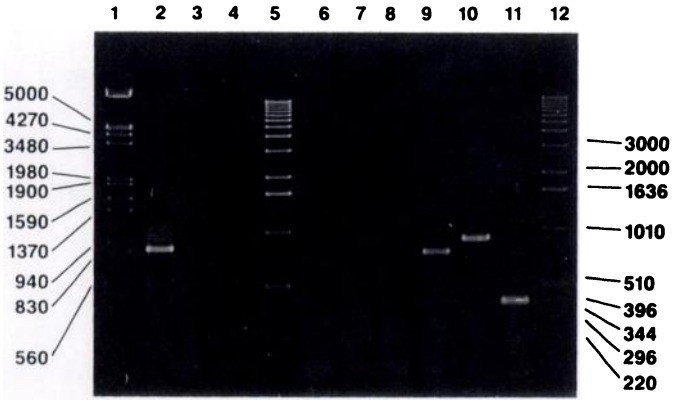


Fig. 3. Detection of ET receptor mRNA in MMQ cells by RT-PCR. Total RNA was isolated from MMQ cells or rat kidneys and RT-PCR was conducted as described in Experimental Procedures. Samples were analyzed by using a 1.4% agarose gel and ethidium bromide staining. *Lane 1*, DNA size markers (*Eco*RI/*Hind*III-digested λ DNA); *lane 2*, MMQ RNA with ET_A primers (set 1); *lane 3*, MMQ RNA without addition of primers; *lane 4*, MMQ RNA without addition of reverse transcriptase; *lane 5*, DNA size markers (1.0-kilobase DNA ladder; GIBCO BRL); *lanes 6*, *7*, and *8*, MMQ RNA with ET_B primer sets 2, 3, and 4, respectively; *lanes 9*, *10*, and *11*, rat kidney RNA with ET_B primer sets 2, 3, and 4, respectively; *lane 12*, DNA size markers (1.0-kilobase DNA ladder; GIBCO BRL).

the reverse transcriptase was omitted from the reaction mixture (Fig. 3, *lanes 3* and *4*). In contrast, when primers for the ET_B receptor (sets 2, 3, and 4) were used with MMQ RNA, no specific DNA band could be detected (Fig. 3, *lanes 6*, *7*, and *8*). This observation was confirmed by performing RT-PCR with the ET_B receptor primer sets and total RNA prepared from rat kidney, in a series of control experiments. As shown in Fig. 3, *lanes 9*, *10*, and *11*, specific DNA bands migrating at the expected sizes of 774 bp (set 2), 918 bp (set 3), and 388 bp (set 4) were observed. It was reported previously that rat kidney possesses ET_B receptors (25–26, 30). These results demonstrate that MMQ cells express ET_A receptor mRNA but not ET_B receptor mRNA, consistent with the results obtained from binding studies.

The molecular mass of the ET receptor in MMQ cells was determined by cross-linking studies using membranes prepared from cells. Fig. 4 shows that two proteins, with molecular masses of 65 and 44 kDa, were labeled with ^{125}I-ET-1. Fig. 4A shows that labeling of the 65-kDa protein was completely abolished by 0.1 μM ET-1, but labeling of the 44-kDa protein was not affected by 0.1 μM ET-1 and was only partially reduced (36%) in the presence of 1 μM ET-1. We have also tested the inhibitory effect of BQ123 and FR139317 in cross-linking studies. The results (Fig. 4B) are similar to those shown above. Labeling of the 65-kDa protein was completely abolished by 0.1 μM BQ123 or FR139317, but labeling of the 44-kDa protein was not affected. In contrast, ET-3 at 1 μM reduced labeling of both the 65- and 44-kDa proteins by <40% (Fig. 4C). These results suggest that the ET_A receptor in MMQ cells has a molecular mass of 65 kDa.

The function of the ET receptor was tested by examining the effect of ET-1 on phosphatidylinositol hydrolysis. Fig. 5, A and B, shows that ET-1 stimulated phosphatidylinositol hydrolysis in a time- and dose-dependent manner. A 350–450% increase in the formation of total inositol phosphates was observed after cells were incubated with 1 nM ET-1 for 30 min (Fig. 5, A and B). The EC_{50} of ET-1 was 0.1 nM. The formation

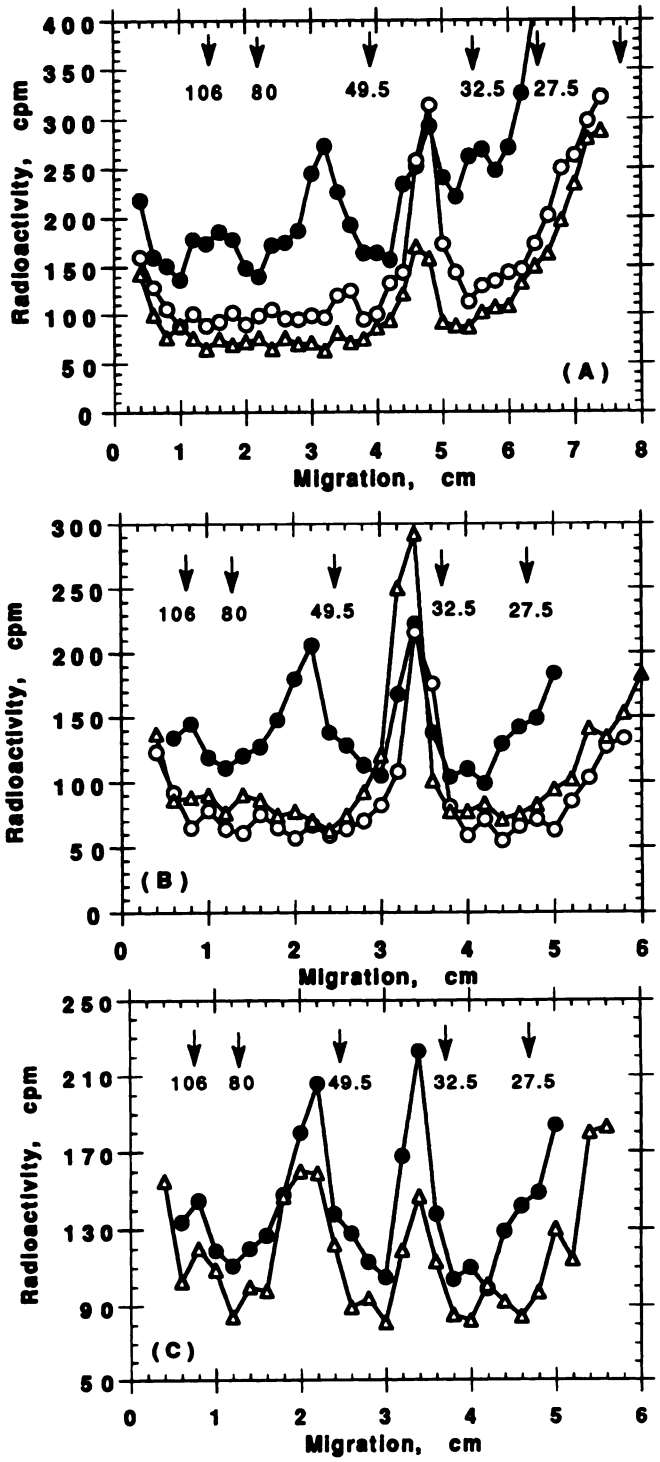


Fig. 4. Cross-linking of ^{125}I-ET-1 to the receptor. Membranes (0.7 mg) prepared from MMQ cells were incubated with 1 nM ^{125}I-ET-1, followed by washing with 1 ml of PBS with protease inhibitors. Bound ^{125}I-ET-1 was cross-linked to the receptor with bis(sulfosuccinimidyl)suberate. A, Samples incubated in the absence (●) or presence of 0.1 μM (○) or 1 μM (△) unlabeled ET-1 were analyzed by 10% SDS-PAGE. B, Samples incubated in the absence (●) or presence of 0.1 μM unlabeled BQ123 (○) or FR139317 (△) were analyzed by 12.5% SDS-PAGE. C, Samples incubated in the absence (●) or presence (△) of 1 μM unlabeled ET-3 were analyzed by 12.5% SDS-PAGE. Radioactivity in the various protein bands was determined by slicing gels into 2-mm pieces, followed by counting in a LKB γ counter. High radioactivity was found near the origins and in the second half of the gel and is not shown.

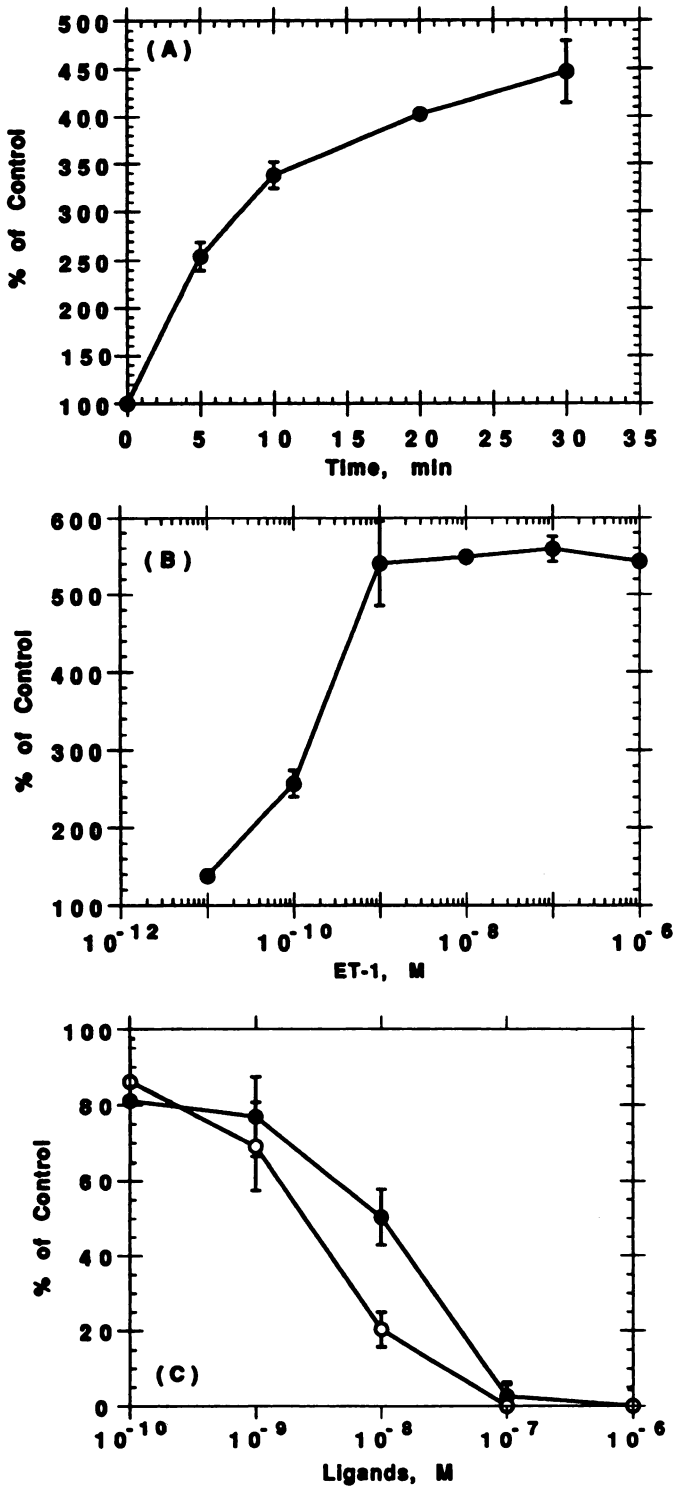


Fig. 5. Effect of ET-1 on phosphatidylinositol hydrolysis. MMQ cells (~2.9 × 10^5 cells/sample) in RPMI were prelabeled with *myo*-[^{3}H]inositol (10 μCi/ml) for 16–24 hr. A, Time course study. Cells (0.3 ml in buffer A with 10 mM LiCl) were challenged with 10 nM ET-1 at 37° for different periods of time. Results were calculated by normalizing AG1-X8-bound radioactivity at each point to that of control (time 0). The amount of inositol phosphates bound to AG1-X8 anion exchanger resin at time 0 (68 ± 3 cpm/sample) was 0.31 ± 0.02% of total extracted radioactivity (22,157 ± 327 cpm). B, Concentration dependence. Cells were challenged for 30 min at 37° with various concentrations of ET-1 in the presence of 10 mM LiCl. Results were calculated by normalizing AG1-X8-bound radioactivity at each point to that of control (no addition of ET). C, Effects of BQ123 and FR139317. Cells were challenged for 30 min at 37° with 1 nM ET-1 in the presence of various concentrations of BQ123 (●) or FR139317 (○). The amount of inositol phosphates bound to AG1-X8 anion exchanger resin without ET-1 treatment was 84 ± 7 cpm/sample. ET-1 at 1 nM without addition of antagonist stimulated the formation of inositol phosphates to 357 ± 12%, compared with samples without ET-1 treatment. Results were calculated by normalizing the percentage of increase in the formation of inositol phosphates at each point to that of control (257% increase with 1 nM ET-1 without addition of antagonists). Each value represents the mean ± standard deviation of three determinations.

of inositol phosphates in the absence of ET was in the range of 60–100 cpm and was 0.30–0.50% of total extracted radioactivity. Fig. 5C shows that the effect of ET-1 on phosphatidylinositol hydrolysis was antagonized by BQ123 and FR139317. When ET-1 at 1 nM was used to challenge cells, both antagonists at 0.1 μM completely inhibited the stimulatory effect of ET-1. The IC_{50} values were 10 and 2.5 nM for BQ123 and FR139317, respectively.

Discussion

Radioligand binding studies have been used to characterize the subtypes of ET receptors in various tissues and cells, including aorta, atrium, cerebellum, kidney, liver, and lung (31). By molecular cloning, two subtypes of ET receptors, ET_A and ET_B, were identified (12–14). ET_A receptors are selective for ET-1 and ET-2, whereas ET_B receptors bind ET-1, ET-2, and ET-3 with equal potencies. In this report, by combining various techniques such as radioligand binding, cross-linking, and RT-PCR techniques, we demonstrate that MMQ cells express predominantly the ET_A receptor. The ET_A receptor in these cells exhibits a molecular mass of 65 kDa and is linked to the intracellular signaling pathway involving phosphatidylinositol hydrolysis.

All the binding studies shown in this report were performed using membranes prepared from cells, to avoid the complication of receptor internalization in intact cells. It was shown previously that, at 37°, ET-1 binds to receptors initially and then is rapidly internalized (19, 27). Even at 4°, receptor internalization is usually 10% of total cellular ET binding (27). By using membranes, we demonstrated that ^{125}I-ET-1 binding in MMQ cells was completely inhibited by unlabeled ET-1 and by the ET_A-selective antagonists BQ123 and FR139317. Based on a K_d value of 0.038 nM for ET-1 (Fig. 2), K_i values for BQ123 and FR139317 were determined to be 2.16 nM and 0.39 nM, respectively. RT-PCR studies using primers for the ET_A receptor detected a specific DNA band in the MMQ cells. However, primers for the ET_B receptor failed to detect any specific DNA band in MMQ cells, although they detected the correct DNA band in rat kidney, which expresses the ET_B receptor (25–26, 30). These results confirm the observation that the ET_A receptor is likely the only subtype expressed by MMQ cells. Nevertheless, we could not exclude the possibility that the ET_B receptor and its mRNA were present at such low levels that they were not detectable in our assays.

The cross-linking results revealed that the ET receptor has an apparent molecular mass of 65 kDa in MMQ cells. Although cross-linking studies using ^{125}I-ET-1 have been conducted to determine the molecular masses of ET receptors in various cells and tissues (31), our report is the first to show the molecular mass of the ET_A receptor in cells that originated from the pituitary. The efficiency of cross-linking in our studies was 0.4%, as determined by the percentage of radioactivity

incorporated into the 65-kDa protein. The release of bound but not cross-linked ^{125}I-ET-1 after samples were solubilized in SDS-PAGE sample buffer may have contributed to the high radioactive background, especially in the beginning and the lower half of the gel (Fig. 4). We have tested free ^{125}I-ET-1 under the same reducing conditions in SDS-PAGE and observed radioactivity accumulation in the lower half of the gel, presumably due to the aggregation of ET after disulfide bond rearrangement. Labeling of the 65-kDa protein was inhibited by ET-1, BQ123, and FR139317, suggesting that this protein is the ET_A receptor. The nature of the 44-kDa protein is not clear at this stage. Because labeling of this protein was not affected by ET-1, BQ123, or FR139317 at high concentrations, it probably represents nonspecific labeling. Labeling of proteins of diverse sizes (32–128 kDa) has been reported for the ET receptor (6, 11). In rat brain two proteins, with molecular masses of 50 and 30 kDa, were labeled, but in rat atrium and rat osteoblastic cells a 70-kDa protein was labeled by ET-1 (31). These results indicate heterogeneity of the ET receptor among different species and tissues, which may result from differential post-translational modifications (6, 11).

Our results indicate that the ET_A receptor in MMQ cells is linked to the intracellular signaling pathway involving phosphatidylinositol hydrolysis. Without any stimulus, the formation of inositol phosphates in MMQ cells was low (in the range of 60–100 cpm/0.3 × 10^6 cells). After treatment of cells with 1 nM ET-1 for 30 min, a ~300% increase in the formation of inositol phosphates was observed. Coupling of the ET_A receptor to phosphatidylinositol hydrolysis and Ca^{2+} mobilization was previously demonstrated in 3-day cultured rat pituitary cells (20). Preliminary studies from our laboratory also indicated that ET stimulated Ca^{2+} mobilization in MMQ cells. These results suggested that MMQ cells are similar to primary cells isolated from pituitary, with respect to the effect of regulatory agents on the intracellular signaling pathway.

The identification of the ET receptor and ET in the pituitary gland (16) has stimulated much interest in studying the potential role of ET in neuroendocrine regulation. However, recent reports using primary cultured cells prepared from anterior pituitary glands to study ET receptor subtypes and the effects of ET on hormone release have been contradictory. For example, some studies suggest that ET stimulates prolactin secretion, but others demonstrate the opposite (17, 19, 21). It remains controversial whether the action of ET on hormone secretion involves Ca^{2+} mobilization (18, 20, 21). Furthermore, it is not known conclusively whether the pituitary cells contain mainly ET_A receptors or both ET_A and ET_B receptors. The fact that ET-1 and ET-3 affect hormone secretion implies that the pituitary cells contain both ET_A and ET_B receptors (18, 21), although Stojilkovic *et al.* (20) have reported that the ET_A receptor may be the more abundant subtype in these cells. Because cultured pituitary cells are heterogeneous, it is difficult to obtain conclusive answers to the questions described above. By using MMQ cells, which are homogeneous in nature, we provide strong evidence that these cells express mainly ET_A receptors. The effects of ET-1 and ET-3 on prolactin secretion in these cells are being investigated. MMQ cells should prove useful for evaluating the effects of ET peptides on hormone secretion and elucidating the mechanisms through which ET exerts its regulatory effects on pituitary cells.

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