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MOLECULAR PHARMACOLOGY, 44:422–429

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Phosphoramidon Modulates the Number of Endothelin Receptors in Cultured Swiss 3T3 Fibroblasts

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Received December 14, 1992; Accepted May 17, 1993

SUMMARY

Endothelin (ET) is generated from prepro-ET by dibasic pair proteolysis, followed by specific proteolytic cleavage between Trp^{21} and Val^{22}. Currently, intense research efforts are focused on the investigation of a metalloprotease-like ET-converting enzyme that is inhibited by phosphoramidon but not by other inhibitors of neutral metalloproteases. In this report, we show that ET binding was increased significantly in cultured Swiss 3T3 fibroblasts after phosphoramidon treatment. Saturation studies using membranes prepared from cells or using intact cells assayed at 4° showed that B_{max} increased from 0.13 pmol/mg or 0.038 pmol/1 × 10^6 cells in untreated cells to 0.66 pmol/mg or 0.22 pmol/1 × 10^6 cells in cells treated with 100 μM phosphoramidon for 24 hr, equivalent to a net increase of 100,000 ET binding sites/cell. The effect of phosphoramidon was time and dose dependent. Other protease inhibitors, such as thiorphan, pepstatin A, E-64, phenylmethylsulfonyl fluoride, bestatin, and leupeptin, failed to exert a similar effect. Reverse phase high performance liquid chromatography analysis indicated that the effect of phosphoramidon was not due to inhibition of ^{125}I-ET-1 degradation. The effect of phosphoramidon remained evident after cells were treated with actinomycin D or cycloheximide to inhibit protein synthesis, suggesting that the phenomenon was not due to the effect of phosphoramidon stimulating the synthesis of ET receptors. Degradation studies suggested that the effect of phosphoramidon was due to inhibition of a protease responsible for degrading the ET receptor. The fact that Swiss 3T3 cells treated with phosphoramidon exhibit an increase in the number of ET receptors is likely to complicate the interpretation of results when phosphoramidon or related compounds are used to block the putative ET-converting enzyme.

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, the pharmacological characterization of 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, mesangial cells, and fibroblasts (5–8). Binding of ET to its receptor triggers a variety of intracellular signaling pathways, including phosphatidylinositol hydrolysis (9–11). Two ET receptor subtypes have been characterized (6, 12) and their cDNAs and/or genes have been cloned and sequenced (13–15).

ET is thought to be generated from its precursor, prepro-ET. A biosynthetic pathway has been proposed in which prepro-ET is first processed by dibasic pair proteolysis to produce a 39-amino acid intermediate form (big ET), which is then converted to ET by a specific proteolytic cleavage between Trp^{21} and Val^{22} (1). The identification of the putative converting enzyme has been the focus of intense research, under the hypothesis that the development of inhibitors for the converting enzyme will provide an effective means for preventing the production of ET and thereby modulating the pathogenic effects of ET (16). Presently, the primary emphasis is on the investigation of a metalloprotease-like converting enzyme that is specifically inhibited by phosphoramidon but not by other known inhibitors of neutral metalloproteases. The development of phosphoramidon-like inhibitors for this metalloprotease is being aggressively pursued. Although the effect of phosphoramidon in inhibiting the generation of ET from big ET has been examined in crude enzyme assays, in cultured cells, and in animal studies (17–21), no detailed studies have been conducted to address the question of whether the phosphoramidon treatment has any effect on ET receptors. Frequent use of phosphoramidon in the investigation of the pathophysiological roles of ET prompted us to investigate the effect of phosphoramidon on ET receptors. Using cultured Swiss 3T3 cells as a model, in this report we demonstrate that cells exposed to phosphoramidon for 24 hr have significantly increased numbers of ET binding sites per

ABBREVIATIONS: ET, endothelin; Gpp(NH)p, guanylimidodiphosphate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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cell. This effect is unique for phosphoramidon, because other protease inhibitors, including thiorphan, fail to exert a similar effect.

Experimental Procedures

Materials. ^{125}I-ET-1 (2000 Ci/mmol) was obtained from Amersham Corp. ET-1 was purchased from American Peptide Company (Sunnyvale, CA). Phosphoramidon was from Sigma Chemical Co. Other reagents were of analytical grade.

Cell culture. Swiss 3T3 cells were plated in 48-well plates at an initial density of 0.05 × 10^6 cells/well and were grown in Dulbecco's modified minimal essential medium containing 10% fetal calf serum (GIBCO). Phosphoramidon or other protease inhibitors were added to confluent monolayer cells (without changing the medium) at a final concentration of 100 μM, or as indicated, for 24 hr at 37°. Cell viability was examined by the trypan blue exclusion method.

^{125}I-ET-1 binding to cells. Cells with or without pretreatment with protease inhibitors were incubated for 1 hr at 37° with 0.2 ml/well of 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 ^{125}I-ET-1 (4 nM). To determine B_{max} and K_d values, cells with or without pretreatment with 100 μM phosphoramidon for 24 hr were incubated for 6 hr at 4° with 0.2 ml/well of buffer A containing various concentrations of ^{125}I-ET-1, in the presence of protease inhibitors (5 μg/ml pepstatin A, 0.01 mM phosphoramidon, 0.01 mM PMSF, and 0.025% bacitracin). After the incubation, cells were washed with PBS (0.5 ml/well), followed by solubilization in 0.5 ml of 0.1 N NaOH. Nonspecific binding was assayed in the presence of 1 μM ET-1.

Reverse phase HPLC. Cells with or without phosphoramidon treatment (100 μM, 24 hr) were incubated for 1 hr at 37° with 0.2 ml/well of buffer A containing ^{125}I-ET-1 (4 nM). After the incubation, supernatants were collected and concentrated to dryness by using a SpeedVac (Savant). Samples were redissolved in solvent A (10% acetonitrile/0.1% trifluoroacetic acid) and analyzed on a Microsorb AAAnalysis column (4.6 mm × 100 mm; Rainin), using a Pharmacia-LKB HPLC system (model 2152). The column was eluted with a linear gradient of 100% solvent A at time 0 to 100% solvent B (60% acetonitrile/0.1% trifluoroacetic acid) at 30 min. Fractions (1 ml/min/fraction) were collected and the radioactivity in each fraction was determined.

Preparation of membranes from cells. Confluent monolayers of cells, in T-150 culture flasks, that had been pretreated with or without 100 μM phosphoramidon for 24 hr were scraped into PBS. Cells were collected by centrifugation and then homogenized in 25 ml of 10 mM HEPES, pH 7.4, containing 0.25 M sucrose and protease inhibitors (3 mM EDTA, 100 μM PMSF, and 5 μg/ml pepstatin A), using a microultrasonic 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 20 mM Tris, pH 7.4, with the aforementioned protease inhibitors and was centrifuged again. The final pellet was resuspended in 20 mM Tris, pH 7.4, with the aforementioned protease inhibitors and was stored at −80° until used. In degradation studies, membranes were prepared according to the protocol described above but without the addition of various protease inhibitors. Protein contents were assayed 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 (0.02 mg of protein) prepared from cells with or without the phosphoramidon (100 μM, 24 hr) treatment were incubated for 4 hr at 25° with ^{125}I-ET-1 at the indicated concentrations, in 0.2 ml/well of buffer B (20 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4, with 0.2% bovine serum albumin, 100 μM PMSF, 5 μg/ml pepstatin A, 0.025% bacitracin, and 3 mM EDTA). After the incubation, unbound ligand was separated from bound ligand by a vacuum filtration method using glass fiber filter strips in PHD cell harvesters (Cambridge Technology, Inc.), followed by three 1-ml saline washes. Nonspecific binding was assayed in the presence of 1 μM ET-1.

Results

The effect of phosphoramidon on ET binding to the receptor was first examined in intact cells. Fig. 1A shows that the amount of ^{125}I-ET-1 bound was 0.416 ± 0.037 pmol/1 × 10^6 cells in control cells (without phosphoramidon pretreatment). This amount remained the same in the first 3 hr of phosphoramidon treatment and increased to 0.574 ± 0.069 pmol/1 × 10^6 cells after cells had been treated with 100 μM phosphoramidon for 5 hr. At 24 hr, the amount of ET bound was 0.743 ± 0.020 pmol/1 × 10^6 cells, indicating a net increase of 0.327 pmol/1 × 10^6 cells, compared with untreated cells. The effect of phosphoramidon was not only time dependent but also dose dependent. As shown in Fig. 1B, without any exposure to phosphoramidon the amount of ^{125}I-ET-1 bound was 0.320 ± 0.042 pmol/1 × 10^6 cells. This amount gradually increased when cells were exposed to increasing concentrations of phosphoramidon and reached a plateau at approximately 25 μM phosphoramidon. ^{125}I-ET-1 binding with 100 μM phosphoramidon was 0.573 ±

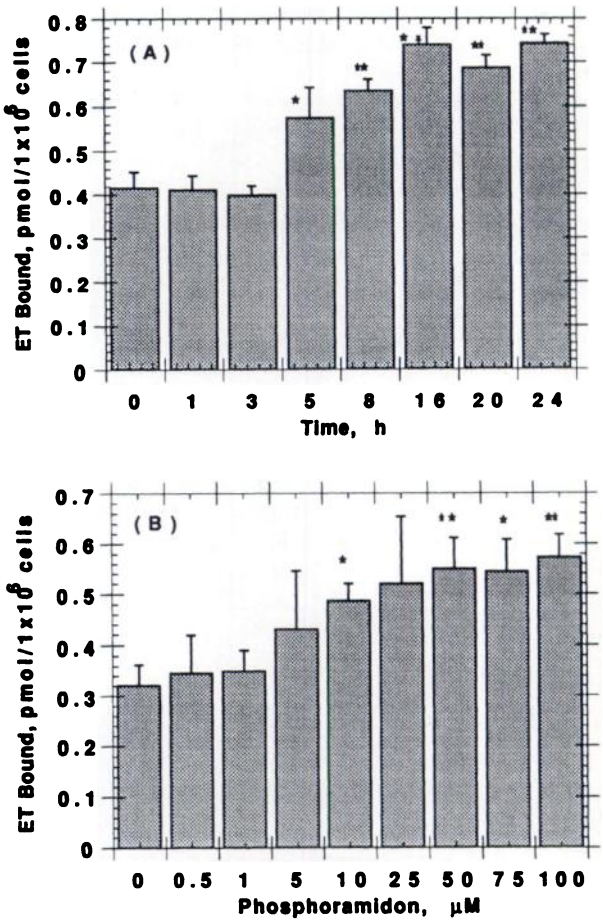

Fig. 1. Effect of phosphoramidon on ^{125}I-ET binding. A, Time course. Confluent monolayers of cells in 48-well plates were pretreated with phosphoramidon at 100 μM for various periods of time before incubation with ^{125}I-ET-1 (4 nM) for 1 hr at 37°. B, Dose-dependent studies. Cells were pretreated with phosphoramidon at different concentrations for 24 hr before incubation with ^{125}I-ET-1 (4 nM) for 1 hr at 37°. Nonspecific binding was determined in the presence of 1 μM ET-1 and was 0.0180 ± 0.0051 pmol/1 × 10^6 cells for both phosphoramidon-treated and untreated cells. Values shown were obtained by subtraction of nonspecific binding from total binding. Each value represents the mean ± standard deviation of at least three determinations. *, $p < 0.05$; **, $p < 0.01$, when data were compared with control (untreated cells) using the paired *t* test in StatView II (Abacus Concepts, Inc., Berkeley, CA).

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0.045 pmol/1 × 10^6 cells, indicating a net increase of 0.253 pmol/1 × 10^6 cells. Phosphoramidon, at doses ranging from 10 to 100 μM, has been used to inhibit the conversion of big ET to ET in cultured cells (16–21). The numbers of viable cells in untreated and phosphoramidon-treated wells were compared, and no difference was observed.

To explore whether other protease inhibitors exert similar effects, ET binding to receptors was compared after 3T3 fibroblasts were treated with various protease inhibitors for 24 hr. Although the efficacies of different inhibitors toward their target proteases are different, for the purpose of comparison and to maximize the potential effect each inhibitor was tested at 100 μM. ^{125}I-ET-1 binding to cells without phosphoramidon treatment was 0.388 ± 0.027 pmol/1 × 10^6 cells. In phosphoramidon-treated cells, the binding was increased to 0.680 ± 0.057 pmol/1 × 10^6 cells, exhibiting a net increase of 0.292 pmol/1 × 10^6 cells, compared with cells without phosphoramidon treatment. Other protease inhibitors, such as bestatin, PMSF, E-64, thiorphan, and pepstatin A, failed to exert a similar effect (Fig. 2). It is important to note that thiorphan, another metalloprotease inhibitor, also failed to cause a similar increase.

To elucidate whether the increase in ET-1 binding was the result of phosphoramidon inhibiting ^{125}I-ET degradation, the integrity of ^{125}I-ET-1 after incubation with untreated or phosphoramidon-treated cells was compared by reverse phase HPLC. First, ^{125}I-ET-1 (0.8 pmol) that had not been exposed to cells was analyzed. A typical result is shown in Fig. 3A, in which 90% of the radioactivity was eluted in two peaks, a minor one at 3 min and a major one at 22 min (three experiments). The distribution of radioactivity was 13 and 77% for the minor and major peaks, respectively. The radioactivity eluted at 3 min may be due to free radioactive iodine or a degradation product of ^{125}I-ET-1 produced during storage. Next, ^{125}I-ET-1 was incubated with control cells (without phosphoramidon pretreatment) for 1 hr at 37°, and then the supernatant was retained for analysis by HPLC. The result (Fig. 3B) was essentially the same as that shown in Fig. 3A. Furthermore, ^{125}I-ET-1 was incubated with phosphoramidon-treated cells (100 μM, 24 hr) for 1 hr at 37°, and the supernatant was retained for analysis (Fig. 3C). The result was similar to that obtained with untreated cells. Supernatants from phosphoramidon-treated cells, perhaps due to increased ^{125}I-ET-1 binding to cells, contained ~10% less radioactivity than did those from untreated cells. The amounts of ^{125}I-ET-1 bound were 0.559 ± 0.058 and 0.220 ± 0.034 pmol/1 × 10^6 cells for phosphoramidon-treated and untreated cells, respectively. Taken together, these results

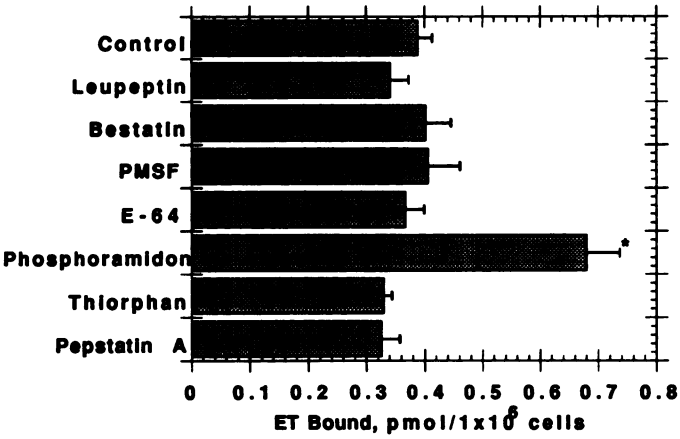

Fig. 2. Effect of protease inhibitors on ^{125}I-ET binding. Cells in 48-well plates were pretreated with various protease inhibitors at 100 μM for 24 hr before incubation with ^{125}I-ET-1 (4 nM) for 1 hr at 37°. Nonspecific binding was determined in the presence of 1 μM ET-1 and was 0.0183 ± 0.0089 pmol/1 × 10^6 cells for both treated and untreated cells. Values shown were obtained by subtraction of nonspecific binding from total binding. Each value represents the mean ± standard deviation of at least three determinations. *, $p < 0.05$, when data were compared with control (untreated cells) using the paired t test.

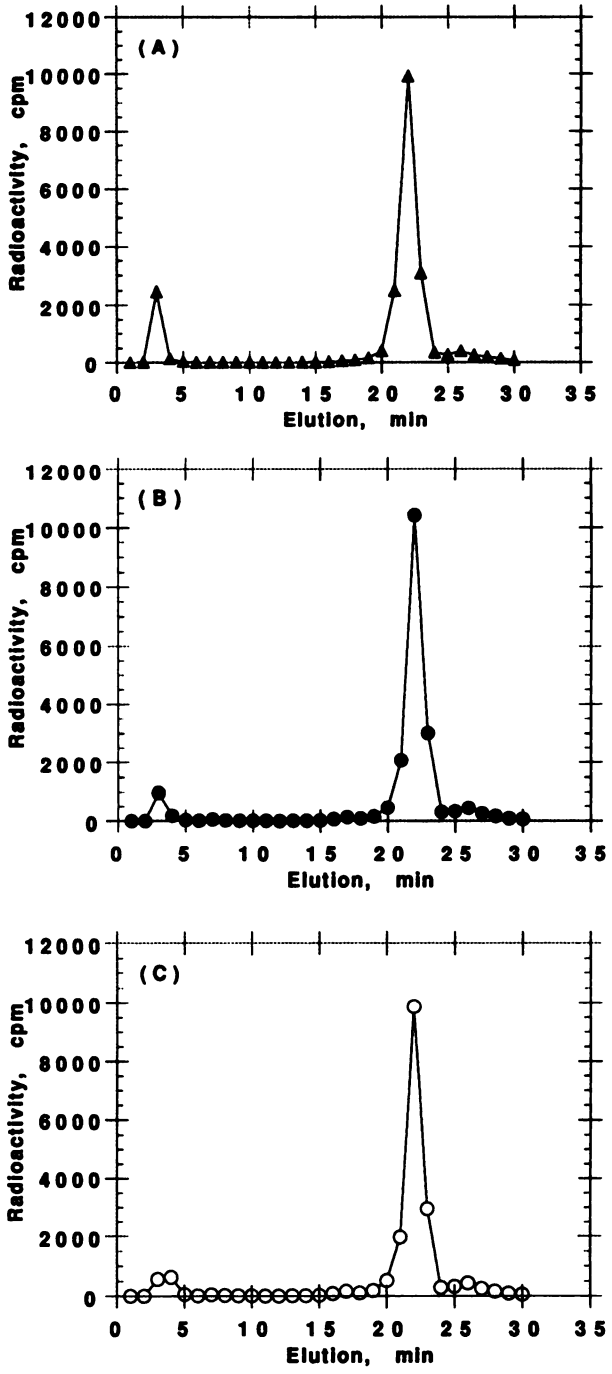

Fig. 3. Reverse phase HPLC analysis of ^{125}I-ET-1. A, ^{125}I-ET-1 (4 nM, 200 μl) without preincubation with cells. B and C, ^{125}I-ET-1 (4 nM, 200 μl) was incubated at 37° for 1 hr with cells that were pretreated without (B) or with (C) phosphoramidon for 24 hr. The supernatants were collected and analyzed as described in Experimental Procedures.

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indicate that nonspecific degradation of ^{125}I-ET-1 during the 1-hr assay period at 37° in both untreated and phosphoramidon-treated cells was minimal and could not account for the effect of phosphoramidon in elevating ^{125}I-ET-1 binding.

The binding studies described above were conducted at 37° using intact cells. At 37°, ET-1 has been shown to bind to receptors initially and then be rapidly internalized (22). To avoid the complication of receptor internalization, ET-1 binding was further studied in membranes prepared from cells. In time course studies, ET binding reached a plateau after 100 min of incubation at 25° in membranes prepared from phosphoramidon-treated or untreated cells (Fig. 4A). ^{125}I-ET-1 binding to phosphoramidon-treated membranes was significantly higher than that to membranes prepared from untreated cells. Under both conditions, addition of 1 μM ET-1 at time 0 completely blocked ^{125}I-ET-1 binding (data not shown). However, once ^{125}I-ET-1 had bound, addition of 1 μM ET-1 plus 500 μM Gpp(NH)p at 150 min for up to 200 min dissociated bound ^{125}I-ET-1 by <10% (Fig. 4A). Similar results were observed for membranes prepared from rat cerebellum, bovine atrium, and rat liver (data not shown). The irreversible binding of ET-1 has also been reported in plasma membranes prepared from rat liver cells (23). Until more studies become available to explain this phenomenon, saturation studies and Scatchard analysis are routinely used to study ^{125}I-ET-1 binding in the absence of more acceptable methods (23). We have conducted saturation studies in membranes prepared from untreated cells and cells treated with phosphoramidon (100 μM) for 24 hr. Fig. 4B shows that ^{125}I-ET-1 binding to membranes in both cases was saturable. Scatchard analysis (Fig. 4C) yielded straight lines, indicating the presence of one predominant receptor type. The results were fitted by the radioligand binding analysis programs EBDA and LIGAND (Biosoft) to obtain values for the Hill coefficient of 0.981 and 0.987 for untreated and phosphoramidon-treated membranes, respectively. Values of B_{max} and K_d were 0.13 pmol/mg and 0.06 nM for membranes from cells without phosphoramidon treatment and 0.66 pmol/mg and 0.09 nM for membranes from phosphoramidon-treated cells, respectively. The calculated numbers of receptors were 7.8×10^{10} and 39.7×10^{10} sites/mg of protein for membranes from untreated and treated cells, respectively, indicating a 408% increase or a net increase in ET receptors of 31.9×10^{10} sites/mg (0.53 pmol/mg) in membranes prepared from phosphoramidon-treated cells. The protein content was similar in membranes prepared from phosphoramidon-treated and untreated cells and was 0.25 mg/10^6 cells. Based on this number, the calculated receptor sites were 19,500 and 99,250 sites/cell for untreated and phosphoramidon-treated cells, respectively. Similar results were obtained from saturation studies performed on whole cells at 4° for 6 hr, in which values of B_{max} and K_d were 0.038 pmol/1 $\times 10^6$ cells and 0.18 nM for untreated cells and 0.22 pmol/1 $\times 10^6$ cells and 0.32 nM for cells treated with 100 μM phosphoramidon for 24 hr, respectively (data not shown). The estimated B_{max} and K_d values obtained in whole-cell studies are in agreement with published results (8, 24). The calculated numbers of receptors were 23,000 and 133,000 sites/cell for untreated and phosphoramidon-treated cells, respectively, again indicating a 478% increase or a net increase in the receptor number of 110,000 sites/cell (0.182 pmol/1 $\times 10^6$ cells) after the phosphoramidon (100 μM) treatment. These data are consistent with numbers obtained from studies done in membranes and

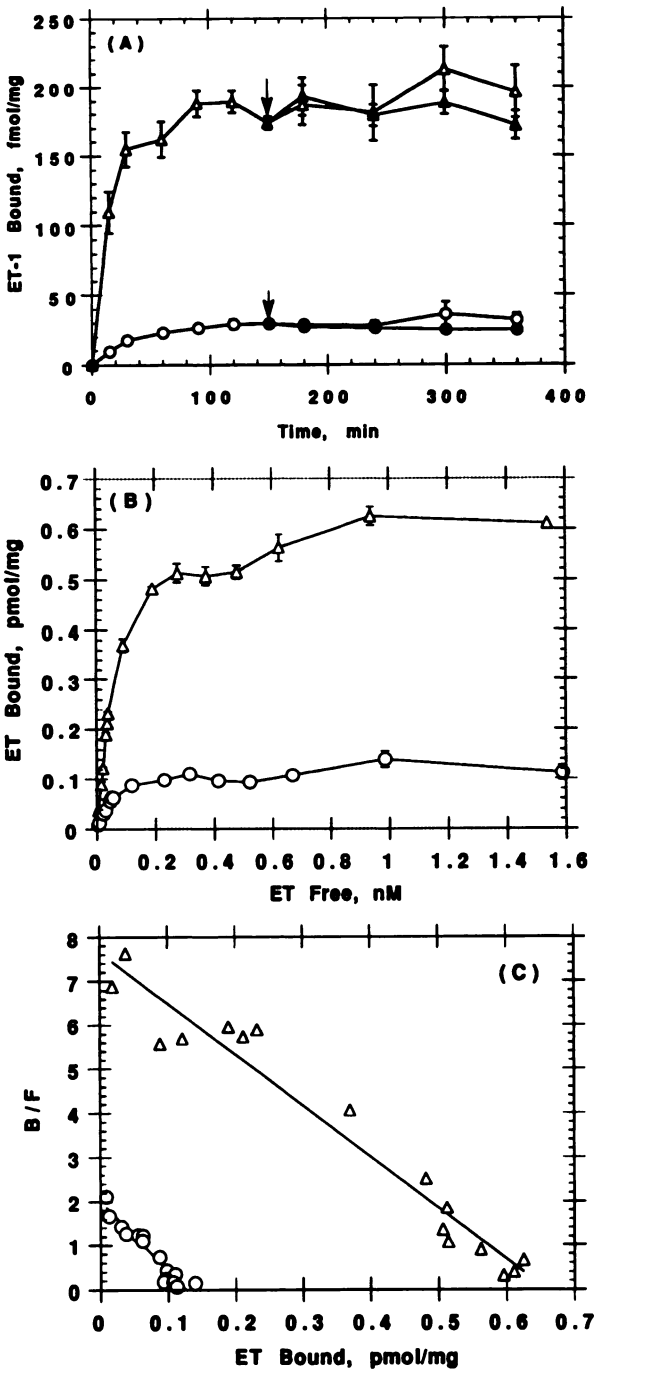

Fig. 4. Binding of ^{125}I-ET-1 to membranes. Membranes were prepared from 3T3 cells that had been pretreated with (*triangles*) or without (*circles*) phosphoramidon for 24 hr. A, Time course study. Membranes were incubated with 0.1 nM ^{125}I-ET-1 in 0.2 ml of buffer B at room temperature for different periods of time (open symbols). *Arrow*, time at which 1 μM ET-1 and 500 μM Gpp(NH)p were added (*closed symbols*). B, Saturation study. Membranes were incubated with different concentrations of ^{125}I-ET-1 in 0.2 ml of buffer B at room temperature for 4 hr. Nonspecific binding was determined in the presence of 1 μM ET-1 and was similar (in the range of 0.002–0.269 pmol/mg) for membranes from phosphoramidon-treated and untreated cells. Specific binding was determined by subtraction of nonspecific binding from total binding. C, Scatchard analysis of data in B. Each value represents the mean ± standard deviation of three determinations. Most *points* have error bars smaller than the symbols.

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strongly suggest that phosphoramidon treatment causes an actual increase in the number of ET receptors in 3T3 cells without affecting the binding affinity.

Phosphoramidon has never been shown to have effects on transcriptional and/or post-transcriptional regulation of the expression of a gene. Thus, it is not likely that phosphoramidon would stimulate the synthesis of ET receptors, resulting in an increase in ET binding. Nevertheless, to exclude the possibility that the phosphoramidon treatment may affect the synthesis of ET receptors, we have attempted to study ET receptor mRNA in 3T3 fibroblasts by using the reverse transcription-polymerase chain reaction technique. Because the mouse ET_A receptor has not been cloned, we prepared primers (5′-TCCTTTTGGCTGGCCCTGGTG-3′ and 5′-CAATCCGCA-AGCTCCCATTCCT-3′) based on published sequences for the rat ET_A receptor. Our control experiments using total RNA isolated from rat kidney revealed a specific DNA band migrating at the expected size of 852 base pairs on an agarose gel (data not shown). However, we failed to detect any specific band using total RNA prepared from either untreated or phosphoramidon-treated Swiss 3T3 cells. This result was not totally unexpected, because Swiss 3T3 fibroblasts originated from mouse, which may have a different sequence for the ET_A receptor.

An indirect approach was then taken to address the question of whether the phosphoramidon treatment stimulates the synthesis of ET receptors. To inhibit the biosynthesis of ET receptors, cells were treated with different concentrations of cycloheximide or actinomycin D for 24 hr before being assayed for ET binding. As shown in Fig. 5A, with no addition of cycloheximide ^{125}I-ET-1 binding was 0.727 ± 0.006 pmol/1 × 10^6 cells in phosphoramidon-treated cells versus 0.266 ± 0.023 pmol/1 × 10^6 cells in untreated cells. The cycloheximide treatment caused a decrease in ^{125}I-ET-1 binding in both phosphoramidon-treated and untreated cells, in a dose-dependent manner. With 0.4 μg/ml cycloheximide, ^{125}I-ET-1 binding decreased to 0.382 ± 0.117 and 0.070 ± 0.006 pmol/1 × 10^6 cells in phosphoramidon-treated and untreated cells, respectively. However, it was evident that phosphoramidon-treated cells exhibited higher ^{125}I-ET-1 binding, with a net increase of 0.312 pmol/1 × 10^6 cells. Similarly, although with 2.0 μg/ml cycloheximide ^{125}I-ET-1 binding decreased by 75% and 87% in phosphoramidon-treated and untreated cells, respectively, binding in phosphoramidon-treated cells was higher than that in untreated cells, with a net difference of 0.147 pmol/1 × 10^6 cells. Fig. 5B shows that the effect of actinomycin D on ^{125}I-ET-1 binding was also dose dependent. With 0.2 μg/ml actinomycin D, although ^{125}I-ET-1 binding decreased to 0.268 ± 0.030 and 0.075 ± 0.010 pmol/1 × 10^6 cells in phosphoramidon-treated and untreated cells, respectively, binding in phosphoramidon-treated cells was higher than that in untreated cells (a net increase of 0.193 pmol/1 × 10^6 cells). It is important to note that both cycloheximide and actinomycin D affect cell growth. The number of viable cells as determined by the trypan blue exclusion method was 0.33 ± 0.03 × 10^6 cells/well (three experiments) for control wells (no addition of actinomycin D or cycloheximide). The cell numbers were 0.32 ± 0.03, 0.22 ± 0.02, and 0.18 ± 0.02 × 10^6 cells/well with 0.08, 0.4, and 2 μg/ml cycloheximide, respectively. The cell numbers were 0.23 ± 0.02 and 0.22 ± 0.03 × 10^6 cells/well with 0.04 and 0.2 μg/ml actinomycin D, respectively. No difference was observed for

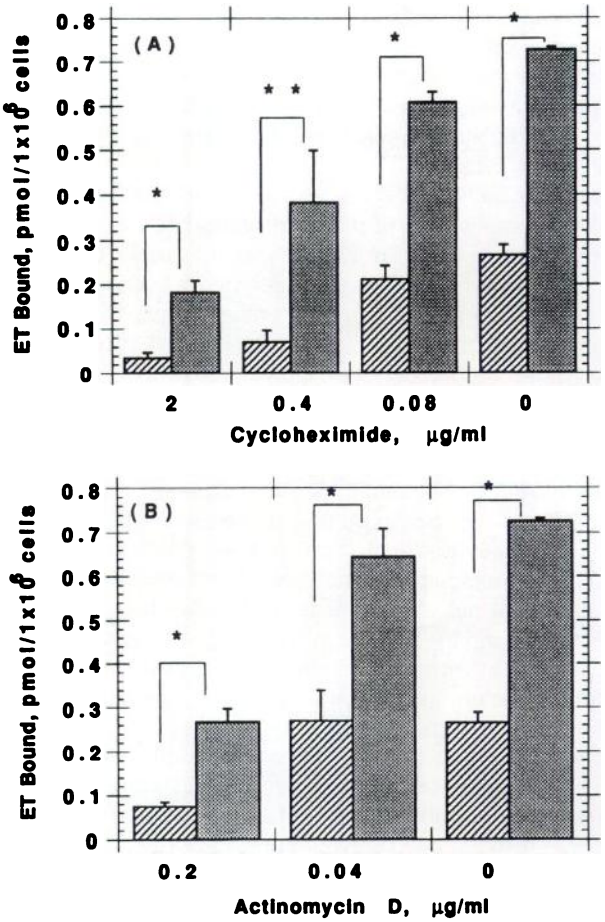

Fig. 5. Effect of cycloheximide or actinomycin D on ^{125}I-ET binding. Confluent monolayers of cells were treated for 24 hr with (■) or without (▨) 100 μM phosphoramidon in the presence of different concentrations of cycloheximide (A) or actinomycin D (B). Cells were washed with 1 ml of PBS and then incubated with 4 nM ^{125}I-ET-1 for 1 hr at 37°. The viability of cells was examined by the trypan blue exclusion method. Nonspecific binding was determined in the presence of 1 μM ET-1 and was 0.031 ± 0.004 pmol/1 × 10^6 cells for both treated and untreated cells. Values shown were obtained by subtraction of nonspecific binding from total binding. Each value represents the mean ± standard deviation of three determinations. *, $p < 0.01$; **, $p < 0.05$, using the paired t test.

the number of viable cells in phosphoramidon-treated and untreated wells. The amounts of ^{125}I-ET-1 binding shown in Fig. 5 were obtained after the difference in cell numbers was normalized.

Previous studies have shown that a degradation product of the ET receptor with a smaller molecular mass is often purified from solubilized membranes, especially in the absence of EDTA and phosphoramidon (6, 26). Taken together with the fact that the increase in ET binding becomes evident after cells are treated with phosphoramidon for 5 hr, we hypothesize that the effect of phosphoramidon on the ET receptor may be due to its inhibition of a protease responsible for degrading the ET receptor. To study this hypothesis, we induced receptor degradation in membranes prepared from cells by incubating membranes at 25° for different periods of time. First, membranes prepared from cells without phosphoramidon treatment were studied. As shown in Fig. 6A, when membranes were exposed to 25° for 24 hr ^{125}I-ET-1 binding decreased to 44% of that in membranes that either were freshly prepared or had been stored at −80° until use. It is important to note that ^{125}I-ET-1 binding was

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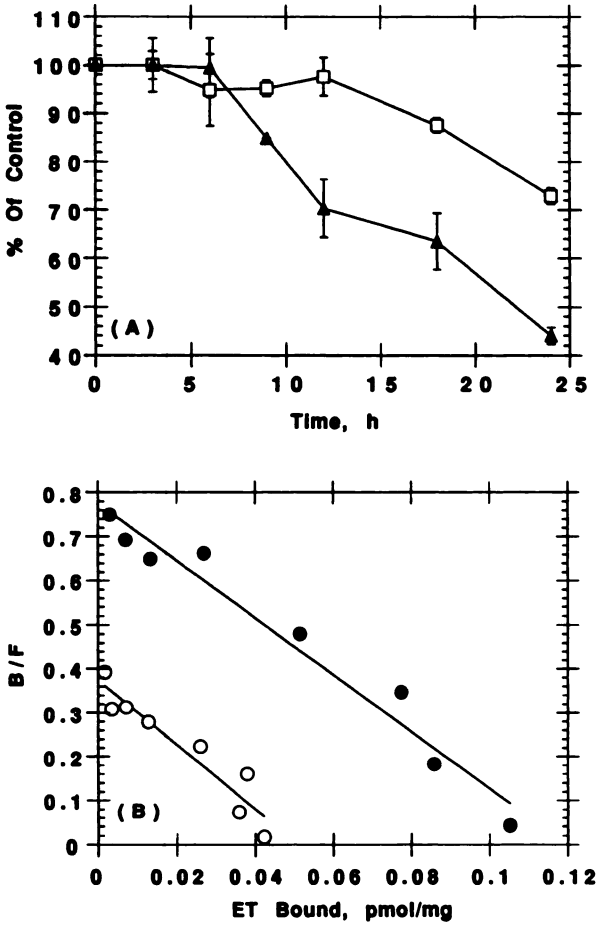

Fig. 6. Degradation studies. A, Membranes prepared from 3T3 cells that had been pretreated with (□) or without (▲) phosphoramidon (100 μM, 24 hr) were exposed to 25° for different periods of time to induce protein degradation. Membranes were then incubated with 0.1 nM ^{125}I-ET-1 in 0.2 ml of buffer B in the presence of protease inhibitors, as described in Experimental Procedures. Nonspecific binding was determined by addition of 1 μM ET-1 and was 0.006 pmol/mg. Specific ^{125}I-ET-1 binding at time 0 was 0.0423 ± 0.00.3 pmol/mg and 0.1892 ± 0.0187 pmol/mg for membranes from untreated and phosphoramidon-treated cells, respectively. Results are expressed as percentage of control (specific binding at time 0). B, Membranes prepared from control 3T3 cells (no phosphoramidon treatment) were incubated at 25° for 24 hr with (●) or without (○) addition of 100 μM phosphoramidon at the beginning of the incubation. Membranes were then incubated with 0.05 nM ^{125}I-ET-1 in 0.2 ml of buffer B with protease inhibitors, in the presence of increasing concentrations of unlabeled ET-1 (0–5 nM), as described in Experimental Procedures. Nonspecific binding was determined by addition of 1 μM ET-1 and was 11% of total binding for both phosphoramidon-treated and untreated membranes. Specific binding was calculated by subtraction of nonspecific binding. Data were analyzed by Scatchard plot. Each value represents the mean of three determinations.

assayed in the presence of protease inhibitors, as described in Experimental Procedures. The decrease in ^{125}I-ET-1 binding was presumably due to the degradation of ET receptors after exposure of membranes to 25°. Next, membranes from phosphoramidon-treated and untreated cells were incubated at 25° for different periods of time in the absence of protease inhibitors before being assayed for ^{125}I-ET-1 binding (Fig. 6A). As shown in Fig. 6A, ^{125}I-ET-1 binding remained the same in the first 6 hr of exposure to 25° for both sets of membranes. When the incubation was longer than 6 hr, ^{125}I-ET-1 binding started to decrease. However, the decrease was approximately 2-fold slower in membranes from phosphoramidon-treated cells than in membranes from untreated cells. The rate of decrease in ^{125}I-ET-1 binding was calculated to be 1.25%/hr and 2.85%/hr for membranes from cells with or without phosphoramidon treatment, respectively. This result was further confirmed in a separate experiment in which membranes prepared from control cells (no phosphoramidon treatment) were studied. To induce protein degradation, membranes from untreated cells were exposed to 25° for 24 hr with or without the addition of 100 μM phosphoramidon at the beginning of the incubation. After 24 hr, membranes were assayed for ^{125}I-ET-1 binding in the presence of protease inhibitors, as described in Experimental Procedures. Scatchard analysis of binding data yielded two parallel lines (Fig. 6B). For membranes that were incubated at 25° for 24 hr in the absence of phosphoramidon, values of B_{max} and K_d were 0.051 pmol/mg and 0.14 nM, respectively. When phosphoramidon was added before membranes were incubated at 25° for 24 hr, values of B_{max} and K_d were 0.12 pmol/mg and 0.15 nM, respectively (Fig. 6B). The B_{max} and K_d values for fresh membranes that had not been exposed to 25° for any length of time before being assayed for ^{125}I-ET-1 binding were 0.13 pmol/mg and 0.06 nM, respectively (Fig. 4C). These results are consistent with the hypothesis that a membrane-bound phosphoramidon-sensitive protease is involved in degrading the ET receptor.

Discussion

Results from these studies show that 1) ET binding sites were increased by >110,000 sites/cell (0.182 pmol/1 × 10^6 cells) after cells were treated with 100 μM phosphoramidon for 24 hr, 2) the effect of phosphoramidon was dose and time dependent, and 3) the phenomenon was specific for phosphoramidon, because other protease inhibitors, including thiorphan, failed to exert a similar effect. Furthermore, studies in which membranes were exposed to 25° for a prolonged period of time to induce protein degradation (Fig. 6) suggest that a membrane-bound protease may be involved in the degradation of ET receptors. By treating cells with phosphoramidon to inhibit this protease, ET receptors were preserved and an increase in ET binding was observed. Our results agree well with previous studies showing that a degradation product of the ET receptor with a smaller molecular mass is often purified from solubilized membranes, especially in the absence of EDTA and phosphoramidon (6, 26). Because the effect was specific for phosphoramidon and thiorphan did not show a similar effect, possibly a protease other than the neutral metalloprotease 24.11 is involved in degrading ET receptors. Additional studies using antibodies to the ET receptor to compare the turnover rates of the receptor in cells pretreated with or without phosphoramidon will provide more evidence to explain this mechanism.

The HPLC analysis clearly demonstrates that the effect of phosphoramidon in elevating ET-1 binding is not due to inhibition of ^{125}I-ET-1 degradation in the assay (Fig. 3). In addition, studies using cycloheximide or actinomycin D to block protein synthesis suggest that the effect of phosphoramidon is not due to stimulation of ET receptor synthesis (Fig. 5). The effects of cycloheximide and actinomycin D are complex and nonspecific. For example, these two agents not only block the transcription and translation of the ET receptor but also inhibit the synthesis of other proteins, including the putative phosphoramidon-sensitive protease responsible for degrading the ET receptor. However, it is reasonable to assume that, if certain amounts of the

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ET receptor and its degradation protease are still present in cells after the cycloheximide and actinomycin D treatment, then inhibiting this residual amount of the degradation protease with phosphoramidon should result in an increase in ^{125}I-ET-1 binding. Our results from Fig. 5 are consistent with this idea, supporting the hypothesis that the effect of phosphoramidon is not in stimulating the synthesis of the ET receptor but in inhibiting the degradation of the ET receptor.

It was shown previously that Swiss 3T3 fibroblasts do not secrete ET, as determined by specific radioimmunoassys or sandwich enzyme-linked immunosorbent assays (28, 29). Therefore, it was not possible that the effect of phosphoramidon was due to its inhibiting the formation of ET, resulting in a decrease in the amount of ET accumulated in the culture medium and subsequent up-regulation of the ET receptor. Furthermore, even if an undetectable amount of ET was secreted by the 3T3 cells, it is unlikely that the phosphoramidon treatment under our experimental conditions would alter the amount of ET accumulated in the medium enough to result in receptor up-regulation. Under our experimental conditions cells had been incubated in the same medium for at least 24 hr before addition of phosphoramidon, and phosphoramidon was added directly to cells without a change in medium. The effect of phosphoramidon was evident after 5 hr of treatment. It is unlikely that the amount of ET accumulated in the medium would be altered so much in this 5 hr of phosphoramidon treatment that it would result in receptor up-regulation with a net increase in ET-1 binding of 0.2 pmol/1 $\times$ 10^6 cells. The third piece of evidence against this unlikely possibility came from studies using cycloheximide and actinomycin D to inhibit protein synthesis. The formation of ET in both phosphoramidon-treated and untreated cells, if there was any, should also have been blocked after inhibition of protein synthesis. However, phosphoramidon still caused a significant increase in ET binding. Lastly, we used a rabbit antibody (27) to the carboxyl-terminal pentapeptide (Leu-Asp-Ile-Ile-Trp) of ET-1 (ET antibody) to treat cells for 24 hr, to capture any ET released by cells. Cells were then washed twice with PBS (1 ml/well) and assayed for ET-1 binding. The results were compared with those from cells treated with ET antibody for 0 hr or cells treated with a control antibody (obtained from rabbits before immunization) for 24 hr. No difference in ET binding could be observed in cells pretreated with ET antibody for 24 hr. We conclude that the effect of phosphoramidon in increasing ET-1 binding is not due to its inhibitory effect on the formation of ET in Swiss 3T3 fibroblasts.

The effect of phosphoramidon in increasing ET binding is not unique for Swiss 3T3 fibroblasts. Previously Liu *et al.* (25) reported in preliminary studies that tissues such as heart and kidney isolated from phosphoramidon-treated rats exhibited an increase in the number of ET receptors and that thiorphan did not exert a similar effect. Also, Fujitani *et al.* (30) reported that human umbilical vein endothelial cells exhibited an increase in ET binding after cells were treated with phosphoramidon. In addition, Clozel[1] (F. Hoffmann-La Roche Ltd., Basel, Switzerland) observed a similar phenomenon in rat mesangial cells. The mechanisms for the effect of phosphoramidon on these tissues and cells are not fully understood. Because both human umbilical vein endothelial cells and rat mesangial cells secrete ET during culture (30, 32) and because the effect of phosphoramidon in inhibiting the ET-converting enzyme (16) and the ET-degrading enzyme (31) may result in receptor up- or down-regulation, using these two cell types to study the mechanism of the effect of phosphoramidon on the ET receptor may be more complicated than using Swiss 3T3 fibroblasts. It is possible that phosphoramidon modulates the number of ET receptors by more than one mechanism in ET-secreting cells.

Phosphoramidon has been shown to block the conversion of big ET to ET in cultured cells and the vasoconstrictor effect of big ET *in vivo*, presumably due to its effect in inhibiting a metalloprotease-like ET-converting enzyme (16). However, based on the present findings, the increase in ET binding after phosphoramidon treatment is likely to complicate the interpretation of results from studies that use phosphoramidon to evaluate the pathophysiological role of ET. The involvement of phosphoramidon in inhibiting the putative ET-converting enzyme and the ET-degrading protease, plus its effect in modulating the number of ET receptors, suggests that a phosphoramidon-sensitive enzyme is involved not only in the synthesis and degradation of ET but also in the regulation of its receptor. Determination of the physiological implications of the phosphoramidon-sensitive enzyme(s) in the regulation of ET effects awaits additional studies.

Acknowledgments

We would like to thank Terry Pederson for providing the Swiss 3T3 cells and Drs. Chun W. Lin and Chi-Ming Lee for their comments on our manuscript.

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