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Novel Selective Quinazoline Inhibitors of Endothelin Converting Enzyme-1

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PD 069185 is a highly selective and structurally novel inhibitor of endothelin converting enzyme-1 (ECE-1). PD 069185 is a trisubstituted quinazoline with an IC_{50} value of 0.9 ± 0.1 μM for inhibition of human ECE-1 from the solubilized membrane fraction of CHO cells stably transfected with human ECE-1 cDNA. Kinetic analysis revealed that PD 069185 is best fit with a competitive inhibition model with a K_i value of 1.1 ± 0.1 μM and binds in a reversible manner. The closely related enzyme, ECE-2, is not inhibited at up to 100 μM PD 069185. In addition, PD 069185 at 200–300 μM has little effect on other metalloproteases, such as neutral endopeptidase 24.11, stromelysin, gelatinase A, and collagenase, showing a high ECE-1 specificity. Data are also presented to show that this series of inhibitors are effective in inhibiting ECE-1 in intact cells and in attenuating the increase in perfusion pressure induced by big ET-1 in isolated rat mesentery. These non-peptidic ECE-1 inhibitors should serve as a valuable tool to study the pathophysiological role of endothelin and the therapeutic potential of ECE-1 inhibitors.

Key Words: **endothelin converting enzyme; endothelin; big endothelin; quinazoline.**

Endothelin-1 (ET-1) was first discovered in 1988 by Yanagisawa *et al.* in the culture media of porcine endothelial cells and shown to be a 21-amino acid peptide with the most potent vasoconstricting activity known [1]. Subsequently, three distinct genes encoding three closely related peptides, ET-1, ET-2, and ET-3, have been identified [2].

Endothelins are produced from peptide precursors of approximately 200-amino acid residues. They are first processed by prohormone processing enzyme(s) [3] into 38-41-amino acid intermediates called big ET-1, -2, and -3. Big endothelins are then cleaved between Trp^{21} and Val^{22}/Ile^{22} to yield 21-amino acid endothelins by endothelin converting enzyme (ECE) [1].

Evidence indicates that the physiologically relevant ECE is inhibited by phosphoramidon, a non-selective peptidic metalloprotease inhibitor. Phosphoramidon has been shown to inhibit the pressor and airway contractile effects of big ET-1 *in vivo* [4,5] and to suppress the secretion of ET-1 from cultured endothelial cells [6,7]. Other biochemical studies and characterization of ECE have shown that ECE from endothelial cells and other tissue sources is a membrane-bound metalloprotease. It has also been shown that ECE is insensitive to inhibitors of other metalloproteases such as thiorphan (neutral endopeptidase 24.11, NEP) and captopril (angiotensin converting enzyme) [8-14].

ECE-1 has been purified to homogeneity [9,10]. The cloning of its gene revealed that ECE-1 is a type II integral membrane protein and is structurally related to NEP and the human Kell blood group protein [11,12,15]. More recently, another ECE family member was cloned and termed ECE-2. ECE-2 was shown to have an overall 59% amino acid identity to ECE-1 and to have an acidic pH optimum in contrast to the neutral pH optimum for ECE-1 [16]. Moreover, two isoforms of ECE-1 with distinct N-terminal tails have been identified and termed ECE-1a and ECE-1b. ECE-1a and ECE-1b have been shown to be encoded by the same gene through the use of two promoters [17,18]. The amino acid sequences of ECE-1 were shown to be highly homologous among mammalian species. Human ECE-1a has 93 and 95% amino acid identity to rat and bovine ECE-1a, respectively. Similarly, human ECE-1b has 96 and 95% amino acid identity to rat and bovine ECE-

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Abbreviations: ET, endothelin; ECE, endothelin converting enzyme; NEP, neutral endopeptidase 24.11.

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1b, respectively [11,12,15]. ECE-1 has been shown to be localized either on the cell surface or in the Golgi depending on the tissue source of the enzyme [19-21]. The biochemistry and molecular pharmacology of ECE have been reviewed [22,23].

Recent studies using endothelin receptor antagonists suggest that endothelins may play important roles in a number of pathological conditions including stroke [24], chronic heart failure [25], and hypertension [26]. In addition, disruptions of ET-1 [27], ET-3 [28], and ET_B receptor genes [29] have demonstrated important roles for endothelins in the development of neural crest-derived tissues.

Because the vasoconstrictor activity of big ET-1 is over 100-fold less than that of ET-1 [30], the conversion from big ET-1 to ET-1 appears to be essential for biological activity. Therefore, specific and potent non-peptidic ECE inhibitors will be an invaluable tool in understanding the pathophysiological role of endothelin and in evaluating the therapeutic potential of ECE inhibition. To date, several non-peptidic ECE-1 inhibitors with IC_{50} (50% inhibitory concentration) values have been reported [31-34]. However, they are either not selective for ECE [31-32, 34] or difficult to modify synthetically [33].

Here we report PD 069185 and its analogs, which are structurally novel and highly selective for ECE-1. We have used the recombinant human ECE-1 and human ECE-2 obtained from stable transfectant cell lines, CHO/ECE-1 and CHO/ECE-2, as enzyme sources. Since CHO cells have minimal endogenous ECE-like activity, the ECE-1 or ECE-2 activity from each cell line will represent the true ECE-1 or ECE-2 activity [12,16]. In this report the inhibitory mechanism and binding mode of PD 069185 are discussed. Data are also presented here to show that these inhibitors are effective in inhibiting the enzyme in the cell-based assay and in attenuating the increase in perfusion pressure induced by big ET-1 in isolated rat mesentery.

MATERIALS AND METHODS

Materials. Human big ET-1 (1-38) was purchased from Peptides International (Louisville, KY). Phosphoramidon, pepstatin A, phenylmethylsulfonyl fluoride and leupeptin were from Boehringer Mannheim. The enzyme-linked immunosorbant assay (Elisa) kit for the measurement of ET-1 was from Amersham and used as instructed by the manufacturer. All reagents for tissue culture were purchased from Gibco unless otherwise indicated. A highly purified crystallized preparation of trypsin (Sigma, T7418) was used for all the tissue culture procedures as described [12].

ECE-1 assay. For determination of IC_{50} values, the reaction mixture (100 μl) contained 0.1 μM big ET-1 (1-38), 100 mM Hepes-KOH (pH 7.0), 50 mM NaCl, 50 μM pepstatin A, 100 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, the indicated concentration of the inhibitor (DMSO for control), and the solubilized membrane fraction of CHO/human ECE-1 cells. The final concentration of DMSO was 1.5%. After incubation for 1h at 37 °C, the reaction was stopped by adding EDTA to give a final concentration of 5 mM. This final mixture was then directly analyzed for the amount of ET-1 by Elisa. Under the assay conditions, the production of ET-1 is linear with an incubation time for several hours. The data were plotted as percent of control vs inhibitor concentration and fit with the equation, $y = 100/1 + (x/IC_{50})^z$, using KaleidaGraph (Synergy Software, Reading, PA), where IC_{50} is the inhibitor concentration at 50% inhibition and z is the slope of the inhibition curve.

Other enzyme assays. The membrane fraction of CHO/human ECE-2 cells, rat kidney cortex membrane homogenate, human stromelysin catalytic domain, human gelatinase A catalytic domain, human fibroblast collagenase, human interleukin-1β converting enzyme, and human thrombin were used for the ECE-2, NEP, stromelysin, gelatinase A, collagenase, interleukin-1β converting enzyme, and thrombin assays, respectively, and the assays were performed essentially as described [16,35-39].

Cell culture and preparation of membrane fractions. Stable transfectant CHO cells were cultured in monolayers in HamF-12 and DMEM (1:1 mixture) medium supplemented with 10% fetal bovine serum and 1 mg/ml G418. All cells were grown in a humidified incubator at 37 °C in an atmosphere of 5% CO_2/95% air. The membrane fractions from CHO cells were prepared as described [12]. The cloning of human ECE-1 and ECE-2, and the preparation of stable transfectant CHO cell lines will be described elsewhere (Yanagisawa *et al.*, manuscript in preparation). The preparation of a stable transfectant cell line, CHO/ human prepro-ET-1, has been described [12,16].

Coculture assay. Approximately 5×10^4 cells of each CHO/ECE-1 and CHO/prepro-ET-1 cells were seeded into 24-well plates. After approximately 16 h, the cells were washed twice with the media and treated with medium containing the indicated concentrations of inhibitors or DMSO (0.3 ml/well). The final DMSO concentration for all experiments was 0.5 %. After incubation for the indicated period of time, the medium was collected and centrifuged at 10, 000 $\times$ g for 10 min in order to remove cell debris. The resulting supernatant was used for the measurement of ET-1 by Elisa.

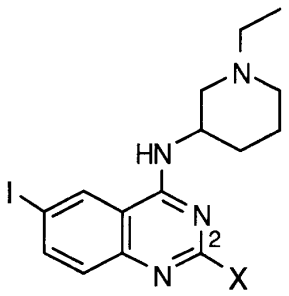
Cellular toxicity assay. After removal of the supernatant from the cells in each well as described above, the remaining cells were used to measure cellular toxicity using the aqueous cell proliferation assay as instructed by the manufacturer (Promega, G5421).

Isolated perfused rat mesentery preparation. Male Sprague Dawley rats (350-400 g) were anesthetized with pentobarbital (60 mg/Kg i.p.), and heparinized (100 units i.v.). The abdomen was opened and the pyloric, colonic, and ileocecal branches of the superior mesenteric artery were ligated. The superior mesenteric artery was cannulated and immediately perfused single-pass with a constant flow (5 ml/min) of prewarmed (37 °C) and oxygenated (95% O_2/5% CO_2) Krebs-bicarbonate buffer at an initial perfusion pressure of 30 mmHg. The rat was killed by exsanguination and the mesenteric vasculature with adhering small intestine was excised and suspended by the mesenteric artery in a climate controlled chamber. Changes in perfusion pressure were measured with an in-line pressure transducer. After a 15 minute equilibration period, the mesentery was perfused for an additional 20 minutes with either DMSO (0.1%) or inhibitor in the buffer. The mesentery was then challenged with increasing concentrations of either ET-1 (0.3 to 30 nM) or big ET-1 (3 to 100 nM). Each peptide was perfused at the indicated concentration until perfusion pressure was stabilized (approximately 15 minutes), before evaluation at the next concentration.

RESULTS AND DISCUSSION

PD 069185, a Highly Selective ECE-1 Inhibitor

A structurally novel trisubstituted quinazoline, PD 069185 (Fig. 1), was found to be an inhibitor of ECE-1



X= CCl_3 (PD 069185, IC_{50}: 0.9 ± 0.1 μM)
X= CF_3 (PD 159790, IC_{50}: 2.6 ± 0.3 μM)

FIG. 1. Structure of PD 069185.

through screening of the Parke-Davis compound library. The IC_{50} value for the inhibition of human recombinant ECE-1 was determined to be 0.9 ± 0.1 μM. The high selectivity of PD 069185 for ECE-1 inhibition is shown in Table 1. ECE-2, which has a 59% amino acid identity with ECE-1 [16], was not inhibited by PD 069185 at concentrations up to 100 μM. Furthermore, PD 069185 showed more than 200-fold selectivity for ECE-1 over NEP, which has a 58% amino acid identity within the C-terminal one-third of the extracellular domain of ECE-1 [12]. The selectivity of this compound was further tested by examining the inhibition of other metalloproteases such as stromelysin, gelatinase A, and collagenase using the assays as described under Materials and Methods. PD 069185 had little or no effect on these three metalloproteases at up to 300 μM, indicating a high ECE-1 specificity. Moreover, PD 069185 showed no effect on interleukin-1β converting enzyme and thrombin, cysteine and serine protease, respectively.

Other non-peptidic ECE inhibitors have been reported previously. De Lombaert *et al.* reported compounds, CGS 26303 and CGS 31447, with IC_{50} values of 1.1 μM and 17 nM, respectively, for ECE-1 inhibition. However, CGS 26303 and CGS 31447 inhibit NEP with approximately 1000- and 3-fold higher affinity, respectively (IC_{50} values of 0.9 and 4.8 nM, respectively, for NEP inhibition) [31-32]. Tsurumi *et al.* reported FR 901533, isolated from fungal broth, which was shown to have an IC_{50} value of 0.14 μM for ECE-1 inhibition with selectivity against NEP [33]. In addition, the same authors also reported WS 75624A and B which were isolated from fungal broth and shown to have IC_{50} values of 0.03 μg/ml for ECE-1 inhibition. However, these inhibitors also showed inhibition of other metalloproteases (collagenase and NEP with IC_{50} values of 1 μg/ml) [34]. A thiol-containing inhibitor with an IC_{50} value of 1.7 μM was reported [40]. However, the selectivity of this inhibitor has not been reported.

PD 069185, a Competitive and Reversible Inhibitor of ECE-1

Studies on the inhibitory mechanism and binding mode of the previously reported ECE inhibitors are largely unavailable. In this present work, kinetic studies were undertaken, since binding mode and true affinity (K_i) of inhibitors are not revealed by IC_{50} values.

Studies were carried out to determine whether PD 069185 acts in a reversible or irreversible manner. PD 069185 at 40 μM was preincubated with the enzyme for 20 min at 37 °C. At this inhibitor concentration, enzyme activity is expected to be inhibited by >95 %. An aliquot of the enzyme and inhibitor was then diluted 10-fold into the reaction mixture containing substrate and incubated for the indicated period of time. The observed ECE-1 activity was then compared to a control where the enzyme was incubated without the inhibitor for 20 min and diluted 10-fold into the reaction mixture containing substrate and 4 μM of PD 069185. As shown in Fig. 2, dilution of the inhibitor clearly restores the enzyme activity to the same level as the control, and therefore PD 069185 inhibits ECE-1 in a reversible manner. The extent of inhibition was not dependent on the preincubation time from 2 to 40 min, indicating that this reversible inhibitor has reached an equilibrium ($E + I \rightleftarrows EI$) within less than 2 min. However, a faster time scale would be needed to determine the exact binding rates.

The mode of protease inhibition by PD 069185 was examined and the results are shown as a double reciprocal plot in Fig. 3. The best fit of these data was obtained with a competitive inhibition model. When the data were fit to the equation for competitive inhibition, $V_i = V_{max}[S]/([S] + K_m(1 + [I]/K_i))$, by a nonlinear least-squares algorithm using KinetAsyst (IntelliKinetics, Princeton, NJ), a K_i value of 1.1 ± 0.1 μM was obtained. The K_m value for big ET-1 (1-38) was determined to be

TABLE 1

Selectivity of PD 069185[a]

Enzyme	Inhibition (%)
ECE-2	0.0[b]
NEP 24.11	25.3[c]
Stromelysin	19.4[d]
Gelatinase A	13.0[d]
Collagenase	0.8[d]
Interleukin-1β converting enzyme	0.9[b]
Thrombin	5.0[b]

[a] PD 069185 was tested at up to the highest concentrations that were soluble in each assay. Each value represents the mean of two to three experiments. The standard errors associated with these measurements were typically ± 10-15%.
[b] At 100 μM.
[c] At 200 μM.
[d] At 300 μM.

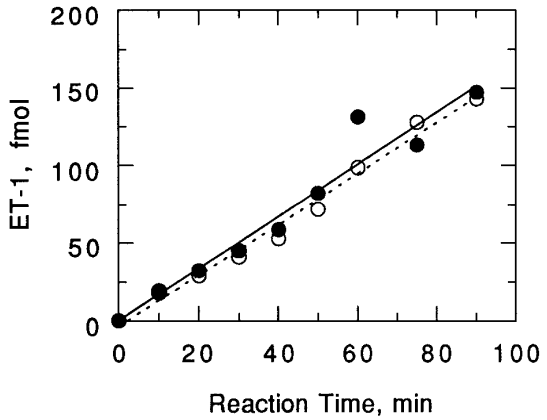


FIG. 2. Determination of the reversibility of PD 069185 for inhibition of ECE-1. The solubilized membrane fraction of CHO/ECE-1 cells was preincubated in the absence (○) or in the presence (●) of PD 069185 at 40 μM at 37°C for 20 min. The aliquot of enzyme and inhibitor (E + I) was diluted 10-fold into the assay buffer containing 0.1 μM big ET-1, and the reaction mixture was further incubated for the indicated periods of time. For the control reaction (○), PD 069185 at 4 μM was added after the preincubation. Each point represents the mean of three experiments.

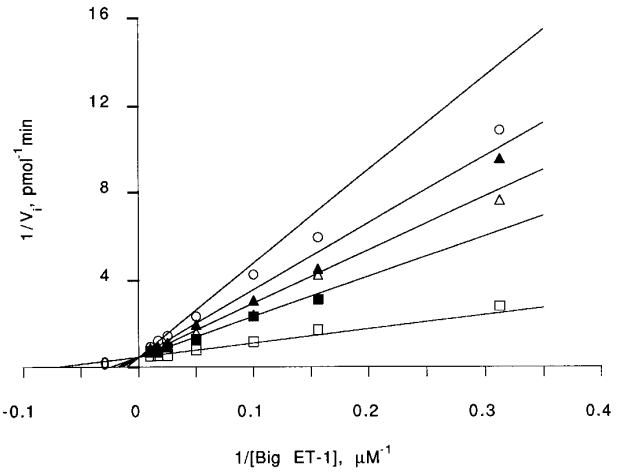


FIG. 3. Double reciprocal plot for inhibition of ECE-1 by PD 069185. The enzyme reactions were performed under the conditions described under Materials and Methods, except that the substrate concentrations used were 3.2, 6.4, 10, 20, 40, 60, and 100 μM. Inhibitor concentration used were 0 (□), 2 (■), 3 (△), 4 (▲), and 6 (○) μM. The data were shown to fit the equation for competitive inhibition by a nonlinear least-square algorithm. Each point represents the mean of two experiments.

14.5 ± 1.9 μM, close to the value reported using the recombinant human enzyme [15]. This K_i value is in good agreement with the IC_{50} value of 0.9 ± 0.1 μM, as should be the case under conditions of competitive inhibition when the substrate concentration is well below the K_m value [41].

Cellular Activity

Studies were designed to evaluate whether the inhibitors are as effective in the intact cells as they are for the isolated enzyme. To determine inhibitor efficacy for inhibiting the ability of CHO/ECE-1 cells to convert exogenously supplied big ET-1, CHO/ECE-1 cells were cocultured with a separate CHO cell line that was stably transfected with the prepro-ET-1 gene (CHO/prepro-ET-1) in a 1:1 ratio using a method similar to that described previously [12]. Both PD 069185 and PD 159790 were tested for further biological evaluation. The replacement of $-CCl_3$ (PD 069185) by $-CF_3$ (PD 159790) at the 2 position exhibited higher aqueous solubility of its salt without affecting the selectivity of the inhibitor.

As shown in Fig. 4, both PD 069185 and PD 159790 after an incubation for 6 h at 37 °C inhibited the production of ET-1 from CHO/ECE-1 cells in a dose-dependent manner with EC_{50} values of 3.8 ± 0.4 and 11.5 ± 2.1 μM, respectively. This inhibition was not time-dependent, i.e., similar potencies were obtained at both shorter and longer incubation time (1 and 12 h). Under the same tissue culture conditions, a 50 % cellular toxicity concentration (TC_{50}) of approximately 56 μM was obtained for PD 069185 and no toxicity was observed at up to 100 μM for PD 159790 when the cellular toxicity was measured by the procedure described under Materials and Methods. These TC_{50} values are in each case at least an order of magnitude higher than EC_{50} values.

The potencies in the cell-based assay were reduced by 4.2- and 4.4-fold for PD 069185 and 159790, respectively, compared to those with the isolated enzyme (Fig. 1). Under the same experimental conditions, a similar reduction in potency in the cell-based assay was also

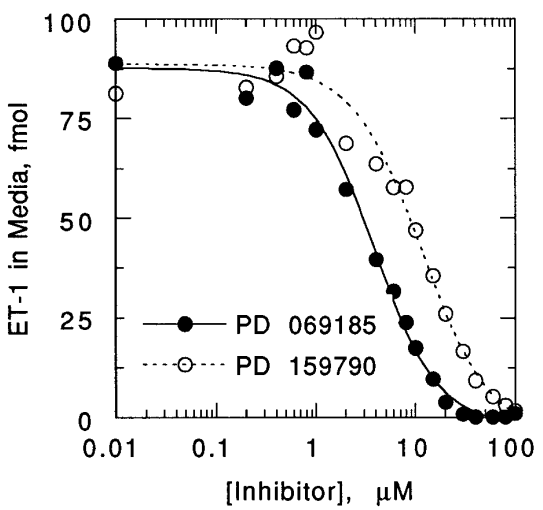


FIG. 4. Effects of PD 069185 (●) and PD 159790 (compound 7) (○) on the production of ET-1 by 1:1 cocultures of CHO/ECE-1 and CHO/prepro-ET-1 cells after incubation for 6 h. Each point represents the mean of three experiments.

observed for phosphoramidon, a non-selective metalloprotease inhibitor, compared to that with the isolated enzyme (IC_{50} and EC_{50} values of 0.6 ± 0.04 and 1.3 ± 0.2 μM, respectively). Lower potency in the cell-based assay for PD 069185 and 159790 is not due to the degradation of the inhibitors since these inhibitors are stable under the assay conditions. A 3.0-fold potency difference between PD 069185 and PD 159790 in the cell-based assay compared well with that observed with the isolated enzyme (2.9-fold, Fig. 1). In conclusion, both PD 069185 and PD 159790 inhibited ECE-1 in the live cell assay with potencies in the low μM range.

Studies to determine the capacity of the above inhibitors to inhibit intracellular ECE-1 using either CHO/ECE-1 cells transfected with the prepro-ET-1 gene or endothelial cells were carried out using methods previously described [12,14]. In this assay, a dose-dependent inhibition of ET-1 production with concomitant increase in big ET-1 levels is the expected pattern from specific ECE-1 inhibition as described for phosphoramidon [12,14]. PD 069185 and PD 159790, after an incubation of 12 h, inhibited ET-1 production in a dose-dependent manner with EC_{50} values of 11.1 and 28.1 μM, respectively. However, the big ET-1 levels also decreased in a pattern resembling that observed for ET-1 (EC_{50} values of 8.7 and 37.5 μM for PD 069185 and PD 159790, respectively). Presumably, the decrease in both ET-1 and big ET-1 levels is mostly due to cytotoxicity of the compounds, since TC_{50} values of 19.6 and 89.0 μM were obtained for PD 069185 and 159790, respectively. A 2-fold difference between the TC_{50} and EC_{50} values is not sufficient to clearly separate inhibition from cytotoxicity. Similar EC_{50} and TC_{50} values were obtained when PD 069185 and PD 159790 were tested in human umbilical vein endothelial cells, EA.hy926, using the method described [14]. Obviously, inhibitors with higher potencies and/or less cytotoxicity will be required to conclude a successful test of intracellular inhibition using the double-transfection system.

Effectiveness of PD 159790 in Attenuating the Increase in Perfusion Pressure in the Perfused Rat Mesentery.

The average basal perfusion pressure of the perfused rat mesentery was 30 ± 3.3 mmHg (n = 37). As shown in Fig. 5, both big ET-1 and ET-1 induced increases in perfusion pressure in a concentration-dependent manner. ET-1 was approximately 5-fold more potent than big ET-1, which is consistent with reports by others [42]. The maximal increase in perfusion pressure was 150 ± 2.8 mm Hg for both peptides (n = 12). When PD 159790 was infused for 20 min prior to peptide challenge, the increase in perfusion pressure induced by big ET-1 was shifted to the right in a concentration-dependent manner. In the presence of 10 μM PD 159790, an increase in big ET-1 concentration of approximately 2-fold was required to achieve 50% of the maximum increase in perfusion pressure (75 mmHg) (Fig. 5A). In contrast, the response to ET-1 was not affected by 10 μM PD 159790 (Fig. 5B). Neither vehicle in concentrations up to 0.1% DMSO or PD 159790 at 10 μM had any effect on perfusion pressure. However, PD 159790 at 30 μM increased basal perfusion pressure

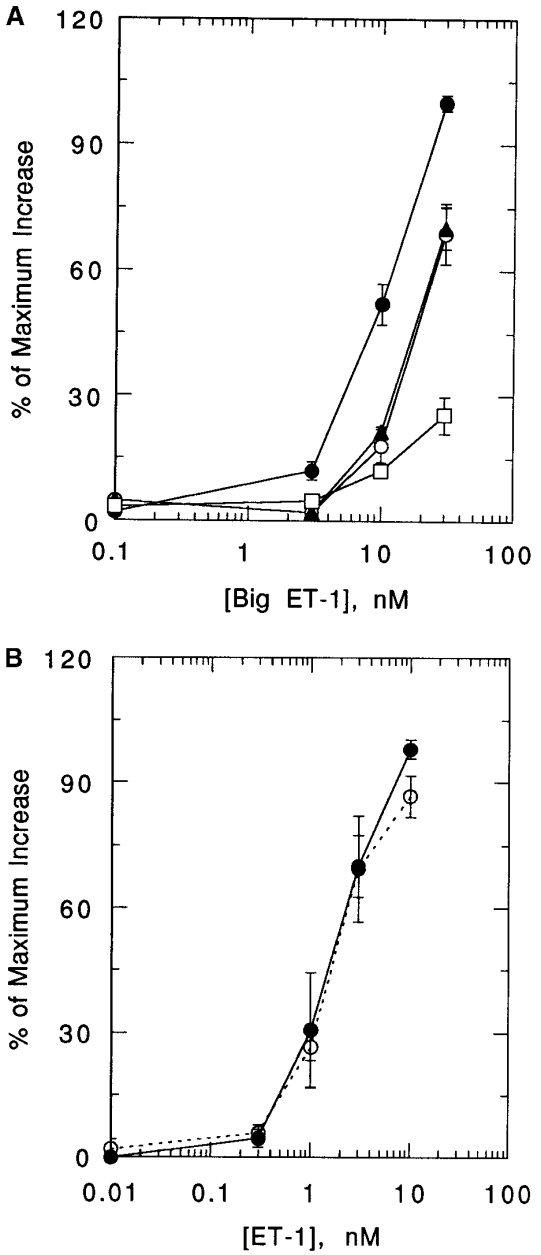


FIG. 5. Effect of 20 min infusion of PD 159790 (compound 7) on the increase in perfusion pressure induced by increasing concentrations of big ET-1 or ET-1 in isolated rat mesentery. For big ET-1 infusion (A), PD 159790 was at 0 μM (●), 10 μM (○), 30 μM (□), and 10 μM in the presence of 1 μM thiorphan (▲). For ET-1 infusion (B), PD 159790 was at 0 μM (●) and 10 μM (○). Each point represents the mean ± standard error of three to six experiments.

from 30 ± 3.3 to 52 ± 2 mmHg which then remained constant for the 20 min pretreatment period. The mechanism for this increase in the basal pressure by PD 159790 at 30 μM is unclear but may be related to some unknown alternate physiological pathway and/or to a subtle cytotoxic effect. Even though no cytotoxicity was observed at up to 100 μM PD 159790 (Because of the solubility limit of PD 159790, the cytotoxicity was tested only up to 100 μM) under similar conditions (See cytotoxicity measurement obtained from the coculture experiment), it is possible that the concentration used, 30 μM PD 159790, is not far enough from the TC_{50} value to be completely free of any cytotoxic effect.

Similar attenuation of the increase in big ET-1 induced perfusion pressure was obtained with 3 μM phosphoramidon; i.e., an increase in big ET-1 concentration of approximately 3-fold was required to achieve 50% of the maximum increase in perfusion pressure without affecting ET-1 pressor activity.

When thiorphan, a specific NEP inhibitor, was coinfused with PD 159790, the effectiveness of PD 159790 in shifting the big ET-1 concentration response curve was not altered (Fig. 5A). The only reported compound able to attenuate the increase in perfusion pressure induced by big ET-1 in the rabbit kidney has been the big ET-1 analog, big ET-1 [19-37, Phe^{22}]. However, big ET-1 [19-37, Phe^{22}] was effective only in the presence of a specific NEP inhibitor, SQ 28603, presumably due to the degradation of the peptide analog by NEP [43]. Unlike this peptidic analog, PD 159790 was effective in the absence of a NEP inhibitor.

The highly selective ECE-1 inhibitors presented here could prove to be useful tools for elucidating the physiological and pathological roles of ECE(s) as well as for developing inhibitors of greater potency.

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