Biochemical Pharmacology, Vol. 43, No. 4, pp. 845–853, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

ROLE OF MAST CELL CHYMASE IN THE EXTRACELLULAR PROCESSING OF BIG-ENDOTHELIN-1 TO ENDOTHELIN-1 IN THE PERFUSED RAT LUNG

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(*Received* 12 *July* 1991; *accepted* 8 *October* 1991)

Abstract—Previous studies of endothelin-1 (ET) synthesis have shown that some cultured endothelial cells secrete an intermediate product, big-endothelin-1 (bigET), suggesting that the processing of secreted bigET to ET may be physiologically significant. In this study, two pertinent ET converting enzyme activities, mast cell chymase I (EC 3.4.21.39) and a phosphoramidon-sensitive, neutral metalloprotease, were identified in a rat lung particulate fraction. We perfused rat lungs with bigET and chymostatin or phosphoramidon to study the relevance of these two proteases to the processing of extracellular bigET *in vivo*. Addition of compound 48/80 (a compound which activates mast cells, causing degranulation and release of chymase) to the perfusion buffer greatly increased hydrolysis of exogenously added bigET to ET. ET formation was inhibited completely by 32 μM chymostatin, whereas inhibition by 50 μM phosphoramidon was incomplete and variable. Perfusate histamine levels were used to monitor the extent of mast cell degranulation, and inhibition of ET production by phosphoramidon was attributed to inhibition of degranulation, *per se*. There was a direct correlation between perfusate ET and histamine levels in both control and phosphoramidon-treated (but not chymostatin-treated) lungs. Our results suggest that chymase from lung mast cells is capable of physiologically relevant extracellular processing by bigET to ET in the perfused rat lung.

Endothelin-1 (ET†) is a potent vasoconstrictor peptide synthesized and constitutively secreted from cultured endothelial cells [1]. Based on cDNA studies, ET (porcine) is derived from specific cleavage of a larger 203-residue precursor protein. Cleavages at two typical dibasic processing sites have been proposed to yield an intermediate product, big-endothelin-1 (bigET) [1]. Additional cleavage of bigET (Fig. 1) at an atypical, non-basic residue processing site, Trp^{21}-Val^{22}, is necessary to yield ET. Several candidate ET converting enzymes responsible for this latter cleavage have been identified, and attention has focused on a neutral metallo-endoprotease as a likely intracellular bigET processing enzyme [2, 3]. This protease from endothelial cell lysates converts bigET to ET and is inhibited by phosphoramidon. Relevance to intracellular processing rests on the observation that 0.1 mM phosphoramidon blocks processing of bigET in intact cells [4, 5].

Various endothelial cell lines secrete both bigET and ET into culture medium [4, 6, 7], indicating that

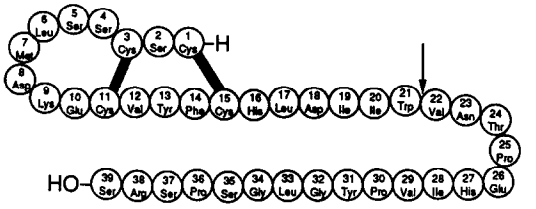

Fig. 1. Schematic diagram of the sequence for porcine bigET. Cleavage at the arrow yields ET as the amino terminal fragment.

putative ET converting enzymes do not fully process the available bigET intracellularly. The molar ratio of secreted bigET/ET can be as high as 1:1. Although bigET is a considerably less potent vasoconstrictor than ET *in vitro*, its effects *in vivo* are comparable, likely due to conversion of bigET to ET [8, 9]. Since bigET is not recognized by ET receptors, and no receptors specific for bigET have been identified, extracellular processing of bigET may be physiologically relevant.

Phosphoramidon also blocks the *in vivo* effects of exogenously added bigET, suggesting that a metalloendoprotease may be involved in both intracellular and extracellular processing of bigET [10, 11]. We propose, however, that chymase, a chymotrypsin-like protease released upon activation of mast cells, can be a major contributor to the extracellular processing of bigET to ET. Chymase readily processed bigET to ET *in vitro*. Additionally, we observed efficient processing of exogenous bigET in perfused rat lungs only after the activation of mast

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† Abbreviations: bigET, big endothelin-1, Cys^1-Ser^{39}; BSA, bovine serum albumin; CPAE, calf pulmonary arterial endothelial; DMSO, dimethyl sulfoxide; ET, endothelin-1, Cys^1-Trp^{21}; HBSS, Hanks' buffered salt solution; IgG, immunoglobulin G; IgE, immunoglobulin E; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sucFPF-pNA, succinyl-Phe-Pro-Phe-*p*-nitroanilide; and Taps, *N*-tris-[hydroxymethyl] methyl-3-amino-propanesulfonic acid.

cells. Processing of bigET was inhibited completely when chymostatin was included in the perfusion buffer. Although phosphoramidon partially blocked extracellular processing of bigET in this system, the results suggest that this is most likely an effect on the activation of mast cells rather than a direct inhibition of an extracellular processing enzyme. We chose compound 48/80 to activate mast cell degranulation in these experiments since degranulation in response to this agent, but not immunoglobulin E (IgE), is insensitive to chymostatin [12].

MATERIALS AND METHODS

Preparation of lung particulate fraction. Five pairs of lungs from Sprague–Dawley rats (250–350 g) were washed in 50 mM sodium phosphate, pH 7.5 buffer, minced, and then homogenized in 25 mL of buffer using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min, and the resulting supernatant was centrifuged at 300,000 *g* for 15 min. The final pellet was suspended in buffer and assayed for bigET hydrolytic activity.

Preparation of purified chymase from mast cell granules. Mast cells were obtained from peritoneal cavity washes of six male Sprague–Dawley rats (250–350 g) as described [13]. Hanks' buffered salt solution (HBSS; Gibco, Grand Island, NY) was used throughout as buffer. Mast cells were purified from other peritonial cells using an albumin gradient [14]; mast cells were located at the bottom of the gradient. The isolated cells were collected, washed twice, and frozen at −20° overnight in 0.5 mL of HBSS. The preparation was thawed, sonicated (30 sec, 30% power), and centrifuged (100 *g*, 8 min, 4°) to remove nuclear debris. Mast cell granules were obtained after further centrifugation (1250 *g*, 20 min, 4°), suspended in 0.5 mL of HBSS, and sonicated again. Octyl-Sepharose 4B hydrophobic chromatography was used to purify chymase from the granule preparation [14]. Purity of enzyme preparations was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

BigET and ET hydrolytic assays. Enzyme and 0.1 mM substrate [bigET (Peptides International, Louisville, KY) or ET (Peninsula Laboratories, Belmont, CA)] were incubated in 50 μL of 0.16 M *N*-tris-[hydroxymethyl]methyl-3-amino-propanesulfonic acid (Taps), pH 8.5, at 25°. The reaction was stopped by the addition of 40 μL of 0.25 M HCl in 75% CH_3CN, followed by incubation at 90° for 5 min. Seventy microliters of 0.14 M NaOH in 0.43 M sodium phosphate, pH 6.8, was added and the reaction was analyzed by HPLC as described previously [15]. When included, pepstatin was 20 μM and chymostatin was 30 μM. One unit of enzyme activity catalyzes the hydrolysis of 1 μmol of substrate/min at 25° at pH 8.5.

For the pH profile of bigET hydrolytic activity, enzyme and substrate (10 μM) were incubated in 50 mM buffers [pH 2 and 10, glycine; pH 3, sodium citrate; pH 4 and 5, sodium acetate; pH 6 and 7, bis-Tris; pH 8, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes); pH 9, Taps; pH 11, piperidine] containing 2 μM phosphoramidon, and the reaction was analyzed as above. In this case, one unit of enzyme activity catalyzes the formation of 1 μmol of ET/min at 37° at the given pH.

BigET and ET concentration determination by ELISA. Sample aliquots were coated on EIA/RIA plates (Costar, Cambridge, MA) overnight at 4°. Following blocking with polyethylene glycol, rabbit bigET antiserum (Peptides International) or rabbit ET antiserum (Peninsula Laboratories) was added. The second antibody was goat anti-rabbit IgG(F_c) conjugated with alkaline phosphatase (Promega, Madison, WI). Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) as the substrate. The bigET antiserum was at least 1000-fold specific for bigET vs ET, whereas the ET antiserum recognized both ET and bigET with approximately equal affinity.

ET concentration determination by receptor binding. For routine and specific quantitation of ET, a 96-well assay was developed using ET membrane receptors. This assay has the advantage that the receptor requires intact ET for high affinity binding and is therefore more specific than antibody assays, which normally recognize a more limited epitope. A detailed characterization of this assay will be presented elsewhere (manuscript in preparation).

Cerebellar membranes were prepared at 4° [16] with modifications as noted. Three bovine cerebella (Pel-Freez, Rogers, AR; 125 g total) were minced and then homogenized in 750 mL of homogenization buffer (5 mM Tris, 0.32 M sucrose, pH 7.5), using a Polytron homogenizer (Brinkmann, Westbury, NY) at maximum power for 1 min. The homogenate was centrifuged at 4000 *g* for 15 min, and the resulting supernatant was centrifuged at 27,000 *g* for 45 min. The final pellet was suspended in 150 mL of homogenization buffer, and placed on ice for 15 min. The suspension was then further homogenized for 30 sec and centrifuged as before. The loose tan-colored portion of the pellet was suspended gently in 10 mL of 50 mM Tris, pH 7.5 buffer, excluding the bottom, dark brown portion of the pellet. The suspension was diluted to 5 mg/mL protein and stored at −120°.

The membrane suspension was diluted 50-fold in 10 mM sodium phosphate, 150 mM NaCl, pH 7.5, buffer and dounced (Potter–Elvehjem) several times. Aliquots (0.1 mL) were pipeted into each well of an EIA/RIA plate (Costar), and the plate was centrifuged (800 *g*, 10 min, 4°). The membranes were fixed on the plate at 4° overnight. Non-specific binding to the plate was blocked by preincubation with 5 mg/mL bovine serum albumin (BSA) in the dilution buffer (30 min, 25°).

Unlabeled test ligands were pre-mixed with ^{125}I-ET (Amersham, Arlington Heights, IL) prior to assay. The mixture added to each well contained 25 μL of assay buffer (10 mM Tris, 1 mM $CaCl_2$, 1 mg/mL BSA, pH 7.5), 25 μL of 180 pM ^{125}I-ET (0.01 μCi) in assay buffer, and 50 μL of sample in Media 199 (Gibco). The plate was incubated subsequently for 90 min at 25°. The unbound ligand was aspirated, and the wells were washed with assay buffer. The bound ligand was extracted by the addition of 0.5% SDS, 10 mM Tris, pH 7.5 buffer (10 min, 25°) and counted.

Lung perfusion procedure. Tail veins from male

Sprague–Dawley rats (300–400 g) were dilated, 25 mg of Nembutal (Abbott Laboratories, Chicago, IL) in 0.5 mL of alcohol:polyethylene glycol:water (10:40:50) was injected as anaesthetic, and 300 U of heparin in 0.3 mL sterile water was injected to aid in clearing blood from the lungs. The trachea was cannulated with a 16-gauge intramedic adapter and tied in place using 4-0 silk. The lung was ventilated with room air at 70 bpm, 1.5 mL/stroke. The abdominal aorta and vena cava were severed to exsanguinate the rat. After exposing the thoracic cavity and removing the thymus gland, an incision was made in the right ventricle, and the pulmonary artery was cannulated with silastic tubing. The left atria was cut open to allow collection of perfusate, and 5 mL of Krebs buffer was flushed into the pulmonary circulation at 0.5 mL/min. The lung was disconnected from the respirator, remaining lung and trachea connections were severed, and the lung was placed in an organ chamber at 37° and reconnected to the respirator. The lungs were kept moist by covering the top of the organ chamber with polyvinyl film. Perfusion was begun at a flow rate of 1 mL/min using perfusion buffer (0.11 M NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 12 mM dextrose, 5% Ficoll, 1 μM diphenhydramine, 1 μM ranitidine, 10 μM indomethacin, 100 U/mL superoxide dismutase) gassed with 95% O_2/5% CO_2 at 37°.

Lungs were flushed with buffer for an initial control period of 15 min. The end of the control period was designated t_0, and perfusate fractions (1 min) were collected subsequently for a total of 30 min. BigET (20 nM) and compound 48/80 (0.1 mg/mL, a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde; Sigma) were added to the perfusion buffer at 10 and 20 min, respectively. After 30 min, edema in the lung often precluded further perfusate collection. When included, 50 μM phosphoramidon or 32 μM chymostatin was added to the perfusion buffer at t_0. All additions to the perfusion buffer were maintained at the noted concentrations throughout the remaining course of perfusion. BigET and ET were added as solutions in dimethyl sulfoxide (DMSO); the final DMSO concentration in the perfusion buffer was <0.6%. In control experiments, DMSO was used alone.

*Succinyl-Phe-Pro-Phe-*p*-nitroanilide (sucFPF-pNA) hydrolytic assay.* Enzyme and substrate (0.1 mM sucFPF-pNA; Bachem, Switzerland) were incubated in 150 μL of 0.16 M Taps, pH 8.5, in a 96-well microplate at 25°. Formation of pNA product was monitored at 405 nm. One unit of enzyme activity catalyzes the formation of 1 μmol of pNA product/min at 25°, pH 8.5.

Histamine concentration determination. Histamine methyltransferase was partially purified [17] through the ammonium sulfate precipitation and subsequent dialysis steps, and used with *S*-[^{3}H]adenosylmethionine (New England Nuclear, Boston, MA) as a cofactor to measure histamine [18].

Protein concentration determination. Protein concentrations were determined using the Bradford protein assay according to the manufacturer's instructions (Bio-Rad, Richmond, CA) or estimated based on the extent of silver-staining of protein bands after SDS–PAGE analysis.

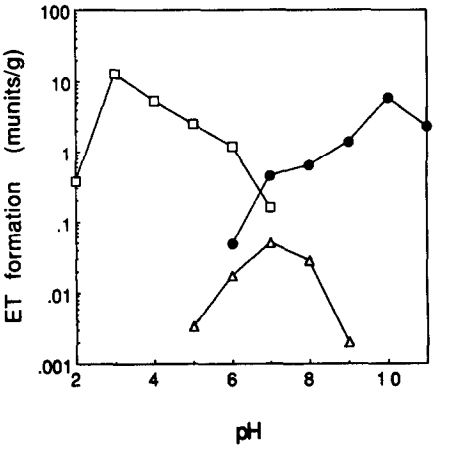

Fig. 2. pH profile for the conversion of bigET to ET in the particulate fraction of lung homogenates. Enzyme and bigET were incubated at 37° at the indicated pH, and reactions were subjected to reverse-phase HPLC analysis. Units are defined in Materials and Methods, and activity is normalized per gram of tissue. Three distinct peaks of activity are revealed in the presence of various protease inhibitors. Key: (□) 2 μM phosphoramidon alone, (●) plus 20 μM pepstatin and (△) plus pepstatin and 30 μM chymostatin.

RESULTS

pH profile for hydrolysis of bigET by lung homogenate. The conversion of bigET to ET in the lung particulate fraction as a function of pH is shown in Fig. 2. ET product identity was confirmed at each pH by coelution with synthetic ET on reverse-phase HPLC [15], as well as recognition by both ET antibodies and the ET receptor. Quantitation by HPLC peak height, ELISA and the receptor binding assay agreed. Two major peaks of activity as well as a minor peak of activity were observed using combinations of protease inhibitors as noted. Inhibition profiles for the three peaks of activity are shown in Table 1. Phosphoramidon (2 μM) was included in all assays to block possible degradation of ET by neutral endopeptidase 24.11 [19].

The large peak with an acidic pH optimum is analogous to previously identified pepstatin-sensitive aspartic protease(s) with converting enzyme activity [20–22]. A second large peak of activity with a broad, alkaline pH optimum was also observed. This protease was inhibited by chymostatin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride (PMSF), but not by EDTA or *p*-chloromercuribenzoate (PCMB), indicating that it is a serine protease (Table 1).

In the presence of both pepstatin and chymostatin, a third minor peak of activity with a neutral pH optimum was detected. This protease was inhibited by EDTA and phosphoramidon, and is analogous to a previously identified phosphoramidon-sensitive protease from cultured endothelial cells [2, 3]. The

Table 1. Inhibition profiles for lung particulate fraction endothelin converting enzyme activities

Inhibitor	Concentration	% Inhibition		
		pH 3.5	pH 7.5*	pH 10†
EDTA	1.0 mM		57	(−20)‡
Phosphoramidon	1.0 mM		92	
Pepstatin	0.015 mM	97		
Chymostatin	0.07 mM		27†	99
Soybean trypsin inhibitor	10 μg/mL			98
PMSF	5.0 mM		(−90)‡	64
PCMB	0.1 mM		(−16)‡	17
N-Ethylmaleimide	1.0 mM		2	
Leupeptin	0.01 mM		8	0

See Fig. 2 for control values.
* Assayed in the presence of 2 μM phosphoramidon, 20 μM pepstatin and 30 μM chymostatin.
† Assayed in the presence of 2 μM phosphoramidon and 20 μM pepstatin.
‡ Negative numbers indicate increased yields of ET.

activity of this neutral protease increased 2-fold when 2 μM phosphoramidon was omitted from the assays. The apparent increase in ET formation in the presence of PMSF was attributed to inhibition of a PMSF-sensitive protease that degrades ET in the tissue homogenates.

Both the acidic and neutral proteases were found at comparable specific activities in calf pulmonary arterial endothelial (CPAE) cells. However, the alkaline serine protease was not detected in these endothelial cells or in three other cell lines examined: RBL-1 cells, rat lung fibroblasts, and human peripheral neutrophils. This serine protease was detected exclusively in the particulate fraction of lung homogenates and was solubilized using 1 M KCl. Attempts to solubilize the enzyme from the particulate fraction using 8 M urea or various detergents at 1 mg/mL (e.g. Triton X-100, Tween 20, Brij 58, cholate, or deoxycholate) were ineffective. Based on its pH, inhibitor and solubilization profiles, the enzyme was tentatively identified as chymase from mast cells in the lung; this identification was confirmed as described below.

Mast cell granule chymase: Purification and hydrolysis of various synthetic peptide substrates. Chymase was purified from rat peritoneal mast cells [13, 14] to electrophoretic homogeneity ($M_r \sim 29{,}000$ by SDS–PAGE). Chymase hydrolyzes a standard substrate, sucFPF-pNA, at the F-pNA bond yielding the chromogenic product pNA. BigET and ET were also cleaved by chymase. A comparison of the hydrolytic activity in various chymase preparations against these three substrates is shown in Table 2. Hydrolysis of all substrates was linear with time and enzyme concentration. Homogeneous chymase was purified 27-fold relative to the mast cell granules, and the ratio of turnover of bigET and ET relative to the standard sucFPF-pNA substrate did not change with purification, indicating that the hydrolytic activity detected was due to chymase rather than a contaminating activity.

Time course for hydrolysis of bigET by purified chymase. Purified chymase cleaved bigET at the Trp^{21}-Val^{22} bond and at three other sites detected by reverse-phase HPLC analysis. Formation of these products was followed as a function of time (Fig. 3). Products were identified by combinations of amino terminal sequence analysis, mass spectroscopy, and coinjection with synthetic peptide standards. As bigET was hydrolyzed, concomitant formation of ET and the carboxy terminal fragment bigET(Val^{22}-Ser^{39}) was evident, with maximal concentration at about 5 min. ET was subsequently cleaved at the Tyr^{13}-Phe^{14} and Phe^{14}-Cys^{15} bonds to yield the corresponding ET internally-clipped peptide products, both of which must contain an intact disulfide bridge at least at Cys^{1}-Cys^{15} since the peptide products contain both the amino and carboxy terminal fragments based on sequence analysis. The rate constant for degradation of ET was nearly equal to its rate of formation, consistent with the data in Table 2. The carboxy terminal fragment from the initial cleavage of bigET, bigET(Val^{22}-Ser^{39}) was subsequently cleaved at the Tyr^{31}-Gly^{32} bond. This bond in bigET itself was also cleaved, but at a rate <5% of the rate of ET formation.

Chymotrypsin is similar to chymase in its substrate specificity, and does convert bigET to ET [23]. The product profile for chymase is qualitatively similar to that for chymotrypsin [24]; but we found that cleavage of bigET by chymotrypsin at the Tyr^{31}-Gly^{32} bond was 180 times faster than cleavage at the Trp^{21}-Val^{22} bond. Formation of ET by chymotrypsin is, therefore, a stepwise process via an intermediate peptide, bigET(Cys^{1}-Tyr^{31}). The specific activities for cleavage at the Trp^{21}-Val^{22} bond were approximately equal for the two proteases. Therefore, the major difference between the two proteases was in the rate of hydrolysis of the Tyr^{31}-Gly^{32} bond. ET was also degraded by chymotrypsin by cleavage at the Tyr^{13}-Phe^{14} bond, but at <5% of the rate of ET formation.

Conversion of bigET to ET in perfused rat lungs. The effects of protease inhibitors on ET levels in rat lungs perfused with bigET are shown in Fig. 4. The data in the figure are the means of 3–5 rat lungs for each condition. BigET and compound 48/80 were

Table 2. Specific activity of chymase preparations

Enzyme preparation	Specific activity (U/mg)			Activity ratios	
	bigET*	ET*	sucFPF-pNA	bigET/sucFPF-pNA	ET/sucFPF-pNA
Lung particulate fraction	0.005		ND†		
Mast cell granule homogenate	1.1	0.74	5.3	0.28	0.19
Purified chymase	30	20	84	0.35	0.23

* Activity was measured as the rate of loss of substrate.
† Activity was undetectable (<0.0001 U/mg).

added to the perfusion buffer at 10 and 20 min, respectively. The collected perfusate fractions can be divided into three 10-min regions: region I represents conditions before bigET perfusion, region II represents bigET perfusion prior to mast cell activation, and region III represents bigET perfusion after activation of mast cells. In all experiments, the ET concentration in region I was constant at ~0.2 nM. This represents secretion of endogenous ET. After addition of bigET, there was an initial increase (about 4-fold) in the amount of ET detected in region II. In region III after addition of compound 48/80, a much larger increase in ET concentration was seen within 1 min. Maximal ET concentration in region III was ~4 nM. In controls in which bigET was omitted from the perfusion buffer, compound 48/80 itself did not induce an increase in ET levels. The large standard errors in ET concentrations, especially in region III, were due to variations between lungs from different animals; the precision of measurement for individual perfused fractions was ±3%.

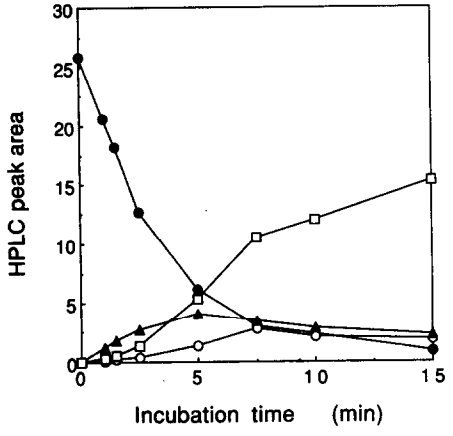

Fig. 3. Time-dependent hydrolysis of bigET by purified chymase. Key: (●) bigET, (▲) ET, (□) ET cleaved at Tyr^{13}-Phe^{14} plus ET cleaved at Phe^{14}-Cys^{15}, and (○) bigET(Cys^{1}-Tyr^{31}).

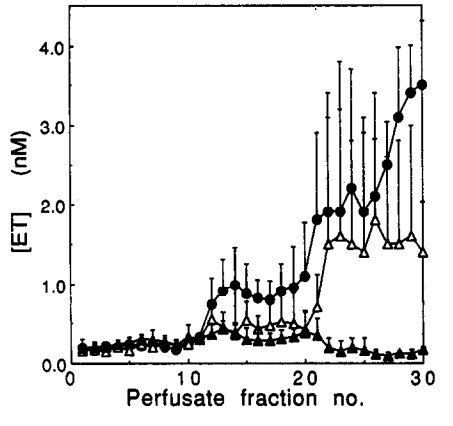

Fig. 4. Conversion of bigET to ET in perfused rat lungs. BigET and compound 48/80 were added to the perfusion buffer at 10 and 20 min, respectively. ET concentration was measured using the membrane-binding assay, and results shown are means ± SEM from three to five rats for each condition. Key: (●) control, (△) 50 μM phosphoramidon and (▲) 32 μM chymostatin.

Attempts were made to inhibit conversion of bigET to ET in the isolated lung preparations using protease inhibitors. After addition of 32 μM chymostatin to the perfusion buffer, there was no increase in ET levels in response to addition to either bigET or compound 48/80 (Fig. 4). In contrast, addition of 50 μM phosphoramidon to the perfusion buffer yielded variable results. Phosphoramidon partially inhibited formation of ET in both regions II and III, but in no case was inhibition as complete as with chymostatin.

In control experiments, ET (6 nM) instead of bigET was perfused into the lung; only 49 ± 13% ET was recovered in region II, and addition of compound 48/80 did not affect recovery of ET so that comparable recoveries were obtained in region III. Essentially the same level of recovery of ET from isolated, perfused guinea pig lungs has been reported and characterized previously [25, 26]. When ET was perfused in the presence of chymostatin, a similar recovery of ET was observed, 36 ± 7%.

Histamine release in perfused rat lungs. Since we are proposing a role for activated mast cells in the conversion of bigET to ET in the lung, sources of variability include the number of mast cells in the lung and the efficiency of their degranulation by compound 48/80. As a marker for the number of activated mast cells in the rat lungs, we measured release of histamine to the perfusate. In control experiments, the background level of histamine in both regions I and II was 70 ± 30 nM. The histamine concentration in region III after addition of compound 48/80 increased about 20-fold to

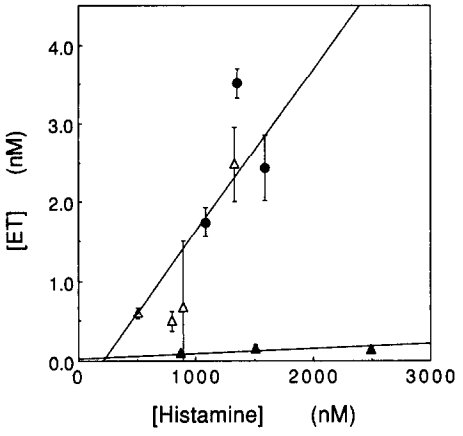

Fig. 5. Comparison of histamine and ET levels for individual lungs in pooled perfusate fractions from region III. Perfusate fractions 21–30 from region III after addition of compound 48/80 were pooled for individual lungs and assayed to determine histamine and ET concentrations. Values shown are means ± SEM, N = 10. Key: (●) control, (△) 50 μM phosphoramidon and (▲) 32 μM chymostatin.

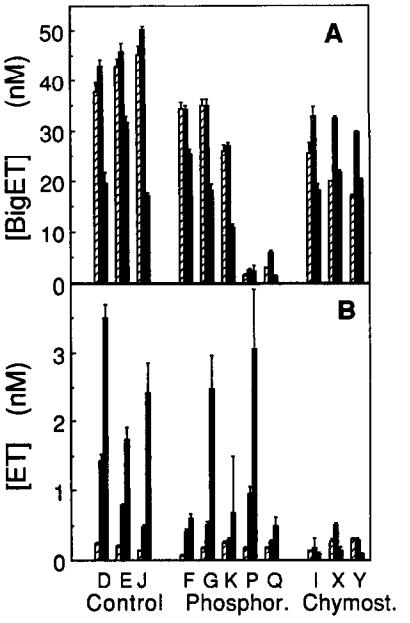

Fig. 6. BigET and ET concentrations in perfusates for individual lungs from pooled regions I, II and III. The bigET (panel A) and ET (panel B) concentrations were averaged for perfusate fractions 1–10, 11–20 and 21–30 from regions I (▨), II (▨) and III (■), respectively. Values are means ± SEM. Region I includes the initial perfusate fractions. Region II includes fractions after addition of bigET, and region III includes fractions after the addition of compound 48/80. Individual rat lungs were assigned letters as noted on the X-axis. Larger error bars in region III are due to the fact that concentrations were changing with time across the region.

1300 ± 200 nM. ET and histamine levels in region III are compared for individual lungs in Fig. 5. In both control and phosphoramidon-treated lungs, the levels of ET correlated directly with levels of histamine. In contrast, in chymostatin-treated lungs, ET formation was blocked without a corresponding effect on histamine levels.

BigET concentrations in the lung perfusate. Average bigET and ET concentrations for each of the three regions are presented for individual rat lung preparations in Fig. 6. As noted previously, the ET levels in region III were always greater than those in region II, and those in region II always greater than those in region I. BigET levels in each region did not follow such a clear-cut pattern. Surprisingly, the bigET levels detected in region I were higher than expected, though variable, ranging from 2 to 45 nM. The identity of the immunoreactive-bigET in both regions I and II was confirmed by reverse-phase HPLC; recovery was quantitative (100–110%) in a single peak with the same retention time as synthetic bigET. In addition, the immunoreactive-bigET was subjected to pepsin digestion [27], and quantitative conversion to ET was confirmed using the ET receptor assay.

The amount of bigET in region II was also variable when compared to the amount in region I. We expected a 20 nM increase in bigET levels; but, although some rats displayed a 7–13 nM increase (e.g. I, X and Y), most levels increased only slightly or remained constant. BigET levels in region III (i.e. after mast cell degranulation) were always lower than those found in region II for individual lungs, but the amount of ET detected in region III did not correlate with either bigET levels in region II (e.g. especially in experiment P) or the decrease in bigET levels from region II to region III (e.g. in chymostatin vs control perfusates).

DISCUSSION

We have attempted to identify and characterize ET converting enzymes from rat lungs. Initial pH profile studies of bigET hydrolysis by the lung particulate fraction delineated three peaks of activity. The large peak of bigET hydrolytic activity with an acidic pH optimum was an aspartic protease. A minor peak of activity with a neutral pH optimum was a metalloprotease similar to that proposed to be responsible for intracellular bigET processing. Both of these proteases have been shown previously to process bigET [2, 3, 20–22].

The third large peak of bigET hydrolytic activity with a basic pH optimum was a serine protease based on its inhibition profile. It was found in rat peritoneal mast cells, but not in four other cell types: CPAE cells, fibroblasts, neutrophils or RBL-1 cells. The ET converting enzyme activity in mast cells was associated with the granules and copurified with mast cell chymase I. When the activity of crude ET converting enzyme in mast cell granules was compared to that in the particulate fraction of lung homogenates, the specific activity in the lung was 0.5% of that in mast cells. This finding is consistent with previous measurements of mast cell densities in lung [28]. These results together strongly suggest that the serine protease activity in the lung is attributable to chymase.

Since the pressor response in rats injected with

bigET is blocked by phosphoramidon, a role for a phosphoramidon-sensitive protease in extracellular processing of bigET has been proposed [10, 11]. We used the inhibitors phosphoramidon and chymostatin to compare the possible physiological relevancies of the neutral metalloprotease and chymase, respectively, to the maturation of circulating bigET in perfused rat lungs. Compound 48/80 was used to activate mast cells in order to expose perfused bigET to chymase.

Basal levels of ET in our lung perfusates were ~0.2 nM. For comparison, previous studies have shown that in rat and pig the lung has one of the highest tissue levels of ET or ET mRNA; ET tissue levels in porcine lung are 0.2 to 1.5 pmol/g tissue [29–31]. In our lung preparations, the basal ET levels in region I were not affected by addition of phosphoramidon or chymostatin, and therefore do not appear to result from processing of endogenous extracellular bigET.

When 20 nM bigET was added to the perfusion buffer, ET levels increased to 0.8 nM, and subsequently reached as high as 4 nM upon stimulation by compound 48/80. The increase in ET levels in response to compound 48/80 represents turnover of exogenously added bigET; no increase was observed in control experiments without addition of bigET.

When chymostatin was present in the perfusion buffer, there was no significant increase in the amount of ET formed in response to the addition of either bigET or compound 48/80. Thus, chymostatin totally inhibited the extracellular ET converting enzyme activity in rat lung preparations. These results suggest that mast cell chymase is responsible for turnover of exogenous bigET to ET in this system.

The highest concentration of ET in the perfusates from region III represented a 20% yield from the added bigET. If corrections are made for about 50% overall recovery of ET from the lung determined from control experiments, then the efficiency of bigET conversion was as high as 40%. This was higher than the efficiency of ET formation by chymase *in vitro* since the purified enzyme both generates and degrades ET, and only an ~15% maximum yield of ET is obtained at steady state. Near maximal efficiency of ET formation in the perfused lung is therefore implied, and selective protection of ET from degradation may be also occurring.

When phosphoramidon was added to the perfusion buffer, the results were variable. In three experiments, there was approximately 40% inhibition of bigET conversion to ET; yet in two other experiments, there was no significant difference from controls. However, in both phosphoramidon and control experiments, lower ET levels clearly correlated with lower histamine levels in region III, and lower levels of histamine in region III may indicate low levels of mast cells or incomplete mast cell degranulation. Therefore, phosphoramidon may affect the perfused lung preparations by inhibition of mast cell degranulation rather than by direct inhibition of an ET converting enzyme. This conclusion is fully consistent with previous observations that 25 μM phosphoramidon inhibits compound 48/80-induced histamine release from mast cells by nearly 30% [32]. The mechanism of this inhibition is not known.

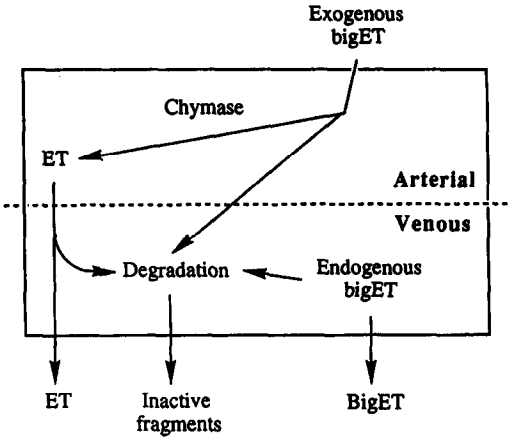

Fig. 7. Schematic diagram of the proposed localization of bigET processing and degradative enzymes in the capillary bed of the lung.

BigET levels detected in region I, before bigET was added to the perfusion buffer, were unexpectedly high and variable. This endogenous bigET appears to be distinguishable from exogeneous bigET added to the perfusion buffer with respect to its ability to be hydrolyzed to ET in the lung. There was no evidence for chymostatin-sensitive formation of ET in region I, before addition of exogenous bigET; there was no correlation between ET levels in region III and levels of endogenous bigET in region I; and when exogenous bigET was omitted from the perfusate in control experiments, no ET was formed in response to compound 48/80.

We know of no precedent to explain these anomalies, but we speculate that processing of exogenous bigET by chymase may occur at the arterial side of the capillary bed, whereas secretion of endogenous bigET may occur on the venous side. In this way, only exogenously added bigET would come in contact with chymase in the perfused lung. This model is described pictorially in Fig. 7. It is important in this respect that chymase complexes with heparin proteoglycans and other proteins after degranulation of mast cells and remains associated with the cells [25, 33]. Therefore, rather than actually releasing chymase, stimulation of mast cells with compound 48/80 merely exposes chymase to the extracellular milieu while at the same time maintaining the localization of the enzyme in the tissue.

The above explanation assumes that if endogenous bigET were converted to ET, then the ET would appear in the perfusates. An alternative explanation would be that ET arising from endogenous bigET is selectively and quantitatively retained by the tissue. The data do not distinguish between these possibilities.

Another apparent anomaly in the bigET levels is the observation that addition of 20 nM exogenous bigET to the perfusion buffer did not result in a concomitant 20 nM increase in bigET perfusate levels. We suggest, therefore, that if the exogenous bigET was not metabolized to ET on the arterial side of the capillaries, it was degraded rapidly to peptides that were not detected by our ET membrane receptor assay (Fig. 7). Furthermore, bigET levels in the perfusate uniformly decreased in region III after stimulation by compound 48/80. This decrease in bigET levels did not correlate with ET levels in region III, and occurred equally well in the presence or absence of chymostatin. Thus, the decrease in bigET levels does not simply represent ET formation and may in addition represent degradation of endogenous bigET to undetected peptides.

The results presented in this paper suggest that extracellular bigET in the lung can be processed efficiently to ET after degranulation of mast cells and subsequent exposure to chymase. If extracellular processing of bigET is physiologically relevant, then processing by chymase in the lung must clearly be considered. In contrast, a phosphoramidon-sensitive enzyme is not likely to be involved in extracellular bigET processing in this system. Our results do not necessarily extrapolate to the hypertensive effect observed when bigET is injected *in vivo*. They do suggest, however, that inhibition of bigET processing by phosphoramidon *in vivo* does not necessarily require that phosphoramidon is acting by directly inhibiting an ET converting enzyme. Further, the results caution that it may be misleading to interpret the physiological role of endogenous bigET based on *in vivo* effects of exogenous bigET. The metabolism of extracellular bigET in the lung appears to represent a complex balance between secretion of bigET, maturation to ET and degradation to inactive products, with each process possibly occurring at spatially distinct sites.

Acknowledgements—We thank Dr. Harry Levine for development of the ET bovine cerebellar membrane receptor assay and Kanak Singh for technical assistance.

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