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Effect of Angiotensinase Inhibitors on Angiotensin Receptors in Rabbit Aorta

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SUMMARY

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Caproic acid (1 cM) and EDTA (10 mM) blocked the cell membrane angiotensinase of rabbit aorta. Caproic acid significantly increased the maximal response to angiotensin and delayed the relaxation time of contracted aortic strips. EDTA decreased the maximal response and had no effect on relaxation. This was attributed to EDTA chelating capacity for Ca ions. The binding studies revealed an increased angiotensin receptor binding in the presence of antiotensinase inhibitors. The increased maximal response and delayed relaxation time observed with caproic acid might be due to uncovering of additional angiotensin receptors and increased agonist availability at the receptor site after angiotensinase blockade. This in turn stimulates some post-receptor mechanism leading to increased intracellular Ca^{2+}.

INTRODUCTION

It is generally accepted that angiotensin receptors in vascular smooth muscle cells are bound to the cell membrane (1, 2). Recently, we were able to demonstrate the existence of angiotensinase activity in the cell membrane fraction of guinea-pig aorta (3). This is in disagreement with Meyer *et al.* (2) who did not find any significant angiotensinase activity in the microsomal fraction of rabbit aortic tissue. They suggested that angiotensin receptors are homogeneous molecular entities, functioning independently (non-cooperatively) from angiotensinases. Our present results provide evidence that angiotensinase is involved in angiotensin receptor function.

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MATERIALS AND METHODS

Female New Zealand albino rabbits (1.5–2.5 kg) were stunned and exsanguinated by section of the carotid arteries. The aortae were removed, freed of any connective tissue, and placed in ice-cold histidine buffer (histidine-HCl 10 mM, KCl 100 mM, $MgCl_2$ 5 mM, pH 7.4). For the isolated organ studies, only the thoracic aorta was used; in the remaining experiments, the entire aorta was used.

Binding studies. The aortae were homogenized in a Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland) at maximum speed for one minute. The homogenate was passed through cheesecloth and the plasma membrane fraction isolated by the method of Fitzpatrick *et al.* (5). The fraction was used either immediately, or stored no more than 18 hours at −20°.

Aliquots of the plasma membrane frac-

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tion, resuspended in histidine buffer (200–300 μg protein/ml), were incubated at 37° for 5 min with varying amounts of labeled angiotensin, both in the presence and absence of a large excess (1 μM) of unlabeled angiotensin or 8-leu angiotensin. The total assay volume was 1.0 ml, the various components being present in the following proportions: A) 0.5 ml membrane suspension, 0.2 ml histidine buffer, 0.1 ml labeled angiotensin, 0.2 ml unlabeled angiotensin, or B) 0.5 ml membrane suspension, 0.4 ml histidine buffer, 0.1 ml labeled angiotensin. For the experiments involving the angiotensinase inhibitors, solutions of 10 mM EDTA and 1 cM caproic acid were made up in the histidine buffer and pH adjusted to 7.4. The incubation was terminated by the addition of 4 ml ice-cold histidine buffer, and the sample was vacuum filtered through Millipore Millex filters, pore size 0.45 μm (Millipore-Worthington). The assay tubes were washed twice with 5 ml histidine buffer and this wash was also filtered. The filters were then dissolved in 10 ml Aquasol (New England Nuclear) and counted in a Packard Liquid Scintillation counter.

Specific binding of [^{3}H]angiotensin was defined as the portion of total binding inhibited by a relative excess (1 μM) of nonlabeled angiotensin or 8-leu-angiotensin.

Nonspecific binding to filters was found to be 1.5%. The linearity of specific binding vs. protein concentration has been previously established (1). Protein determinations were done by the method of Lowry (10).

Marker enzymes. To evaluate the degree of purity of the plasma membrane fraction, marker enzyme activity was determined. 5'-Nucleotidase activity was measured according to the method of Emmelot and Boss (7). Glucose-6-phosphatase was determined by the method of Marchand *et al.* (8). The method of Pennington (9) was used for the determination of succinic-INT-reductase. The enzyme activities found in the final high-speed supernatant and plasma membrane fraction are presented in Table 1. As can be seen, the membrane fraction is devoid of mitochondria, enriched in plasma membranes, and possesses a low level of endoplasmic reticular contamination.

Angiotensinase activity. The angiotensinase activity of both the plasma membrane and circulating enzyme was determined according to the method of Jelinek *et al.* (11), in which the pressor effect of residual angiotensin is measured on rat blood pressure preparation.

Isolated organ studies. The aortic strips were prepared and studied as previously described (12). The oil immersion technique of Kalsner and Nickerson (13) was used to study the relaxation time and disposition mechanisms.

Reagents. Angiotensin amide (Hypertensin, Ciba), 8-leucine angiotensin (gift from Dr. W. K. Park, University of Sherbrooke), [^{3}H]angiotensin 40 Ci/mM (New England Nuclear), EDTA (disodium salt, British Drug House, Ltd.), epsilon-amino-*n*-caproic acid (Schwarz-Mann Comp.), norepinephrine HCl (Sigma Corp.).

RESULTS

Effect of angiotensinase inhibitors on reactivity of rabbit aortic strips to angiotensin. Two well known angiotensinase inhibitors, EDTA and caproic acid, were used in our studies. Single concentrations of 0.1 mM EDTA and 1 cm caproic acid were chosen since they have been found to have the maximal inhibitory effect on angioten-

TABLE 1

Specific activities of marker enzymes in subcellular fractions of aortic smooth muscle

Fraction	5'-Nucleotidase	Succinic-INT-reductase	Glucose-6-phosphatase
	(μmol p_i/mg protein/hr)	*(μmol INT reduced/mg protein/hr)*	*(μmol p_i/mg protein/hr)*
Supernatant	133.8 ± 22.9	0.0	25.2 ± 6.1
Pellet	2741.5 ± 128.6	0.0	162.1 ± 71.8

Values represent mean ± SEM of six experiments.

sinase activity in aortic tissue (14, 15). Figure 1 shows the dose-response curves for angiotensin and norepinephrine in the presence of angiotensinase inhibitors. Caproic acid significantly potentiated the response of aortic strips to angiotensin and produced an increased maximal response. EDTA decreased maximal response of the strips. Neither of these substances significantly altered the apparent affinity constant for angiotensin (10 mM) as judged from the ED_{50}. The effects of both substances are abolished if applied together.

In order to determine the specificity of the observed effects, the reactivity of aortic strips to norepinephrine was examined. Caproic acid depressed, and EDTA potentiated the reactivity to norepinephrine.

Effect of angiotensinase inhibitors on angiotensin disposition. Using the method of Kalsner and Nickerson (13), the time required for complete relaxation of maximally contracted aortic strips was measured. As can be seen from Figure 2, caproic acid significantly delayed relaxation time for strips stimulated by angiotensin but had no effect on norepinephrine-stimulated strips. Conversely, EDTA did not affect angiotensin-stimulated strips but extended the relaxation time for strips stimulated by norepinephrine.

Effect of EDTA and caproic acid on angiotensin inactivation by membrane fraction and by high-speed supernatant. The angiotensinase activity was assayed by the method of Jelinek *et al.* (11) on rat blood pressure preparation. Figure 3 illustrates the results. In the plasma membrane fraction, caproic acid consistently inhibits the angiotensinase, while EDTA does so only after a protracted delay. In the supernatant, both compounds inhibit the angiotensinase activity.

Binding studies. Table 2 summarizes the results obtained in binding studies. Control and 8-leu angiotensin results are almost identical, suggesting that nonlabeled angiotensin and its antagonist compete for the same receptor site. We have found essentially the same results in guinea pig aorta (1). The saturation of binding occurs at 0.5 μM. In the presence of EDTA and caproic acid, the specific angiotensin binding was increased throughout the range of concentrations used.

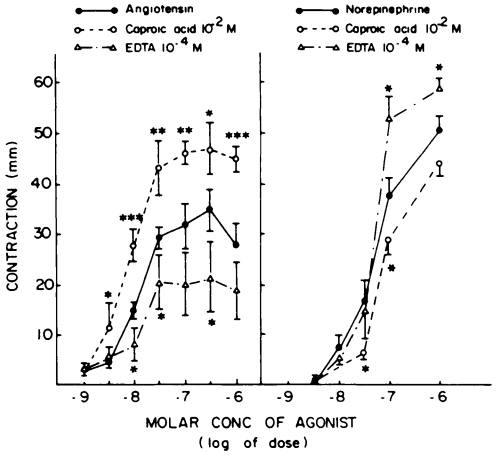

FIG. 1. *Dose response curves to angiotensin and norepinephrine in the presence of angiotensinase inhibitors*

Mean of 6–8 experiments ± S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

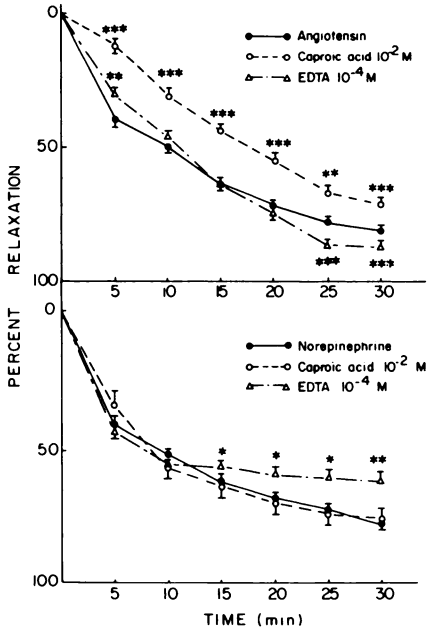

FIG. 2. *Effect of angiotensinase inhibitors on relaxation time of contracted rabbit aorta*

The maximal contraction was produced with 0.1 μM angiotensin. Mean of 6–8 experiments ± S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DISCUSSION

Previously we have demonstrated that heating of aortic strips at 47° for 20 min

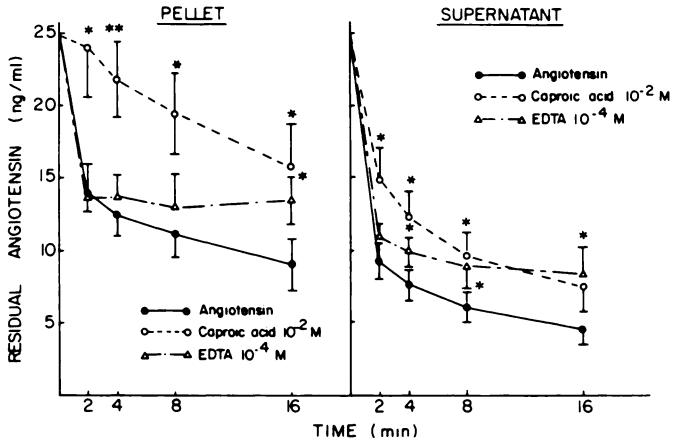

FIG. 3. *Effect of caproic acid and EDTA on angiotensinase activity in high-speed supernatant and plasma membrane fraction of rabbit aorta*

The pressor effect of residual angiotensin after incubation for different time periods was measured on rat blood pressure preparation. 0.25 nM angiotensin was incubated with 100–150 μg of protein/ml of supernatant or plasma membrane fraction. * $p < 0.05$.

TABLE 2

Specific [^{3}H]angiotensin binding (DPM/mg of protein) to plasma membrane fraction of rabbit aorta in the presence of angiotensinase inhibitors

Aliquots of plasma membrane fraction (100–150 μg of protein) were incubated with varying concentration of [^{3}H]angiotensin after preincubation with 1 μM of nonlabeled angiotensin or 8-leu angiotensin. The values are means ± standard errors of 4–8 experiments, each with duplicate determination.

[^{3}H]angiotensin concentration (molarity)	Control	8-leu-angiotensin	EDTA	Caproic acid
2.5×10^{-8}	288 ± 22	225 ± 27	545 ± 32***	598 ± 39***
5×10^{-8}	782 ± 142	535 ± 59	1,169 ± 102**	2,510 ± 148***
10^{-7}	2,679 ± 196	2,288 ± 189	4,230 ± 1,089*	3,894 ± 144*
5×10^{-7}	19,400 ± 2,074	24,308 ± 2,532	24,680 ± 1,285*	27,633 ± 3,722*
10^{-6}	20,090 ± 1,527	22,238 ± 1,916	26,250 ± 721**	33,922 ± 3,827**

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

would increase the maximal response to angiotensin (16), inhibit membrane angiotensinase activity, but would not affect angiotensin receptor binding (3). We explained these findings on the basis of an assumption that inactivation of membrane angiotensinase would increase the availability of angiotensin at the receptor sites. These experiments suggested a rather close functional relationship between angiotensin membrane receptors and membrane angiotensinase. If this is true, then one should be able to observe similar effects with angiotensinase inhibitors. We have chosen two known inhibitors, EDTA and caproic acid, in concentrations known to have inhibitory effect on angiotensinase (14, 15). Our results show that these two compounds blocked the angiotensinase activity in plasma membrane and supernatant fraction of rabbit aorta. Similar effects by these substances on angiotensinase activity from different tissue sources were reported by Itskowitz and Miller (14).

Only caproic acid significantly prolongs the relaxation time of aortic strips, suggesting a definite blockade of angiotensin disposition mechanism, through which it is possible to influence the duration of stimulation and detachment of angiotensin from

receptor sites. This observation is in agreement with the study of Khairallah *et al.* (17) on the relationship between angiotensinase activity and duration of angiotensin tachyphylaxis. These authors have found that increase in angiotensinase activity would shorten the angiotensin tachyphylaxis. The reactivity of aortic strips to angiotensin was potentiated and an increased maximum was obtained with caproic acid, while EDTA diminished the response to angiotensin. The ED_{50}, which remained unchanged, indicated that the affinity of receptors for angiotensin did not change under the influence of angiotensinase inhibitors. This suggests that caproic acid and EDTA indeed act at sites different from angiotensin receptors. According to Kalsner (21), an increased maximal response would indicate a post-receptor mechanism, most probably an increase in intracellular Ca at the contractile apparatus. A further indication that caproic acid possibly increases the intracellular Ca^{2+} is provided by the evidence that EDTA abolishes the potentiating effect of caproic acid. Also, EDTA, being a Ca^{2+} chelating agents, diminished the maximal response to angiotensin, thus suggesting again the possible involvement of Ca^{2+} ions in the regulation of the magnitude of maximal contraction. The effect of EDTA on norepinephrine response is similar to one observed by Furchogott (18).

Based on the occupation theory of receptors (19), the potentiation of pharmacological response by caproic acid could be explained by two possible mechanisms: 1) An induction or activation of additional receptors or 2) Increased efficacy or "intrinsic" activity of angiotensin in the presence of caproic acid. At the molecular level, both of these two mechanisms are poorly understood. An analogy to enzyme activators or inhibitors might be applied (19, 20).

Our binding experiments reveal an increased receptor binding in the presence of angiotensinase inhibitors. Thus, it is possible that caproic acid and EDTA by inhibiting membrane bound angiotensinase participate in "uncovering" or activation of additional angiotensin receptors. Recently, it has been suggested by Kunos (22) that change in efficacy for an agonist "reflects events beyond receptor activation." Indeed, in order to explain the increased maximal response and delayed relaxation time observed with caproic acid, one could postulate an increase in agonist availability at receptor site after angiotensinase blockade, which in turn stimulates some post-receptor mechanism leading to increased intracellular Ca^{2+}. Thus, we suggest that the role of membrane-bound angiotensinase is related to the regulation of availability of agonist at receptor site and to the process of activation of additional receptors. Once the enzyme is inactivated, a process of activation of receptors is set in motion. This, linked with increased concentrations of agonist at the receptor site, would bring about an increased maximal response.

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