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Effects of peptidase inhibition on angiotensin receptor agonist and antagonist potency in rabbit isolated thoracic aorta

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1 Experiments were performed with peptidase inhibitors on rabbit aortic strip preparations, to determine whether endogenous peptidase activity can influence the potency estimates for angiotensin receptor agonists and antagonists in this tissue.

2 Angiotensin II (A II) and angiotensin III (A III) both induced concentration-related contractions of rabbit aortic strip preparations. A III was approximately 38 fold less potent than A II, and the gradient of the A III concentration-response curve (1.00 ± 0.04) was significantly more shallow than that (1.76 ± 0.05) of the A II curve.

3 Neither the aminopeptidase-A and -M inhibitor, amastatin, nor the aminopeptidase-B and -M inhibitor, bestatin, affected the potency of, or the maximum response to, A II. In contrast, the potency of A III was increased by both amastatin and bestatin. Amastatin had the most marked effect and at 10 μM caused approximately a 12 fold increase in the potency of A III (EC_{50} values, 102 nM and 8.6 nM in the absence and presence of amastatin, respectively), and also significantly steepened the gradient of the A III concentration-response curve. Amastatin did not affect the position or shape of the concentration-response curve to the α_1-adrenoceptor agonist, phenylephrine. Finally, the carboxypeptidase-N inhibitor, D-L-mercaptomethyl-3-guanidine-ethylpropanoic acid (MERGETPA) did not change the position or shape of the concentration-response curves to either A II or A III.

4 In the presence of amastatin, the potency of the peptide angiotensin receptor antagonist, Ile^7-A III (100 nM–1 μM), was increased approximately 13 fold (pA_2, with A II as the agonist, 7.0 ± 0.1 and 8.1 ± 0.1, in the absence and presence of amastatin, respectively). However, the potency of the non-peptide angiotensin receptor antagonist, DuP 753 (30–300 nM), was little affected by amastatin (pA_2, 8.2 ± 0.1 and 8.1 ± 0.1 in the absence and presence of amastatin, respectively).

5 The results of this study suggest that endogenous aminopeptidase activity in the rabbit thoracic aorta can profoundly affect estimates of the potency of peptide angiotensin receptor agonists and antagonists. A suitable aminopeptidase inhibitor should therefore be included in studies, using this tissue, which aim to classify angiotensin receptor subtype(s) based on the rank order of peptide angiotensin receptor agonist and/or antagonist potencies.

Keywords: Angiotensin II, III; peptidase inhibition; rabbit aorta; DuP 753; Ile^7-A III; amastatin

Introduction

The renin-angiotensin system exerts its many effects predominantly via angiotensin II (A II). This octapeptide, which plays a major role in the maintenance of blood pressure, and in salt and water retention, is formed following the action of angiotensin converting enzyme on the weakly active precursor, angiotensin I (Reid *et al.*, 1978; Ferrario, 1990). The potent contractile effect of A II on vascular smooth muscle both *in vitro* and *in vivo* has been well documented (Peach, 1977).

The degradative enzyme, aminopeptidase A, can cleave the N-terminal amino acid (aspartate) from A II (Ahmad & Ward, 1990), resulting in formation of the heptapeptide, angiotensin III (A III). This can be further degraded at its N-terminal by aminopeptidase-B or aminopeptidase-M (Sullivan *et al.*, 1988; Ahmad & Ward, 1990; see Figure 1)to produce a hexapeptide with less biological activity (Peach, 1977; Dostal *et al.*, 1990). A II and A III may also be cleaved at the C-terminal by the action of carboxypeptidase enzymes (Peach, 1977; Magnan & Regoli, 1977). Although A III retains considerable activity as a contractile agent on vascular smooth muscle, estimates of its potency relative to A II have varied (e.g. see Waugh & Bales, 1978; Trachte & Peach, 1983).

It is common practice to investigate receptor heterogeneity by comparing the relative potencies of agonists and/or antagonists. Despite this, few studies have been carried out to determine the extent to which differential degrees of enzymatic degradation may have influenced the estimates of the potency of A II or A III (e.g. Peach, 1977; Trachte & Peach, 1977; Freer *et al.*, 1980). Moreover, until recently, the only angiotensin receptor antagonists available have been peptides based closely on the structures of A II and A III. Consequently, these antagonists are also likely to be subject to degradation by peptidase enzymes.

Current angiotensin receptor classification is defined by the selectivity of the non-peptide ligands, DuP 753 (losartan) and PD 123177 for what have been ascribed AT_1 and AT_2 receptors (Bumpus *et al.*, 1991). The vast majority of functional responses to A II appear to be mediated via AT_1 receptors (see Wong *et al.*, 1990). Clues to the possible locations of further subtypes of the AT_1 receptor may be obtained by differences in the relative potencies of agonists or antagonists in various tissues and vascular beds. Thus, if progress is to be made in the future sub-classification of angiotensin receptors, it is essential to investigate whether endogenous peptidase activity can influence these potency estimates. The aim of the present work was to address this question using the rabbit isolated thoracic aortic strip preparation.

In this study, the contractile effects of A II and A III in rabbit aortic strips were compared in the absence and presence of either the aminopeptidase-A and -M inhibitor, amastatin (Aoyagi *et al.*, 1978; Palmieri *et al.*, 1989; Ahmad & Ward, 1990), the aminopeptidase-B and -M inhibitor,

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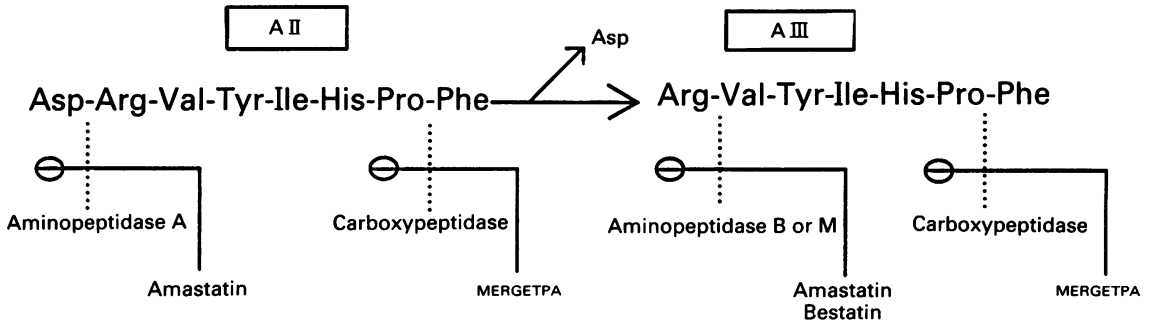

Figure 1 Putative pathway for the breakdown of angiotensin II and III (A II and A III) by various peptidases, and how peptidase inhibitors may interfere with the degradation processes. Dotted lines show the proposed point of cleavage by enzymes. ⊖ shows sites where amastatin, bestatin or D-L-mercaptomethyl-3-guanidino-ethylpropanoic acid (MERGETPA) are believed to interfere with these enzymes.

bestatin (Umezawa *et al.*, 1976; Sullivan *et al.*, 1988), or the carboxypeptidase-N inhibitor, D-L-mercaptomethyl-3-guanidino-ethylpropanoic acid (MERGETPA) (Ward *et al.*, 1987). In addition, the potencies of two angiotensin receptor antagonists were determined, against first A II and then A III-induced contractions, in both the presence and absence of amastatin. One of the antagonists used, Ile^7-A III, is a peptide. The other, DuP 753 (losartan), has a non-peptide heterocyclic structure, and has high affinity for A II binding sites in a variety of animal and human tissues including vascular smooth muscle, adrenal cortex and kidney cortex (see Timmermans *et al.*, 1991). A preliminary account of these findings has been published in abstract form (Robertson *et al.*, 1991).

Methods

Preparation of the rabbit aorta

Male, New Zealand White rabbits (2–3 kg) were killed by captive bolt. The descending thoracic aorta was rapidly removed on a perspex rod, then cleaned of connective tissue in a Petri dish containing physiological salt solution (at room temperature) of the following composition (mM) :Na^+ 143.4, K^+ 5.9, Mg^{2+} 0.6, Ca^{2+} 1.3, Cl^- 124.5, $H_2PO_4^-$ 1.2, SO_4^{2-} 0.6, HCO_3^- 25 and glucose 11.1, bubbled with 95% O_2/5% CO_2. The physiological salt solution also contained indomethacin (30 μM, to prevent the formation of endogenous cyclo-oxygenase products) and ascorbic acid (100 μM, as an anti-oxidant). Each aorta, with the endothelium removed, was cut into a spiral strip as described by Furchgott & Bhadrakom (1953), and then cut into 4–8 smaller strips each measuring 1.5–2.0 cm in length and 3–4 mm in width. Individual strips were suspended vertically in glass tissue chambers (10 or 20 ml) containing the physiological salt solution at 37°C (pH = 7.4). Each strip was connected by a cotton thread to a force displacement transducer (Dynamometer, UF1) and a resting tension of 0.5 g was applied to each tissue. Changes in isometric tension were displayed on an 8-channel pen recorder (Lectromed, MT8P).

Experimental design: construction of angiotensin II and III concentration-response curves

Fifteen minutes after adjusting the applied tension on the tissues to 0.5 g, KCl (80 mM) was added to the bathing fluid to depolarize the smooth muscle and thereby determine the contractile viability of the preparations. Once the contraction had reached a plateau, the tissues were washed. After 10 min, the tension was mechanically re-adjusted to 0.5 g and 5 min later, a cumulative 10 fold priming concentration-response curve to A II (0.1–100 nM) or to A III (1 nM–10 μM) was constructed. After washing, the preparations were allowed to equilibrate for 30 min before re-adjusting the basal tension to 0.5 g. After a further 15 min, a cumulative 3 fold concentration-response curve to A II (0.3–100 nM) or A III (1 nM–10 μM) was constructed. After washing and equilibrating as before, another cumulative 3 fold concentration-response curve to A II or A III was obtained (pretest curve) and the concentration of agonist required to elicit a 50% maximum response (EC_{50}) in the pretest curve was calculated.

Effects of peptidase inhibitors on contractile responses to angiotensin II and III

The pretest curves for either A II or A III were constructed as described above. After washing, three preparations were then exposed to a different concentration of either amastatin (3, 10 or 30 μM), bestatin (3, 10 or 30 μM), MERGETPA (10, 30 or 100 μM), or a combination of amastatin (10 μM) and bestatin (10 μM), whilst a fourth preparation was exposed to vehicle alone. Forty-five minutes later, a final cumulative, 3 fold A II or A III concentration-response curve (test curve) was constructed. In separate experiments, the specificity of action of amastatin (3, 10 or 30 μM) was assessed. The protocol in these studies was the same as described above, with the exception that phenylephrine (10 nM–30 μM) was used to contract the tissues in place of A II or A III.

Determination of angiotensin receptor antagonists potencies in the absence or presence of amastatin

The pretest curve for A II or A III was constructed as before. After washing, three preparations were then exposed to different concentrations of angiotensin receptor antagonist DuP 753, (30, 100 or 300 nM) or Ile^7- A III (100, 300 or 1000 nM), whilst a fourth preparation was exposed to vehicle alone. Forty-five minutes later a final cumulative 3 fold A II or A III concentration-response curve (test curve) was constructed. In this series of experiments and those described in the previous paragraph there was a tendency for the maximal response to A II and A III in the test curves to increase slightly in vehicle-treated preparations, although the concentration-range over which each agonist was effective was unchanged. Where angiotensin curves obtained in the presence of the antagonists were parallel with the control curves, angiotensin concentration-ratios were calculated. To do this, the concentration of agonist in the test curve, which produced the same response as that caused by the EC_{50} in the pretest curve, was divided by the EC_{50}. The concentration-ratio values from the drug-treated preparations were divided by the concentration-ratio value from the vehicle-treated preparations to correct for any spontaneous changes in sensitivity. These results were plotted graphically in the form of a Schild plot as log (agonist concentration-ratio − 1) versus log antagonist concentration (mol l^{-1}), and pA_2 values were derived (Arunlakshana & Schild, 1959). In some experiments

with A III as the agonist, curves obtained in the presence of the antagonists, although parallel with each other, were not parallel with the control A III curve. However, to obtain an approximate measure of their potency, pA_2 values were calculated as described above. The potency of each antagonist was also determined in the presence of amastatin. In these experiments, antagonist pA_2 values were obtained by use of the protocol described above, with the exception that amastatin (10 μM) was added to all the tissues following the KCl challenge at the beginning of the experiment, and was then present at this concentration in the bathing fluid for the remainder of the study.

Expression of results and statistical analysis

Mean EC_{50} values and mean concentration-ratios were expressed in the results as geometric means with 95% confidence intervals. For each individual preparation, the contractile responses to A II or A III in the test curve were expressed as a percentage of the maximum response to A II or A III in the pretest curve, and the results plotted graphically. pA_2 values and Schild plot slopes, and mean changes in contractile tension are expressed as the arithmetic mean ± s.e.mean. The gradients of the A II and A III concentration-response curves are calculated by use of the non-linear unweighted ALLFIT curve-fitting programme (De Lean *et al.*, 1978). Differences between groups of pA_2 values were tested for statistical significance by Student's unpaired t test. In all analyses the level of statistical significance was taken as $P<0.05$.

Drugs used

A III, amastatin hydrochloride, bestatin hydrochloride, phenylephrine hydrochloride, Ile^7-A III, indomethacin, and ascorbic acid were obtained from Sigma. A II and MERGETPA (D-L-mercaptomethyl-3-guanidino-ethylpropanoic acid) were obtained from Novabiochem and Calbiochem, respectively. DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2'(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) potassium salt, was a gift from DuPont de Nemours (Delaware, USA). All drugs were dissolved in distilled water with the exception of indomethacin which was dissolved in 100 μl NaOH (2 M). The stock solutions of amastatin, bestatin and MERGETPA were all 10 mM. A II and A III were stored frozen as aliquots (1 mM or 10 mM), which were defrosted as required on the day of experimentation. Dilutions of all drugs were made in distilled water. All chemicals for the physiological salt solution were of Analar grade and obtained from BDH Ltd., England.

Results

Contractile effects of angiotensin II and III

In most experiments, the maximum contractile response to both A II and A III became progressively greater in consecutive curves. This was particularly apparent between the first and second A II or A III curve. On a few occasions, consecutive A II and A III curves became smaller. These experiments were abandoned. Rarely, all aortic preparations from a single rabbit failed to respond to either A II or A III after having shown a normal contractile response to the depolarizing KCl solution.

Cumulative addition of A II (0.3 nM – 100 nM) or A III (1 nM – 10 μM) to the rabbit isolated aortic strips caused concentration-related contractions (Figure 2) with a similar maximal contractile response (A II, 0.99 ± 0.07 g, $n = 33$; A III, 1.03 ± 0.05 g, $n = 36$). The concentration (geometric mean and 95% confidence interval) of A III in the test curve, required to elicit a response equal to 50% of the maximum response observed in the pretest curve, was 139.9 (106.5–183.9) nM ($n = 36$). The corresponding concentration of A II was 3.7 (3.0–4.4) nM ($n = 33$). Thus, A III was approximately 38 fold less potent than A II. Figure 2 also illustrates that the effects of A III occurred over a wider concentration-range than those of A II. Statistical comparison of the gradients of the concentration-response curves for A II and A III revealed that the mean gradient of the A III curve (1.00 ± 0.04), was significantly lower ($P<0.001$, unpaired t test) than that of the A II curve (1.76 ± 0.05). Thus, only 3 or 4 cumulative three fold concentrations of agonist were typically required to produce the maximum contractile response for A II, whilst 6 or 7 concentration increases were needed for A III. Additionally, as shown in Figure 3, the peak contractile response to each concentration of A III was achieved more rapidly than that to A II.

Effects of peptidase inhibitors on contractile responses to angiotensin II and III

Neither the potency, nor the maximum contractile response to A II, was affected by the aminopeptidase-A and -M inhibitor, amastatin (3–30 μM, Figure 2a). In contrast, the potency of A III was increased to a similar extent in the presence of 3, 10, or 30 μM amastatin (Figure 2b). The geometric mean concentration of A III, required to cause 50% of the maximum response (relative to the pretest curve), before and after amastatin (10 μM) was 102.2 (40.0–260.0) nM and 8.6 (3.6–20.5) nM, respectively. Thus, amastatin (10 μM) increased the potency of A III by approximately 12 fold. Ama-

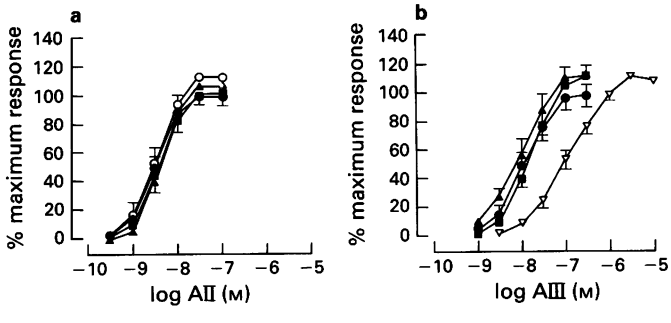

Figure 2 Concentration-response curves to (a) angiotensin II (A II) and (b) A III in separate rabbit aortic strip preparations, before (○, A II; ▽, A III), and after treatment with amastatin (■, 3; ▲, 10 and ●, 30 μM) $n = 5$–7. Results are shown as mean response to A II or A III in the test curve, expressed as a % of the maximum response to A II or A III, respectively, in the pretest curve. Vertical bars show s.e.mean.

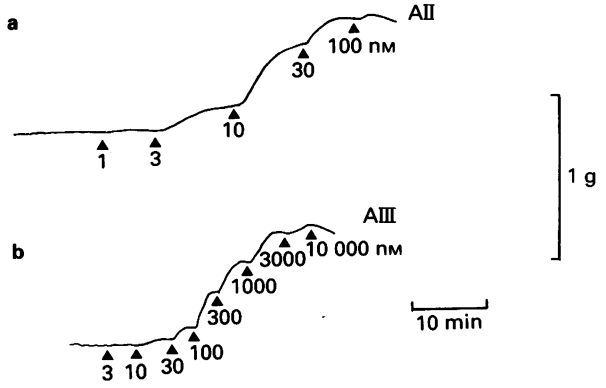

Figure 3 Representative tracings from two rabbit aortic strip preparations showing that less cumulative concentrations of angiotensin II (A II) (a) than A III (b) are required to produce their respective maximal contractile responses. Each change in the bath concentration of A II or A III is indicated (▲).

statin also caused a significant increase in the mean gradient of the A III concentration-response curve, although it was still significantly less ($P<0.05$:paired t test) than the mean gradient of the A II curve:the mean gradients ($\pm$ s.e.mean) of the A III concentration-response curves, before and after amastatin (10 μM, $n=7$), were 0.92 ± 0.06 and 1.40 ± 0.13, respectively. Additionally, in the presence of amastatin, the time to peak response for each concentration of A III was increased, such that the contractile responses to A III more closely resembled those to A II. Amastatin (3–30 μM) had no effect on the position (<2 fold displacement), shape or size of the α_1-adrenoceptor agonist, phenylephrine, concentration-response curve (data not shown).

Like amastatin, although the aminopeptidase -B and -M inhibitor, bestatin (3, 10 or 30 μM), did not affect the potency of A II (Figure 4a), it did significantly ($P<0.05$) increase the potency of A III (Figure 4b). The increase in the potency of A III was similar (approximately 3–10 fold) at each concentration of bestatin tested. Incubation of tissues with a combination of both amastatin (10 μM) and bestatin (10 μM) had no effect on the potency of A II, and produced no greater increase in the potency of A III than was observed in the presence of amastatin (10 μM) alone.

Finally, the carboxypeptidase-N inhibitor, MERGETPA (10–100 μM; $n=4$–5) had no effect on the potency (<2 fold displacement), or maximum contractile effect, of either A II or A III (data not shown).

Potency of angiotensin receptor antagonists in the absence or presence of amastatin

The previous experiments showed that of the peptidase inhibitors tested, amastatin (10 μM) caused the greatest increase in A III potency. For this reason, the potency of the peptide angiotensin receptor antagonist, Ile^7-A III, and the non-peptide angiotensin receptor antagonist, DuP 753 (Chiu *et al.*, 1990), as antagonists of A II- and A III-induced contractions, were determined in the absence and presence of amastatin (10 μM). The pA_2 and slope estimates are shown in Table 1.

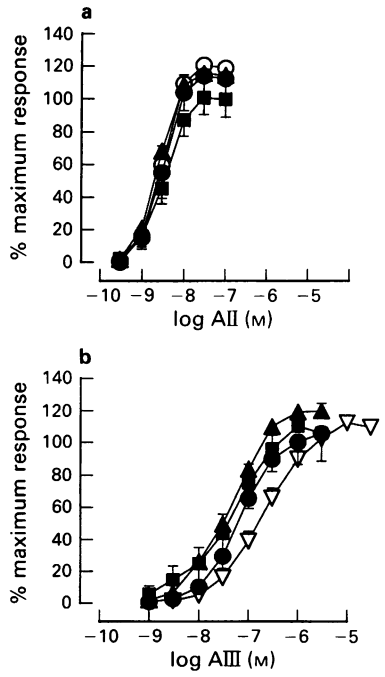

Figure 4 Concentration-response curves to (a) angiotensin II (A II) and (b) A III in rabbit aortic strip preparations, before (○, A II; ▽, A III), and after treatment with bestatin (■, 3; ▲, 10 and ●, 30 μM), $n=4$–6. Expression of results is as for Figure 2.

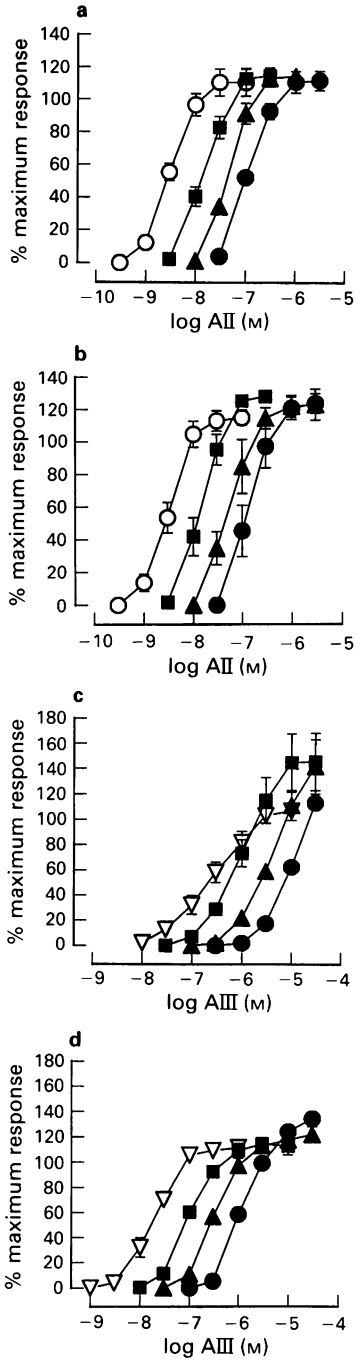

Figure 5 The effect of DuP 753 on concentration-response curves to (a) angiotensin II (A II), (b) A II in the presence of amastatin (10 μM), (c) A III and (d) A III in the presence of amastatin (10 μM). For each panel, curves shown are in the absence (○ A II, ▽ A III) or presence of DuP 753 (■, 30 nM; ▲, 100 nM and ●, 300 nM), $n=4$. Results are shown as mean response to A II or A III in the test curve, expressed as a % of the maximum response to A II or A III, respectively, in the pretest curve. Vertical bars show s.e.mean.

DuP 753

DuP 753 (30, 100 or 300 nM) caused concentration-related, parallel, rightward displacements of A II concentration-response curves without change in the maximum response, in both the absence (Figure 5a) and presence (Figure 5b) of amastatin (10 μM).

Similarly, over the same concentration-range, DuP 753 also caused concentration-related rightward shifts of A III concentration-effect curves in the absence (Figure 5c) and

Table 1 Angiotensin receptor antagonist potency in the absence and presence of amastatin (10 μM)

	DuP 753		*Ile⁷-A III*	
	− Amastatin	*+ Amastatin*	*− Amastatin*	*+ Amastatin*
A II	8.2 ± 0.1	8.1 ± 0.1	7.0 ± 0.1	8.1 ± 0.1
	SL = 1.1 ± 0.1	SL = 1.0 ± 0.1	SL = 1.4 ± 0.1	SL = 1.2 ± 0.1
A III	7.9 ± 0.2	8.1 ± 0.1	7.2 ± 0.2	7.9 ± 0.03
	SL = 1.2 ± 0.05	SL = 1.1 ± 0.05	SL = 1.1 ± 0.1	SL = 1.0 ± 0.2

Values are pA_2 with slope (SL) calculated as arithmetic mean ± s.e.mean ($n = 4$).

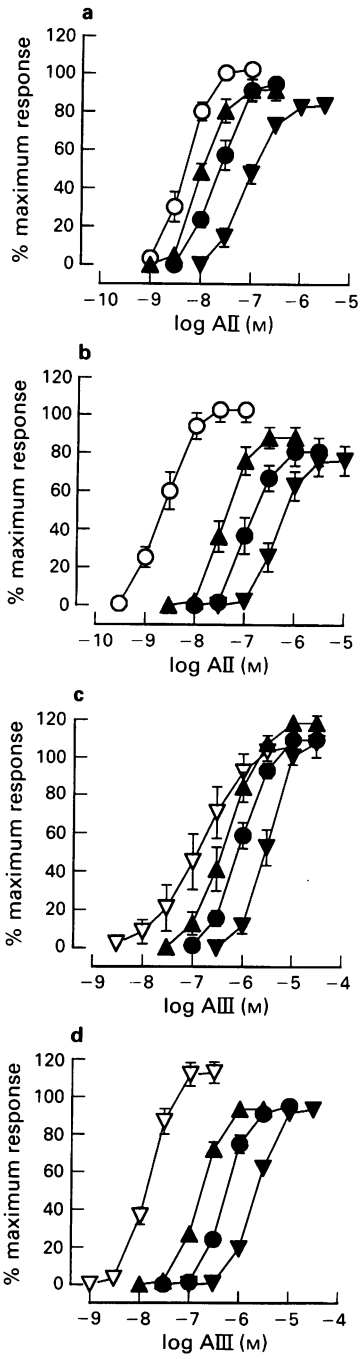

Figure 6 The effect of Ile⁷-A III on concentration-response curves to (a) angiotensin II (A II), (b) A II in the presence of amastatin (10 μM), (c) A III and (d) A III in the presence of amastatin (10 μM). For each panel, curves shown are in the absence (○ A II, ▽ A III) or presence of Ile⁷-A III (▲, 100 nM; ●, 300 nM; and ▼, 1000 nM), $n = 4$. Expression of results is as for Figure 5.

presence (Figure 5d) of amastatin (10 μM). In the presence of amastatin, A III concentration-response curves were shifted in a parallel manner by DuP 753. In contrast, Figure 5c shows that, although A III curves were shifted to the right by DuP 753 in the absence of amastatin, the gradient of the curves also appeared to increase, as did the maximum response (by approximately 40%). Thus, in the presence of DuP 753, A III curves were parallel with each other, but were not parallel with the A III curves generated from the corresponding vehicle-treated preparations. Despite this, calculated at the pretest curve EC_{50} level, the affinity estimate (pA_2 values, Table 1) for DuP 753 against A III was not significantly altered by the addition of amastatin.

Table 1 also shows that the potency of DuP 753 was similar against both A II and A III, in the absence or presence of amastatin.

Ile⁷-A III

Ile⁷-A III (100, 300 and 1000 nM) caused concentration-related, rightward displacements of A II concentration-response curves, with some suppression (up to about 20%) of the maximum response, in both the absence (Figure 6a) and presence (Figure 6b) of amastatin. In contrast to DuP 753, the potency of Ile⁷-A III against A II was significantly increased ($P < 0.005$), approximately 13 fold (Table 1), in the presence of amastatin.

Ile⁷-A III (100, 300 and 1000 nM) also caused rightward, concentration-related displacements of A III concentration-response curves in the absence (Figure 6c) and presence (Figure 6d) of amastatin. In the presence of amastatin, Ile⁷-A III caused parallel shifts in A III curves with some suppression (approximately 20%) of the maximum response (Figure 6d). However, as had been observed with DuP 753, in the absence of amastatin, Ile⁷-A III seemed to cause more displacement at the bottom, than at the top, of A III concentration-response curves, and did not suppress the maximum response (compare Figures 6c and 5c).

As with A II, the potency of Ile⁷-A III against A III-induced contractions was significantly ($P < 0.05$) increased (approximately 6 fold) in the presence of amastatin (Table 1). Hence, similar to DuP 753, the potency of Ile⁷-A III was independent of the agonist used (A II or A III), in the absence or presence of amastatin.

Discussion

The physiological and pathophysiological importance of the renin-angiotensin system in cardiovascular homeostasis is established. It is accepted that the main biologically active components of this system, A II and A III, exert their effects via specific angiotensin receptors. In the conventional pharmacological manner, attempts to classify angiotensin receptors have often been carried out by determining relative potencies of agonist and/or antagonists within a given tissue (e.g. Samenen *et al.*, 1988) or between tissues (Moore *et al.*, 1976; Trachte & Peach, 1983). However, for the results of such experiments to be meaningful, it is important to ensure that, as far as possible, potency measurements are obtained

under optimal and uniform conditions (Furchgott, 1972).

In the current study, the possibility that endogenous peptidases may degrade angiotensin receptor agonists and antagonists, and therefore interfere with estimates of their potency, was considered with respect to the rabbit isolated aorta. This preparation has been used widely to investigate contractile responses to A II and A III, largely because the preparation is robust, and consecutive agonist concentration-response curves do not readily develop tachyphylaxis (e.g. see Trachte & Peach, 1983).

Initial results of the present study showed that A II and A III each elicited concentration-related contractions of rabbit aortic strips, producing a similar maximum response. However, in addition to A III being less potent (approximately 38 fold) than A II, the gradient of the A III concentration-response curve was found to be shallower than that of the A II curve. Furthermore, the time to peak response for each concentration of A III was shorter than for A II (Figure 3).

One explanation for these differences is simply that the concentration of A III (especially at lower concentrations), but not that of AII, declines rapidly following penetration into the tissue biophase because of its enzymatic degradation. This steady removal of A III from the biophase could also explain why each A III-induced contraction reaches a plateau more quickly than A II. In contrast A II will penetrate further into the biophase and therefore take longer to reach equilibrium. This hypothesis is supported by two further observations in the present study. Firstly, responses to A II were unaffected by amastatin. Thus, in rabbit isolated aorta, A II does not appear to be metabolized by aminopeptidase-A. Secondly, in contrast to its lack of effect against A II, amastatin markedly enhanced the potency of A III, steepened the A III concentration-response curves, and increased the time to peak response for each concentration, making A III- and A II-induced contractions appear similar. These findings may be explained by the ability of amastatin to inhibit aminopeptidase-M. This enzyme is present on vascular smooth muscle membranes, can metabolize A III but not A II, and is inhibited by amastatin (Palmieri *et al.*, 1989). The present data are consistent with those of Ahmad & Ward (1990), who found that pressor responses to A III, but not A II, were enhanced in the presence of amastatin. Finally, amastatin was found not to affect contractile responses to the α_1-adrenoceptor agonist, phenylephrine. This provided further confirmation of the specificity of this peptidase inhibitor.

Like aminopeptidase-M, the enzyme aminopeptidase-B can also degrade A III at the N-terminal amino acid. Bestatin, an inhibitor of both aminopeptidase-B and -M, had, like amastatin, no effect on the potency of A II, but increased the potency of A III. Again, this effect of bestatin is probably due to its ability to inhibit aminopeptidase-M (rather than aminopeptidase-B), since a combination of amastatin and bestatin caused no greater enhancement of the potency of A III than did amastatin alone (not shown).

In some tissues, angiotensins are also susceptible to metabolism at the C-terminal by carboxypeptidase enzymes (Peach, 1977). Based on chromatographic evidence, Magnan & Regoli (1977) suggested that carboxypeptidase activity was important in the metabolism of A II in rabbit aorta. Furthermore, Regoli *et al.* (1986) have shown that MERGETPA (8 μM) completely inhibits the contractile effect of bradykinin in rabbit aorta, where the contractile activity depends on the conversion of bradykinin to the metabolite, des-Arg^9-bradykinin, by carboxypeptidase-N. However, the present results showed that MERGETPA, (up to 100 μM) was without effect on contractile responses to A II or A III. It is therefore possible that A II and A III (unlike bradykinin) are poor substrates for carboxypeptidase-N; however, it cannot be ruled out that a C-terminal specific carboxypeptidase, other than carboxypeptidase-N, but capable of degrading angiotensin, is present in rabbit aorta.

To summarise, the present results are consistent with the presence of aminopeptidase-M in isolated strips of the rabbit thoracic aorta. This enzyme appears to be capable of degrading A III, but not A II. Failure to inhibit this enzyme results in an underestimation of both the potency of A III, and the gradient of the A III concentration-response curve.

Since the data indicated that A III was subject to metabolism by aminopeptidase-M, experiments were subsequently carried out to determine whether this enzyme could also degrade angiotensin receptor antagonists. Thus, using A II and A III separately as agonists, the effects of amastatin on the antagonist potencies of the peptide, Ile^7-A III, and of the non-peptide, DuP 753, were measured. In the absence of amastatin, a pA_2 value of 8.2 was obtained for DuP 753 with A II as the agonist, which is comparable with data published in recent literature (Chiu *et al.*, 1990). Unlike DuP 753, Ile^7-A III tended to reduce the maximum of the A II concentration-response curve. This insurmountable antagonism of A II responses in smooth muscle preparations by peptide antagonists is well documented (e.g. Freer *et al.*, 1980; Chiu *et al.*, 1988).

DuP 753 and Ile^7-A III, as well as causing a rightward displacement of A II and A III concentration-response curves, also steepened the A III curves, and increased the maximum response to A III. In amastatin-treated tissues, however, these phenomena were not apparent. Here, the gradient of the pre-antagonist A III curve was already increased as a consequence of aminopeptidase inhibition. It is tempting to speculate, therefore, that the angiotensin receptor antagonists can, like amastatin, reduce the degradation of A III. However, this is unlikely to constitute the whole explanation because neither amastatin nor bestatin increased the maximum response to A III. Thus, at present, the reason for the angiotensin receptor antagonist-induced increase in the maximum response to A III is uncertain. Although the potency of DuP 753 was little affected by amastatin, the potency of Ile^7-A III was markedly increased. This is not particularly surprising since Ile^7-A III differs in structure from A III only by the replacement of the C-terminal amino acid, phenylalanine, by isoleucine. Consequently, Ile^7-A III may well be subject to metabolism by aminopeptidase-M in this preparation. As one might expect, it would appear that the non-peptide structure of DuP 753 makes it a poor substrate for peptidase enzymes. Whether amastatin was present or not, the respective potencies of both antagonists were independent of the agonist used. However, it should be emphasised that in the absence of amastatin, the antagonist affinities may be slightly underestimated because the antagonist-induced displacements of the A III concentration-response curves lacked parallelism. In the presence of amastatin, the antagonist-induced displacements of A II and A III concentration-response curves are more in keeping with that expected for competitive antagonism (Figures 5b, 5d, 6b and 6d). In this situation, the antagonist affinity estimates are consistent with the idea that A II and A III act via a single class of angiotensin receptors in rabbit thoracic aorta.

In conclusion, the study has demonstrated that endogenous aminopeptidase activity can profoundly influence estimates of peptide agonist and antagonist potency at angiotensin receptors in rabbit aorta. Future studies using this preparation to assess either peptide agonist or antagonist potency should routinely include amastatin (or another suitable aminopeptidase-M inhibitor) in the assay system. The present results also serve as a reminder that for any study of angiotensin receptors *in vitro*, an assessment should first be made of whether endogenous peptidase enzymes are active in the tissue being studied. This seems essential before valid comparisons of agonist or antagonist potencies can be made between different preparations, and thus allow meaningful receptor subclassification.

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