Vascular actions of thrombin receptor-derived polypeptides: structure-activity profiles for contractile and relaxant effects in rat aorta

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1 Using endothelium-denuded and intact rat aortic rings, we have determined the contractile and relaxant structure-activity profile for a series of thrombin receptor-derived polypeptides (TRPs) based on the human and rat receptor sequences: SFLLR (P5), SFLLR-NH₂ (P5-NH₂), SFLLR (Rat P5), SFLLR-NH₂ (Rat P5-NH₂), SFLLRNP (P7), SFLLRNP-NH₂ (P7-NH₂), SFLLRNP (Rat P7), SFLLRNP-NH₂ (Rat P7-NH₂), and SFLLRNPNDKYEPF (P14).

2 A contractile response to thrombin and the TRPs in the endothelium-denuded aortic tissue was minimal or absent in preparations obtained from animals weighing less than 180 g (<6 weeks of age), but increased with animal size, plateauing in tissues derived from animals weighing between 320 and 420 g (about 9 to 14 weeks of age). In contrast, the contractile responses to KCl and noradrenaline did not differ in the tissues and relaxant responses to the TRPs in endothelium-intact aortic preparations were comparable for tissues obtained from either young (≤ 180 g) or older (≥ 320 g) animals.

3 The contractile response of the endothelium-denuded preparation to thrombin and the TRPs showed marked cross-desensitization: the relaxation response of the intact rings did not desensitize to the TRPs.

4 The relative potencies for the TRPs in the aortic contraction assay were comparable to those for the relaxation assay, but were distinct from the relative potencies we measured previously in a rat gastric longitudinal muscle contraction assay. Further, P5 behaved as a partial agonist in the aortic contraction assay, whereas it had been observed to be a full agonist in the gastric contraction assay.

5 The contractile activity of P5-NH₂ in endothelium intact aortic rings was low or absent, but in the presence of the nitric oxide synthase inhibitor, N^o-nitro-L-arginine-methyl ester (L-NAME), the contractions in the intact preparation were equivalent to the response of the endothelium-denuded preparation in the absence of L-NAME.

6 The contractile response of the endothelium-denuded aortic preparation to P5-NH₂ was inhibited by nifedipine and the kinase C antagonist, chelerythrine, but was resistant to the action of indomethacin, tetrodotoxin and the tyrosine kinase inhibitor, genistein.

7 We conclude that the receptor system for the TRPs in the aortic smooth muscle elements, responsible for the contractile response, is similar to the aortic endothelial cell receptor responsible for the relaxation response, but is distinct from the receptor that we have previously characterized in gastric longitudinal smooth muscle, results pointing to the presence of receptor subtypes in the vascular and gastric smooth muscle elements.

Keywords: Thrombin; thrombin receptor; thrombin receptor-derived peptides; rat aorta

Introduction

Thrombin, apart from its widely recognized role as a coagulation factor, *via* the proteolytic cleavage of fibrinogen and the activation of platelets, has also been known for some time to regulate vascular contractility (White *et al.*, 1980; DeMey *et al.*, 1982; Rapoport *et al.*, 1984; White *et al.*, 1984; Haver & Namm, 1984; Walz *et al.*, 1986). Depending on the vascular preparation, thrombin can cause either an endothelium-dependent relaxation (DeMey *et al.*, 1982; Rapoport *et al.*, 1984; White *et al.*, 1984; Walz *et al.*, 1985) or an endothelium-independent contraction (Haver & Namm, 1984; White *et al.*, 1984; Walz *et al.*, 1985). The proteolytic mechanism whereby thrombin activates target tissues such as the platelet has been elucidated by Coughlin and colleagues (Vu *et al.*, 1991; Coughlin *et al.*, 1992), who in parallel with others (Rasmussen *et al.*, 1991) cloned the G-protein-coupled receptor for thrombin and demonstrated that proteolytic activation of the receptor involves the exposure of an amino-terminal sequence (beginning with serine-42 in the human

receptor), that acts as an 'anchored' or 'tethered' receptor-activating moiety. Remarkably, peptides derived from the thrombin-revealed amino-terminal sequence of the receptor (TRPs), beginning with serine and ranging from five (P5) to fourteen (P14) amino acids, have been found to mimic the actions of thrombin in a variety of target tissues ranging from platelets (Chao *et al.*, 1992; Hui *et al.*, 1992; Sabo *et al.*, 1992; Vassallo *et al.*, 1992) to vascular and gastric smooth muscle (Yang *et al.*, 1992; Muramatsu *et al.*, 1992; deBlois *et al.*, 1992).

In our own work studying the action of TRPs in rat aortic tissue derived from relatively young rats (<200 g; 6 weeks or younger), we observed that the principal response of the tissue to thrombin and to the TRPs was an endothelium-dependent relaxation (Muramatsu *et al.*, 1992; Yang *et al.*, 1992); we were unable to detect a thrombin-dependent contraction in endothelium-free rat aortic tissue, although guinea-pig aortic tissue contracted in response to thrombin and to the fourteen amino acid human TRP, P14 (Muramatsu *et al.*, 1992). In subsequent studies with rat vascular and gastric tissues, using a variety of TRPs ranging

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in length from five (P5) to seven (P7) amino acids, both in their carboxylate (P5, P7) and amidated (P5-NH₂, P7-NH₂) forms (Hollenberg *et al.*, 1993), we observed distinct structure-activity profiles for the endothelium-dependent aortic relaxation response (potency order: P7-NH₂ ≥ P5-NH₂ > Rat P5-NH₂ > P5 ≈ P7), compared with the gastric longitudinal muscle contractile response (potency order: Rat P5-NH₂ > P7-NH₂ > P5-NH₂ > P7 > P5), so as to suggest the existence of functional receptor subtypes. We were puzzled to learn from a study appearing after the completion of our structure-activity work, that the TRPs, P7 and P14, could elicit a contractile response in endothelium-denuded rat aortic rings (Antonaccio *et al.*, 1993), whereas our own work with rat aortic preparations failed to detect such a response; and we were intrigued to see that the human TRPs, P7 and P14, were apparently equipotent in terms of the rat aortic contraction assay (Antonaccio *et al.*, 1993), whereas our own work indicated that P7 and P14 exhibit distinct potencies in other bioassay systems (Hollenberg *et al.*, 1992; 1993). The study we describe here was done with two main aims: first, to determine conditions under which a reproducible contractile response to TRPs could be observed in the endothelium-free rat aortic ring preparation; and second, to determine the structure-activity relationships for a spectrum of TRPs in the rat aortic contraction assay. In addition to comparing the structure-activity relationships for the contraction assay, with results obtained in parallel in the endothelium-dependent aortic relaxation assay, we were especially interested to compare the results using the aortic contraction assay with data we obtained previously using a rat gastric contractile assay (Hollenberg *et al.*, 1993). Our data demonstrated that a contractile response to the TRPs was observed reliably in tissues derived from older animals (240 to 420 g; 7.5 to 14 weeks), but was barely detectable in young animals (<180 g; <6 weeks). Further, although there were only subtle differences between the TRP structure-activity profiles for the two vascular assays (contraction and relaxation), there was a clear difference between the structure-activity profile for the vascular contractile assay and the profile we observed in the previous study with the rat gastric longitudinal smooth muscle contractile assay.

Methods

Bioassay procedures

Procedures for the humane treatment of animals, according to the Declaration of Helsinki and as approved by an institutional committee on animal care were followed. Male Sprague-Dawley rats (140 to 430 g, as indicated) were killed by cervical dislocation, and the thoracic aorta was removed for the preparation of ring segments (2 mm × 3 mm) to be used for bioassay purposes. Rings were used either intact or after mechanical disruption of the endothelium by rolling the interior aspect of the ring gently over the inserted end of a fine forceps. Rings were mounted in a plastic organ bath (4 ml total capacity) and were bathed at 37°C in gassed (95% O₂/5% CO₂) Krebs-Henseleit buffer (pH 7.4) of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2 and glucose 10. Tissue was equilibrated for about 1 h before the start of an experiment and the tension (base line, 1.0 g) was monitored isometrically with Grass force-displacement transducers. The presence (or absence) of an intact endothelium was ascertained by monitoring the relaxation response to 1 µM acetylcholine in a preparation that was submaximally contracted with 0.1 µM noradrenaline in the presence of 0.1 mM ascorbic acid (to preserve the catecholamine). For intact preparations, only tissues yielding a relaxation concentration-response curve between 0.01 and 10 µM acetylcholine (maximal relaxation of 70–90%, relative to the tension developed with 0.1 µM noradrenaline) were used to evaluate the actions of the

TRPs. The relaxation responses to the TRPs were measured (e.g. see Figure 2) in tissues that were pre-contracted with 0.1 µM noradrenaline at 30 min time intervals. Tissues were washed approximately 5 min after the application of TRPs. In previous work (Hollenberg *et al.*, 1993) it was observed that 215 µM (137 µg ml⁻¹) P5 caused a maximal relaxation of the pre-contracted endothelium-intact rat aortic preparation. Therefore, as in previous work (Hollenberg *et al.*, 1993), to normalize data between individual tissue preparations and to construct concentration-effect curves, the relaxation responses to P5 and to the other TRPs were expressed as a percentage (% P_{5,215}) of the relaxation caused by 215 µM P5. Concentration-response curves for relaxation were carried out on tissues obtained from animals weighing from 320 to 420 g. As will be described in the results section, large animals (320 to 420 g) were required for monitoring a contractile response; and the contractile response exhibited desensitization towards repeated exposures to the TRPs. Therefore, in the endothelium-free ring preparations, the contractile response to the TRPs was monitored only once, after assessing the tissue response to 50 mM KCl (1.4 ± 0.4 g; mean ± standard error for *n* = 195). Thus, to construct concentration-response curves for the TRPs and to normalize data between different tissue preparations, the contractile responses to the TRPs were expressed as a percentage (% KCl) of the response elicited by 50 mM KCl, which concentration was at the plateau of the KCl concentration-response curve. For purposes of comparison, the same stock solutions of the individual TRPs were evaluated in both the contractile and relaxation assays.

Peptides and other reagents

The TRPs based on the human receptor sequence (P5, SFLLR; P5-NH₂, SFLLR-NH₂; P7, SFLLRNP; P7-NH₂, SFLLRNP-NH₂; P14, SFLLRNPNDKYEPF) and on the rat receptor sequence (Rat P5, SFFLR; Rat P5-NH₂, SFFLR-NH₂; Rat P7, SFFLRNP; Rat P7-NH₂, SFFLRNP-NH₂), prepared by standard solid phase synthesis procedures, were provided either by BioChem Therapeutic Inc., Laval PQ, Canada, with the assistance of Dr John DiMaio or by the Core Peptide Synthesis Laboratory at the Department of Biochemistry, Queens University, Kingston, ON, Canada. Peptides were >95% pure by chromatographic criteria (h.p.l.c.). Weighed peptide samples were routinely dissolved in 50 mM phosphate buffer, pH 7.4 to yield stock solutions, from which volumes of up to 0.2 ml were added directly to the organ bath; the concentrations and amino acid compositions of all TRP stock solutions used were verified by quantitative amino acid analysis of aliquots. Acetylcholine, indomethacin, noradrenaline, nifedipine, N^ω-nitro-L-arginine methyl ester (L-NAME), chelerythrine and thrombin (1000 units mg⁻¹; catalogue No. T3010: 1 clotting unit of thrombin ml⁻¹ is equivalent to approximately 10 nM pure enzyme) were from Sigma (St. Louis, MO, U.S.A.); genistein was from ICN Bio Chemicals (Costa Mesa, CA, U.S.A.) and tetrodotoxin was from Calbiochem (San Diego, CA, U.S.A.). Indomethacin was dissolved in ethanol, such that the final ethanol concentration in the organ bath was ≤ 0.01% (v/v); this concentration of ethanol alone did not affect the bioassay systems. Stock solutions of genistein were prepared with dimethylsulphoxide, which alone upon dilution in the organ bath to a concentration lower than 0.01% v/v did not affect the bioassay.

Results

Characteristics of the contractile response to TRPs: roles of animal size and desensitization

In keeping with our previous observations (Muramatsu *et al.*, 1992), we were not able to observe a reproducible contractile

response either to the TRPs or to thrombin in tissues obtained from rats weighing less than 180 g (≤ 6 weeks old, data not shown). In view of the findings of Antonaccio *et al.* (1993), we decided to evaluate the responsiveness of tissues obtained from older animals, using body weight as an index of age, according to a chart provided by the animal supplier. When we examined tissues from animals more than 7 weeks old (≥ 240 g), it proved possible to monitor a contractile response. Upon studying tissue responsiveness as a function of animal weight, we determined that there was a marked increase in contractile responsiveness to the TRPs between animals weighing from 180 to 240 g (6 to 8 weeks of age); a plateau in responsiveness was observed in tissues obtained from animals weighing between 320 and 420 g (9 to 14 weeks of age) (Figure 1). In contrast, there was no appreciable difference in the relaxation response to the TRPs of endothelium-intact preparations obtained from either young (≤ 180 g) or older (≥ 320 g) animals. Moreover, no such

Figure 1 Contractile response of endothelium-denuded aorta rings as a function of animal age. The contractile responsiveness, of 10 μ M P5-NH₂ (●) and 0.1 μ M noradrenaline (■) of preparations obtained from animals ranging in weight from 140 g (approximately 5 weeks old) to 420 g (about 12 to 14 weeks old) was measured (% KCl) relative to the contractile response of the same tissues to 50 mM KCl. Values for the contractile responses represent averages $\pm$ s.e. (vertical bars) for observations done with eight or more individual tissue strips obtained from each of 3 to 5 different animals, for which the average weights ($\pm$ s.e.: horizontal bars) are shown on the abscissa scale.

Figure 2 Relaxant and contractile responses of intact and endothelium-denuded aortic preparations. Representative relaxant (upper tracings) or contractile (lower tracings) responses to sequential replicate applications of 10 μ M P5-NH₂ (●) are shown for individual tissue strips, exposed to P5-NH₂ at 30 min intervals for the relaxation assay and at 70 min intervals for the contraction assay, followed by washing (W, arrows) and re-equilibration in fresh buffer. The relaxant response was monitored in a tissue that was pre-contracted with 0.1 μ M noradrenaline (NA, Δ). The scales for time and tension are shown to the right of the tracings.

change in the contractile response with animal age of the gastric longitudinal muscle response to the TRPs had been noted in connection with our previous study. Further, in the aortic tissue, in contrast to the change in responsiveness to the TRPs, the magnitude of the contractile response to 50 mM KCl of tissues derived from the younger (1.4 ± 0.2 g, mean $\pm$ s.e. for $n = 8$) and older animals (1.4 ± 0.6 g, mean $\pm$ s.e. for $n = 15$) did not differ, nor did the response to 0.1 μ M noradrenaline (Figure 1). In view of these observations, subsequent work was done only with tissues obtained from animals weighing 320 g or more (≥ 9 weeks of age), in which both the contractile and relaxant responses were measured.

We had previously observed that the contractile response of guinea-pig aortic tissue desensitized both to thrombin and to the TRP, P14 (Muramatsu *et al.*, 1992). Since we wished to obtain concentration-effect curves for the contractile actions of the TRPs, we first wished to determine if desensitization would also occur for the contractile action of the TRPs in the rat aortic preparation. As illustrated in Figures 2 and 3, the contractile response did desensitize towards the repeated exposure of the tissue to the TRP, P5-NH₂, resulting in a greater than 60% desensitization after 3 exposures. In keeping with our previous observations (Hollenberg *et al.*, 1993), the relaxant response did not desensitize to P5-NH₂ (Figure 3) but did so to thrombin (not shown). Thrombin (3 μ l ml^{-1} ; 30 nM) also caused a contractile response in the endothelium-free preparations from animals weighing from 320 to 420 g in contrast with the lack of response to thrombin of tissues derived from young animals (≤ 180 g). However, once exposed to thrombin, the responsive tissue became completely refractory to subsequent exposures to the enzyme (data not shown). Although refractory to thrombin, a thrombin-treated tissue was still able to contract in response to P5-NH₂ (not shown). Nonetheless, prior treatment of a

Figure 3 Desensitization of the contractile but not the relaxation response. The contractile (●) and relaxant (○) responses of intact (○) or endothelium-denuded (●) aortic preparations were measured in tissues exposed sequentially to P5-NH₂ (10 μ M) as illustrated in Figure 2. The average responses ($\pm$ s.e.) of 4 to 6 individual tissue preparations, relative to the first contractile or relaxant response (% control) are shown for three consecutive exposures to P5-NH₂.

tissue with thrombin (3 u ml^{-1} ; 30 nM) reduced the contractile response of the tissue (% KCl) to P5-NH₂ ($10 \text{ }\mu\text{M}$) from $98 \pm 10\%$ (mean $\pm$ s.e. for $n=7$) in untreated tissues to $57 \pm 8\%$ (mean $\pm$ s.e. for $n=6$) of the contractile response caused by 50 mM KCl. Similarly, in a tissue that was partially desensitized by multiple exposures to P5-NH₂ (see Figure 3), the contractile response (% KCl) to 3 u ml^{-1} (30 nM) thrombin was also reduced from $27 \pm 7\%$ (mean $\pm$ s.e. for $n=4$) to $3 \pm 1\%$ (mean $\pm$ s.e. for $n=5$) of the contractile response to 50 mM KCl. Thus, there was cross-desensitization of the contractile response caused by thrombin and P5-NH₂. In tissues that were desensitized either to thrombin or the TRPs, there was no desensitization of the response to either KCl (50 mM) or noradrenaline ($0.1 \text{ }\mu\text{M}$) (not shown).

Concentration-effect curves

For the construction of concentration-effect curves, it was necessary to take into account desensitization properties described for the contractile assay in the previous section. Thus, the contractile responsiveness of an endothelium-denuded preparation was standardized using 50 mM KCl as a stimulus and the first response of the tissue either to the TRPs or thrombin was then measured, relative to the KCl-induced contraction. As shown in Figure 4, TRPs based both on the

human and rat receptor sequences yielded concentration-effect curves over a concentration-range of 1 to $200 \text{ }\mu\text{M}$. In the contraction assay, the order of potencies of the peptides was: P7-NH₂ = P5-NH₂ > Rat P7-NH₂ $\geq$ P14 = Rat P5-NH₂ > P7 $\geq$ Rat P7 $\geq$ P5 > Rat P5. Although all of the peptides appeared to be full agonists in the relaxation assay (see below: Figure 4b), Rat P5 and human P5 behaved only as partial agonists in the contraction assay. The maximal contractile response to P5 was equivalent to the maximal contractile effect caused by thrombin. However, the maximal contractile effect of the other TRPs was 3 to 4 fold greater than that caused by either thrombin or P5 (Figure 4). The comparatively low response to thrombin, which would presumably be the physiological agonist, may have been due to its desensitizing properties, as described above.

It was necessary to repeat with tissues from the older animals ($\geq 320 \text{ g}$), the relaxation assays done previously with tissues obtained from younger animals, to permit a direct comparison with the contractile assay done in the present study. For the relaxant concentration-effect curves, as in previous work (Hollenberg *et al.*, 1993), tissues were exposed to TRPs at 30 min intervals and the relaxation response was expressed as a percentage of the maximal relaxant effect in the same tissue of $215 \text{ }\mu\text{M}$ P5 (% P₂₁₅). In the relaxation assay done with tissues from the older animals, the relative order of potencies of the peptides examined was: P7-NH₂ $\geq$ P5-

Figure 4 Concentration-effect curves for TRPs and thrombin in the contractile (a) and relaxant (b) assays. Tissues were exposed to increasing concentrations of the TRPs to obtain responses such as those shown in Figure 2. Contractile preparations were exposed only once to each TRP and the responses (% KCl) were measured relative to the contraction caused by 50 mM KCl in the same preparation. The relaxation responses in individual pre-contracted rings ($0.1 \text{ }\mu\text{M}$ noradrenaline) were quantitated as a percentage (% P₂₁₅) of the relaxation caused by $215 \text{ }\mu\text{M}$ P5 (boxed open circle) ($48 \pm 3\%$ relaxation, mean $\pm$ s.e. for $n=20$). The concentration-response curves for each agonist represent data from assays done with tissue from three or more animals, using five or more independent tissues in each assay. The figure shows the average response at each concentration, with the s.e. ($n=5-12$) represented at each point with a bar. The symbols used for thrombin and the various TRPs are: (○) P5; (◇) Rat P5; (●) P5-NH₂; (◆) Rat P5-NH₂; (□) P7; (▽) Rat P7; (■) P7-NH₂; (▼) Rat P7-NH₂; (△) P14 and (▲) thrombin.

$\text{NH}_2 > \text{Rat P7-NH}_2 > \text{P14} \geq \text{Rat P7} > \text{P7} \simeq \text{P5}$, entirely in keeping with our previous work.

As we observed previously with a contractile assay using rat gastric tissue (Hollenberg *et al.*, 1993), establishing a single maximum response level in the aortic contractile assay was problematic because of differences in apparent agonist efficacies. Thus, the precise determinations of the EC_{50} values were not attempted. Rather, for comparative quantitative purposes for this study and for comparison with our previous work, the potencies of the TRPs were expressed relative to the activity of P5-NH_2 . By use of at least three response levels on the linear portions of the concentration-effect curves, a potency ratio (R_{EC}) for each TRP was calculated, relative to the concentrations of P5-NH_2 causing an equivalent contractile or relaxant response (i.e. $R_{\text{EC}} = \text{EC}_{\text{TRP}} \div \text{EC}_{\text{P5-NH}_2}$). With this approach, it was possible to calculate the relative equi-effective concentration values (R_{EC} values, Table 1) for all agonists evaluated in the present study and to place in the table, calculated values obtained from our previous study of the aortic relaxation assay and the gastric longitudinal muscle contraction assay (Hollenberg *et al.*, 1993). A comparison of the values in the table revealed a pattern of concordance between potency profiles of the TRPs in the two vascular assays, but a clear distinction between the values for the aortic contraction assay and the gastric contractile assay.

Role of peptide proteolysis

Both in a human platelet aggregation assay and in a rabbit vascular contractile assay, the aminopeptidase inhibitor, amastatin, has been found to potentiate the actions of TRPs (Coller *et al.*, 1992; Godin *et al.*, 1994). In contrast, in our own work with the rat vascular assay system (Hollenberg *et al.*, 1993), we found by h.p.l.c. analysis that the TRPs were not subject to proteolysis in the organ bath during the course of an assay and we observed that, functionally, the addition of amastatin to the organ bath did not potentiate the action of P5 in the vascular relaxation assay. Nonetheless, we wished to rule out the possibility that the low intrinsic activity of P5 in the aorta contractile assay (Figure 4a) might be due to the presence of aminopeptidase activity. Therefore, the contractile activity of P5 (100 μM) was measured both in the absence and presence of 10 μM amastatin. Relative to the contractile response of preparations to 50 mM KCl, 100 μM P5 caused a $34 \pm 3\%$ (mean $\pm$ s.e. for $n = 7$) response in the absence of amastatin and a $38 \pm 10\%$ (mean $\pm$ s.e. for $n = 4$) response in the presence of the aminopeptidase inhibitor. Thus, aminopeptidase activity did not appear to be a factor in the rat aortic contractile assay and did not appear to account for the relatively low contractile response of the tissue to P5, compared with the other TRPs.

Signal transduction pathways

In our previous work comparing the aortic relaxation assay with the guinea-pig and rat gastric longitudinal smooth muscle assays (Hollenberg *et al.*, 1993), we established that both genistein and indomethacin could attenuate the contractile, but not the relaxant actions of P5-NH_2 , thereby suggesting roles for tyrosine kinase and cyclo-oxygenase pathways in the gastric smooth muscle contractile response. Further, we observed that the calcium channel blocking reagent, verapamil could inhibit the contractile action of P14 in a guinea-pig aortic preparation (Muramatsu *et al.*, 1992). Accordingly, we evaluated the effects of genistein, indomethacin and the calcium channel blocker, nifedipine, on the contractile action of P5-NH_2 in rat aortic contraction assay. Further, we assessed the effects of the kinase C inhibitor, chelerythrine and the neuronal toxin, tetrodotoxin. As summarized in Table 2, indomethacin was without effect and genistein caused only a 25% inhibition in the rat aortic contractile assay, whereas we previously determined that these two reagents markedly affected the gastric contractile assays (Hollenberg *et al.*, 1993). Tetrodotoxin also had no effect. In contrast, nifedipine and chelerythrine caused a 60 to 70% inhibition of the contractile activity of P5-NH_2 in the endothelium-denuded aortic preparation.

Role of endothelial nitric oxide synthase

Although the predominant action of P5-NH_2 in the endothelium-intact aorta preparation was to cause a relaxation of pre-contracted tissue (see Figure 2), we also observed a small but consistent increase in tension in this preparation when a comparatively high concentration of P5-NH_2 alone ($> 10 \mu\text{M}$) was added to the organ bath (Figure 5 and data

Table 2 Effects of antagonists on P5-NH_2 -induced contractions

Addition	Contractile response (% KCl)
None	98 ± 10 (7)
Genistein (15 μM)	74 ± 5 (4)
Indomethacin (3 μM)	94 ± 12 (4)
Nifedipine (1 μM)	38 ± 2 (4)
Tetrodotoxin (10 μM)	96 ± 11 (5)
Chelerythrine (5 μM)	30 ± 6 (4)

The contractile response to 10 μM P5-NH_2 was measured, relative to the action of 50 mM KCl (% KCl), either for untreated tissues (no addition) or for tissues treated with the indicated antagonist for 20 min prior to the addition of P5-NH_2 . Values represent the means $\pm$ s.e. for the number of estimates shown in parentheses.

Table 1 Relative potencies of TRPs in the aortic and gastric bioassay systems

Peptide	Designation	R_{EC} value (relative to P5-NH_2) ^a		
		Aortic relaxation	Aortic contraction	Gastric contraction ^b
SFLLR	P5	7.5 ± 0.1	29 ± 3^c	5.0
SFLLR-NH ₂	P5-NH_2	1	1	1
SFFLR	Rat P5	44^b	55 ± 2	5.0
SFLLR-NH ₂	Rat P5-NH_2	2.0^b	2.9 ± 0.04	0.35
SFLLRNP	P7	6.0 ± 0.02	11 ± 0.6	1.5
SFLLRNP-NH ₂	P7-NH_2	0.69 ± 0.04	1.0 ± 0.01	0.5
SFFLRNP	Rat P7	4.0 ± 0.4	14 ± 1^c	1.5
SFLLRNP-NH ₂	Rat P7-NH_2	1.6 ± 0.3	2.3 ± 0.8	ND
SFLLRNPNDKYEPF	P14	2.7 ± 0.2	3.0 ± 0.3	ND

^aThe potencies of the TRPs, relative to P5-NH_2 , as expressed by the R_{EC} values, were calculated from the concentration-effect curves shown in Figure 4, as outlined in the text, according to the formula: $R_{\text{EC}} = \text{EC}_{\text{TRP}} \div \text{EC}_{\text{P5-NH}_2}$. Values represent the means $\pm$ s.e. mean for R_{EC} values calculated from measurements at 3 to 4 levels of response along linear portions of the concentration-response curves for the various TRPs and P5-NH_2 . An R_{EC} value > 1 denotes a TRP with a potency lower than that of P5-NH_2 . ^bValues calculated from Table 1 of Hollenberg *et al.*, 1993. ^cPartial agonist. ND = not determined.

Figure 5 Effect of N^o-nitro-L-arginine methyl ester (L-NAME) on the contractile response of endothelium-intact aortic rings. The response of preparations with an intact endothelium (+E) to 10 μ M P5-NH₂ was measured in the absence (stippled column) or in the presence of either L-NAME (100 μ M: hatched column) or D-NAME (100 μ M: cross-hatched column). Responses of the intact preparations were compared with the response to 10 μ M P5-NH₂ of endothelium-denuded preparations (-E) in the absence of L-NAME (solid column). Contractile responses, representing the average $\pm$ s.e. for 4 to 8 individual tissue strips, were measured as a percentage (% KCl) of the tissue response to 50 mM KCl.

not shown). Since the relaxant effect of the TRPs in the aortic preparation is due to the production of nitric oxide (Muramatsu *et al.*, 1992), we wished to determine the degree to which the inhibition of nitric oxide synthase by L-NAME in an endothelium-intact aorta preparation could unmask the contractile action of P5-NH₂. As illustrated in Figure 5, in the presence of 100 μ M L-NAME, the contractile action of P5-NH₂ in the endothelium-intact preparation was equivalent to the contractile effect of P5-NH₂ in the absence of L-NAME in an endothelium-denuded preparation. In contrast, the D-isomer of the synthase inhibitor, D-NAME, was ineffective in unmasking a contractile effect of P5-NH₂ in the endothelium-intact preparation.

Discussion

Our work has revealed two significant factors that are relevant to a study of the contractile activity of thrombin and the receptor-derived TRPs in rat vascular tissues. First, it is evident that the vascular contractile response to the TRPs is dependent on animal age, as reflected by body weight. Second, it appears that both thrombin and the TRPs cause desensitization of the contractile response, whereas the relaxant response of the vascular preparation to the TRPs does not desensitize. The desensitizing property of thrombin, which may explain its action only as a partial agonist in the contractile assay, may be of physiological relevance because the thrombin-mediated signal at a site of injury would be short-lived. Since tetrodotoxin did not block the action of the TRPs in the aortic contractile assay, and since the aortic tissue is minimally innervated, the desensitization process would appear to occur at the level of the smooth muscle rather than nerve elements in the aortic tissue. The dependence of the contractile response on the age of the animal would explain the apparent discrepancies between our earlier work using vascular tissues from young rats (<200 g),

wherein thrombin failed to elicit a contractile response (Muramatsu *et al.*, 1992), and the more recent results of Antonaccio *et al.* (1993), who observed a contractile response to TRPs in endothelium-denuded vascular preparations obtained from older animals (14 weeks; >420 g). The marked increase in contractile responsiveness to TRPs of the aortic tissue from rats between about 6 and 9 weeks of age points to interesting changes in the receptor system in the vascular smooth muscle elements that merit further study.

One main outcome of our work was that by using aortic preparations from older animals and by measuring the non-desensitized contractile responses to thrombin and the TRPs, it was possible to obtain a contractile structure-activity profile for comparison with the profile obtained using the endothelium-dependent relaxation assay and with the profile we determined previously for the gastric longitudinal muscle contraction assay (Hollenberg *et al.*, 1993). The main finding (Figure 4 and Table 1) was that the order of peptide potencies in the aortic contractile assay were in keeping with the potency order observed in the endothelium-dependent relaxation assay, but did not agree with the potency order we observed previously in the gastric longitudinal muscle contraction assay (see Figure 3 in Hollenberg *et al.*, 1993). For instance, for both the aortic contraction and relaxation assays, the order of potency of the TRPs, P5-NH₂, Rat P5-NH₂ and P7 was: P5-NH₂ > Rat P5-NH₂ > P7 (R_{EC} values for contractile assay, 1:2.9:11; and for the relaxation assay, 1:2.0:6.0), whereas the order of potency of these three peptides measured previously in the gastric contractile assay was distinctly different: Rat P5-NH₂ > P5-NH₂ $\geq$ P7 (R_{EC} values, 0.35:1:1.5). Further similarities between the endothelial and smooth muscle receptor systems conferring relaxation and contraction in the aorta preparation can be seen in the comparable potencies, relative to P5-NH₂ (R_{EC} values, Table 1), of Rat P7-NH₂, P7-NH₂ and P14. In this context it can be pointed out that in both of the aortic tissue assays done according to our procedures, P7 and P14 had distinct relative potencies (P14 > P7 for both assays) as opposed to the equal potencies observed for P14 and P7 by Antonaccio *et al.* (1993), who used a cumulative concentration-effect procedure for the aortic contraction assay. We believe the potency differences between P14 and P7 observed by us in the aortic contractile assay are due to our conduct of the assay in a manner that took into consideration the ability of the TRPs to cause receptor desensitization.

In addition to the distinct structure-activity profiles for the TRPs, differences between the receptor system responsible for the contractile response of the aortic tissue and the contraction of the gastric longitudinal muscle preparation (Hollenberg *et al.*, 1993) can be seen in: (1) the desensitization of the contractile response to TRPs in the aortic tissue but not in the gastric preparation, (2) the inability of P5 to act as a full agonist in the contractile aorta preparation, compared with its high degree of intrinsic activity in the gastric contraction assay and (3) the differences in the signal transduction system used in the aortic contraction assay (see Table 2), which, as opposed to the gastric contractile assay, does not appear to involve either tyrosine kinase or cyclooxygenase pathways, but rather appears to depend on a kinase C-mediated pathway, linked to the influx of extracellular calcium. Given these differences between the contractile assays employing the aortic tissue on the one hand and the gastric tissue on the other, particularly in terms of the distinct TRP structure-activity profiles, it is possible to suggest, according to classical criteria (Ahlquist, 1948), that the vascular and gastric smooth muscle element possess distinct thrombin receptor subtypes, with the vascular smooth muscle receptor closely resembling the one present in vascular endothelial cells. Unfortunately, antagonists of the vascular and endothelial thrombin receptors are not yet available to evaluate this possibility by a bioassay procedure. As an alternative to rationalizing the different structure-activity

profiles in the gastric and vascular smooth muscle tissue in terms of distinct receptor subtypes, it could be suggested on the theoretical grounds (Kenakin *et al.*, 1989), that the coupling of the same thrombin receptor to distinct transducer proteins in the gastric and vascular tissue might alter the relative potencies of the agonists. Further work will thus be required to determine the molecular basis for the differences in the receptor systems present in vascular and gastric tissue. Nonetheless, from a practical point of view, the data we have

obtained point to the possibility of synthesizing tissue-specific agonists or antagonists that may target selectively either the gastric or the vascular receptor for the TRPs.

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