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ACCELERATED COMMUNICATION

Proteinase-Activated Receptor-2 in Rat Aorta: Structural Requirements for Agonist Activity of Receptor-Activating Peptides

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SUMMARY

We measured in rat aorta rings the relaxant activity of a number of peptides derived from the activating sequence (SLIGRL, or PP6) of the proteinase-activated receptor-2 (PAR-2). The relaxant action of PP6-NH_2 mimicked the action of low concentrations of trypsin (0.5–1 unit/ml; 1–2 nM), was dependent on an intact endothelium, and was blocked by *N*-ω-nitro-L-arginine methyl ester but not by *N*-ω-nitro-D-arginine methyl ester. The relaxant actions of PP6, SLIGRL-NH_2 (PP6-NH_2), SLIGR (PP5), and SLIGR-NH_2 (PP5-NH_2) were comparable in magnitude, with relative potencies of PP6-NH_2 ≥ PP6 > PP5-NH_2 > PP5. Peptides lacking either a leucine at position 2 (SAIGRL) or an arginine at position 5 (SLIGAL) exhibited markedly reduced or no relaxant activity; nevertheless, the tetrapeptide LIGR-NH_2 exhibited low but detectable intrinsic activity. With the use of reverse-transcriptase/polymerase chain reaction, we documented the presence of PAR-2 mRNA in aorta tissue and determined that the rat aorta amino-terminal receptor-activating sequence was the same as that reported for the murine PAR-2 receptor. We concluded that the rat aorta tissue has a PAR-2 receptor that can be activated by peptides as short as four amino acids; the leucine and arginine at positions 2 and 5, respectively, of the proteolytically revealed PAR-2 receptor-activating sequence play key roles in regulating receptor function.

The serine protease thrombin is known to activate target tissues via a proteolytically activated G protein-coupled receptor (1–3). By cleaving the receptor at Arg^{41} of the human receptor sequence, thrombin reveals an amino-terminal domain that acts as an anchored ligand to stimulate receptor function. Remarkably, peptides based on the revealed amino-terminal sequence, ranging from five (SFLLR, or P5) to 14 amino acids (SFLLRNPNDKYEPF), have been found to activate the thrombin receptor so as to mimic the actions of thrombin in a variety of tissues, including platelets (1) and vascular and gastric smooth muscle (3–6). In vascular preparations, the TRAPs can cause either an endothelium-dependent relaxation (6, 7) or an endothelium-independent contraction (7, 9).

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Recently, a reduced stringency survey of a mouse genomic library using a neurokinin receptor probe resulted in the identification of a novel G protein-coupled receptor that displayed limited homology with the thrombin receptor (10, 11). Like the thrombin receptor, this newly identified receptor had in its amino-terminal sequence a target sequence for serine protease action, but the receptor was preferentially activated by low concentrations of trypsin (1–2 units/ml or 2–4 nM) rather than by thrombin. Like the thrombin receptor, this new receptor (PAR-2) possesses a proteolytically revealed amino-terminal activating sequence (SLIGRL) that is believed to act as a tethered ligand. A synthetic peptide based on this sequence has been found to activate the PAR-2 receptor expressed in oocytes and cultured mammalian cells (10, 11). However, the PAR-2-AP (SLIGRL) lacks a critical phenylalanine pharmacophore at position 2, which is essen-

ABBREVIATIONS: ACh, acetylcholine; A_2PP6, SAIGRL; A_5PP6, SLIGAL; L-NAME, *N*-ω-nitro-L-arginine methyl ester; D-NAME, *N*-ω-nitro-D-arginine methyl ester; PE, phenylephrine; PAR-2, protease-activated receptor type 2; PAR-2-AP, PAR-2-activating peptide; P5-NH_2, SFLLR-NH_2; PP4-NH_2, LIGR-NH_2; PP5, SLIGR; PP5-NH_2, SLIGR-NH_2; PP6, SLIGRL; PP6-NH_2, SLIGRL-NH_2; TRAP, thrombin receptor-activating peptide; PCR, polymerase chain reaction; PF, forward primer; PR, reverse primer; RT, reverse-transcriptase; bp, base pair(s).

tial for interacting with and activating the thrombin receptor (5, 12, 13). It was thus possible to document the activation of the expressed PAR-2 receptor by SLIGRL (PP6) in a cell (COS) that concurrently possesses receptors for thrombin (11). In view of our previous observations of the vasorelaxant actions of TRAPs(6), we wondered whether the PAR-2-APs might also affect vascular tissue in a similar manner. We thus synthesized a series of PAR-2-derived peptides (PP6, PP6-NH_2, PP5, PP5-NH_2, PP4-NH_2, A_2PP6, and A_5PP6) and evaluated the actions of these PAR-2-related peptides in a rat aorta ring assay system (6). Furthermore, with the use of RT/PCR, we sought to document the presence of the PAR-2 receptor mRNA in rat aorta tissue. Our results indicate that the PAR-2 receptor has a functional role in regulating vascular tension. Also, with our structure/activity study, we identified two key pharmacophores in the PAR-2-APs and delineated a tetrapeptide PAR-2 activating sequence that retains intrinsic vasorelaxant activity.

Materials and Methods

Bioassay procedures. Animals were cared for and treated in accordance with the recommendations of the Canadian Council on Animal Care. Male albino Spague-Dawley rats (200–250 g) were killed by cervical dislocation, the thoracic aorta was removed, and aortic rings (2 mm × 3 mm) were prepared as described previously (6). Rings were mounted in a plastic organ bath (4 ml) and were equilibrated in a gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer, pH 7.4, composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose. The presence of an intact endothelium was determined through monitoring of a relaxation response to 1 μM ACh in a preparation that had been precontracted with 1 μM PE. The endothelium was removed from some preparations by rolling the ring over a fine forceps. PE was added to the organ bath at ~30-min intervals, followed by the addition of PAR-2-AP, trypsin, or TRAPs at the plateau of the PE-induced contraction. The relaxant response was allowed to stabilize (~5 min), and the tissue was then washed free from all agonists and reequilibrated in fresh buffer. The relaxant responses to PAR-2-AP, trypsin, and the TRAP P5-NH_2 were monitored isometrically with Statham or Grass force transducers and expressed as a percentage of the relaxant response caused by 1 μM ACh.

Peptides and other reagents. All peptides were prepared according to standard solid-phase synthesis methods by either the Core Peptide Laboratory at the Department of Biochemistry, Queen's University (Kingston, Ontario, Canada) or through the courtesy of Dr. John DiMaio, BioChem Therapeutic (Laval, Quebec, Canada). Stock solutions of peptides were prepared using 50 mM sodium phosphate buffer, pH 7.4. The concentrations and compositions of stock solutions were verified with the use of quantitative amino acid analysis. PCR primers were synthesized by the Core DNA Services Facility at the University of Calgary, Faculty of Medicine (Calgary, Alberta, Canada). ACh, PE, L-NAME, D-NAME, and highly purified porcine trypsin (14,900 units/mg, catalog No. T7418) were obtained from Sigma Chemical Co. (St. Louis, MO).

PCR detection of PAR-2 and thrombin receptors and cloning of PAR-2 receptor. RNA was isolated from freshly dissected aorta tissue, prepared exactly as for a bioassay, using the TRI-reagent (Molecular Research Center, Cincinnati, OH), and reverse-transcribed with a first-strand cDNA synthesis kit using pd(N) 6 primer (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to manufacturer's recommendations at 37° for 60 min. Of this solution, 3 μl was used for PCR amplification with primer pairs targeted to either the PAR-2 or thrombin receptors based on the published mouse PAR-2 receptor sequence (10, 11) and the rat thrombin receptor sequence (14). Amplification was allowed to proceed for 35 cycles, beginning with a 1-min denaturation period at 94° followed by a 1-min reannealing time at 55° and a primer extension period of 1 min at 72°. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized with the use of ethidium bromide staining. The PCR primers used to screen aorta tissue for the PAR-2 receptor were PF1 5′ CAACAGTAA AGGGAGAA GTC3′, PR1 5′ AGCACATCA/GTGACAGGTA/GGTG3′, and PR2 5′ ACGCTGAGGC AGGTCATGAA3′. PF1 and PR1 were targeted to the amino-terminal putative proteolytic cleavage site (NSKGR/SL) and to the second extracellular loop of the PAR-2 receptor (TTCHDVL), respectively. PR2 was nested between PR1 and PF1. A final set of PAR-2 receptor primers were used to obtain a portion of the murine receptor sequence for library screening: PF2 5′ CACCACCTGTCACGATGT GCT3′ and PR3 5′ CCCGGGCTCAGTA GGAGGTTTTAACAC3′. The PCR primers for the thrombin receptor were TF1 5′ CCCGCT-CATTTT TTCTCAGGAA3′ and TR1 5′ CAAT CGGTGCCG-GAGAAGT3′. These primers were targeted to the thrombin receptor-activating sequence (SFFLR) and to a region of the receptor just beyond the second transmembrane domain. The PCR signals yielded by the thrombin and PAR-2 receptor primer pairs were normalized to the PCR signal yielded by an intron-spanning actin primer pair (15): PF 5′ CGT GGG CCG CCC TAG GCA CCA3′ and PR 5′ TTG GCC TTA GGG TTC AGG GGG3′. The detection of a 243-bp actin PCR product using this primer pair can confirm the absence of intron-derived cDNA in the RT product obtained from tissue RNA.

A λ ZAP II rat neonatal intestinal cDNA library (Stratagene, La Jolla, CA) was screened with a PCR-generated PAR-2 receptor fragment cloned from mouse intestinal cDNA with the use of primer pairs PF1 and PR1 (see above). The identity of the ~3-kb cDNA cloned from the rat intestinal library was confirmed using a one-way nested PCR approach with primer pairs PF1 and PR2. Partial sequencing of the rat intestinal cDNA, subcloned into pBluescript SK-phagemid, was done using the dideoxynucleotide sequencing method (16); PCR products were also sequenced directly from the separating gel using the Sequenase sequencing kit (United States Biochemical, Cleveland, OH).

Results

Characteristics of the relaxation responses to PAR-2-APs. In the precontracted rat aorta ring, PP6 and PP6-NH_2 caused reproducible relaxation responses that mimicked the effect of low concentrations of trypsin (0.5 unit/ml, ~1 nM) and were comparable to the relaxation responses caused by either the TRAP P5-NH_2 or 1 μM ACh (Fig. 1A). Qualitatively, the relaxation in response to the PAR-2-APs differed from that caused by P5-NH_2 in that the TRAP caused a transient relaxation that returned to the PE-stimulated tension within 5 min, whereas the PAR-2-APs caused a more prolonged relaxation (Fig. 1, A and B). The PAR-2-APs, PP5, and PP5-NH_2 caused relaxation responses comparable to that of PP6-NH_2, albeit at higher concentrations (Figs. 1B and 2). In the endothelium-intact preparation, the relaxation in response to PP6-NH_2 was blocked by L-NAME but not by D-NAME (Fig. 1C). Moreover, in an endothelium-free preparation, PP6-NH_2 caused neither a relaxation nor a contraction (Fig. 1E).

Structure/activity relationships and concentration/response curves. In addition to evaluating the relaxant activity of the pentapeptide PAR-2-AP sequences PP5 and PP5-NH_2 (Fig. 1B), we examined the activity of the tetrapeptide PP4-NH_2 (Fig. 1, F and F′). We also assessed the activity of the hexapeptide PAR-2-APs in which either leucine at position 2 or arginine at position 5 was replaced with alanine: A_2PP6 and A_5PP6 (Fig. 1D). Peptides containing leucine at

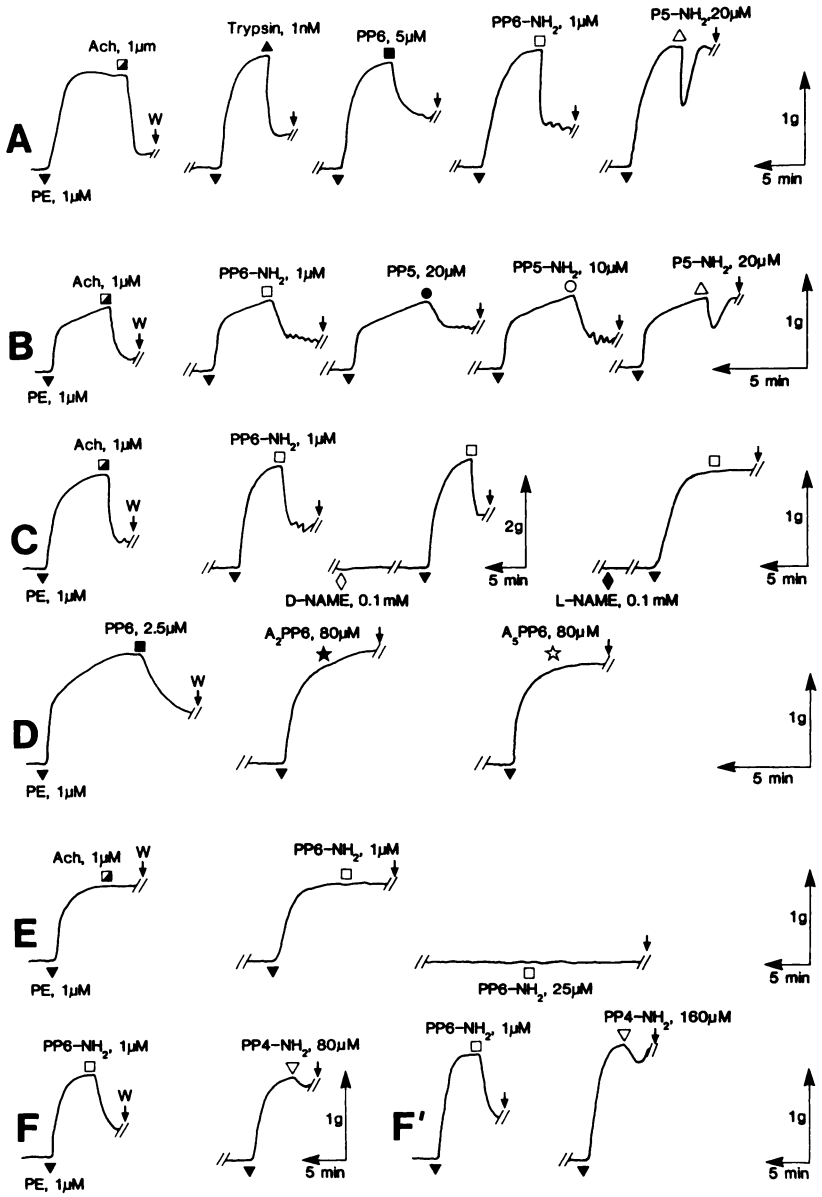

Fig. 1. Relaxant actions of PAR-2-APs showing representative responses, endothelium dependence, and inhibition by L-NAME. A and B, In PE (1 μM, ▼)-precontracted rings, the relaxant effects of PP6 (5 μM, ■), PP6-NH_2 (1 μM, □), PP5 (20 μM ●), and PP5-NH_2 (10 μM ○) were monitored and compared with the actions of ACh (1 μM, ◪), trypsin (0.5 unit/ml or 1 nM, ▲), and P5-NH_2 (20 μM, △). C and E, Inhibition of PP6-NH_2-mediated relaxation by L-NAME (0.1 mM, ♦) but not D-NAME (0.1 mM, ◇) (C) and the lack of a relaxant or contractile response to PP6-NH_2 (□, 25 μM) in an endothelium-free preparation (E). D, Lack of activity of 80 μM A_2PP6 (★) and A_5PP6 (☆) compared with 2.5 μM PP6 (■). F and F', Relaxant activity of the tetrapeptide PP4-NH_2 (80–160 μM, ▽). Each tracing (A to F'), showing the response of an individual tissue preparation, is representative of three or more independent experiments. *Right*, scales for time and tension. *W and arrows*, tissue wash.

position 2 and arginine at position 5 were active; the two alanine replacement analogues (A_2PP6 and A_5PP6) showed little if any activity (Figs. 1D and 2). Furthermore, we observed that the shortened peptide, PP4-NH_2, possessed low but detectable relaxant activity (Figs. 1, F and F', and, Fig. 2). The relative potencies for PP6, PP6-NH_2, PP5, and PP5-NH_2 in the relaxation assay were determined to be PP6-NH_2 ≥ PP6 > PP5-NH_2 > PP5 (Fig. 2). It was not possible to observe a true plateau of the concentration/effect curves for the PAR-2-APs PP6 and PP6-NH_2 because at 10 μM, these peptides caused a near-maximal relaxation of the preparation to base-line tension. In this regard, PP6 and PP6-NH_2 appeared to exhibit higher intrinsic activity than the pentapeptides (PP5, PP5-NH_2), which did not reduce the tension to base-line, even at the plateau of their concentration/effect curves (Fig. 2). The maximum relaxant effects of PP5 and PP5-NH_2 were equal to those of trypsin (Fig. 2).

PCR detection of PAR-2 and thrombin receptors and partial sequencing of the aorta PAR-2 receptor. We wanted to establish unequivocally that the PAR-2 receptor was present in the aorta assay tissue along with the thrombin receptor. To this end, we used primer pairs PF1/PR1 for the PAR-2 receptor and TF1/TR1 for the thrombin receptor to detect RT/PCR products for both receptors from aorta-derived RNA (Fig. 3, *lane A, bands P and T*). The sizes of the amplified products corresponded to the predicted oligonucleotide sequence length for both receptors (~600 bp for PAR-2 and ~400 bp for the thrombin receptor). To establish the identity of the PAR-2 receptor RT/PCR product obtained from aorta RNA with primer pairs PF1/PR1, we used the first PCR product to perform a second round of amplification with a seminested primer pair: PF1/PR2. The second amplified product was also of the predicted size (~420 bp, not shown). We sequenced this second PCR product over a sufficient length to establish its identity with the mouse PAR-2 sequence (90 nucleotides) and to compare its sequence with a partial nucleotide sequence of the PAR-2 receptor clone obtained from a rat neonatal λ ZAP II cDNA library. In addition, with the use of primer pairs PF1/PR1, we compared directly the PCR product obtained from the intestinal cDNA

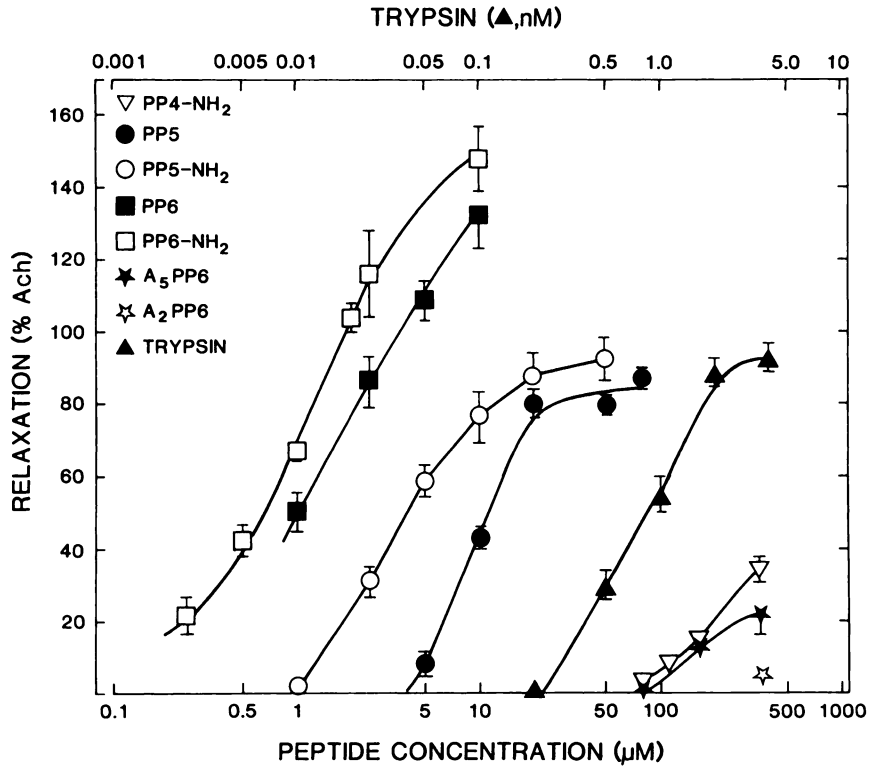

Fig. 2. Concentration/effect curves for PAR-2 receptor-derived peptides. PE-precontracted rings were exposed to increasing concentrations of receptor-derived peptides as well as trypsin, and the relaxation response relative to that caused by 1 μM ACh (% ACh) was monitored: ■, PP6; □, $PP6\text{-}NH_2$; ●, PP5; ○, $PP5\text{-}NH_2$; ▽, $PP4\text{-}NH_2$; ★, A_5PP6; ☆, A_2PP6; and ▲, trypsin. Values represent the mean ± standard error for measurements obtained for 4–50 or more independent tissue preparations coming from at least two different animals.

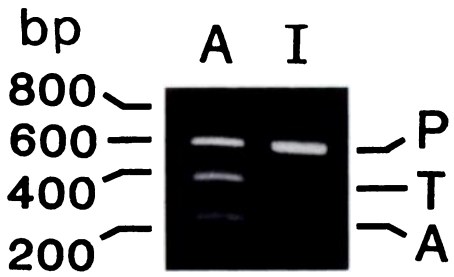

Fig. 3. Detection of PAR-2 and thrombin receptor by RT-PCR in rat aorta showing comparison with intestinal library PAR-2 receptor clone. cDNA samples obtained from rat aorta (*lane A*) and from the rat intestinal library (*lane I*) were subjected to PCR amplification using primer pairs PF1/PR1 for the PAR-2 receptor and (aorta sample only) primer pairs TF1/TR2 for the thrombin receptor. In parallel, the aorta cDNA was probed with the two actin primer pairs (see Materials and Methods). *Right*, positions of the PCR products obtained with the PAR-2 primer pairs (*P*), the thrombin receptor primer pair (*T*), and the actin primer pair (*A*). *Left*, positions of the oligonucleotide markers (in bp).

TABLE 1

Translated partial amino acid sequences of the rat and mouse PAR-2 receptors

The sequences of the rat and mouse receptor are compared, beginning with the putative receptor-activating domain (bold type) revealed by trypsin cleavage (/). Numbering is shown according to the published mouse sequence (11). After Asn^{34}, the dots indicate identity of amino acid residues between the rat and mouse receptors.

Receptor source	Sequence
Rat aorta RNA	NSKGR/**SLIGRL**DTPPPITGKGAPVEPGFSV
Mouse genomic DNA	N^{34}.../.....E.Q.....V.....I^{63}

clone (Fig. 3, *lane I*) with the PCR product obtained from aorta RNA (Fig. 3, *lane A*). The PCR products from aorta and intestinal cDNA were the same. Moreover, the translated amino acid sequence determined over a region of 30 residues for the aorta PCR product (primer pairs PF1/PR2) matched exactly the partial sequence over the same translated 30 amino acid region of the ≅3-kb PAR-2 receptor clone obtained from the rat intestinal library. A comparison of the translated partial amino acid sequence determined for the rat aorta PAR-2 receptor with that for the murine PAR-2 receptor, beginning with the putative receptor-activating domain, is shown in Table 1. Our RT/PCR and sequence data established that the rat aorta tissue did contain the previously reported PAR-2 receptor. In the limited region we sequenced to establish this identity, there was a high degree of sequence identity between the rat and mouse receptors. Nevertheless, four amino acid substitutions over a 30-amino-acid residue region were observed, raising the possibility that the rat receptor we identified may differ from the mouse receptor by as much as 12%, possibly representing a distinct PAR-2 receptor subtype.

Discussion

The main finding of our study was that the PAR-2-APs exhibited a nitric oxide-mediated endothelium-dependent vascular relaxant activity that depended on the leucine and arginine residues at positions 2 and 5 of the SLIGRL-activation sequence. Furthermore, the tetrapeptide $PP4\text{-}NH_2$ retained low but detectable intrinsic activity in the relaxation assay. The partial receptor sequence obtained from aorta RNA unequivocally demonstrated that the PCR product shown in Fig. 3 represented the rat PAR-2 receptor. Significantly, we found that the putative receptor-activating sequence in the rat aorta PAR-2 receptor was the same as that previously reported for the murine PAR-2 receptor (10, 11).

Because our RT/PCR data demonstrated a comparable abundance of PAR-2 and thrombin receptor mRNA in the

aorta preparation, an important question to consider was whether the relaxant activity of the PAR-2-APs in the aorta preparation could have been due to the activation of the thrombin receptor rather than the PAR-2 receptor. For several reasons, we believe that the results were due to the activation of the PAR-2 receptor. It has been amply demonstrated in previous studies that TRAP peptides lacking an aromatic residue at position 2 (e.g., the PAR-2-AP SLIGRL) are unable to activate the thrombin receptor in either platelets (12, 13) or smooth muscle assay systems (6). In addition, in general, the PAR-2-APs PP5 and PP5-NH_2 ($EC_{50} < 10$ μM) are much more potent than the corresponding pentapeptide TRAPs P5 and P5-NH_2 characterized previously in the aorta relaxation assay ($EC_{50} \geq 10$ μM) (6, 9). In this regard, the maximal relaxant effects of trypsin (at ~5 nM, Fig. 2) were observed well below concentrations (>50 nM) required to activate the thrombin receptor (1). Taken together, our data support the conclusion that the relaxant actions of the PAR-2-APs were due to the activation of the PAR-2 receptor and not the thrombin receptor. The activation of the vascular PAR-2 receptor might provide a rationale for the hypotensive action of trypsin when administered intravenously in amounts equal to thrombin (17).

Despite the apparent independence of the PAR-2 and thrombin receptors in the aorta system, there is a remarkable similarity between the structural requirements for activation of the two receptor systems by their putative tethered peptide sequences. First, a relatively short (four or five residues) peptide motif appears to be sufficient for activation of both receptors (Ref. 5 and this study). Second, for both receptors, the amino acid at position 2 and the arginine at position 5 of the tethered receptor-activating sequence appear to be critical for receptor activation despite different specific residue requirements at position 2 for activating the PAR-2 and thrombin receptors. Finally, for both receptors, the incorporation of an amide at the carboxyl terminus of receptor-activating peptides increases peptide potency (6) (e.g., for PAR-2 receptor, PP5-NH_2 > PP5; for thrombin receptor, P5-NH_2 > P5). It remains to be seen whether a free primary amino group is important for the action of PAR-2-APs, as is the case for TRAPs (6, 12). Given these comparable structural requirements for the PAR-2-APs and TRAPs, one can predict that the mode of interaction between the putative proteolytically revealed anchored ligands and the domains responsible for receptor triggering may turn out to be quite similar in the two receptor systems. Future work comprising the completion of the entire sequencing of the rat PAR-2 receptor (presently ongoing in our laboratory) and a detailed comparison of the domains in the thrombin (18) and PAR-2 receptors required for receptor activation should confirm or disprove the above prediction. Furthermore, the completion of the rat PAR-2 receptor sequence should indicate whether it represents a receptor subtype distinct from that detected in murine tissue.

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