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Role of the Amino- and Carboxyl-Terminal Domains of Thrombin Receptor-Derived Polypeptides in Biological Activity in Vascular Endothelium and Gastric Smooth Muscle: Evidence for Receptor Subtypes

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SUMMARY

Using guinea pig gastric longitudinal muscle (GLM) and rat gastric longitudinal muscle (RLM) contractile assays and a rat aortic ring (RA) endothelium-dependent relaxation assay, we have examined the biological activities of a number of human and rat thrombin receptor-derived polypeptides (TRPs) modified at amino-terminal and carboxyl-terminal residues. Our study focused primarily on the human pentapeptide [$S_{42}FLLR_{46}$ (P5)], previously shown to retain full thrombin-like activity. Whereas *N*-acetylation (*N*-acetyl-P5) abolished biological activity in the GLM and RA assays, amidation or esterification of the carboxyl-terminal carboxyl group [P5-NH_2, P5-OCH_3, or $S_{42}FLLRNP_{48}$-NH_2 (P7-NH_2)] enhanced peptide potency by about 10-fold in both the GLM and RA assays, compared with the unmodified TRPs (P5 and P7). Removal from P5 of either the amino-terminal hydroxyl group of serine (to yield $A_{42}FLLR_{46}$) or both the amino-terminal hydroxyl group and the primary amino group of P5 [to yield propionyl-$F_{43}LLR_{46}$ (Pr-P4)] produced peptides that were active in both the GLM and RA assays. Substitution of the carboxyl-terminal guanidinium group of P5 with a less basic primary amino acid residue ($S_{42}FLLK_{46}$) resulted in a peptide with a lower potency than that of P5 in the GLM and RA assays, whereas substitution of D-arginine for L-arginine at the carboxyl terminus abolished biological activity. Substitution of norleucine for arginine (S_{42}FLLNorleuN) resulted in a peptide active in the GLM but not in the RA assay. For selected agonists (Pr-P4, P5, P7, and P7-NH_2), the potencies in the GLM and RA assays, relative to that of P5, differed; for the GLM the order was P7-NH_2 > P7 > P5 ≃ Pr-P4, whereas for the RA the order was P7-NH_2 ≥ Pr-P4 ≫ P5 ≥ P7. Data comparable to those obtained with the GLM assay were also obtained with a RLM assay, wherein the potency series was P7-NH_2 > P7 > P5. The relative potencies of pentapeptides based on the rat receptor sequence [SFFLR (Ra-P5), SFFLR-NH_2 (Ra-P5-NH_2), and SFFLRNP (Ra-P7)] differed in the RLM and RA assays. In the RLM the order was Ra-P5-NH_2 > P7-NH_2 > P5-NH_2 > Ra-P7 = P7 > P5 = Ra-P5, whereas in the RA the relative potency series was P7-NH_2 > P5-NH_2 > Ra-P5-NH_2 > Ra-P7 > P5 ≥ P7 > Ra-P5. Using P5 and P5-NH_2 as representative agonists, no appreciable peptide proteolysis was detected by high performance liquid chromatography analysis during the course of the RA or LM assays. In the GLM and RLM assays, the contractile activities of the TRPs were selectively blocked either by the tyrosine kinase inhibitors genistein and tyrphostin or by the cyclooxygenase inhibitor indomethacin. In contrast, the TRP-induced relaxation in the RA assay was not blocked by these enzyme inhibitors. The data hightlight the importance of the charge properties of the amino- and carboxyl-terminal residues of the TRPs for biological activity. Furthermore, the different relative potencies of the TRPs in the GLM and RLM systems, compared with the RA assay, as well as the distinct signal transduction pathways used by the TRPs in the gastric versus the arterial assays, point strongly to the existence of distinct thrombin receptor subtypes in the gastric smooth muscle elements and in the arterial endothelial cells.

In addition to its role as a coagulation factor, thrombin is now known to regulate cell function via the proteolytic activation of a specific guanine nucleotide-binding protein-linked cell surface receptor (1–4). The novel mechanism whereby thrombin activates a target cell involves the proteolytic exposure of an amino-terminal sequence of the receptor; this revealed amino-terminal domain (beginning with serine-42 in the human receptor) is then believed to act as an "anchored" or "tethered" ligand that activates the receptor (2). Remarkably, it has been demonstrated that a peptide comprising only the first 14 amino acids of the thrombin-revealed amino-terminal sequence of the human receptor (P14) can, on its own, activate the thrombin receptor, mimicking many of the cellular actions of thrombin in a variety of target tissues (1, 3, 5–8). In our own work (5), we have established that the human TRP, P14, possesses thrombin-mimetic activity in both vascular and non-vascular smooth muscle preparations. In accord with the action

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ABBREVIATIONS: P14, S_{42}FLLRNPNDKYEPF$_{55}$ (single-letter code is used for amino acids); NA, noradrenaline; Nleu-P6, S_{42}FLLNorleuN; P3-NH_2, $S_{42}FL_{44}$-NH_2; Pr-P4, propionyl-$F_{43}LLR_{46}$; Pr-P4-NH_2, propionyl-$F_{43}LLR_{46}$-NH_2; P5, $S_{42}FLLR_{46}$; PK5, $S_{42}FLLK_{46}$; P5-NH_2, $S_{42}FLLR_{46}$-NH_2; P5-OCH_3, $S_{42}FLLR_{46}$-OCH_3; P7, $S_{42}FLLRNP_{48}$; P7-NH_2, $S_{42}FLLRNP_{48}$-NH_2; RA, rat aortic ring(s); Ra-P5, $S_{42}FFLR_{46}$; Ra-P5-NH_2, $S_{42}FFLR_{46}$-NH_2; Ra-P7, $S_{42}FFLRNP_{48}$; R_{EC}, equi-effective concentration ratio (EC_{TRP} ÷ EC_{P5}); RLM, rat gastric longitudinal muscle; TRP, thrombin receptor-derived peptide; TP, tyrphostin (RG 50864, also designated AG213); ACh, acetylcholine; *N*-Ac-P5, *N*-acetyl-$S_{42}FLLR_{46}$; AP5, $A_{42}FLLR_{46}$; D-Arg-P5, $S_{42}FLLr_{46}$; F-P5, $F_{43}LLRN_{47}$; GLM, guinea pig gastric longitudinal muscle; GS, genistein; LM, longitudinal muscle; HPLC, high performance liquid chromatography.

of thrombin itself (9–13), we observed that P14 can cause either a direct contractile response in gastric or vascular smooth muscle (5, 14) or an endothelium-dependent relaxation of selected vascular smooth muscle preparations, such as that derived from rat aortic tissue (5). We used the GLM strip contractile assay (15) and the RA relaxation assay as reliable and convenient bioassays to establish that only the first five amino acids of P14 (i.e., P5) are required to mimic the actions of thrombin on smooth muscle preparations (14). With P5 we observed further that the carboxyl-terminal arginine-46 and phenylalanine-43 play critical roles in activating the thrombin receptor in the GLM and RA tissues. P5 has also been shown to exhibit thrombin-like activity in fibroblasts (3) and in platelets (8, 16, 17). Structure-activity studies have demonstrated that, in platelets as well, the phenylalanine-43 and arginine-46 residues are important for the biological activity of the TRPs (7, 8, 16).

In the structure-activity studies TRPs done by us (14) and by others (7, 8), we were struck by two principal observations. 1) The potency of the TRPs first increased upon shortening of the peptide from 14 (TRP_{42-55}) to six residues (TRP_{42-47}), followed by a marked reduction in potency upon shortening of the hexapeptide further to P5 (TRP_{42-45}) and then by an abrogation of activity upon shortening of the peptide to the 4-mer SFLL. 2) Acetylation of the amino-terminus abolished the activity of otherwise active TRPs. These data pointed to the importance of the amino- and carboxyl-terminal regions of the pentapeptide for its intrinsic thrombin-mimetic biological activity. The study described in this communication was therefore undertaken to explore further the contribution of the amino-terminal and carboxyl-terminal residues to the biological activities of the TRPs, as evaluated in the GLM and RA preparations that we characterized previously (14, 15). We also wished to establish whether data obtained in the GLM preparation would be directly comparable to assays done using a RLM preparation. Our data enlarge upon the importance of the amino- and carboxyl-terminal residues for the biological action of TRPs, and our results suggest that modifications of the amino- and carboxyl-terminal residues of the active TRPs may serve to discriminate between the receptors present in gastric smooth muscle and those present in aortic endothelial cells.

Materials and Methods

Bioassay procedures. For all animal-derived tissues, 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. The GLM strips and the RA preparations were isolated as described previously (14, 15). In brief, gastric LM strips (3 × 10 mm), obtained from male albino Hartley strain guinea pigs, were equilibrated for about 1.5 hr at 37° in an aerated (95% O_2/5% CO_2) Krebs-Henseleit buffer (3 ml) of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; and glucose, 10. The pH was maintained at 7.4. Rat gastric LM strips (3 × 10 mm) were obtained, as for the GLM strips, from male albino Sprague-Dawley animals (250–300 g). The stomach, opened along the greater curvature, was stripped free of all mucosal tissue under microscopic visualization and longitudinal tissue strips were cut exactly at right angles to the visible circular muscle bundles. Tissue strips were pre-equilibrated in the buffer described above. Each tissue was challenged twice each in succession, at timed intervals, with 50 nM carbachol (20-min intervals), 1 μM angiotensin II (1-hr intervals), and 95 μM (60 μg/ml) P5 (25-min intervals for GLM; 30–35 min intervals for RLM); tissues were washed 10 and 15 min after the additions of agonists. Contractions were monitored isometrically using Statham force-displacement transducers. Only tissues responding well to all three agonists were used for the evaluation of the biological activities of P5 and the other peptide agonists. For the GLM and RLM assays, concentration-effect curves for all peptides were constructed by expressing the response of each agonist as a percentage (percentage of $P5_{95}$) relative to the contraction caused by 95 μM (60 μg/ml) P5 (0.9 ± 0.1 g of tension, mean ± standard error for $n = 12$ in the GLM; 1.6 ± 0.9 g of tension, mean ± standard error for $n = 36$ in the RLM).

Thoracic RA (2 mm × 3 mm), obtained from male albino Sprague-Dawley rats, were equilibrated (about 1 hr at 37°) in the same organ bath buffer as was used for the LM tissue, and tension (base line, 1.0 g) was monitored isometrically using either Statham or Grass force-displacement transducers. The presence of an intact endothelium was ascertained by monitoring the relaxation response to ACh (1 μM) in a preparation that was submaximally (EC_{75}) contracted with 0.1 μM NA in the presence of 0.1 mM ascorbic acid (to preserve the catecholamine). Only tissues yielding a relaxation concentration-response curve between 0.01 and 10 μM ACh (maximal relaxation of 70–90%, relative to the tension developed with 0.1 μM NA) were used for the evaluation of the TRPs. The assay of TRP responsiveness was done (for example, see Fig. 1) with tissues that were precontracted with 0.1 μM NA at 30-min time intervals. Tissues were washed approximately 5 min after the application of TRPs. In preliminary work studying the concentration-effect curve for P5 (see below), it was observed that 215 μM (137 μg/ml) P5 yielded a nadir of relaxation in the RA preparation (on average, 61% relaxation of the NA-induced contractile response). Therefore, to construct concentration-effect curves and to normalize data between individual preparations, the relaxation responses to P5 and to the other TRPs were expressed as a percentage of the relaxation caused by 215 μM (137 μg/ml) P5 (percentage of $P5_{215}$). For purposes of comparison, the same stock solutions of individual peptides were evaluated, relative to P5, in both the RA and LM bioassays. When possible, both assays were done simultaneously on the same day.

Peptides and other reagents. The TRPs (based on the human receptor sequence) P3-NH_2, Pr-P4, Pr-P4-NH_2, P5, PK5, D-Arg-P5, AP5, *N*-Ac-P5, P5-NH_2, P5-OCH_3, F-P5, Nleu-P6, P7, and P7-NH_2 were prepared by standard solid-phase synthesis procedures either in our own laboratory (18) (*tert*-butoxycarbonyl procedures for P7, Nleu-P6, P5-OCH_3, and P3-NH_2) or in the Core Peptide Synthesis Laboratory at the Department of Biochemistry, Queens University (Kingston, Ontario, Canada) (9-Fluorenylmethoxycarbonyl procedures for Pr-P4, Pr-P4-NH_2, P5, AP5, P5-NH_2, F-P5, and P7-NH_2). The rat receptor sequence TRPs, Ra-P5 and Ra-P5-NH_2 were also from the Queens University Laboratory; the heptapeptide based on the rat receptor sequence, Ra-P7, was prepared in our own laboratory using a *tert*-butoxycarbonyl procedure, as for P7. Pr-P4 was prepared using propionic acid/dicyclohexylcarbodiimide to react with resin-bound P4 before deprotection; P5-OCH_3 was prepared by treating the resin-attached peptide with 50 mEq of triethylamine in 20 ml of methanol. The peptide ester released from the resin was deprotected with anhydrous HF. Peptides released from the resin and deprotected were then purified to homogeneity (single peak by analytical reverse phase HPLC with a Vydac RPC18 column and an acetonitrile gradient in 0.1% trifluoroacetic acid). Peptide compositions were confirmed by amino acid analysis and, where appropriate (Pr-P4 and Pr-P4-NH_2), by mass spectral analysis. 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 of all stock solutions used were verified by quantitative amino acid analysis of aliquots. Pr-P4 and Pr-P4-NH_2 were dissolved in 40 mM phosphate buffer, pH 7.4, containing 20% (v/v) absolute ethanol (i.e., 8 volumes of the aforementioned 50 mM phosphate buffer plus 2 volumes of ethanol). Control aliquots of the ethanol-containing phosphate buffer alone had no effect on either of the bioassay systems.

ACh, carbachol, indomethacin, NA, angiotensin II, and thrombin (human, 3000–4000 units/mg; 1 unit/ml ≃ 10 nM) were from Sigma Chemical Co. (St. Louis, MO); GS was from ICN Biochemicals (Costa Mesa, CA). TP was obtained through the courtesy of Dr. R. R. Swillo, Rhône-Poulenc Rorer (Collegeville, PA). GS and TP were dissolved in dimethylsulfoxide to yield a stock solution that, when diluted, resulted in a concentration of dimethylsulfoxide in the organ bath (<0.01%, v/v) that alone had no effect on the bioassay systems. 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 had no effect on the bioassay systems.

Results

Characteristics of the responses of the GLM and RA preparations to TRPs. Our initial focus was on the biological activities of peptides representing modifications of the amino and carboxyl termini of the human P5 sequence. Because of our previous experience with the GLM and RA bioassays (14, 15, 19) and with the human TRP sequence, the majority of our study was done with the GLM and RA assays, in which peptides based on the human TRP sequence were evaluated. The responses of the GLM and RA preparations to all of the human receptor sequences tested, except for Pr-P4, are typified by the data for P5-NH_2 shown in Fig. 1; concentration-effect curves for the GLM and RA preparations are shown in Fig. 2. As anticipated from previous results with *N*-acetylated TRPs, *N*-Ac-P5 (≤200 μM) was not active in either the GLM or RA assay, either as an agonist or as an antagonist of P5 (Fig. 2 and data not shown). Simply removing the P5 amino-terminal hydroxyl group of serine (to form AP5) yielded a peptide with activity comparable to that of P5 (see Fig. 2). This result was in keeping with our previous observations with a nonapeptide TRP beginning with alanine in position 42 (15). Our next step involved deletion of the amino-terminal amino group of AP5, to yield Pr-P4. Because we had already established that the tetrapeptide P4 was inactive in the LM assay (14), we were surprised to observe that Pr-P4 exhibited a thrombin-mimetic action in both the LM and RA bioassays (Figs. 1 and 2). Nonetheless, the responsiveness of the two tissues to Pr-P4 differed somewhat, in comparison with the responses to P5 and P5-NH_2. First, the GLM assay exhibited desensitization in response to the repeated administration of Pr-P4 (Fig. 1B), but not P5-NH_2 or P5. Therefore, to measure a concentration-effect curve for Pr-P4, tissues yielding a standard response to 95 μM (50 μg/ml) P5 were exposed to only a single concentration of Pr-P4. As discussed below, the concentration-effect curve suggested partial agonist activity for Pr-P4 in the GLM tissue. When a tissue was desensitized by repeated exposure to Pr-P4, a diminished but measurable response towards P5 could still be monitored (Fig. 1B). It was not possible to estimate with confidence the degree of codesensitization caused by Pr-P4 for either P5 or P5-NH_2. A second qualitative difference between the actions of Pr-P4 and P5 was observed in the RA assay system (Fig. 1), wherein Pr-P4 caused a persistent relaxation of the tissue, as opposed to the transient relaxation caused by P5, P5-NH_2, and the other TRPs tested. In the RA assay, Pr-P4 did not cause desensitization (data not shown) and appeared to act as a full agonist (Fig. 2).

In contrast to acetylation of the amino terminus of P5 (a substitution that abolished biological activity), esterification or amidation of the carboxyl terminus of P5 resulted in an active peptide that was even more potent than that having a free carboxyl group (Figs. 1 and 2). Conversely, amidation of the carboxyl terminus of Pr-P4 (to yield Pr-P4-NH_2) attenuated the activity of the peptide (Fig. 2). As an additional control, we also ascertained that P3-NH_2 was inactive (data not shown), to indicate that amidation *per se* did not result in an active peptide. In view of the importance of the carboxyl-terminal arginine for the activity of P5 (14), we tested the activity of the

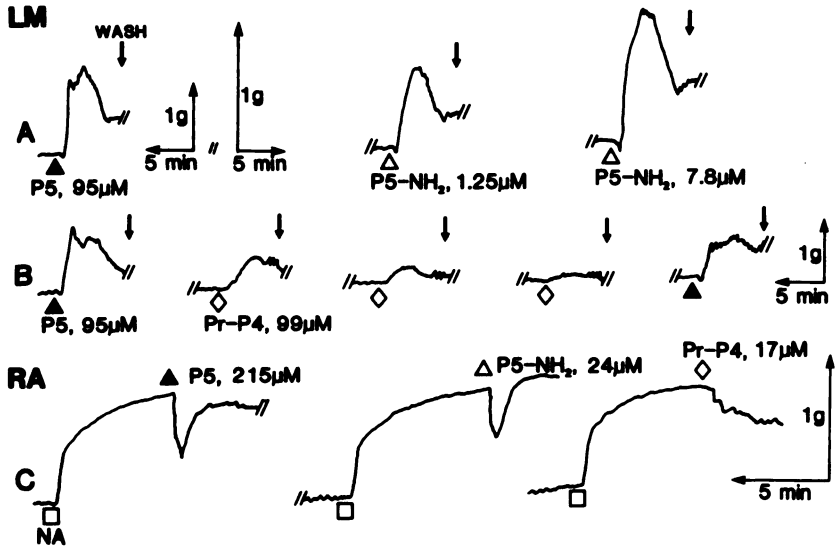

Fig. 1. Representative responses of the GLM and RA preparations to receptor peptide analogues. Tissue strips were exposed to the receptor-derived peptide analogues P5 (▲), P5-NH_2 (△), and Pr-P4 (◇) at a dosing interval of 25 min; tension was measured isometrically. The increased tension (*upward deflection*) that developed in the GLM preparation (A and B, *upper tracings*, *LM*) was measured directly. The relaxation (*downward deflection*) of the RA preparation (C, *lower tracing*, *RA*) was monitored after precontraction of the tissue with 0.1 μM NA (□). A, Sequential responses to P5 (60 μg/ml, 95 μM) and P5-NH_2 (0.82 μg/ml or 1.25 μM and 5 μg/ml or 7.8 μM) in a single tissue preparation; B, sequential responses in a single tissue to P5 (95 μM) and to repeat doses of the same concentration of Pr-P4 (60 μg/ml, 99 μM), followed again by P5 in a preparation separate from the one shown in tracing A; C, sequential relaxation responses in a single tissue to P5 (137 μg/ml, 215 μM) and P5-NH_2 (15 μg/ml, 24 μM), as well as a representative response of a separate preparation to Pr-P4 (10 μg/ml, 17 μM). The concentrations of peptides were selected to produce an optimal typical response in each of the tissue preparations. The data are representative of six or more independently conducted experiments, using tissues from four or more separate animals, for which the variability of response is illustrated by the *error bars* in Fig. 2. The scales for time and tension for each assay are shown for each series of tracings; note the change of tension sensitivity in tracing A. Tissue strips were washed and equilibrated for 25 min between each exposure to the agonists, as outlined in Materials and Methods.

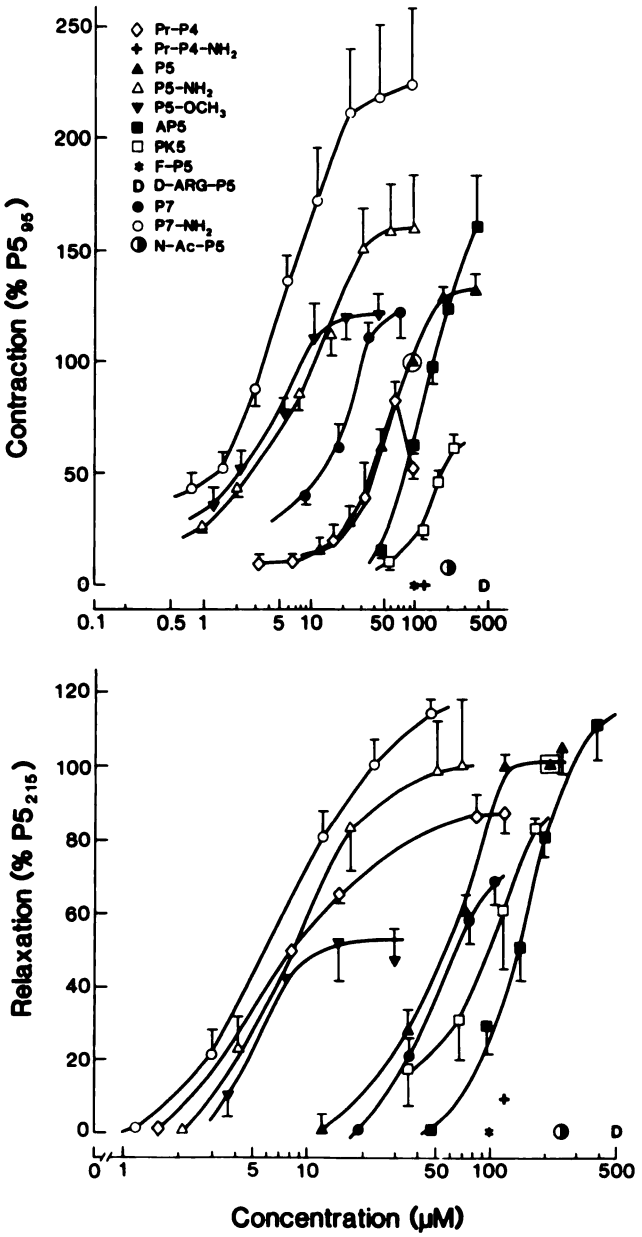

Fig. 2. Concentration-effect curves for receptor peptide analogues in the GLM (*upper*) and RA (*lower*) assays. Tissue strips were exposed to increasing concentrations of the indicated peptides, to obtain responses such as those shown for the GLM and RA tissues in Fig. 1. The contractile responses in individual GLM tissue strips were quantitated as a percentage (% $P5_{95}$) of tension developed in the same tissue strip in response to 95 μM (60 μg/ml) P5 (*circled triangle*) (0.9 ± 0.1 g of tension, mean ± standard error for $n = 12$). The relaxation responses in individual precontracted (0.1 μM NA) RA rings were quantitated as a percentage (% $P5_{215}$) of the relaxation caused in the same preparation by 215 μM (137 μg/ml) P5 (*boxed triangle*) (61 ± 3% relaxation, mean ± standard error for $n = 18$). The concentration-response curves for each agonist represent data from assays done with tissue from three or more animals, using three or more independent tissue strips in each assay. The figure shows the average response at each concentration, with the standard error ($n = 5$–12) represented at each *point* with a *bar*. The responses to peptides with little or no activity (F-P5, Pr-P4-NH_2, D-Arg-P5, and *N*-Ac-P5) are shown along with the responses to active peptides (Pr-P4, P5, P5-NH_2, P5-OCH_3, PK5, AP5, P7, and P7-NH_2). The scales for concentration (μM) are on the *x*-axes; the response scales for contraction (GLM) or relaxation (RA) are shown on the *y*-axes.

peptide in which the L-arginine at position 46 was replaced with D-arginine. No response to this analogue at concentrations as high as 400 μM was observed in either the LM or RA assay, and the analogue did not exhibit antagonist activity. The pentapeptide in which the carboxyl-terminal arginine residue was replaced with lysine (PK5) was active in both the LM and RA assays but exhibited a lower potency than did P5 (Fig. 2). However, replacing the arginine with norleucine, to yield a peptide without a basic side chain, Nleu-P6, resulted in an agonist for which preliminary work indicated an activity comparable to that of P5 in the GLM assay but no activity in the RA assay at concentrations up to 120 μM (data not shown). Because of difficulties with peptide solubility, it was not possible to pursue work with Nleu-P6 further.

P7, representing the human receptor motif, is a sequence that is highly conserved among the four thrombin receptors that have been cloned to date. The hamster and rat equivalent of P7, SFFLRNP, has a phenylalanine for leucine substitution at a position (position 3) that has been observed to allow an alanine substitution without appreciably affecting peptide activity (7, 8, 16). In all of the receptors cloned to date there is appreciable sequence variation beyond the proline residue (1, 3, 4, 20). We thus wished to compare the biological activity of the carboxyl-terminally extended version of P5 with that of P5 itself. We also wished to evaluate the consequence of carboxyl-terminal amidation of P7 for comparison with the results obtained with P5-NH_2. As indicated in Fig. 2, P7 and P7-NH_2 were both active in the GLM and RA assay systems and P7-NH_2 was more potent than either P5 or P7 in the two assays. A portion of this conserved sequence (F-P5), reported previously to be an antagonist in platelets (7), was inactive as either an agonist or antagonist in the GLM and RA assays at concentrations up to 200 μM.

Concentration-response curves in the GLM and RA preparations. Our objective for all of the peptides was to obtain concentration-response data that could be compared with the activities of P5 in both the GLM and RA assays. We thus sought a standard basis for normalizing assays done on different days in separate tissues. In our previous work with the GLM assay (14), we expressed the contractile actions of the TRPs relative to the contractile response caused by 50 nM carbachol. Upon continued use of the GLM assay system, we observed that preparations responding well to carbachol did not necessarily respond well to a convenient test concentration (95 μM, 60 μg/ml) of P5. As an alternative to using a standard carbachol response for normalizing each assay, we considered using a test concentration of angiotensin II (1 μM), because this agonist appears to work in the LM tissue via a signal transduction pathway akin to the one used by P5 (14, 19). Although tissue responsiveness to 1 μM angiotensin II (1.9 ± 0.4 g of tension, mean ± standard error for $n = 12$ in the GLM assay) proved to be a somewhat better indicator of tissue responsiveness to P5 than was responsiveness to carbachol, we still encountered appreciable intertissue variation, i.e., some preparations responding with a robust contraction to 1 μM angiotensin II did not necessarily respond well to P5, and *vice versa*. Because of this apparent intertissue variability in P5 responsiveness, we decided to use the response to a standardized test concentration of P5 itself in both the GLM and RA assays for the evaluation of the relative activities of the other peptides. In the GLM assay, as was found previously (14), a maximum

contraction in response to increasing concentrations of P5 (see Fig. 2) was observed only at quite high concentrations of P5 (between 200 and 400 μM). Therefore, for reasons of economy, we selected a P5 concentration (95 μM, 60 μg/ml) that was on the upper portion of the concentration-effect curve (Fig. 2) to use as a standard test concentration in the routine LM assays. Hence, for the concentration-effect curves in the GLM assay, the contractile responses of all peptides were expressed as a percentage relative to the contraction (0.9 ± 0.1 g of tension, mean ± standard error for $n = 12$) caused by 95 μM P5 (percentage of $P5_{95}$; Fig. 2). In the RA assay, as in the GLM assay, the response to ACh (relaxation) was not necessarily a good indicator of the relaxation response to a test dose of P5. In pilot studies done in RA tissue that had been precontracted with 0.1 μM NA, it was observed that concentrations of P5 between 100 and 250 μM caused a maximal relaxation [61 ± 3% ($n = 18$), relative to the tension developed in response to 0.1 μM NA; see Figs. 1 and 2]. Because of the nature of the relaxation assay, it was deemed best to use a maximally effective concentration of P5 (215 μM, 137 μg/ml) for comparative purposes in the RA assay (Fig. 2). The concentration-effect curves in the RA assay for all TRPs were, therefore, expressed as a percentage relative to the relaxation caused by 215 μM P5 (percentage of $P5_{215}$; Fig. 2).

The strategy outlined in the paragraph above made possible the construction of comparative concentration-effect curves for the active agonists in all assay systems. As observed in our previous study, establishing a single maximum response level in the GLM assay was problematic, because some agonists appeared to exhibit greater efficacy than others. Previously, we also noted that the TRPs caused a greater response in the GLM assay than did thrombin itself (14). Precise determinations of the EC_{50} values were, therefore, not attempted. Rather, for comparative purposes it was possible to obtain an evaluation of the potencies of the various TRPs, relative to that of P5, by using the linear portions of the concentration-effect curves to calculate for several response levels the average R_{EC} of an individual TRP, relative to the concentration of P5 that caused the same contractile response (i.e., $R_{EC} = EC_{TRP} + EC_{P5}$). Using this approach to calculate the relative effective concentration values (R_{EC} values; Table 1), the relative order of potencies of the agonists in the GLM assay (Fig. 2, *upper*, *left* to *right*) was P7-NH_2 ≥ P5-OCH_3 ≃ P5-NH_2 > P7 > Pr-P4 (partial agonist) ≃ P5 > AP5 > PK5. In the RA assay, a similar approach to the estimate of relative potencies (R_{EC} values) was used, even though all agonists, except for P5-OCH_3, appeared to cause equivalent maximal relaxation (Fig. 2, *lower*). In the RA assay, the relative order of potencies (Fig. 2, *lower*, *left right*) was P7-NH_2 ≥ P5-NH_2 ≃ Pr-P4 ≥ P5-OCH_3 (partial agonist) > P5 ≥ P7 > PK5 ≥ AP5. Numerically, the relative potencies, as expressed by the R_{EC} values, relative to P5 are summarized in Table 1.

Characteristics of the RLM assay, concentration-effect curves, and comparison with the RA assay. The majority of our study was done with the GLM and RA tissues, because of our previous experience in validating these assays for a variety of peptide agonists (5, 14, 15) and because of the necessity of comparing our newly acquired data with our previous observations (14, 15). Because of the anatomic distribution of muscle fibers in the rat stomach, it is rather difficult to obtain a contractile rat gastric preparation that contains only intact LM elements, without any active circular muscle activity. Nonetheless, it was possible, by cutting dissected rat gastric tissue exactly at right angles to the visible circular muscle striation, to obtain a RLM muscle strip that was comparable to the GLM preparation that we had used for the majority of our assays. In contrast to the GLM, thrombin caused considerable desensitization of the RLM, such that it was not possible to obtain a concentration-effect curve in a single tissue preparation (data not shown). Based on the initial responses of a tissue to thrombin in a preparation that had been standardized with P5 (95 μM, 60 μg/ml), we determined that the contractile response to thrombin plateaued at a concentration of 1–3 units/ml (10–30 nM); this plateau response at 1 unit/ml thrombin was 52 ± 20% (mean ± standard error for $n = 5$) of the contractile response caused by 95 μM P5. As seen below, the RLM preparation was similar to the GLM preparation in terms of its sensitivity to tyrosine kinase inhibitors but was less sensitive than the GLM assay in terms of the inhibitory action of indomethacin. Notwithstanding these minor differences in responsiveness, the RLM preparation, like the GLM tissue, was sensitive to the TRPs, so as to provide for a bioassay akin to the one we had already developed for the GLM (Fig. 2).

Because the sequences of the guinea pig and rat thrombin receptors may well differ, we wished to determine whether the peptide potency differences that we had observed between the GLM and RA assays (Fig. 2) might also be observed when RLM and RA assays were compared. To this end, we selected the TRPs P5, P5-NH_2, P7, and P7-NH_2 for assay in the RLM tissue, because the relative EC_{50} values of these peptides (especially P5 and P7) discriminated between the GLM and RA preparations. Because of the complex properties of Pr-P4 in the GLM assay (a desensitizing partial agonist), we decided not to test this peptide in the RLM. Provided that the intervals of exposure of the RLM tissue to the TRPs were >30-min, the contractile responses to the TRPs were reproducible (see Fig.

TABLE 1
Relative potencies of TRPs in the LM and RA assays

Peptide	Designation	R_{EC} value (relative to P5)[a]		
		GLM	RLM	RA
Propionyl-$F_{43}LLR_{46}$	Pr-P4	1[b]		0.1
Propionyl-$F_{43}LLR_{46}$-NH_2	Pr-P4-NH_2	NA[c]		A
$S_{42}FLLR_{46}$	P5	1	1	1
$S_{42}FFLR_{46}$	Ra-P5		1.0	4.4
$S_{42}FLLK_{46}$	PK5	4.5		1.3
$S_{42}FLLr_{46}$	D-Arg-P5	NA		NA
$A_{42}FLLR_{46}$	AP5	1.8		1.7
$F_{43}LLRN_{47}$	F-P5	NA		NA
N-Acetyl-$S_{42}FLLR_{46}$	*N*-Ac-P5	NA		NA
$S_{42}FLLR_{46}$-NH_2	P5-NH_2	0.1	0.2	0.1
$S_{42}FFLR_{46}$-NH_2	Ra-P5-NH_2		0.07	0.2
$S_{42}FLLR_{46}$-OCH_3	P5-OCH_3	0.1		0.1[b]
$S_{42}FLLNorleuN_{47}$	Nleu-P6	A	NA	
$S_{42}FLLRNP_{48}$	P7	0.4	0.3	1.1
$S_{42}FFLRNP_{48}$	Ra-P7		0.3	0.6
$S_{42}FLLRNP_{48}$-NH_2	P7-NH_2	0.04	0.1	0.08

[a] The relative potencies for all agonists were determined by using several points on the linear portions of the concentration-effect curves shown in Figs. 2 and 3 to estimate for each agonist an average concentration ratio (R_{EC}), relative to a concentration of P5 that caused an equal response, as outlined in the text. The numbering of amino acid residues is based on the human receptor sequence.
[b] Partial agonist properties.
[c] NA, not active; A, active but not able to determine R_{EC}.

6), so as to enable us to obtain the concentration-effect curves illustrated in Fig. 3 (*upper*). The relative EC_{50} values of P5, P5-NH_2, P7, and P7-NH_2 (P7-NH_2 > P5-NH_2 > P7 > P5) in the RLM assay closely paralleled the relative potencies in the GLM assay (Table 1; Fig. 2) but differed from the relative potencies of these same polypeptides in the RA assay (P7-NH_2 ≥ P5-NH_2 >P5 ≥P7) (Fig. 3, *lower*).

Because the amino-terminal pentapeptide sequence of the anchored rat receptor revealed by thrombin cleavage (SFFLR) differs from the revealed pentapeptide sequence of the human receptor (SFLLR), it was of interest to compare the activities of the rat P5 sequences (i.e., Ra-P5 and Ra-P5-NH_2) and the activity of Ra-P7 with the activities of the human P5 and P7 sequences in the RLM and RA bioassay systems. In keeping with previous structure-activity data for P5, in which a substitution at position 3 (i.e., SFALR) had little impact on the activity of the pentapeptide in a platelet assay (16), our data revealed equivalent activities for Ra-P5 relative to P5 and for Ra-P7 relative to P7 in the RLM assay (Fig. 3, *upper*). As with the human receptor P5 sequence, carboxyl-terminal amidation also increased the activity of Ra-P5 in the RLM assay but with an even more dramatic effect on potency than was observed for P5 (Fig. 3). Indeed, Ra-P5-NH_2 was the most potent of all of the peptides we examined in the RLM assay. In contrast to the activities of the rat receptor sequence peptides in the RLM assay, as indicated by the data in the next paragraph, Ra-P5 and Ra-P5-NH_2, were considerably less potent than the comparable human receptor sequences in the RA assay. Significantly, Ra-P5 was > 4-fold less potent than P5 in the RA assay (Fig. 3, *lower*; Table 1), whereas P5 and Ra-P5 were equipotent in the RLM assay (Fig. 3, *upper*).

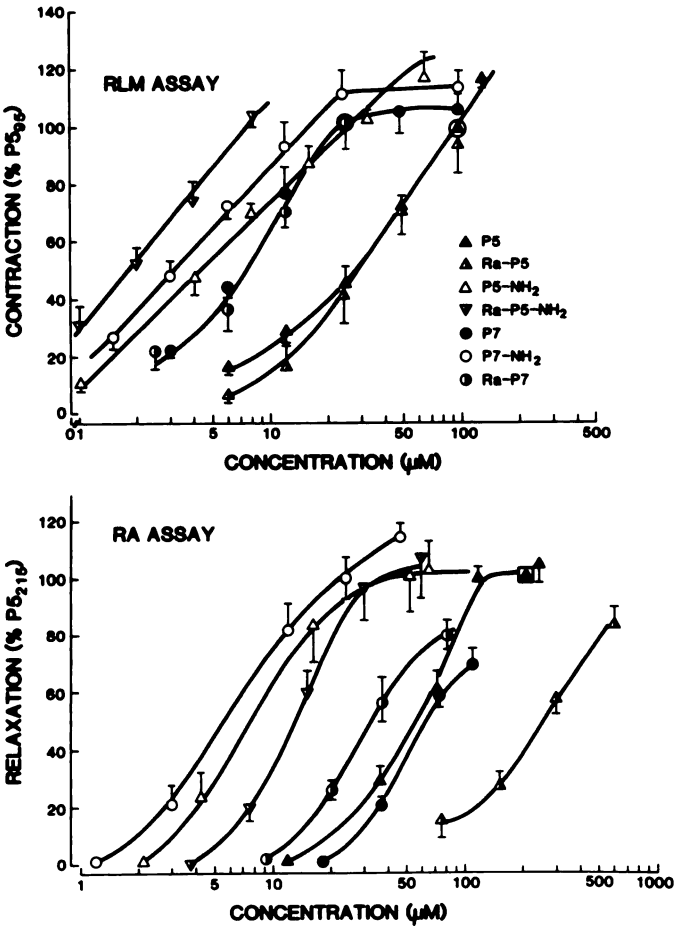

Fig. 3. Concentration-effect curves for receptor peptide analogues in the RLM (*upper*) and RA (*lower*) assays. Tissue strips were exposed to increasing concentrations of the indicated peptides, to obtain responses such as those shown for the RLM and RA tissues in Figs. 1 and 6. The contractile responses in individual RLM tissue strips (*upper*) were quantitated as a percentage (% $P5_{95}$) of tension developed in the same tissue strip in response to 95 μM (60 μg/ml) P5 (*circled triangle*) (1.6 ± 0.9 g of tension, mean ± standard error for $n = 36$). RLM tissue was exposed to agonists at 30–35 min intervals, followed by washing and reequilibration. The relaxation responses (% $P5_{215}$) of the RA (*lower*) were quantitated exactly as outlined in the legend to Fig. 2, relative to a standard response to P5 (*boxed triangle*) (215 μM, or 137 μg/ml). For clarity, data obtained for the human sequence peptides, shown in Fig. 2, are replotted for comparison with the responses of the RA to the rat sequence-derived peptides. The figure shows the average response at each peptide concentration, relative to that caused by P5 in the same preparation, with the standard error (n = 4–12) represented at each *point* by a *bar*. The scales for concentration are on the *x*-axes; the response scales for contraction (RLM, *upper*) or relaxation (RA, *lower*) are shown on the *y*-axes.

Taken together, our data for P5, Ra-P5, P5-NH_2, Ra-P5-NH_2, P7, Ra-P7, and P7-NH_2 revealed quite distinct orders of potencies for the RLM and RA assays. Relative to P5, the potencies of these compounds in the RLM assay, as summarized in Table 1, were as follows: P5, 1.0; Ra-P5, 1.0; P5-NH_2, 0.2; Ra-P5-NH_2, 0.07; P7, 0.3; Ra-P7, 0.3; P7-NH_2, 0.1. This yields a potency series of Ra-P5-NH_2 > P7-NH_2 > P5-NH_2 > P7 = Ra-P7 > P5 = Ra-P5. In the RA assay the potency ratios for these same agonists relative to P5 (from Table 1) were as follows: P5, 1.0; Ra-P5, 4.4; P5-NH_2, 0.1; Ra-P5-NH_2, 0.2; P7, 1.1; Ra-P7, 0.6; P7-NH_2, 0.08. This yields a potency series of P7-NH_2 > P5-NH_2 > Ra-P5-NH_2 > Ra-P7 > P5 ≥ P7 > Ra-P5. Thus, in summary, the relative potency series in the LM tissue, obtained using human or rat sequence-derived peptides in either the GLM (human sequence peptides) or RLM (both human and rat sequence peptides), were quite distinct from the order of potencies of the same agonists in the RA assay system.

Evaluation of peptide proteolysis. In a platelet assay, aminopeptidase activity has been observed to affect the activity of TRPs (21). Because vasoactive peptides can be metabolized by smooth muscle-derived proteases, such as aminopeptidase M (22), potentially to alter agonist potencies in a smooth muscle bioassay, (23), it was relevant to our study to determine whether the TRPs were degraded in the organ bath during their course of action (about 5–10 min). To deal with this issue, a number of approaches were used, with P5 as a model peptide that might be degraded by either aminopeptidase or carboxypeptidase activity. First, a bath-transfer experiment was done, wherein the organ bath solution (about 60 μg/ml P5) from one GLM tissue strip was removed just after the peak of contraction and added to a second test strip, for which the sensitivity to P5 was known. As demonstrated by the response of this second recipient tissue to the organ bath solution transferred from the first tissue, there was no appreciable loss of contractile activity upon incubation of the P5 solution for 5–10 min with the first tissue. This bath-transfer experiment was not possible with the RA preparation. In a second set of experiments, the action of P5 was measured in the absence and presence of the aminopeptidase M inhibitor amastatin (10 μM). The protease inhibitor did not potentiate the action of P5 (60 μg/ml, 95 μM) in either the LM or RA assay. Finally, as illustrated in Fig. 4 (*tracings C* and *D*), the organ bath solution withdrawn from either a

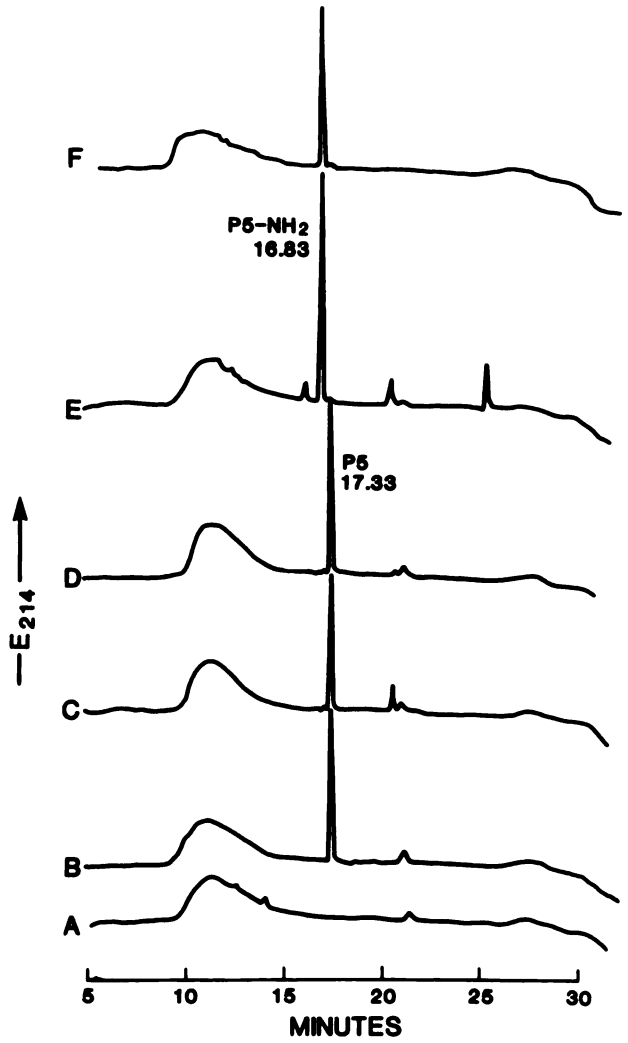

Fig. 4. Chromatographic analysis of P5 and P5-NH_2 recovered from the RLM and RA bioassay medium. Either P5 (final concentration, 30–60 μg/ml; 47.5–95 μM) or P5-NH_2 (final concentration, 5–10 μg/ml; 8–16 μM) was added to the RLM and RA assay baths (3.0 ml) exactly as for a bioassay procedure, and tissue response was monitored over a 10-min period (e.g., see Figs. 1 and 6). After the peak of tissue response (at about 5–10 min) the tissue bath medium was withdrawn and quick-frozen for HPLC analysis. Parallel samples of tissue bath medium, except without added TRPs, were also withdrawn for analysis and for use in preparing standard stock solutions of peptides that were compared with stock solutions made up with bioassay buffer alone. Bioassay bath samples either were diluted in distilled water or were applied directly to the HPLC column (Vydac RPC18, analytical column), at a flow rate of 1 ml/min, in a volume of 180 μl containing 1–2 μg of peptide. A 20-min gradient of acetonitrile (final concentration, 60% in 0.1% trifluoroacetic acid in water) began with a gradient from 0 to 18% acetonitrile over 3 min, followed by a linear gradient of 18% to 39% acetonitrile over 15 min and finally a gradient from 39% to 60% acetonitrile over the final 2 min. With this gradient, P5-NH_2 and P5 were eluted as sharp peaks between 16.5 and 17.5 min; smaller peptides had earlier elution times (data not shown). *Tracing A,* analysis of 180 μl of gastric LM assay bath medium; this same pattern was observed for the analysis of RA assay medium. *Tracing B,* analysis of a standard P5 solution (1.2 μg) made up in organ bath medium and analyzed immediately; an identical chromatogram was observed for a standard sample made up in distilled water. The broad peak at 10–12 min represents a gradient-shift artifact. *Tracing C,* analysis of P5 recovered from the RLM organ bath (expected amount, 1.1 μg). *Tracing D,* P5 recovered from the RA organ bath. *Tracing E,* P5-NH_2 recovered from the RLM assay. *Tracing F,* P5-NH_2 recovered from the RA assay. The elution times shown for P5 (17.33 min) and P5-NH_2 (16.83 min) were consistent for the entire series of analyses, with P5-NH_2 eluting slightly before P5.

RLM or RA assay (30–60 μg/ml P5, 47.5–95 μM) just after the peak of tissue response was subjected to analysis by HPLC and compared with a standard solution of P5 made immediately before HPLC analysis, either in buffer alone or in buffer that had been exposed to tissue under the conditions of a bioassay. The HPLC analysis (Fig. 4) demonstrated essentially quantitative recovery of the peptide from the RLM organ bath, without appreciable degradation (compare Fig. 4, *tracing B*, 1.2 μg from a P5 stock solution, with *tracing C*, wherein a calculated amount of 1.1 μg of P5 was expected from the organ bath solution). Quantitative recovery of P5 without appreciable degradation was also observed from the RA assay organ bath (Fig. 4, *tracing D*). Similarly, P5-NH_2 was recovered essentially intact from both the RLM and RA organ baths (Fig. 4, *tracings E* and *F*). In Fig. 4, the peptide degradation products would have been eluted at retention times earlier than those of either P5 (17.33 min) or P5-NH_2 (16.83 min). Our data thus led us to conclude that, during the relatively short time course of the tissue response (5–10 min), peptide proteolysis did not appear to be a factor that might affect the estimates of peptide potencies.

Signal transduction pathways. In our previous study we established that in the GLM preparation P5, like epidermal growth factor-urogastrone, acts via the production of prostanoid metabolites, so as to be blocked by indomethacin (14, 24). Like epidermal growth factor-urogastrone, but unlike bradykinin, the action of P5 in the GLM preparation was also selectively blocked by relatively low concentrations of the tyrosine kinase inhibitors GS and TP (14). We wished to determine whether the active P5 analogues modified at the carboxyl and amino termini (represented by P5-NH_2 and Pr-P4, respectively) would also act in the GLM assay via the prostanoid-linked, tyrosine kinase-associated, signal transduction pathway. We also wished to determine for the RA assay, wherein relaxation is caused by TRPs via the production of nitric oxide (5), whether the response might be sensitive to indomethacin and to the tyrosine kinase inhibitors, as is the case in the GLM assay. As illustrated for the GLM assay in Fig. 5 (*tracings E, F, G,* and *H*), the contractile actions of both P5-NH_2 and Pr-P4 were blocked by either indomethacin or TP; GS was similarly effective (data not shown). Because of the desensitization caused by Pr-P4 in the experiments shown for the GLM in Fig. 5, *tracings G* and *H*, the initial tissue sensitivity to TRPs was evaluated by exposure to P5-NH_2 (5 μg/ml, 7.8 μM) before the effects of TP, GS, and indomethacin on Pr-P4 action were determined (Fig. 5, *tracings G* and *H*). In contrast, the responses of the RA, which were abolished with the nitric oxide synthase inhibitor $N\omega$-nitro-L-arginine methyl ester (data not shown), as observed previously were not inhibited either by the tyrosine kinase inhibitors (Fig. 5, *tracings A* and *C*, and data not shown) or by indomethacin (Fig. 5, *tracings B* and *D*). As illustrated by the representative experiments shown in Fig. 6, the response of the RLM preparation to P5-NH_2 was also quite sensitive to TP (data not shown) and GS (77 ± 3% inhibition, mean ± standard error for $n = 6$) but was only partially affected by indomethacin (40 ± 6% inhibition, mean ± standard error for $n = 6$). In contrast, the contractile action of carbachol was unaffected in the RLM by either GS or indomethacin (Fig. 6). Thus, the actions of the TRPs in the gastric and aortic assays were differentially sensitive to indomethacin and the tyrosine kinase inhibitors.

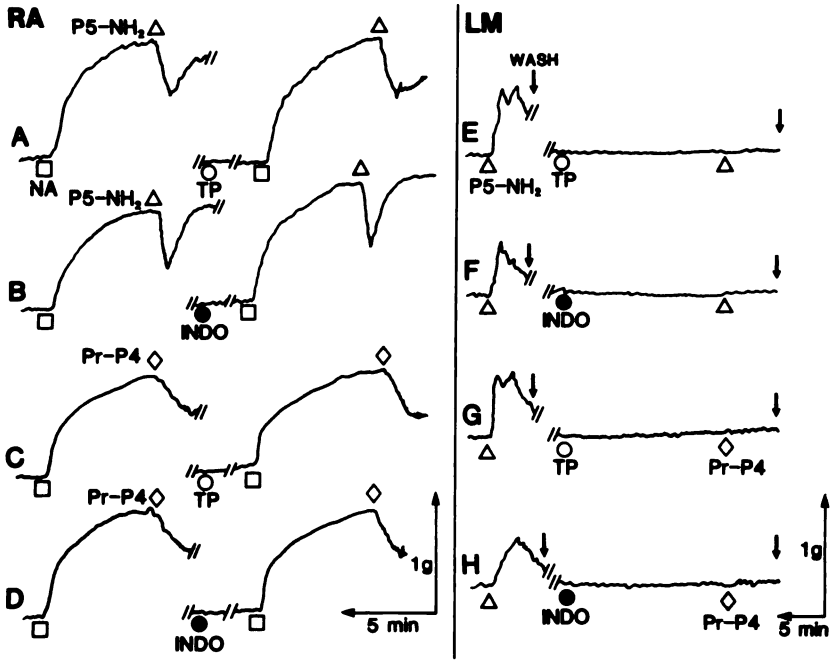

Fig. 5. Effects of TP, (○) and indomethacin (*INDO*) (●) on the actions of P5-NH_2 (△) and Pr-P4 (◇) in the RA (*left*) and GLM (*right*) assays. Responses to either P5-NH_2 (24 μM for RA, 7.8 μM for GLM) or Pr-P4 (17 μM for RA, 66 μM for LM) were measured either before or after treatment of tissues with either indomethacin (3 μM) *tracings B, D, F,* and *H*) or TP (40 μM) *tracings A, C, E,* and *G*). Each *tracing* (*A* through *H*) illustrates typical responses for either the RA or GLM preparations exposed sequentially to the various agonists and inhibitors, as indicated. Because of the Pr-P4-induced desensitization of the LM preparation, the initial tissue sensitivity of the LM was evaluated by exposure to P5-NH_2 before treatment of the tissues with either TP or indomethacin. Tissues were washed and re-equilibrated between each addition of agonist, as outlined in Materials and Methods. The data are representative of three or more independently conducted experiments on tissues derived from different animals, wherein identical results (either 0 or 100% inhibition) were observed. The scales for tension and time are shown on the *right* in each panel. RA preparations were precontracted with noradrenaline (□).

Discussion

In our previous study of the actions of the TRPs in the GLM bioassay (14), we established that the intrinsic activity of the revealed amino-terminal sequence, beginning at residue 42 of the human receptor sequence, resides in the first five amino acids (i.e., P5), wherein phenylalanine-43 and arginine-46 appear to be the major pharmacophores. Within this sequence, the third residue (leucine in the human sequence; phenylalanine in the rodent sequence) has been shown to play a minor role (7, 8, 16). The major finding of the present study was that, although the P5 motif is sufficient for exhibiting thrombin-mimetic activity, the charge properties of the amino- and carboxyl-terminal residues appear to play major roles in terms of the intrinsic activities of the TRPs in both the LM and RA receptor systems. The quantitative recovery of intact peptide from the RA and LM organ baths would appear to rule out peptide metabolism playing any major role in determining the relative potencies of the agonists we have studied. From the observations with P5, Ra-P5, P5-NH_2, Ra-P5-NH_2, P7, and P7-NH_2 in the GLM, RLM, and RA systems, it is clear that, in general, removing the negative charge of the carboxyl-terminal residue of the TRPs by amidation or esterification results in a marked increase in potency (about 10-fold). From these data, one may suggest that the absence of a carboxyl-terminal carboxyl group in the TRP may allow the side chain of amino acid residue 5 of the TRP (arginine) to come into closer proximity to a key carboxyl residue on the receptor. Because of the demonstrated activity of a P5 citrulline analogue (i.e., SFLL-citrulline) in platelets (16) and the contractile activity of Nleu-P6 in the GLM assay, one can suggest that positive charge *per se* is probably not the sole determinant governing the interaction of the residue at position 5 with the receptor. The stereochemical requirements for such an interaction are highlighted by the lack of agonist or antagonist activity of D-Arg-P5 in both the LM and RA assays. In this regard, for the peptide-receptor interaction the conserved aspartic and glutamic acid residues found in the sequences of many guanine nucleotide-binding protein-linked receptors (25) may be of particular significance.

The results of modification of the amino-terminal residue of P5 are less easy to interpret. Because *N*-acetylation abolishes the biological activity not only of P5 (this study) but also of other TRPs (8), one could speculate that a charge-charge interaction between the primary amino group and a receptor carboxyl group may also be involved in the binding of the ligand to the receptor. The importance of the ionization state of the amino-terminal serine for the biological activity of TRPs has been suggested by an NMR study that appeared upon completion of our work (21). Alternatively, it could be argued that a lone-pair electron interaction between the TRP amino group and the receptor (this interaction could be abrogated by *N*-esterification), as has been postulated for opiate-receptor interactions (26), may play a key role in receptor activation. However, the results of the experiments with desamino-AP5 (or Pr-P4) are difficult to interpret in such a context. Clearly, Pr-P4 results in a receptor activation process that is distinct from the one triggered by P5, because 1) Pr-P4 causes desensitization in the LM assay, whereas P5 and P5-NH_2 do not, and 2) carboxyl-terminal amidation markedly enhances the potency of P5 but abolishes the activity of Pr-P4. Nonetheless, the signal transduction pathways triggered by Pr-P4 and P5 in the GLM and RA assays appear to be the same. It is possible that the hydrophobic property of Pr-P4 may account for its unusual desensitization behavior. Because of the anomalous activity in the GLM and RA assays, the Pr-P4 analogue merits further scrutiny. Thus, the mechanism whereby the amino-terminal amino group modulates the activity of the TRPs will require further study, as will the mechanisms whereby Pr-P4 activates the LM and RA tissues.

One main outcome of our study of the structure-activity relationships for the TRPs and of the signal transduction pathways is a clear distinction between the receptor systems present in the gastric GLM and RLM preparations on the one hand and the aortic RA tissue on the other. The distinction

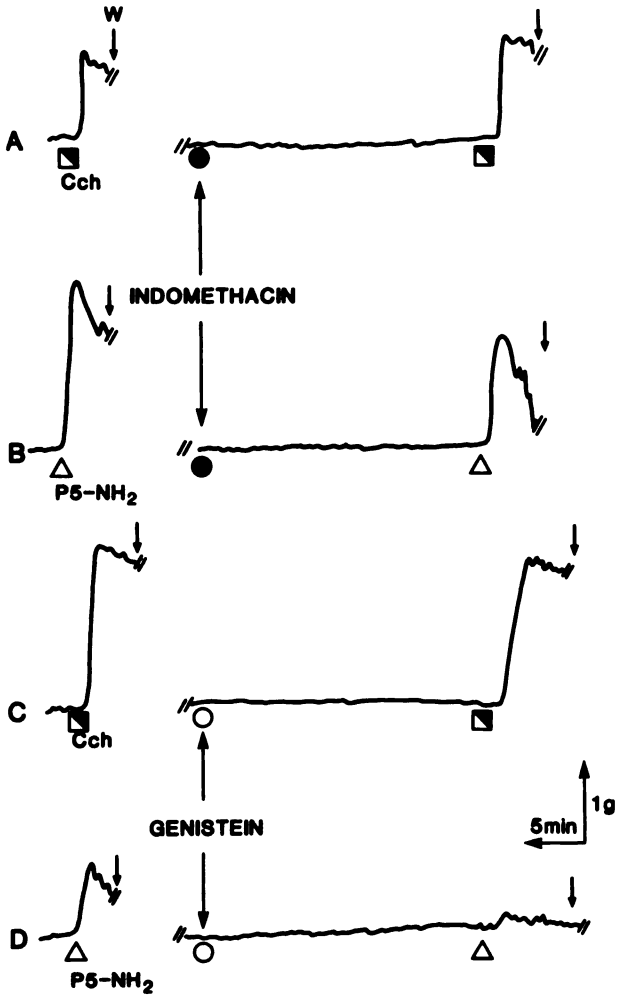

Fig. 6. Effects of GS (○) and indomethacin (●) on the actions of P5-NH_2 and carbachol in the RLM assay. Tissue strips were exposed, at a dosing interval of 1 hr, to either P5-NH_2 (8 μM) (◹) or carbachol (*Cch*) (1 μM) (◹), either before or 20 min after the addition of either indomethacin (3 μM) (*arrows* or GS (15 μM) (*arrows*). Tissues were washed (*W, arrow at top of traces*) and re-equilibrated between each addition of reagents to the organ bath. The data, showing no effect on carbachol-induced contractions and attenuation of P5-NH_2-triggered contractions, are representative of three or more independently conducted experiments using three to six different tissue strips, for which the inhibitory effects (mean ± standard error for $n = 6$) of GS and indomethacin are recorded in the text. *Tracings A* and *B* represent data from the same tissue strip; *tracings C* and *D* were obtained sequentially from a separate tissue preparation.

can be made by evaluating the relative potencies of a series of structurally related agonists in the three assay systems that we have used. This approach, in keeping with a classical structure-activity study of agonist action, does not depend on the selection of the agonists used but rather requires the same agonists be evaluated in the different bioassays. In terms of peptide potencies relative to P5, the activities of P7-NH_2, P7, P5, and Pr-P4 in the GLM assay were 0.04, 0.4, 1, and 1, respectively, yielding an order of potencies of P7-NH_2 > P7 > P5 ≥ Pr-P4 (Fig. 2; Table 1) A comparable order of potencies (P7-NH_2 > P7 > P5) was observed in the RLM assay (Fig. 3). In contrast, in the RA assay the relative EC_{50} values for P7-NH_2, P7, P5, and Pr-P4 were 0.08, 1, 1, and 0.1, respectively, yielding a distinct order of potencies, i.e., P7-NH_2 ≥ Pr-P4 ≫ P5 ≥ P7. It could be suggested that the differences in agonist potencies between the GLM and RA assays might relate to differences between the rat and guinea pig receptor sequences. However, such an argument would not apply to the comparative data obtained with the RA and RLM assays, which show distinct relative potencies for P7-NH_2, P7, and P5 in different tissues from the same species. Moreover, the differences in the potency series between the RLM and RA assays are emphasized by experiments using TRPs derived from the rat receptor sequence (Ra-P5, Ra-P5-NH_2, and Ra-P7). In the RLM assay the potency series was Ra-P5-NH_2 > P7-NH_2 > P5-NH_2 > P7 = Ra-P7 > P5 = Ra-P5, whereas in the RA assay the potency series was P7-NH_2 > P5-NH_2 > Ra-P5-NH_2 > Ra-P7 > P5 ≥ P7 > Ra-P5. In keeping with the distinct potency series for the actions of the TRPs in the LM versus the RA assays, both the GLM and RLM systems were inhibited by cyclooxygenase and by tyrosine kinase inhibitors, whereas these reagents did not inhibit the actions of the TRPs in the RA system. It could be argued that phenotypic differences in the signal transduction pathways present in the two agonist target tissues (gastric smooth muscle cell versus aortic endothelial cell) might account for the differential sensitivities of the RA and LM assays to the enzyme inhibitors. Nonetheless, the distinct order of potencies of the TRPs in the gastric and aortic bioassay systems (especially the RA and RLM preparations), the selective action of Nleu-P6 (GLM and RA assays), and the distinct signal transduction pathways activated by the TRPs in the gastric and aortic tissues argue, as persuasively as does any other structure-activity approach to receptor characterization (e.g., Ref. 27), in favor of the existence of distinct thrombin receptor subtypes in these tissues. Even though Southern blot analysis of restriction enzyme-digested rat genomic DNA suggests the presence of only a single thrombin receptor gene (4), alternative splicing or post-translational modification of the receptor protein could possibly result in receptor subtypes in different tissues. It is of interest that the pharmacological approach has been successful in identifying a thrombin receptor in intact RA preparations (5), whereas the Northern blot analysis of comparable tissue failed (4). Thus, in future work it will very likely prove necessary to use a combined pharmacological/molecular biological approach, possibly employing some of the selective agonists described in the present study, to establish unequivocally the existence of unique thrombin receptor subtypes. We believe that our work, describing the presence of distinct signal transduction pathways in separate tissues and identifying distinct structure-activity relationships for amino-terminally and carboxyl-terminally modified TRPs in the LM and RA assays, provides a good basis for continuing the search for thrombin receptor subtypes.

Acknowledgments

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