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Contractile actions of thrombin receptor-derived polypeptides in human umbilical and placental vasculature: evidence for distinct receptor systems

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1 We studied the structure-activity profiles of four thrombin receptor-derived polypeptides (TRPs) (P5, SFLLR; P5-NH_2, SFLLR-NH_2; P7, SFLLRNP; P7-NH_2, SFLLRN) in contractile human placental artery (PA), umbilical artery (UA) and umbilical vein (UV) preparations and in a human platelet aggregation assay.

2 The contractile actions of the TRPs in the two arterial preparations were endothelium-independent, whereas in the UV tissue a contractile response was observed only in an endothelium-denuded preparation; no endothelium-mediated relaxation responses were observed in any of the vascular preparations.

3 In the three vascular preparations, the contractile responses required extracellular calcium and were attenuated by the tyrosine kinase inhibitor, genistein.

4 The relative contractile orders of potencies of the TRPs in the three vascular preparations were distinct from each other (PA: P7-NH_2 > P7 > P5-NH_2 > P5; UA: P7-NH_2 $\geqslant$ P5-NH_2 $\simeq$ P7 > > P5; UV: P5-NH_2 > > P7-NH_2 = P7 > > P5) and these were in turn distinct from the potency order observed in the platelet aggregation assay (P5-NH_2 $\geqslant$ P7-NH_2 > P7 > > P5).

5 Despite the markedly dissimilar TRP potency orders in the placental artery and umbilical vein preparations, the cDNA sequences for the thrombin receptor obtained by polymerase chain reaction cloning of cDNA from the two tissue sources were identical.

6 We conclude that the four tissues studied possess functionally distinct thrombin receptor systems that interact in a distinct way with agonist peptides. In view of the identity of the thrombin receptor cDNA in the two tissues displaying the most dissimilar structure-activity profiles, we suggest that in different tissues, differences in post-translational receptor processing or differences in receptor-effector coupling interactions may result in unique thrombin receptor systems that can display distinct structure-activity profiles.

Keywords: Thrombin receptor; umbilical artery; placental artery; umbilical vein; platelets

Introduction

It is now widely accepted that the ability of the serine protease, thrombin, to stimulate target cells involves the proteolytic activation of a specific G-protein-coupled thrombin receptor (Rasmussen *et al.*, 1991; Vu *et al.*, 1991; Coughlin *et al.*, 1992). The receptor's proteolytically-exposed amino-terminal sequence, beginning with serine-42 in the human receptor, is thought to act as an activating 'anchored' or 'tethered' ligand. Synthetic peptides based on the revealed N-terminal sequence of the thrombin receptor, containing up to 14 amino acids (amino acids designated by their single letter code) (i.e. S_{42}FLLRNPNDKYEPF or P14) are, on their own, capable of triggering the thrombin receptor, so as to mimic many of the diverse cellular actions of thrombin, ranging from the aggregation of platelets to the regulation of vascular contractility (Davey & Luscher, 1967; White *et al.*, 1980; 1984; DeMey *et al.*, 1982; Haver & Namm, 1984; Rapoport *et al.*, 1984; Walz *et al.*, 1985; 1986; DeBlois *et al.*, 1992; Muramatsu *et al.*, 1992). In structure-activity studies of the thrombin receptor-derived peptides (TRPs) done by us using vascular and gastric smooth muscle bioassay systems (Yang *et al.*, 1992; Hollenberg *et al.*, 1992; 1993) and by others using platelet aggregation assays (Chao *et al.*, 1992; Hui *et al.*, 1992; Sabo *et al.*, 1992; Vassallo *et al.*, 1992) it has become apparent that TRPs ranging in length from five (i.e. SFLLR or P5) to seven (i.e. SFLLRNP or P7) amino acids can exhibit distinct potencies in the different assay systems, equal to or even greater than the potency of the originally described TRP 14-mer, P14. In particular, we have observed that the orders of potencies of selected TRP agonists containing either a free or amidated carboxyl-terminal residue (e.g. P5, P5-NH_2, P7, P7-NH_2) can be used to distinguish between different thrombin receptor assay systems (Hollenberg *et al.*, 1993), so as to suggest the presence of pharmacologically distinct receptor subtypes in different tissues. The pharmacological approach using the TRPs has proved of considerable value for the delineation of functional thrombin receptors in intact vascular tissue (e.g. in rat aorta), wherein molecular probe assays (e.g. Northern blot analysis) have not proved sufficiently sensitive *in vivo* to detect receptor message in the vascular smooth muscle elements (Zhong *et al.*, 1992). Our work (Muramatsu *et al.*, 1992) and that of others (DeBlois *et al.*, 1992; Antonaccio *et al.*, 1993) indicates that TRPs, presumably acting via the thrombin receptor, can regulate contractility in vascular preparations from a number of species via two mechanisms: (1) the TRPs can cause an endothelial cell-dependent nitric oxide-mediated vasorelaxation (Muramatsu *et al.*, 1992), or (2) the TRPs can cause vascular contraction via a direct action on the smooth muscle elements by a mechanism that requires extracellular calcium (Muramatsu *et al.*, 1992; DeBlois *et al.*, 1992; Antonaccio *et al.*, 1993). In a study of the expression of the thrombin receptor in human arteries (Nelken *et al.*, 1992), *in situ* hybridization and immunohistochemical

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methods have localized the thrombin receptor in normal-appearing adult arteries almost exclusively in the endothelial layer.

Since the response profile for TRPs acting on human arterial samples has yet to be evaluated in any depth, the study described in this paper was designed with the following objectives in mind: First, we wished to use a readily accessible human vascular tissue source (placental and umbilical vessels) to evaluate the vasoactive properties of thrombin and the TRPs in human-derived tissues. Second, we wished to use a spectrum of TRP agonist probes to evaluate the structure-activity relationships for these peptides in the umbilical artery, umbilical vein and placental artery preparations; and we sought to compare the relative activities of the peptides in the three vascular preparations with their relative potencies in a platelet aggregation assay. We selected the four TRPs, SFLLR (P5), SFLLRNP (P7) and their carboxyamidated counterparts, SFLLR-NH_2 (P5-NH_2) and SFLLRNP-NH_2 (P7-NH_2) as receptor probes, since these four peptides have proved of value in previous work (Hollenberg *et al.*, 1993) to discriminate pharmacologically between distinct thrombin receptor systems in different tissues from the same species. Our study describes the evaluation of the activities of these polypeptides in the three placental vascular preparations, and in the platelet aggregation assay. Further, we describe the sequencing of cDNA for the thrombin receptor obtained from placental artery and umbilical vein RNA. The data point to the existence of distinct pharmacological receptor systems in the different placental vascular preparations that, nonetheless, contain the same receptor mRNA.

Methods

Preparation of tissue and contractile bioassay procedures

Full term placental tissue, with the attached umbilical cord, was obtained from normal vaginal deliveries, through the courtesy of the Maternity Care Centre of the Foothills Hospital, Calgary, Canada. Tissue was dissected immediately from the placenta and was transported to the laboratory in ice-cold Krebs-Henseleit buffer pH 7.4, of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. Placental artery segments (0.3 × 1 cm) were excised just at the placental surface where their structures arborize and penetrate the placental mass; umbilical cord sections (3 × 5 cm) were cut to facilitate the isolation of intact umbilical artery and umbilical vein samples for the preparation of helical strips. Vessels were trimmed free of Whartons jelly or other adhering material under microscopic visualization and helical strips (0.3 × 1 cm) were cut, routinely with the aid of a fine guide wire threaded through the vessel. In some experiments, the use of a guide wire was omitted to ensure the retention of an intact endothelium. In other experiments, tissue strips were rubbed firmly with damp filter paper to remove the endothelium, as documented previously by electron microscopic observation with umbilical artery preparations (Xie & Triggle, 1994). Tissues were mounted in a plastic organ bath (3 to 4 ml) under 2 g tension for equilibration at 37°C in the above described buffer, gassed with 95% O_2/5% CO_2 to maintain the pH at 7.4. Placental artery strips and umbilical artery strips were allowed to equilibrate 2–5 h until the baseline tension had stabilized; umbilical vein strips required 3–4 h for the stabilization of baseline tension. Tension was monitored isometrically with either Statham or Grass force-displacement transducers. The contractile integrity of each preparation was routinely assessed by challenging the tissue with a test concentration of 50 mM KCl, which caused a contractile response of 1.3 ± 0.1 g in the placental artery strip (mean ± s.e.mean for $n = 126$), of 1.0 ± 0.1 g in the umbilical artery strip (mean ± s.e.mean for $n = 120$) and a response of 1.7 ± 0.1 g in the umbilical vein preparation (mean ± s.e.mean for $n = 93$). The response of each tissue to 50 mM KCl was routinely used as a reference standard for normalizing the responses of different preparations to the TRPs, for which responsiveness was expressed as a percentage (% KCl) of the 50 mM KCl-mediated contraction. To assess the role of extracellular calcium, tissues were switched to a calcium-free Krebs-Henseleit buffer containing 0.1 mM EGTA, 10 min before the addition of agonist; after the addition of agonist, the buffer was replenished with calcium (2.5 mM) and the tissue was washed and re-equilibrated in calcium-containing buffer. In some experiments, a contractile response to 0.1 μM noradrenaline was also used to monitor tissue integrity. Reagents from stock solutions were added directly to the organ bath and concentrations were calculated accordingly. In order to avoid desensitization of the tissues to the TRPs, the following dosing intervals were used for the three vascular preparations: placental artery, 40–60 min; umbilical artery, 1–2 h; umbilical vein, 1–2 h. Because of desensitization of the umbilical vein preparation to the TRPs, concentration-effect curves were obtained by exposing each tissue only once to each TRP and expressing the response (% KCl) relative to the contraction caused by 50 mM KCl. This concentration of KCl was just at the top of its concentration-effect curve. Tissues were washed free from agonist at the plateau of the contractile response (about 3 to 5 min after adding agonist to the organ bath) and were washed 2–3 times further during the re-equilibration period between additional exposures to contractile agonists. Concentration-response curves for each TRP agonist in the three different vascular preparations were obtained with multiple tissue strips from over 120 tissue donors. In each experiment, using replicate tissue strips from an individual donor (4 to 7 strips per assay), the contractile responses to increasing concentrations of TRPs were normalized, as indicated above, as a percentage (% KCl) of the response elicited by 50 mM KCl. Data from 4–9 individual experiments for each concentration of TRP were pooled to construct the concentration-response curves.

Platelet aggregation assays

Platelet-rich plasma suspensions (300×10^6 ml^{-1}), anticoagulated with sodium citrate (1 ml of 0.105 M citrate per 9 ml of blood), were obtained by differential centrifugation of venipuncture samples obtained from healthy donors who denied taking either medications or alcohol for two weeks prior to the assays. Replicate platelet suspensions (0.2 ml) were constantly stirred (1,200 r.p.m.) at 37°C in a 4-channel aggregometer and reagents in a volume of 25 μl, were added to initiate the aggregation reaction. Turbidity was monitored constantly and both the rate and extent of aggregation were automatically recorded during the aggregation reaction. Measurements of aggregation were done minimally in triplicate for each TRP concentration; all experiments were done in the presence of 100 μM amastatin to minimize degradation of the TRPs. The response to a fixed concentration of P5-NH_2 (5 μM) was used as an internal standard to compare assays done with different platelet preparations. Concentration-effect curves for the four TRPs were constructed using the initial rate of aggregation as an index of platelet response. A semi-quantitative comparison of the platelet response to increasing concentrations of the four TRPs was also done using the initial microaggregation response (primary aggregation wave) as a monitor of platelet reactivity. This initial microaggregation response, detected by a transient decrease in turbidity was observed prior to the full platelet aggregation response, even in the absence of a full platelet aggregation reaction.

Peptides and other reagents

Thrombin receptor-derived peptides, based on the human receptor sequences, SFLLR (P5), SFLLR-NH_2 (P5-NH_2), SFLLRNP (P7) and SFLLRNP-NH_2 (P7-NH_2), were obtained from the Core Peptide Synthesis Laboratory at the Queens University, Department of Biochemistry, Kingston, ON, Ca-

nada, and through the courtesy of Dr. J. DiMaio, BioChem Therapeutic Inc., Laval, PQ, Canada. Stock peptide solutions, prepared in 50 mM sodium phosphate buffer, pH 7.4, were verified for peptide compositions and concentrations by quantitative amino acid analysis and by h.p.l.c. analysis. Lyophilized thrombin from human plasma, free from other clotting factors, possessing a specific activity of 3000 NIH units mg^{-1} (1 u ml^{-1} = 10 nM) was from Sigma, St. Louis, MO, U.S.A. (lot 21H9310, Cat. No. T-6759) as were the reagents, nifedipine, noradrenaline, indomethacin and amastatin. Genistein was from ICN biochemicals, Costa Mesa CA, U.S.A.

H.p.l.c. analysis of peptides

Liquid chromatographic analysis of aliquots ($\simeq$ 100 μl) of both stock peptide solutions and peptide samples (3 to 4 μg peptide) recovered from the organ bath during the course of a contractile bioassay was performed using a Vydac RPC 18 analytical column (0.5 x 30 cm) eluted with a gradient of acetonitrile (0 to 54% v/v) in 0.1% trifluoroacetic acid at a flow rate of 1 ml min^{-1}; absorbance was monitored at 214 nm.

Isolation and sequencing of receptor cDNA clones

Total tissue RNA was isolated from placental artery (PA) and umbilical vein (UV) tissue that was first rapidly dissected and cleaned, as for a bioassay procedure, and then quick-frozen. Four tissues (two PA and two UV samples) were processed for the preparation of RNA. In one instance, the PA and UV tissue came from the same donor; in other preparations, the PA and UV tissues were obtained from two separate donors. RNA was prepared with the use of the TRI-reagent (Molecular Research Centre, Cincinnati, OH, U.S.A.) and was reverse-transcribed (RT) with first strand cDNA synthesis kit and pd (N) 6 primer (Pharmacia) according to manufacturer's recommendations at 37°C for 60 min, followed by denaturing at 93°C for 5 min and flash-cooling to 4°C. The RT product was then diluted 3 x with water and 3 μl of this solution was used with sets of overlapping sequence-specific primers spanning the entire receptor cDNA coding sequence for polymerase chain reaction (PCR) amplification employing 1 unit of Ampli Taq polymerase in a 10 mM Tris HCl buffer, pH 8.3 (0.1 ml final vol), containing $MgCl_2$ (2.5 mM), KCl (50 mM), nuclease-free bovine albumin (5 μg) and 0.6 μM each of the four deoxynucleotide triphosphates.

The PCR primers used were: *Forward primers: (A)* 5' ATG GGG CCG CGG CGG CTG CT 3', targeted to amino acids M_1 to L_7 of the human receptor sequence. (B) 5' CCC GGT CAT TTC TTC TCA GGA A 3' targeted to amino acids P_{40} to N_{47} of the human receptor sequence, but using the rat receptor nucleotide sequence (131–152) for this domain. *Reverse primers:* (C) 5' CCT AAG TTA ACA - GCT TTT TGT ATA T 3' targeted from the stop codon to I_{419} of the human receptor sequence. (D) 5' AAT - CGG TGC CGG AGA AGT 3' targeted to amino acids P_{167}

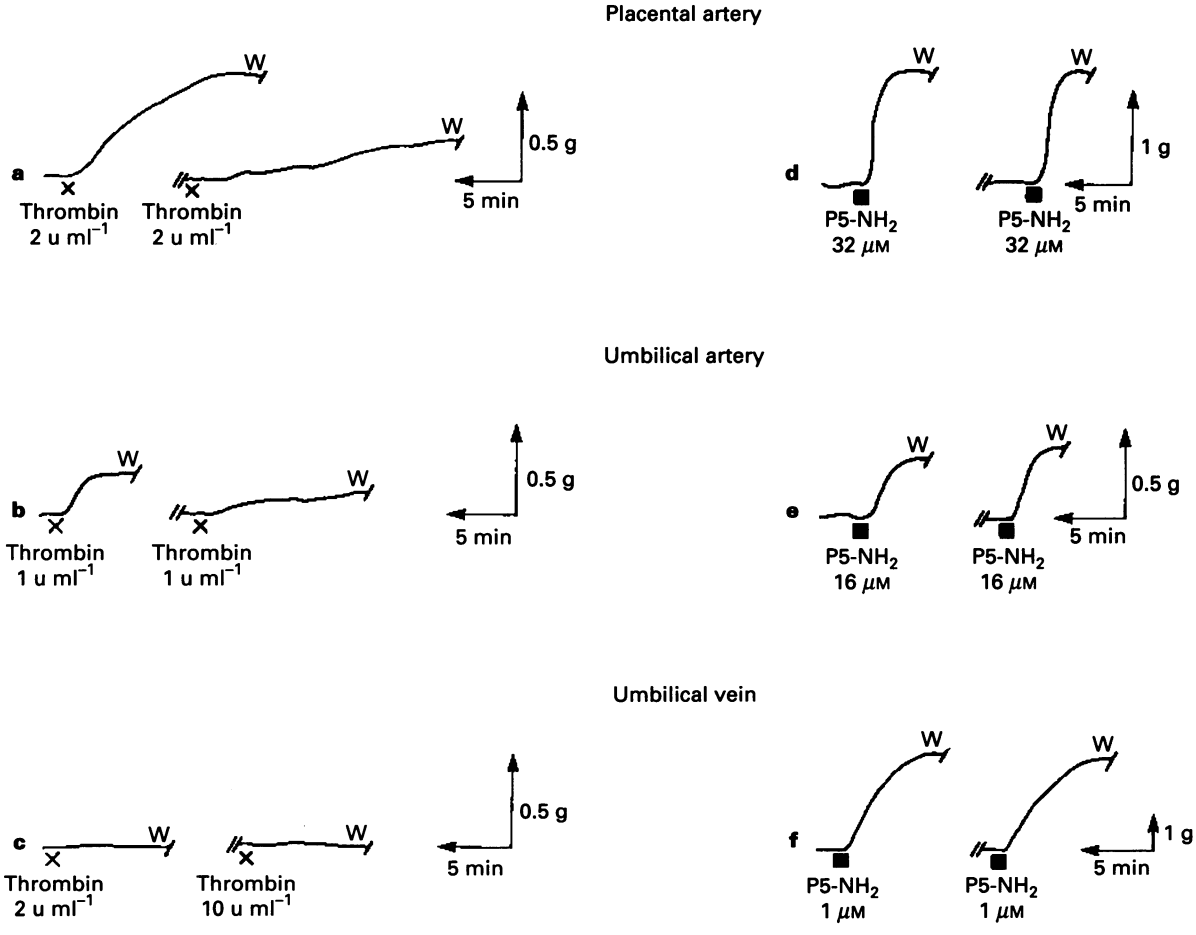

Figure 1 Responses of the placental artery, umbilical artery and umbilical vein to thrombin and P5-NH_2. The three tissue preparations were exposed either to thrombin (2 to 10 u ml^{-1}: left-hand panels a, b and c) or to P5-NH_2 (right-hand panels d, e and f) at 40 to 60 min intervals, followed by washing the tissue (W). Except for tracing (f) where the tissue was rubbed free of endothelium, all preparations possessed an intact endothelium. The scales for time and tension are shown to the right of each tracing. Each tracing (a to f), showing a continuous experiment for an individual tissue strip is representative of three or more independently conducted experiments.

to Y_{162} of human receptor sequence, but using the rat receptor nucleotides sequence (523–505) for this receptor domain. Primers (A) and (C) were used to obtain the complete coding region of the human receptor cDNA. Primers (B) and (C) were used to generate cDNA spanning the thrombin cleavage site up to the end of the coding region. Primers (B) and (D) were used as nested primers to confirm the identity of the PCR products. Amplification was allowed to proceed for 35 cycles, beginning with a 45 s denaturation period at 94°C followed by a 45 s reannealing time at 55°C and a primer extension period of 2 min at 72°C. The PCR products were purified by 1.5% agarose gel electrophoresis, and cloned in the pGEM-T vector (Promega) following transformation of *E. coli* strain DH5α cells. The insert was sequenced with M 13 universal, reverse sequencing primers and primers designed on the basis of human thrombin receptor sequence (Vu *et al.*, 1991), using the dideoxynucleotide sequencing method (Sanger *et al.*, 1977), employing a T7 DNA polymerase sequencing kit (Pharmacia).

Results

General responsiveness of the three vascular preparations to thrombin and P5-NH_2

Initially, we assessed the response characteristics of the placental artery (PA), umbilical artery (UA) and umbilical vein (UV) preparations to thrombin (1–10 u ml^{-1}) and to P5-NH_2, which was selected as a representative receptor-activating TRP. As illustrated in Figure 1 the intact PA and UA preparations exhibited contractile responses to both thrombin (tracings a and b) and P5-NH_2 (tracings d and e). However, as observed by us previously in other vascular preparations (Muramatsu *et al.*, 1992), once exposed to thrombin, the PA and UA tissues were desensitized to a second thrombin exposure (Figure 1a and 1b). Nonetheless, both the PA and UA tissues responded reproducibly to repeated exposures of P5-NH_2, provided the dose intervals were of 1 h or more (Figure 1, tracings d and e). In contrast with the PA and UA tissues, the umbilical vein tissue did not respond to thrombin (2–10 u ml^{-1}) either in intact preparations (Figure 1c) or in preparations rubbed free of endothelium (data not shown). Nonetheless, P5-NH_2 caused a robust reproducible contractile response of the endothelium-free UV preparation (Figure 1f), whereas the intact UV preparation was not responsive to P5-NH_2, even at concentrations as high as 100 μM (data not shown and see Figure 2). Although reproducible contractile responses of the endothelium-free UV preparation to P5-NH_2 were observed at relatively low concentrations ($\leqslant 2$ μM), provided the dose interval was greater than 1 h, higher concentrations ($\geqslant 5$ μM) caused some desensitization of the tissue. Therefore, concentration-effect curves were done by exposing UV preparations only once to a given concentration of the TRPs and expressing the response as a percentage (% KCl) of the tissue response to 50 mM KCl.

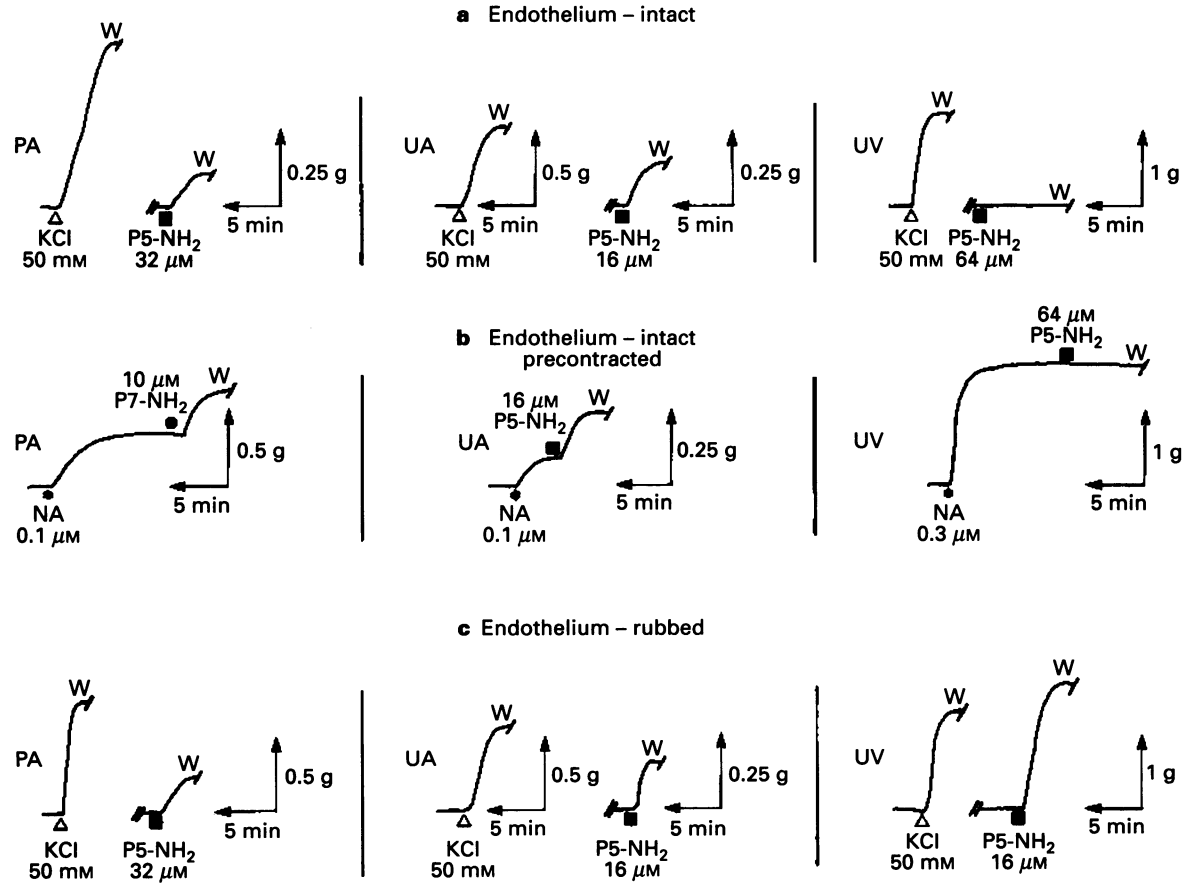

Figure 2 Evaluation of endothelial function. Individual tissue strips of placental artery (PA, left-hand panels), umbilical artery (UA, middle panels) or umbilical vein (UV, right-hand panels) were prepared either with the endothelium intact (top and middle tracings, a and b) or were rubbed free of endothelium (lower tracings, c). Tissue contractility was first monitored for each preparation with a test dose of either KCl or noradrenaline (NA); preparations were then exposed to P5-NH_2 either in the continuous presence of noradrenaline or after washing (W). The responses to P5-NH_2 relative to KCl can be compared in tissues with (top tracings, a) and without (bottom tracings, c) endothelium. The lack of a nitric oxide-mediated relaxation response is illustrated by the middle tracings (b). The tracings showing the responses of individual preparations are representative of three or more independently conducted experiments done with separate tissue strips.

Role of endothelium and endothelium-derived relaxing factors

In other arterial preparations (DeMey *et al.*, 1982; Rapoport *et al.*, 1984; Muramatsu *et al.*, 1992) thrombin or the TRPs can cause either an endothelium-dependent, nitric oxide synthase-mediated relaxation, or an endothelium-independent contraction. What was evident from the action of P5-NH_2 or P7-NH_2 in the PA and UA preparations, was that a contractile response was observed either in the presence or absence of an intact endothelium (Figure 2a and c, PA and UA tracings). Further, as opposed to our observations with rat aortic tissue (Muramatsu *et al.*, 1992), a contractile, rather than a relaxation response was detected in an endothelium-intact preparation that was precontracted with noradrenaline and then exposed to P5-NH_2 (Figure 2b, PA and UA tracings). A lack of a relaxation response to TRP in a noradrenaline-precontracted tissue was also observed for the UV preparation (Figure 2b, UV tracing). In view of the ability of the endothelium-intact UV preparation to release a relaxing factor presumed to be NO (Van de Voorde *et al.*, 1987; Chaudhuri *et al.*, 1991) we also examined the response of this preparation to P5-NH_2 in the presence of the NO-synthase inhibitor, L-NAME (0.1 μM); no contractile response was observed (not shown). Further, replenishing the organ bath with L-arginine, the precursor for NO synthesis, did not enable P5-NH_2 to cause a relaxation response in a UV preparation that was precontracted with 0.3 μM noradrenaline (e.g. see middle panel, Figure 2 and data not shown). Similarly, indomethacin (1 μM) was unable to unmask a contractile action of P5-NH_2 in the UV preparation, indicating that the production of prostanoids (e.g. prostaglandin) that might cause relaxation could not account for the inability of P5-NH_2 to cause a contractile response (also see below). As pointed out above, in the UV preparation, a TRP-induced contraction was only observed when the tissue was denuded of endothelium (Figure 2c, right-hand tracing). In all respects, the results using thrombin as an agonist (not shown) paralleled those obtained with the TRPs, except that in the endothelium-denuded UV preparation that contracted in response to P5-NH_2, no response was observed upon exposure to thrombin (up to 10 u ml^{-1}).

Role of extracellular calcium

In the absence of extracellular calcium, all three vascular contractile preparations failed to respond to P5-NH_2 (Figure 3). Nonetheless, upon replenishing extracellular calcium in the continued presence of the agonist, P5-NH_2, a robust contraction ensued (Figure 3, upper PA, middle UA and lower UV tracings). In keeping with these observations, pretreatment of the three tissues with the calcium channel antagonist, nifedipine (1 μM), attenuated the contractile actions of either P5-NH_2 or P7-NH_2 in the three preparations (response tracings not shown): in the PA and UA preparations, P5-NH_2-mediated contractions were inhibited by 50 ± 11% (mean ± s.e.mean for $n = 7$), in the presence of 1 μM nifedipine, whereas in the endothelium-free UV preparation, contractions caused by P7-NH_2 were virtually abolished by 1 μM nifedipine (not shown). The contractile response of all three tissues to 50 mM KCl was also blocked by 1 μM nifedipine (not shown).

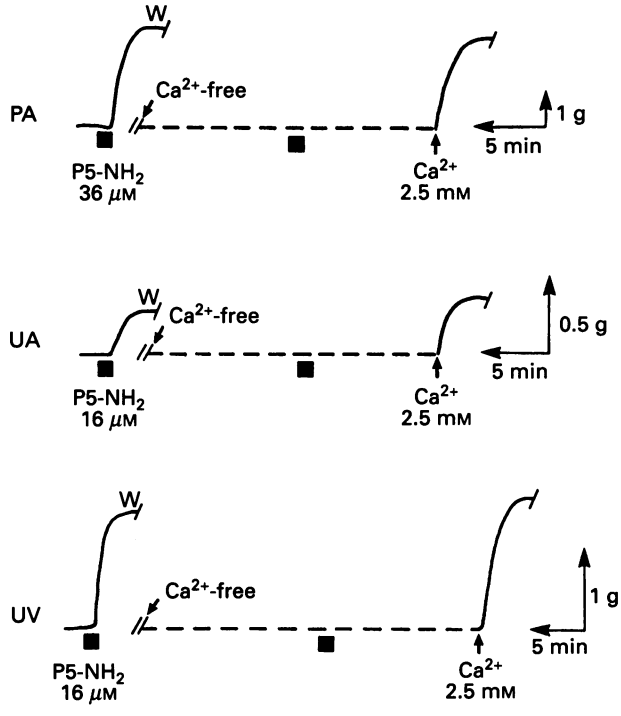

Figure 3 Role of extracellular calcium. Tissue strips were first assessed for responsiveness to P5-NH_2 (■), followed by washing (W) and transfer to a calcium-free buffer containing 0.1 mM EGTA. Tissues were again exposed to P5-NH_2 followed by reconstituting the buffer with 2.5 mM Ca^{2+} (arrow). The endothelium was removed from the umbilical vein (UV) preparation, but was intact in the placental artery (PA) and umbilical artery (UA) preparations.

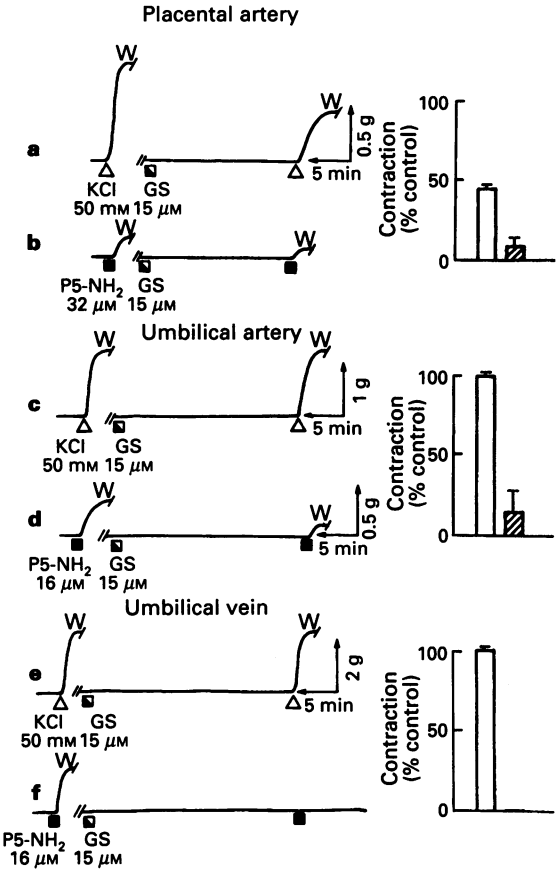

Figure 4 Inhibition of P5-NH_2-mediated contraction by genistein. The responsiveness of individual tissue strips (tracings a to f) was first assessed by exposure to either KCl (△, tracings a, c and e) or P5-NH_2 (■, tracings b, d and f) followed by washing (W). Genistein (□, 15 μM) was then added to the organ bath and the tissues were again challenged with either KCl (△) or P5-NH_2 (■). The histograms to the right show the responsiveness of the tissues to KCl (open columns) and P5-NH_2 (hatched columns) in the presence of genistein, as a percentage of the control response (mean ± s.e.mean for $n = 7$) observed in the absence of genistein.

Effects of inhibitors of cyclo-oxygenase and tyrosine kinase

In our previous work with gastric longitudinal smooth muscle preparations (Hollenberg *et al.*, 1992; 1993), we established that the contractile actions of P5-NH_2 and thrombin could be attenuated by either the cyclo-oxygenase inhibitor, indomethacin (1 μM), or by the tyrosine kinase inhibitor, genistein (15 μM). We were, therefore, interested to evaluate the effects of these enzyme inhibitors in the three placental vessel preparations. Although indomethacin (1 μM) caused a small ($35 \pm 6\%$, mean ± s.e.mean for $n = 7$) inhibition of the P5-NH_2-mediated contraction in the placental artery preparation, without affecting contractions caused by 50 mM KCl, this cyclo-oxygenase inhibitor had no effect on contractions caused by either P5-NH_2 or KCl in the UA or in the endothelium-denuded UV preparation (not shown). In contrast, genistein (GS, 15 μM) was able to attenuate P5-NH_2-mediated contractions in all three preparations, with essentially a complete and selective inhibition of P5-NH_2 action (compared with KCl-induced contractions) in the UV preparation (Figure 4). In the placental artery preparation, the inhibitory effect of genistein on P5-NH_2 action appeared to be partially non-specific, in that contractions caused by KCl were also inhibited, but to a lesser degree than those elicited by P5-NH_2 (Figure 4, top histogram). On the other hand, in the umbilical artery preparation the inhibition by genistein of P5-NH_2-induced contractions was selective (i.e. KCl action was unaffected: middle histogram, Figure 4), but not as complete as the inhibition by genistein in the umbilical vein preparation (lower histogram, Figure 4).

Concentration-effect curves

Concentration-effect curves were obtained for the contractile actions of P5, P5-NH_2, P7 and P7-NH_2 in the three vascular preparations (Figures 5 to 7) and for the platelet aggregation activity of the four polypeptides in a platelet-rich plasma preparation (Figure 8). Although there was some inter-tissue variability in responsiveness, as indicated by the error bars at each point in the several concentration-effect curves, it was possible to assign a relative order of potencies for the four polypeptides in each of the four bioassay systems (see below). Because of the intense desensitization caused by thrombin in the vascular preparations and because of the coagulation activity of thrombin in the platelet assay, it was not possible to compare directly the actions of the four TRPs with the action of thrombin (Figure 1 and data not shown). In the placental artery system, it was evident that the four TRPs did not all exhibit full intrinsic activity, since the maximal contractile forces, relative to the KCl response, differed somewhat (e.g. compare the maximal responses to P5-NH_2 with those of P7-NH_2 in Figure 5). This type of result, seen by us previously in a gastric contractile bioassay (Hollenberg *et al.*, 1992), was also evident to some extent in the umbilical artery preparation (Figure 6) but not in the umbilical vein preparation (Figure 7). Since the platelet response represents essentially an all-or-none phenomenon, it was perhaps not surprising that the intrinsic activity of all four polypeptides appeared to be equivalent (Figure 8).

In the two arterial preparations, P7-NH_2 was the most potent and P5 the least potent of the four polypeptides tested; however in these two preparations, the potency of P5-NH_2, relative to the other peptides, differed considerably (compare

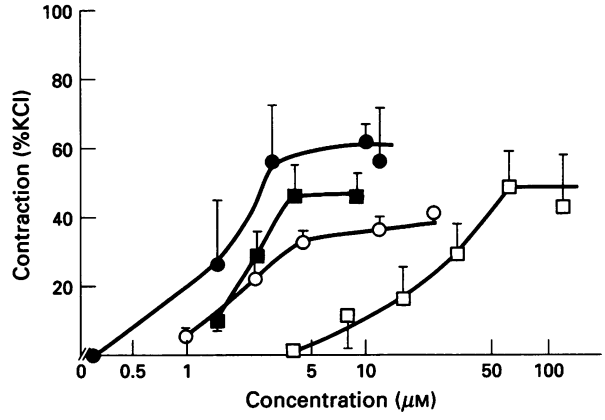

Figure 6 Concentration-response curves for the contractile actions of TRPs in the umbilical artery. The contractile responses of the umbilical artery preparations to different concentrations (μM) of the TRPs (x-axis) were expressed as a percentage (% KCl) of the tissue response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 15 independent observations in different tissue preparations. The relative order of potencies in this system was P7-NH_2, (●) ≥ P5-NH_2, (■) ≃ P7, (○) > > P5 (□).

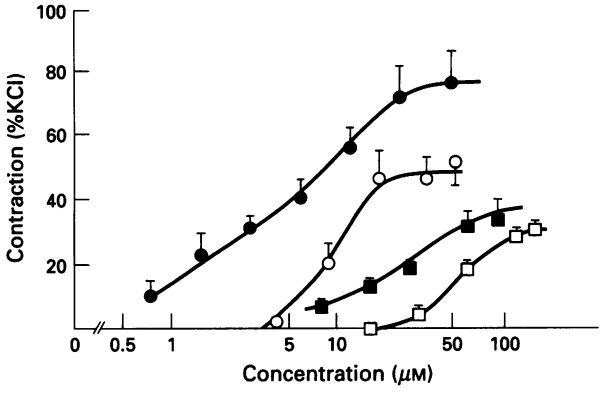

Figure 5 Concentration-response curves for the contractile actions of TRPs in the placental artery. The contractile responses of placental arterial tissues to increasing concentrations (μM) of the TRPs were expressed as a percentage (% KCl) of the tissue response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 21 independent observations on different tissues. The relative order of potencies in this system was P7-NH_2, (●) > P7, (○) > P5-NH_2, (■) > P5 (□).

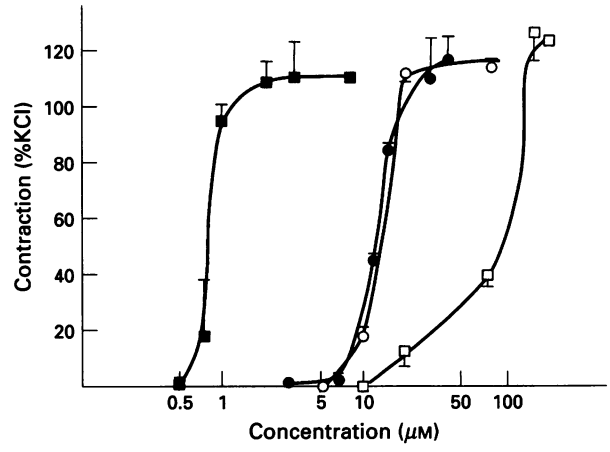

Figure 7 Concentration-response curves for the contractile actions of TRPs in the umbilical vein. The contractile responses of the endothelium-denuded umbilical vein preparation to different concentrations (μM) of the TRPs (x-axis) were expressed as a percentage (% KCl) of the response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 12 independent observations in different tissues. The relative order of potencies in this system was P5-NH_2, (■) > > P7-NH_2, (●) = P7, (○) > > P5 (□).

Figures 5 and 6). In the PA preparation, the relative potency order was: P7-NH_2 > P7 > P5-NH_2 > P5, whereas in the UA preparation, the relative potency order was: P7-NH_2 ≥ P5-NH_2 ≃ P7 > > P5. In contrast, in the umbilical vein preparation, although P5 was, as in the other preparations, the least potent, P5-NH_2 was clearly the most potent of the four TRPs; both P7 and P7-NH_2 were equivalent, but lower in potency than P5-NH_2 (Figure 7). Thus, the relative order of potencies of the four TRPs in the UV preparation was: P5-NH_2 > > P7-NH_2 = P7 > > P5.

The platelet aggregation assays were done in the presence of amastatin (50–100 μM) to minimize peptide proteolysis by plasma amino peptidase (Coller *et al.*, 1992). As in the three vascular assays, in the platelet assay, P5 was the least potent of the four polypeptides (Figure 8). However, as in the UV system, but in contrast with the PA and UA assays, P5-NH_2 exhibited a comparatively high potency, equal to or even slightly greater than that of P7-NH_2 (Figure 8). The relative potency order shown in Figure 8, based on measures of the initial aggregation rate, was also observed when the data were expressed as a percentage of maximum aggregation (not shown). When platelet response was monitored semi-quantitatively, using the initial microaggregation shape change as an index of response, a reaction to P5-NH_2 (microaggregation at concentrations ≤1.25 μM) was routinely observed at a lower concentration than that of P7-NH_2 (microaggregation at concentrations ≥1.25 μM). Thus, the relative order of potencies of the four polypeptides in the platelet assay was P5-NH_2 ≥ P7-NH_2 > P7 > > P5. Quantitatively, it was possible to use the linear portions of the concentration-effect curves (Figures 5 to 8) to estimate the activities of P7-NH_2, P7 and P5-NH_2 relative to the activity of P5, as we have done previously (Hollenberg *et al.*, 1993), by calculating the relative effective concentration (EC) of a given agonist (R_{EC} = EC ÷ EC_{P5}) required to yield the same response as that caused by an equi-effective concentration of P5 (EC_{P5}) (Table 1). For instance, it can be seen that for P7, the R_{EC} values (0.12 to 0.14) relative to P5 were essentially the same in all three vascular preparations, whereas the R_{EC} value for P7 in the platelet assay differed considerably (0.06). In contrast with P7, the R_{EC} values for P7-NH_2 and P5-NH_2 differed markedly in all three vascular preparations. Thus, as summarized in Table 1, the relative biological activities of the four TRPs in the vascular and platelet assays could be seen to be distinct for each assay.

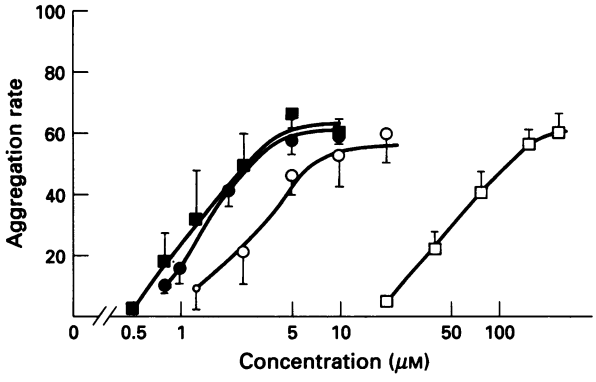

Figure 8 Concentration-response curves for TRP-induced platelet aggregation. The aggregation response of the human platelets to different concentrations (μM) of the TRPs (x-axis) were expressed as the initial rate of aggregation (% min^{-1}, y-axis). The experiments were done in the presence of amastatin (100 μM). Each data point represents the mean ± s.e.mean of 6 to 15 observations made in 3 independent experiments using different platelet samples. The relative order of potencies in this system was P5-NH_2, (■) ≥ P7-NH_2, (●) > P7, (○) > P5 (□).

H.p.l.c. analysis of peptides

As mentioned above, amastatin was added to the incubation medium for the platelet assay, in view of previous observations documenting the ability of this compound to block the degradation of TRPs by plasma aminopeptidase (Coller *et al.*, 1992). However, the protease inhibitor was routinely omitted from the vascular bioassay medium, since its presence at concentrations up to 50 μM was not found to affect the TRP-mediated contractile responses in the PA, UA and endothelium-denuded UV preparations. We had previously observed that, under the conditions of the vascular bioassay, no degradation of TRPs occurred for rat vascular and gastric smooth muscle preparations (Hollenberg *et al.*, 1993). Further, in the endothelium-containing UV preparation, the presence of amastatin (50 μM) failed to reveal a contractile action of P5-NH_2, indicating that endothelial clearance by proteolysis did not appear to be a factor related to the inability of this preparation to contract in response to P5-NH_2. We wished, nonetheless, to confirm with the three vascular preparations that the TRPs could be recovered intact from the organ bath at a time corresponding to the peak of contraction. As illustrated for P5-NH_2 in Figure 9, no degradation was observed during the course of an assay using placental artery or umbilical artery tissue; the same lack of degradation was observed using either

Table 1 Relative activities of the thrombin receptor derived peptide (TRPs) in human placental and umbilical vessels and platelets

	Relative activity (R_{EC})[a] value-relative to P5			
Peptide	*Placental artery*	*Umbilical artery*	*Umbilical vein*	*Platelets*
SFLLR (P5)	1	1	1	1
SFLLR-NH_2 (P5-NH_2)	0.33 ± .07	0.11 ± .04	0.01 ± .000	0.02 ± .001
SFLLRNP (P7)	0.12 ± .02	0.12 ± .02	0.14 ± .01	0.06 ± .001
SFLLRNP NH_2 (P7- NH_2)	0.02 ± .001	0.05 ± 0.001	0.12 ± .01	0.03 ± .001
Relative order of potencies	P7-NH_2 > 7 > P5-NH_2 > P5	P7-NH_2 ≥ P5-NH_2 ≈ P7 > > P5	$P5_2$-NH > > P7-NH_2 ≈ P7 > > P5	P5-NH_2 ≈ P7-NH_2 > P7 > > P5

[a] An activity ratio (R_{EC} = EC_{TRP} ÷ EC_{P5}) was calculated as outlined previously (Hollenberg *et al.*, 1993) and in the text as the ratio of the concentration of a given TRP agonist relative to the concentration of P5 required to cause the equivalent biological response. R_{EC} values were obtained at 3 or more levels of response along linear portions of the concentration-response curves. Values represent the averages ± s.e.mean.

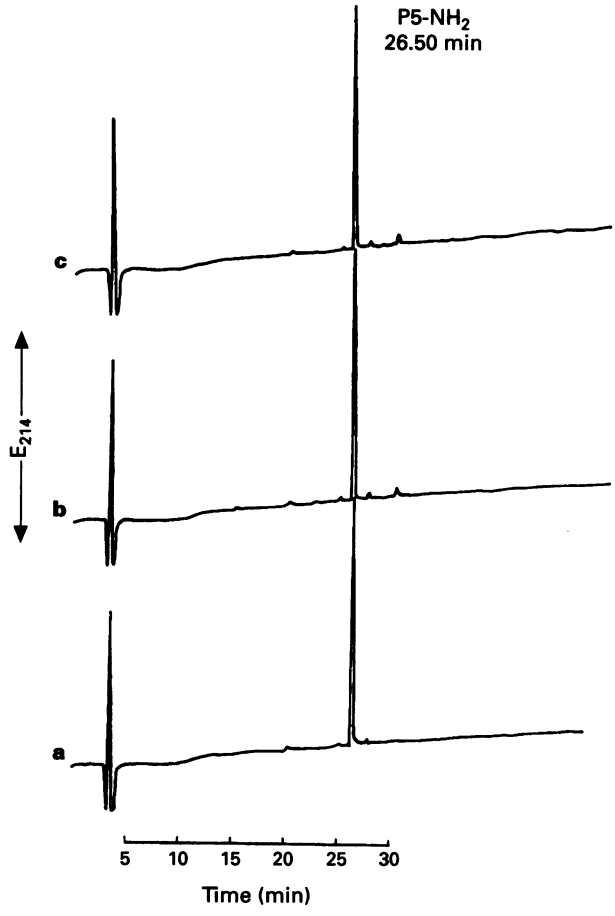

Figure 9 High performance liquid chromatography (h.p.l.c.) analysis of TRPs recovered from the bioassay organ bath. Each panel shows the h.p.l.c. profile for an individual sample of P5-NH_2, recovered from the organ bath in experiments using either placental artery (middle tracings, b) or umbilical artery (upper tracings, c) tissues; the analysis of control peptide solutions is shown in the lower tracing (a).

endothelium intact or endothelium-denuded umbilical vein preparations (not shown). We similarly confirmed that P5, P7 and P7-NH_2 were not degraded during the course of the three vascular bioassays (not shown). Thus, the difference in biological potencies that we observed could not be attributed differences in peptide degradation in the different vascular bioassay systems.

Receptor sequences obtained from PA and UV cDNA

Of the three vascular tissues studied, the umbilical vein and placental artery tissue yielded the most dissimilar structure-activity profiles for the TRPs. We thus selected these two tissues for the preparation of RNA in order to determine the thrombin receptor sequences in the tissues using the RT-PCR approach. The sets of overlapping PCR primers were targeted to the coding region of the thrombin receptor, beginning with the N-terminal methionine and ending at the carboxyterminal threonine (Vu *et al.*, 1991). Somewhat to our surprise, in view of the distinct structure-activity profiles for the TRPs in the PA and UV preparations, we found that the amino acid sequences corresponding to the cDNA sequences were identical in the two tissues and were essentially the same as those observed previously (Vu *et al.*, 1991) for the receptor cloned from a megakaryocyte cell line source. We did, however consistently find two differences in the vascular receptor sequences, compared with the originally published sequence (Vu *et al.*, 1991), with the following nucleotide changes: (1) base pairs 935–936 CG→GC, I_{237} unchanged, V_{238}→L_{238} and (2) base pairs 1315–1316, CG→GC, S_{364}→C_{364}. The base transversion at nucleotides 935–936 was entirely in agreement with previously published results, obtained with a human umbilical vein endothelial cell library (Bahou *et al.*, 1993). The base transversion at nucleotides 1315–1316 was confirmed in all of our experiments done to sequence independent receptor cDNA clones obtained from four independently prepared RNA samples (two from PA; two from UV) that were separately subjected to reverse transcription in four reactions done on two different occasions. The consistent finding of the two base transversions at residues 935–936 and 1315–1316 from 4 independently prepared RNA samples ruled out the possibility that these sequences arose as a result of PCR artifact.

Discussion

The main finding of our study was that all three placental vascular preparations possessed functional receptors for the thrombin receptor-derived peptides and that the relative potencies of P7, P7-NH_2, P5 and P5-NH_2 differed appreciably between the three contractile preparations; further, the relative orders of potencies of the four TRPs in the vascular preparations were all distinct from the one measured in the platelet aggregation assay (see Table 1). For all four bioassay preparations, a relatively consistent concentration-effect curve was observed for P5, the least potent of the four peptides, with responses occurring between 20 to 200 μM of peptide and an EC_{50} between 50 to 100 μM (Figures 5 to 8). Nonetheless, relative to P5, the activities of the other three polypeptides, as expressed by their R_{EC} values, differed substantially between the four bioassay systems, with P5-NH_2 showing the greatest variation, being least potent and closest to P5 in the placental artery assay ($R_{EC}=0.33$) and most potent relative to P5 ($R_{EC}=0.01$) in the umbilical vein preparation. Taken together, the distinct orders of agonist potencies for the same set of agonists in the four different assay systems point to distinct functional receptor subtypes, according to classical structure-activity criteria (Ahlquist, 1948).

In comparison with our previous work using rat and guinea-pig vascular and gastric bioassay systems (Hollenberg *et al.*, 1993), the distinct order of potencies for the four TRPs in the human PA and UV tissues were selected to determine the thrombin receptor cDNA sequences. In spite of the very distinct orders of potencies of the four TRP analogues in the PA and UV tissues, the cDNA sequences for the receptor obtained from PA and UV RNA were the same in both tissues; and these sequences were essentially the same as the one originally cloned from a human megakaryocyte-like cell line (Vu *et al.*, 1991) and from a human umbilical vein endothelial cell cDNA library (Bahou *et al.*, 1993). Of the two base transversions that we have detected in comparison with the originally published sequence (Vu *et al.*, 1991), one (base pairs 935–936, CG→GC) has been detected in a human umbilical vein endothelial cell cDNA library (Bahou *et al.*, 1993). The second transversion that we have detected (base 1315–1316, CG→GC) cannot be attributed to PCR artifact and may, as with the one detected by Bahou and coworkers (1993), represent genetic polymorphism of the thrombin receptor gene. Interestingly, the S_{364}→C_{364} mutation that we have detected would lead to a sequence homology with residues $S_{358}C_{359}C_{360}$ of the *Xenopus* thrombin receptor (Gerszten *et al.*, 1994). Such a substitution in transmembrane domain No. 7 of the human receptor might have a functional consequence.

How, one may ask, might tissues containing the same thrombin receptor mRNA possess functionally distinct receptor subtypes, as indicated by the different structure-activity profiles for the four TRPs? One possibility is that post-translational modification of the product of the same mRNA in different cell types might yield functionally different receptor subtypes. This situation would appear to be the case for the

murine bradykinin receptor, for which the transfection of the same cDNA into COS cells yielded two populations of receptors with distinct ligand binding properties (McIntyre *et al.*, 1993). To our knowledge, our work would represent the first description in intact tissues of a situation akin to the one described *in vitro* by McIntyre and coworkers (1993) for the murine bradykinin receptor, transfected into COS cells. A second possible explanation for the presence of pharmacologically distinct receptors resulting from the same mRNA can by hypothesized on theoretical grounds. Kenakin and coworkers have predicted that the coupling of an individual receptor to single or multiple transducer proteins might alter the relative potencies of agonists (Kenakin & Morgan, 1989). Very possibly, the types and content of G-proteins in the PA and UV preparations may differ sufficiently to alter the potency profiles of the TRPs in these tissues, according to the predictions of Kenakin & Morgan (1989). A third alternative to be considered is the possibility that a TRP such as P5-NH_2, in addition to activating the thrombin receptor, might also be capable of activating an entirely unrelated receptor that may be present in the vasculature, such as the recently cloned protease activated receptor, PAR-2 (Nystedt *et al.*, 1994). Further work will be required to evaluate these several possibilities in the PA and UV preparations. From a practical point of view, our data suggest that studies aimed at developing selective TRP agonists or antagonists for the thrombin receptor may require bioassay evaluations in a number of distinct intact tissues rather than solely in receptor transfection systems such as the frog oocyte (Vu *et al.*, 1991; Gerszten *et al.*, 1994).

The absence of an endothelium-mediated relaxation response to the TRPs in the placental vascular preparations merits comment in view of our own observations of a TRP-stimulated nitric oxide (NO)-mediated relaxation in endothelium-containing rat aortic tissue (Muramatsu *et al.*, 1992) and in view of the ability of thrombin to cause an NO-mediated relaxation in human mammary artery ring preparations (Yang *et al.*, 1994). In the endothelium-intact PA, UA and UV preparations (middle panel, Figure 2) a relaxation response in a precontracted tissue was not observed under conditions where we had readily observed a TRP-mediated relaxation in endothelium-intact rat aortic tissue (Muramatsu *et al.*, 1992; Hollenberg *et al.*, 1993). Further, the presence of L-NAME in the UV preparation at concentrations that would have blocked the synthesis of NO did not unmask a contractile response to P5-NH_2; a complete denudation of the endothelium was required to reveal a contractile response to P5-NH_2 in the UV preparation. Yet the presence of NO synthase has been observed by immunohistochemistry in UA and UV tissue (Buttery *et al.*, 1994). We suggest that although present in the endothelium of the PA, UA and UV tissues, the NO synthase is somehow refractory to activation by the TRPs, so as to yield too low a level of NO to cause a relaxant effect. Possibly, the comparatively high Po_2 to which the placental tissues are exposed in the organ bath may be a factor in the lack of a relaxant response of the preparations to the TRPs (Xie & Triggle, 1994).

In the UV tissue, the lack of a contractile response in endothelium-intact preparations to TRPs could not be attributed to the production of NO or a relaxant cyclo-oxygenase product and could not be explained by rapid degradation of the peptide by aminopeptidase, in contrast with the observations of Godin and coworkers (1994). We are thus unable at present to account for the unmasking of a contractile action of the TRPs in this tissue by removal of the endothelium. Possibly, in endothelium-intact preparations the TRPs cause the production of an as-yet-unidentified relaxing factor that completely offsets the contractile response. Alternatively, the intact endothelium may in some way present a physical barrier that limits access of the TRPs to the contractile elements in the UV tissue. Further work will be required to resolve this issue. Similarly, the inability of the UV preparation to respond to thrombin would suggest the presence of inhibitory factors that may affect the enzymatic activity of thrombin itself or that may in some way modulate receptor activity. Thus, the physiological interpretation of the response of the denuded UV preparation to the TRPs remains an open question.

The signal transduction pathway(s) whereby the TRPs regulate contractility in the PA, UA and UV preparations appear to be similar in some respects to those activated by TRPs in gastric and aorta-derived smooth muscle preparations (Muramatsu *et al.*, 1992; Hollenberg *et al.*, 1993; Antonaccio *et al.*, 1993). Like the contractile response of aortic tissue, the contractile response of the placental vessels to the TRPs all depended on the presence of extracellular calcium. Thus, the TRP-induced activation of phosphoinositide hydrolysis (Hung *et al.*, 1992) with the consequent inositol-tris-phosphate mediated elevation of intracellular calcium (Berridge, 1993), would not appear to be sufficient to generate a TRP-mediated contractile response. Also, like the contractile response caused by TRPs in a gastric tissue preparation, the contractile actions of the TRPs in the PA, UA and UV preparations were inhibited selectively (e.g. compared with KCl-induced contractions) by the tyrosine kinase inhibitor, genistein. In this regard, the TRP-mediated contraction of the UV preparation appeared to be selectively (compared with KCl) and completely inhibited by genistein, whereas the TRP-induced contractions of the UA and PA preparations were only partially blocked by genistein. In contrast with the sensitivity of the contractile actions of the TRPs in the gastric contractile assay to indomethacin (Hollenberg *et al.*, 1993), the contractile action of the TRPs in the placental vessels was essentially resistant to this cyclo-oxygenase inhibitor. Thus, further work appears warranted to explore the similarities and differences between the placental vessels and other contractile smooth muscle preparations, in terms of the signal transduction pathways activated by thrombin and the TRPs especially with a focus on a possible role for a tyrosine kinase pathway. In this respect, the potential ability of the functional receptor subtypes for the TRPs that we have observed in this study to couple to distinct signal transduction pathways in different tissues will be of considerable interest.

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