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Dual endothelium-dependent vascular activities of proteinase-activated receptor-2-activating peptides: evidence for receptor heterogeneity

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1 The vascular actions of the proteinase-activated receptor-2-activating peptides (PAR_2APs), SLIGRL-NH_2 (SL-NH_2) and SLIGKV-NH_2 (KV-NH_2) as well as the reverse-sequence peptide, LSIGRL-NH_2 (LS-NH_2) and an N-acylated PAR_2AP derivative, trans-cinnamoyl-LIGRLO-NH_2 (tcLI-NH_2), were studied in rat intact and endothelium-denuded artery ring preparations, primarily from the pulmonary artery (RPA).

2 In RPA rings with but not without a functional endothelium, SL-NH_2 (but not LS-NH_2) caused either an endothelium-dependent relaxation (at concentrations: $<10\ \mu$M) or (at higher concentrations: $>10\ \mu$M), an endothelium-dependent contraction. No contractile response was observed in endothelium-denuded preparations, that otherwise contracted in response to the PAR_1AP, TFLLR-NH_2.

3 The endothelium-dependent contractile response to SL-NH_2 was not blocked by the α-adrenoceptor antagonist prazosin, the endothelin antagonist BQ123, the angiotensin II antagonist DuP753, by tetrodotoxin; nor by the enzyme inhibitors, N^{ω}-nitro-L-arginine-methylester (NO-synthase), indomethacin (cyclo-oxygenase), SKF-525A (epoxygenase) and MK886 (leukotriene synthesis inhibitor).

4 In the relaxation assay, KV-NH_2 was 5 fold less potent than SL-NH_2, whereas in the contractile assay KV-NH_2 was about equipotent with SL-NH_2. However, the maximal contractile response to KV-NH_2 was lower than that of SL-NH_2.

5 The PAR_2AP analogue, tcLI-NH_2, was as active as SL-NH_2 in the relaxation assay but was inactive as a contractile agonist in the endothelium-intact RPA.

6 The relaxant responses caused by SL-NH_2 and trypsin, as well as the contractile response caused by SL-NH_2, did not desensitize in the course of repeated exposures of the tissue to agonist; whereas the contractile response to trypsin, only observed at concentrations greater than 30 u ml^{-1}, was desensitized by previous exposure of the tissue to either thrombin or trypsin.

7 In a contractile assay, where the tissue was desensitized to a concentration of trypsin that would otherwise cause a relaxant response, the preparation still contracted in response to SL-NH_2. However, the trypsin-desensitized preparations were no longer contracted by thrombin.

8 From the cross-desensitization by thrombin of the contractile response to trypsin (and *vice versa*), we concluded that the contractile effect of trypsin was due to activation of the thrombin receptor and not PAR_2.

9 We concluded that the endothelium-dependent contraction caused by high concentrations of SL-NH_2 is due to an as yet unidentified contracting factor; whereas the endothelium-dependent relaxation response observed at low concentrations of SL-NH_2 ($\leqslant 10\ \mu$M) is mediated by nitric oxide.

10 The distinct structure activity profiles for the contractile response (potency of KV-$NH_2 \leqslant$ SL-NH_2) compared with the relaxant response (potency of KV-$NH_2 <<$ SL-NH_2); the contractile responsiveness to SL-NH_2 of an endothelium-intact RPA preparation, that did not contract in response to trypsin; and the lack of contractile activity of the PAR_2AP analogue tcLI-NH_2, that was as active as SL-NH_2 in the relaxation assay all argue in favour of receptor heterogeneity in the vasculature for the PAR_2APs. It remains to be determined if the distinct endothelial receptor responsible for the contractile action of SL-NH_2 can be proteolytically activated, like PAR_1 and PAR_2.

Keywords: Thrombin receptor; trypsin; protease-activated receptor; pulmonary artery; endothelium; PAR_2; PAR_1

Introduction

Proteinases such as thrombin and trypsin, in addition to acting as activators of proteolytic enzyme cascades (e.g. the participation of thrombin in the coagulation process), are now known to affect target tissues via the proteolytic activation of cell surface G-protein-coupled receptors (Vu *et al*., 1991; Rasmussen *et al*., 1991; Nystedt *et al*., 1994; 1995a, b). A unique property of the stimulation of these proteinase-activated receptors (PARs) relates to the proteolytic unmasking of an N-terminal tethered self-activating neoligand. Further, short synthetic peptides based on the revealed anchored receptor-activating sequences (so-called PAR-activating peptides or PAR-APs) can in isolation stimulate either PAR_1 or PAR_2, so as to mimic the action of either thrombin (PAR_1APs) or trypsin (PAR_2APs) in a variety of tissues ranging from platelets to vascular and gastric smooth muscle (Vu *et al*., 1991; De Blois *et al*., 1992; Muramatsu *et al*., 1992; Simonet *et al*., 1992; Yang *et al*., 1992). In our own work

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leading to the cloning of the rat PAR_2 receptor, we found that the rat vasculature concurrently expresses mRNA for PAR_1 and PAR_2 both in the endothelial and non-endothelial components (Saifeddine *et al*., 1996). We were able to use the PAR_2AP, SLIGRL-NH_2 (SL-NH_2), that selectively activates PAR_2 but not PAR_1 (Nystedt *et al*., 1994; 1995a, b; Blackhart *et al*., 1996; Hollenberg *et al*., 1997) to demonstrate a PAR_2-triggered endothelium-dependent nitric oxide-mediated relaxation of rat aorta rings that were precontracted with phenylephrine (Al-Ani *et al*., 1995; Saifeddine *et al*., 1996). Although PAR_2 receptor mRNA was detected in endothelium-denuded aorta tissue, we were not able to observe a response (either relaxation or contraction) to the PAR_2AP, SL-NH_2, in the endothelium-free aorta preparation, despite its contractile response to thrombin and PAR_1-activating peptides (Al-Ani *et al*., 1995; Saifeddine *et al*., 1996). We were surprised by the lack of response to the PAR_2AP of the aorta tissue that possessed PAR_2 mRNA and we wondered about the possible effects of PAR_2 activation in vascular preparations other than those derived from the aorta. We therefore decided to survey the actions of the PAR_2-selective PAR_2AP, SLIGRL-NH_2 (SL-NH_2, based on the rat and mouse receptor sequence) as well as the PAR_2AP, SLIGKV-NH_2 (KV-NH_2, based on the human receptor sequence: Nystedt *et al*., 1995b; Böhm *et al*., 1996), in arterial rings derived not only from aorta but also from the renal, femoral, mesenteric and pulmonary circulation. In this study, we examined the actions of the PAR_2APs in such preparations with a particular focus on rings derived from the pulmonary artery. To evaluate possible endothelium-derived contributors to the PAR_2AP-induced contractile response, we also assessed the potential inhibitory actions of the endothelin$_A$ receptor antagonist, BQ123, as well as inhibitors of cyclo-oxygenase, leukotriene synthesis or epoxygenase. Further, a preliminary structure-activity profile for the endothelium-mediated contractile and relaxant responses was obtained with the PAR_2APs: SLIGRL-NH_2, SLIGKV-NH_2 and trans-cinnamoyl LIGRLO-NH_2 (tcLI-NH_2).

Methods

Bioassay procedures

Male Sprague-Dawley albino rats (250–350 g), cared for in accordance with the guidelines of the Canadian Council on Animal Care, were anaesthetized with diethyl ether, killed by cervical dislocation and were immediately anticoagulated by the injection of heparin (1000 units in 2 ml isotonic saline) into the left ventricular circulation. Clot-free arterial samples derived from the femoral, renal, mesenteric, aortic and pulmonary circulation were rapidly dissected free from adventitial tissue and ring preparations (about 2 mm × 2 mm) were cut for use in the bioassay. Where indicated, the endothelium was denuded by rolling the arterial ring on the end of a sharp forceps. Rings were equilibrated for 1 h at 37°C in a gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer pH 7.4 (4 ml, in a plastic disposable cuvette) of the following composition (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. A tension of 1 g was applied and the force of contraction was monitored with either Grass or Statham force-displacement transducers. Tissue viability was routinely monitored by observing a contraction in response to 50 mM KCl and 1 μM phenylephrine (PE). The presence of a functional endothelium was monitored by observing a prompt relaxant response to 1 μM acetylcholine (ACh) in a tissue that had been precontracted with 1 μM PE. For the construction of concentration-relaxation curves, the relaxant response to increasing concentrations of PAR_2APs were expressed as a percentage (% ACh) of the relaxation caused by 1 μM ACh; the contractile responses are expressed as a percentage (% KCl) of the contraction caused by 50 mM KCl. Tissues were exposed to agonists at 45 min intervals followed by washing and re-equilibration in fresh buffer. When present, putative receptor or enzyme antagonists were added to the organ bath 20 min before the addition of PAR_2APs.

Peptides and other reagents

The PAR_2APs: SLIGRL-NH_2, SLIGKV-NH_2 and *trans*-cinnamoyl LIGRLO-NH_2, the inactive peptide, LSIGRL-NH_2 as well as the selective PAR_1AP, TFLLR-NH_2, were prepared by standard solid phase synthesis procedures either by the Core Peptide Synthesis Laboratory at The University of Calgary, Faculty of Medicine (Calgary, AB, Canada) under the direction of Dr Denis McMaster or by Immunosystems at BioChem Therapeutic Inc. (Laval, PQ, Canada) with the assistance of Dr Lorraine Leblond. Peptides were >95% pure by chromatographic and mass spectral criteria. The concentration and amino acid composition of stock peptide solutions, dissolved in 25 mM HEPES buffer, pH 7.4, were verified by quantitative amino acid analysis. Acetylcholine, BQ123 (cyclo-[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]), prazosin, ketoconazole, nordihydroguairetic acid, indomethacin, phenylephrine, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and porcine trypsin (14,9000 u mg^{-1}, cat No. T7410), were from Sigma (St. Louis, MO, U.S.A.). A maximum specific activity of 20,000 u mg^{-1} was used to calculate the approximate molar concentration of trypsin in the organ bath. The lipoxygenase inhibitor, MK886(3-[1-(4-chlorobenzyl)-3-t-butyl-thio-t-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid), was kindly provided by Merck-Frosst Canada. (Pointe Claire-Dorval, PQ); the nonspecific cytochromic P450 enzyme inhibitor, SKF-525A (β-diethylaminoethyl-diphenyl-propyl-acetate HCl) was from Sigma (St Louis, MO, U.S.A.) and DuP753 (losartan) was from Merck (Rahway, NJ, U.S.A.). Phenoxybenzamine was from Research Biochemicals International (Natick, MA, U.S.A.).

Results

Contractile action of SLIGRL-NH_2: survey of arterial beds and dependence on endothelium

In our preliminary survey of the action of the PAR_2AP, SLIGRL-NH_2, arterial ring preparations both with an intact and denuded endothelium were obtained from the femoral, renal, mesenteric and pulmonary circulation as well as from the thoracic and abdominal aorta. As found previously with aorta-derived preparations (Al-Ani *et al*., 1995; Hollenberg *et al*., 1996), tissues from all of these vascular regions with (but not without) an intact endothelium, when precontracted with 1 μM phenylephrine, relaxed in response to SLIGRL-NH_2 (2–5 μM), as exemplified by the tracing shown for the pulmonary ring in Figure 1. The relaxation was blocked by L-NAME (not shown). Trypsin 1–4 u ml^{-1}; approx. 8 nM) also caused a relaxation response in the pulmonary artery preparation (Figure 1a). In all preparations with an intact endothelium, except for those from the femoral artery, which otherwise exhibited a relaxation response, we observed that 50 μM SLIGRL-NH_2 caused a contractile response of tissue maintained at baseline tension. The response is typified by the data shown for the pulmonary artery ring preparation in

Figure 1b. In the RPA preparation shown, the tissue did not respond to 20 u ml^{-1} trypsin, even though SLIGRL-NH_2 caused a robust contraction; the relaxation response to acetylcholine in the same preparation demonstrated that the endothelium was intact (Figure 1b). The N-terminal reverse sequence peptide, LSIGRL-NH_2, which is not able to activate PAR_2, did not cause a contractile response in a preparation that was otherwise responsive to SLIGRL-NH_2 (Figure 1d). For reasons we could not determine, this contractile response was not observed in every endothelium-intact preparation obtained from the aorta (e.g. contractile response in 12 of 20 independent preparations), the mesenteric artery (11 of 15 tissues responding) and the renal arterial beds (9 of 10 responding), even though endothelial function was intact in all of these preparations, as assessed by ACh-mediated relaxation. It appeared that tissues obtained from animals larger than 250 g yielded more consistent contractile responses, and that endothelium-intact preparations obtained from the pulmonary artery uniformly exhibited reliable and consistent contractile responses to multiple agonist exposures. Thus, most of the subsequent experiments were done with pulmonary artery rings from animals weighing between 270 and 350 g. No preparation from any arterial region evaluated, in which the endothelium was removed (e.g. Figure 1c and Figure 2d), exhibited a contractile response to the PAR_2AP, like the one observed in the endothelium-intact preparations (compare (b) and (d) with (c) in Figure 1). Notwithstanding, endothelium-denuded preparations that did not contract in response to 50 μM SLIGRL-NH_2 did contract in response to the selective PAR_1AP, TFLLR-NH_2 (Figure 1c).

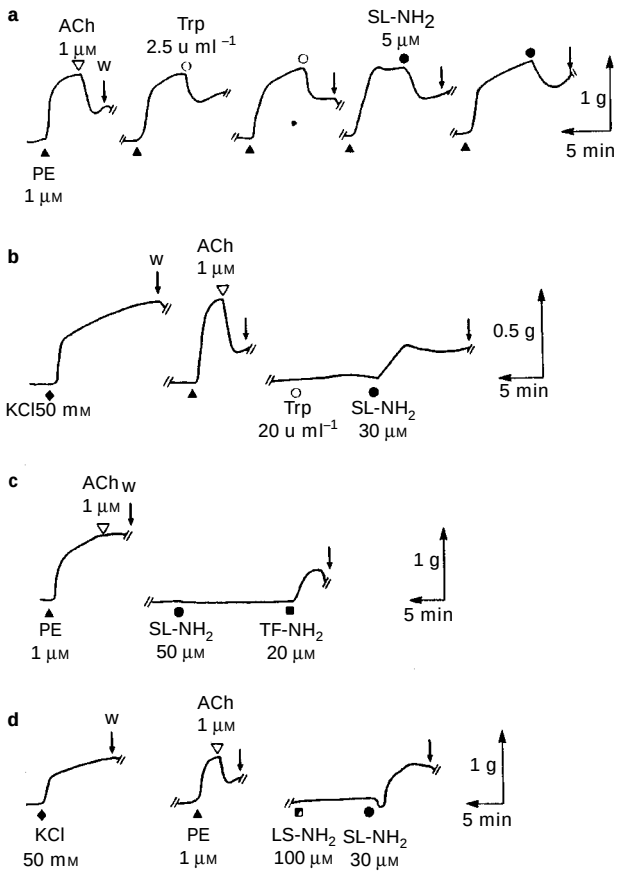

Figure 1 Representative responses of the pulmonary artery ring to trypsin and SLIGRL-NH_2: role of endothelium and SLIGRL-NH_2 peptide sequence. The actions of trypsin (Trp, 2.5 or 20 u ml^{-1}) and SLIGRL-NH_2 SL-NH_2 5 to 50 μM) were monitored in pulmonary artery rings either without (c) or with (a, b, d) an intact endothelium, as monitored by observing a relaxant response to acetylcholine (ACh, 1 μM) in a phenylephrine (PE, 1 μM) precontracted tissue. (a) Reproducible relaxant response to trypsin and SL-NH_2; (b) contraction in response to SL-NH_2, but not Trp; (c) lack of contractile response to SL-NH_2 in an endothelium-free preparation that responded to TFLLR-NH_2 (TF-NH_2, 20 μM); (d) lack of response to LSIGRL-NH_2 (LS-NH_2, 100 μM). The scales for time and tension are shown to the right of the tracings, which are representative of 8 or more independently conducted experiments with tissues coming from 5 separate animals. W = tissue wash (arrows).

Cross-densensitization of the trypsin and thrombin-mediated contractile response does not alter the contractile response to SLIGRL-NH_2

Trypsin, at concentrations sufficient to activate PAR_2 without affecting PAR_1 (i.e. $\leqslant$20 u ml^{-1} or $\leqslant$40 nM: see Vu *et al.*, 1991) was not able to cause a contractile response in endothelium-intact preparations that were otherwise responsive to 30 μM SLIGRL-NH_2 (Figure 1b and Figure 2c) and that demonstrated a relaxant response to even lower concentrations of trypsin (Figure 1a and Figure 2c). Nonetheless, higher concentrations of trypsin, capable of activating the thrombin receptor (50 to 100 u ml^{-1}: approx. 100 to 200 nM), did cause a contractile response in endothelium-intact preparations (Figure 2a and data not shown) as well as in endothelium-free preparations (Figure 2d). Thrombin (10 u ml^{-1} or 100 nM) also caused a contractile response in either intact or endothelium-denuded preparations (Figure 2b and data not shown). In a cross-densensitization assay, the contractile response to either 10 u ml^{-1} thrombin or 50 u ml^{-1} trypsin (but not to SLIGRL-NH_2) was abolished by first pretreating the tissue with 100 u ml^{-1} trypsin (Figure 2a, d and data not shown); similarly, treating the tissue first with thrombin abolished the contractile response to trypsin but not to SLIGRL-NH_2 (Figure 2b). The thrombin-treated tissue was also unresponsive to a second exposure to thrombin (not shown). As mentioned above, in endothelium-intact pulmonary preparations that no longer contracted in response to either thrombin or trypsin, SLIGRL-NH_2 still caused a contractile response (Figure 2a,b). Also, in preparations in which comparatively high concentrations of trypsin ($\geqslant$20 u ml^{-1}) did not cause a contractile response whereas SLIGRL-NH_2 did, both lower concentrations of trypsin (e.g. 4 u ml^{-1} (Figure 2c) and SLIGRL-NH_2 (not shown) caused a relaxation response.

Effects of inhibitors on contractions caused by SLIGRL-NH_2

As endothelium-derived metabolites of arachidonic acid (via cyclo-oxygenase, lipoxygenase or epoxygenase) might be responsible for the PAR_2AP-induced contractions, we evaluated the effects of indomethacin (cyclo-oxygenase inhibitor), nordihydroguairetic acid (lipoxygenase inhibitor), MK886 (inhibitor of leukotriene synthesis), ketoconazole and SKF525A (epoxygenase/cytochrome P450 inhibitors). None of these reagents at enzyme-inhibitory concentrations (3–10 μM) blocked the contractile effect of SLIGRL-NH_2 in the pulmonary arterial ring preparation (Figure 3b,d). Similarly, in an endothelin-1-sensitive preparation, a concentration of BQ123 (1 μM) that was sufficient to block the contractile effect of 10 nM endothelin did not block the contractile effect of SLIGRL-NH_2 (Figure 3a). Neither histamine nor 5-hydroxytryptamine (1 μM) caused a contractile response in the pulmonary arterial ring (not shown), indicating that neither of these agonists is responsible for SLIGRL-NH_2-mediated contractile response; nor did the α_1-adrenoceptor antagonist,

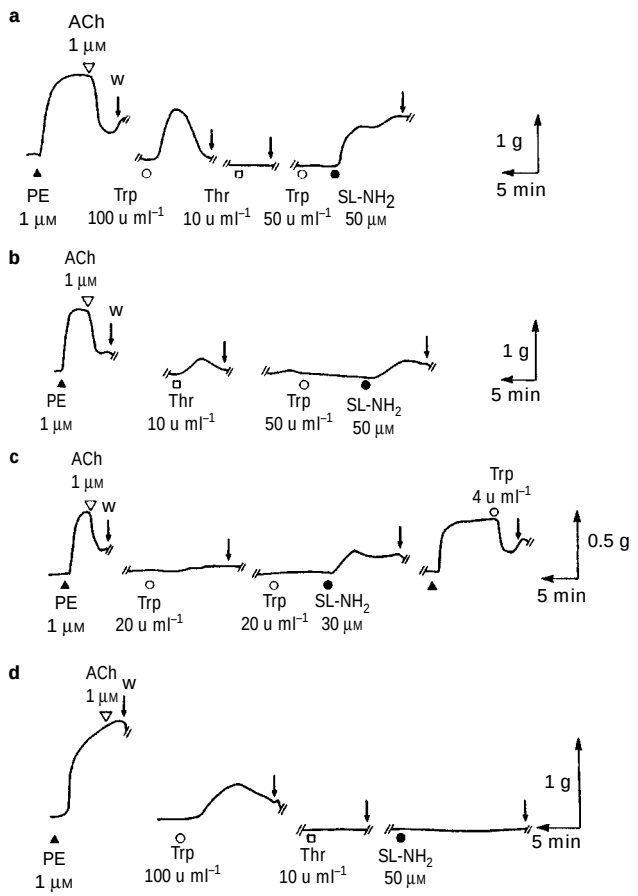

Figure 2 Desensitization of the contractile response to trypsin and thrombin in an endothelium-intact preparation did not abolish the response to SLIGRL-NH_2. Pulmonary artery rings either with (a, b, c) or without (d) an intact endothelium were exposed to either trypsin (Trp, 20 to 100 u ml^{-1}) or thrombin (Thr, 10 u ml^{-1}) followed by washing (W, arrow) and equilibration in fresh buffer. The tissues were again challenged with either trypsin or thrombin, and the enzyme-desensitized preparations were then tested with a contractile concentration of SLIGRL-NH_2 (SL-NH_2 30 or 50 μM). An endothelium-intact preparation that did not contract to 20 u ml^{-1} trypsin, but was responsive to SL-NH_2, did nonetheless exhibit a relaxation response to trypsin (c). The data are representative of experiments done with at least 6 tissue preparations coming from two or more separate animals. The scales for time and tension are shown to the right of each tracing. W (arrows) = tissue wash.

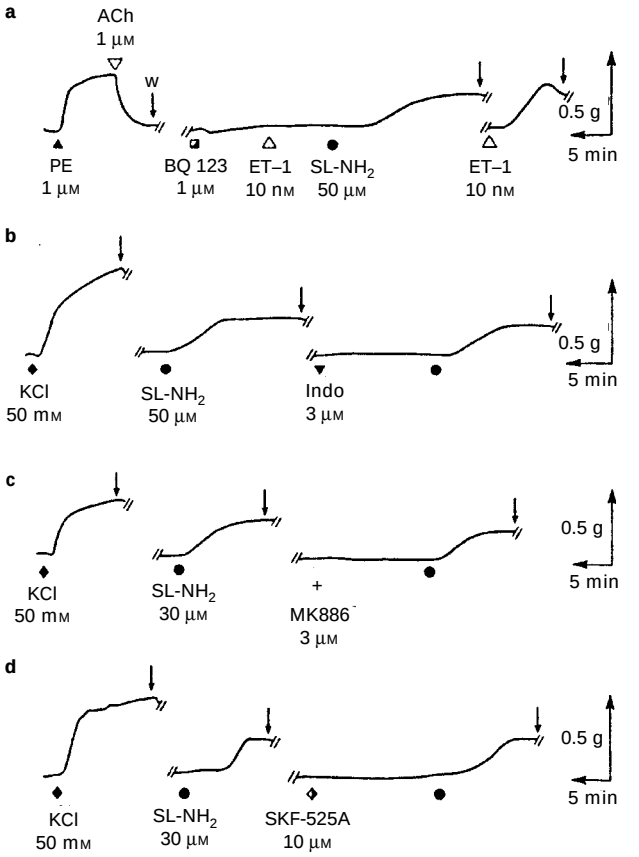

Figure 3 Effects of inhibitors on the pulmonary arterial ring contractile response to SLIGRL-NH_2. In each tissue, the integrity of the endothelium was ascertained by monitoring a relaxation response to acetylcholine (ACh, 1 μM, shown in (a) only) and a control contractile response was then observed for SLIGRL-NH_2 (SL-NH_2, shown for (b) to (d) only). Separately standardized tissues were then treated with the indicated inhibitors for 20 min, followed by a second challenge with SLIGRL-NH_2: (a) BQ123; (b) indomethacin (Indo); (c) MK 886; (d) SKF-525A. The tissue response to endothelin-1 (ET-1) was monitored after washing the tissue free of BQ123 (a). The SL-NH_2-induced contractile responses were compared with contractions caused by either phenylephrine (PE) or KCl. The scales for time and tension are shown on the right. Each continuous tracing (a to d) illustrating the response of a single tissue is representative of results with 8 or more tissue preparations obtained from 3 or more different animals.

prazosin or the angiotensin antagonist, DuP753 (losartan), affect the action SLIGRL-NH_2 (not shown). Likewise, tetrodotoxin, which blocks the release of other agonists from nerve elements, failed to affect the contractile effect of 50 μM SLIGRL-NH_2 (not shown). The presence of the nitric oxide synthase inhibitor L-NAME (100 μM) neither potentiated nor inhibited the contractile response elicited by SLIGRL-NH_2 (data not shown).

Concentration-effect curves for SLIGRL-NH_2 and SLIGKV-NH_2, and differential activity of trans-cinnamoyl LIGRLO-NH_2

Concentration-effect curves (Figure 4) were obtained for the rat PAR_2-derived sequence, SLIGRL-NH_2, and the human PAR_2-derived sequence, SLIGKV-NH_2. Also, a preliminary assessment was made of the activity of the PAR_2AP analogue, trans-cinnamoyl LIGRLO-NH_2, both for the relaxant (Figure 4a) and contractile (Figure 4b) responses of the endothelium-intact pulmonary artery ring. Although the relaxant potency of SLIGKV-NH_2 was about 5 fold lower than that of SLIGRL-NH_2 in the relaxation assay (Figure 4a), the two peptides were very close in potency in the contractile assay (SLIGKV-$NH_2 \leqslant$ SLIGRL-NH_2: Figure 4b and Figure 5). Further, SLIGKV-NH_2 appeared to be a partial agonist in the contractile assay and a full agonist in the relaxant assay (compare Figure 4a and b). The close potency of the two peptides in the contractile assay was more evident when the contractile response to each agonist was normalized to the maximal response at the plateau of the concentration-effect curves (Figure 5). The concentration range over which SLIGRL-NH_2 caused a contractile response was shifted to the right by about an order of magnitude, compared with the concentration range over which a relaxant response was observed (compare Figure 4a and b). In assessing the activity of the PAR_2AP derivative, trans-cinnamoyl-LIGRLO-NH_2, compared with SLIGRL-NH_2, we found that equimolar concentrations (3 μM) of the trans-cinnamoyl peptide and SLIGRL-NH_2 caused a comparable relaxation (Figure 4a and

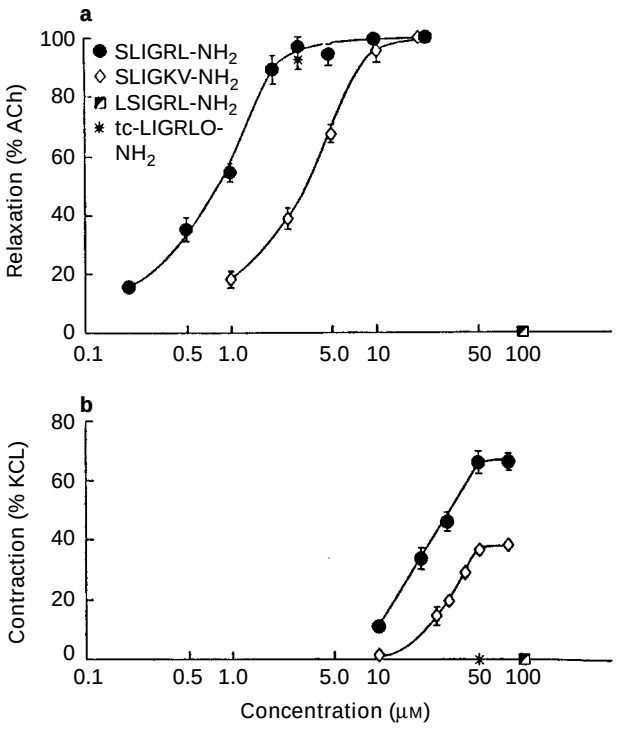

Figure 4 Concentration-effect curves for the relaxant (a) and contractile (b) actions of PAR_2APs in the pulmonary arterial ring. (a) The relaxant responses to PAR_2AP analogues expressed as a percentage (% ACh) of the relaxation caused by 1 μM acetylcholine (ACh) in a phenylephrine (1 μM)-precontracted tissue were measured for increasing concentrations of SLIGRL-NH_2 and SLIGKV-NH_2. The effects of trans-cininamoyl-LIGRLO-NH_2 and LSIGRL-NH_2 were measured at one concentration only. (b) The contractile responses to PAR_2AP analogues, expressed as a percentage (% KCl) of the contractile response to 50 mM KCl, were monitored for increasing concentrations of SLIGRL-NH_2 and SLIGKV-NH_2; only a single concentration of the trans-cinnamoyl (tc-LIGRLO-NH_2) and N-terminal reverse sequence peptide (LSIGRL-NH_2) were studied. All points represent the means for measurements made with 4 to 20 individual tissue preparations coming from 4 or more separate animals; vertical lines show s.e.mean.

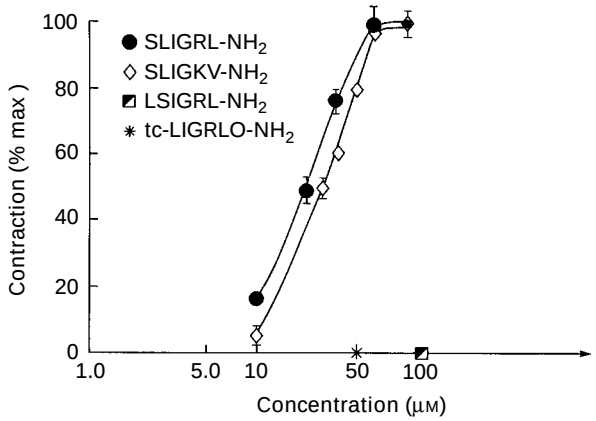

Figure 5 Contractile actions of SLIGRL-NH_2 and SLIGKV-NH_2; normalization of concentration-effect curves. The data shown in Figure 4b were normalized (% max) for each peptide, according to the maximum contractile response observed at concentrations of 50 to 100 μM. The lack of activity of LSIGRL-NH_2 and trans-cinnamoyl LIGRLO-NH_2 are also shown.

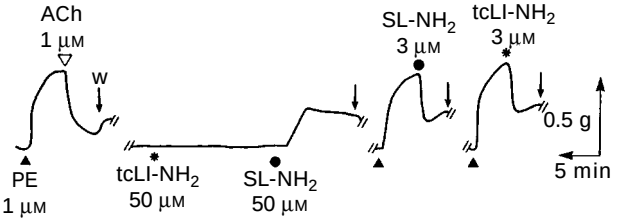

Figure 6 Comparative activity of trans-cinnamoyl LIGRLO-NH_2 in the contractile and relaxation assays: representative tracing. The contractile response of an individual tissue strip possessing an intact endothelium (relaxant response to acetylcholine (ACh, 1 μM) in a phenylephrine (PE, 1 μM) pre-contracted tissue) was first monitored for both trans-cinnamoyl LIGRLO-NH_2 (tcLI-NH_2) and SLIGRL-NH_2 (SL-NH_2) followed by a tissue wash (W, arrows). The relaxant action of the two peptides was then assessed sequentially in the same preparations that were first precontracted with phenylephrine (PE, 1 μM: right-hand tracings). The scale for time and tension is shown to the right of the tracing. The tracing is representative of experiments done with more than 6 different tissue preparations obtained from 5 separate animals.

Figure 6). However, in the contraction assay, 50 μM SLIGRL-NH_2 caused a robust response, whereas 50 μM trans-cinnamoyl-LIGRL-NH_2 had no effect (Figure 4a and b; Figures 5 and 6).

Effect of partial depolarization on the contractile response to SLIGRL-NH_2

To assess whether or not membrane potential might play a role in the tissue response to SLIGRL-NH_2, we examined the effect of subconstrictor levels of elevated potassium (i.e. 10 mM). At a submaximal contractile concentration of SL-NH_2 (30 μM), this slight elevation in KCl concentration potentiated the contractile response by about 2 fold without affecting baseline tension (data not shown).

Discussion

The principle finding of our study was that the PAR_2-activating peptide, SLIGRL-NH_2, but not the partial reverse-sequence peptide, LSIGRL-NH_2, caused an endothelium-dependent contractile response in arterial rings in addition to causing an endothelium-dependent relaxation response, as previously found by us (Al-Ani *et al.*, 1995; Hollenberg *et al.*, 1996) and others (Hwa *et al.*, 1996). In contrast, SLIGRL-NH_2 even at 50 μM was unable to cause a contractile response in endothelium-free preparations that were otherwise responsive to the PAR_1AP, TFLLR-NH_2. In our previous work we did not observe a contractile response to SLIGRL-NH_2, presumably because we did not study in depth the comparatively high concentration range (10 to 100 μM) over which SLIGRL-NH_2 causes a contractile response, compared with the lower concentrations (0.5 to 10 μM) that elicit maximal relaxation in a phenylephrine-precontracted tissue (compare (a) and (b) in Figure 4). The sensitization of the tissue to the contractile action of SLIGRL-NH_2 by 10 mM KCl would suggest the release from the endothelium of a depolarizing factor. We believe that this aspect of the contractile action of SLIGRL-NH_2 merits further study.

Our new data, as well as our previously published results (Hollenberg *et al.*, 1996) are not in agreement with a recent study (Emilsson *et al.*, 1997) that appeared after the completion of our study and after a preliminary account of our new findings (Roy *et al.*, 1997). In none of our experiments

were we able to observe a contractile response to SLIGRL-NH_2 in an endothelium denuded arterial preparation like the very small response described by Emilsson *et al.* (1997). Possibly, differences in the PAR_2 agonist peptides for that study (SLIGRL) and ours (SLIGRL-NH_2) may explain the discrepancy in the two sets of results. It should also be pointed out that the very small and delayed response found by Emilsson *et al.* (1997) differed from the prompt, more robust response we observed (e.g. Figure 1) in the endothelium-intact preparations. Like Emilsson *et al.* (1997) we did nonetheless observe a contraction in response to PAR_1APs.

In view of the finding that the PAR_2AP, SLIGRL, can cause the release of endothelin-1 from rat aorta rings (Magazine *et al.*, 1996), we anticipated that our observed contractile response to SLIGRL-NH_2 might be due to endothelin-1 itself. However, the ability of BQ123 to block the contractile action of endothelin-1 without blocking contractions caused by SLIGRL-NH_2 (Figure 3) would argue against any role for endothelin-1 in the SLIGRL-NH_2-mediated contractile response. Similarly, the inability of the angiotensin receptor antagonist, DuP753 and the α-adrenoceptor antagonist prazosin to block the contractile action of SLIGRL-NH_2 ruled out a role for either tissue-generated angiotensin-II or noradrenaline. In addition, the lack of effect of indomethacin, nordihydroguairetic acid, MK886, ketoconazole and SKF525A suggests that endothelium-derived metabolites of arachidonic acid, via the cyclo-oxygenase, lipoxygenase or epoxygenase pathways are not responsible for the contractile action of SLIGRL-NH_2. Tetrodotoxin was used to evaluate a potential role for nerve-released agonists; the lack of effect of this toxin in blocking the contractions caused by SLIGRL-NH_2 suggest that it is unlikely that released neurotransmitters are responsible for the contractile response. The potentiating action of slightly elevated potassium concentrations on the contractile response to SLIGRL-NH_2 suggests that membrane potential may be an important factor in the effect of the contractile substance(s) released from the endothelium. Vascular preparations studied *in vitro* typically exhibit hyperpolarized membrane potentials and the modest depolarization afforded by 10 mM KCl may be required for further studies of the endothelium-dependent contractile action of SLIGRL-NH_2. The nature of the contractile endothelium-derived factor that mediates the SLIGRL-NH_2 response remains an interesting topic for future work.

The cross-densensitization by trypsin of the contractile response caused by thrombin, and *vice versa* (Figure 2) indicated that the contractile effect of 50 to 100 u ml^{-1} trypsin was due to the activation of PAR_1 and not PAR_2. Thus, we were surprised that a concentration of trypsin (20 u ml^{-1}, or about 40 nM) that should have been more than sufficient to activate PAR_2 (Nystedt *et al.*, 1995a, b), without activating PAR_1, did not cause a contractile response (Figure 2c); whereas the addition of SLIGRL-NH_2 did so. Further, in a preparation that was desensitized to the contractile effect of a high concentration (50 u ml^{-1}) of trypsin, SLIGRL-NH_2 still caused a contractile response (Figure 2b). Nonetheless, in a preparation wherein 20 u ml^{-1} trypsin did not cause a contractile response, a much lower trypsin concentration (4 u ml^{-1}), presumably via activation of PAR_2, elicited relaxation (Figure 2c). We therefore suggest that the contractile response due to SLIGRL-NH_2 in a trypsin-insensitive preparation must be due to a receptor other than the one (PAR_2) activated by trypsin.

Two further pieces of evidence also indicate that SLIGRL-NH_2 activates a receptor other than PAR_2 to cause the endothelium-dependent contractile response. Firstly, the peptides SLIGRL-NH_2 and SLIGKV-NH_2 were approximately equipotent in terms of eliciting the contractile responses, whereas SLIGKV-NH_2 was approximately 1/5th as potent as SLIGRL-NH_2 in causing relaxation. The reduced potency (about 1/5th) relative to SLIGRL-NH_2 in the relaxation assay is consistent with the relative potencies of these peptides at activating either cloned (Blackhart *et al.*, 1996) or naturally occurring PAR_2 in cultured cell assays (Hollenberg *et al.*, 1997). However, these two peptides were equipotent in activating the receptor involved in the contraction assay. Secondly, the PAR_2 AP derivative, trans-cinnamoyl-LIGRLO-NH_2, that was equally active on a molar basis with SLIGRL-NH_2 in the relaxation assay, did not cause a contractile response in preparations that were responsive to SLIGRL-NH_2 and did not antagonize the contractile action of SL-NH_2. It was of interest that tcLI-NH_2 was a full agonist in the PAR_2-mediated relaxation response, since comparable peptides have been found to be PAR_1 antagonists (Bernatowicz *et al.*, 1996). Thus, (1) the distinct relative potencies of SLIGKV-NH_2 and SLIGRL-NH_2 in the contraction assay compared with the relaxation assay, (2) the selectivity of the trans-cinnamoyl peptide derivative, causing a relaxation response but not a contractile response and (3) the contractile response to SLIGRL-NH_2 either in a trypsin-desensitized preparation or in a naive preparation that did not contract in response to 20 u ml^{-1} trypsin, all point to the activation by SLIGRL-NH_2 of a receptor other than PAR_2 to cause the contractile response. Since the newly described thrombin-activated receptor, PAR_3, has been shown not to respond to SLIGRL or to other PAR-APs (Ishihara *et al.*, 1997), our data suggest that yet another member of the proteinase-activated receptor family might be present in the rat vascular endothelium. Whether the endothelial receptor activated by SLIGRL-NH_2 to cause the contractile response is activated by a protease, like PAR_2, or whether the contractile response is due to a proteinase-insensitive receptor is an open question. The physiological significance of the dual action of SLIGRL-NH_2 on the aorta tissue and the apparent heterogeneity of the receptor systems for the PAR_2AP peptides in the endothelium are interesting topics for further study. Additionally, a concerted search for a new member of the PAR family in endothelial tissue would appear to be warranted on the basis of our data.

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