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Evidence for proteinase-activated receptor-2 (PAR-2)-mediated mitogenesis in coronary artery smooth muscle cells

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1 This study investigates, whether in addition to the thrombin receptor (PAR-1), the proteinase-activated receptor-2 (PAR-2) is present in vascular smooth muscle cells (SMC) and mediates mitogenesis. PAR-2 is activated by low concentrations of trypsin and the synthetic peptide SLIGRL.

2 Stimulation of bovine coronary artery SMC by trypsin (2 nM) caused a 3 fold increase in DNLA-synthesis. A similar effect was observed with 10 nM thrombin. Trypsin-induced mitogenesis was inhibited by soybean trypsin inhibitor, indicating that the proteolytic activity of the enzyme was required for its mitogenic effect.

3 The specific PAR-2-activating peptide SLIGRL or the PAR1-activating peptide SFFLRN did not elicit mitogenesis.

4 When the SMC were exposed to SLIGRL (40 nM), a homologous desensitization of cytosolic Ca^{2+} mobilization was found after subsequent stimulation with trypsin (40 nM) but not thrombin (15 nM).

5 Trypsin (2 nM) as well as SLIGRL (100 μM) activated the nuclear factor κB (NFκB) with a maximum response 2 h after stimulation of the SMC. This suggests that both agonists acted *via* a common receptor, PAR-2. Maximum activation of NFκB by thrombin (10 nM) was detected after 4–5 h.

6 These data suggest that PAR-2 is present in coronary SMC and mediates a mitogenic response. Activation of NFκB *via* either PAR-1 or PAR-2 does not predict mitogenesis.

Keywords: PAR-2; vascular smooth muscle cells; NFκB; mitogenesis

Abbreviations: FCS, foetal calf serum; NFκB, nuclear factor κB; PAR-1, proteinase-activated receptor-1; PAR-2, proteinase-activated receptor-2; SBTI, soybean trypsin inhibitor; SMC, vascular smooth muscle cells

Introduction

The proteinase-activated receptor-2 (PAR-2) belongs to the family of seven-transmembrane domaine G-protein coupled receptors and is closely related to the thrombin receptor (proteinase-activated receptor-1, PAR-1) with a 30% overall amino acid sequence identity. Similar to the activation of the thrombin receptor, activation of PAR-2 also involves the proteolytic cleavage of the extracellular N-terminus of the receptor. A new N-terminus is exposed which interacts intramolecularly with other regions of the receptor thereby triggering intracellular signalling (Nystedt *et al*., 1994; Böhm *et al*., 1996b). Synthetic peptides, corresponding to the sequence of the new N-terminus of PAR-1 or PAR-2, can activate their respective receptor directly. Synthetic thrombin receptor activating peptides consisting of 6–14 amino acid residues were shown to mimic many cellular effects of thrombin (Reilly *et al*., 1993; Glusa *et al*., 1996). In the present study a peptide with the sequence SFFLRN, corresponding to the new N-terminus of the cleaved bovine thrombin receptor, was used. PAR-2 is activated by nanomolar concentrations of trypsin and by the synthetic peptides SLIGRL and SLIGKV, representing the sequence of the newly exposed N-terminus of mouse and human PAR-2, respectively (Nystedt *et al*., 1994; 1995; Böhm *et al*., 1996b). These PAR-2 specific peptides can also mimic cellular effects of trypsin (Glusa *et al*., 1997; Saifeddine *et al*., 1996).

There are only a few reports dealing with the tissue distribution and function of PAR-2. The receptor was detected in various tissues including vascular endothelial cells (Storck *et al*., 1996; Mirza *et al*., 1996), keratinocytes (Derian *et al*., 1997) and enterocytes (Kong *et al*., 1997). Possible functions include contraction of intestinal smooth muscle (Saifeddine *et al*., 1996), secretion of prostaglandins from enterocytes (Kong *et al*., 1997) and endothelium-dependent relaxation (Glusa *et al*., 1997; Emilsson *et al*., 1997).

Both, PAR-1 and PAR-2 are involved in the control of cell proliferation. In human keratinocytes, thrombin receptor stimulation enhanced cell growth, whereas activation of PAR-2 led to the inhibition of cell growth (Derian *et al*., 1997). In human umbilical vein endothelial cells, proliferative responses were mediated by both receptors (Mirza *et al*., 1996).

We have previously shown that thrombin is a potent mitogen for bovine coronary SMC (Bretschneider *et al*., 1997; Zucker *et al*., 1998). In the present study we investigated whether, in addition to PAR-1, PAR-2 is also present in SMC and whether this receptor mediates a mitogenic response. To verify the presence of PAR-2 and the receptor specificity of trypsin, desensitization of cytosolic Ca^{2+} mobilization in SMC

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after stimulation with PAR-1 and PAR-2 agonists was investigated. The nuclear transcription factor NFκB was shown to have a key role for the proliferation of SMC (Autieri *et al*., 1995; Bellas *et al*., 1995). Therefore, NFκB activation was also determined after stimulation of SMC by PAR-2 agonists.

Methods

Cell culture

Coronary artery SMC were isolated from adult cows according to Fallier-Becker *et al*. (1990). Cells were cultured in a humidified atmosphere (37°C, 5% CO_2) in 80% HAM's F12-medium and 20% DMEM supplemented with 10% foetal calf serum (FCS), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. SMC were identified by their typical 'hill and valley' growth pattern and by immunostaining with a specific monoclonal α-actin antibody. Cells of passages 4–10 were used for the experiments.

Cell number and viability

SMC were grown in 24-well plates, made quiescent in FCS-free culture medium for 24 h and stimulated by trypsin (0.2–20 nM). After 24 h, SMC were detached from the culture plate by treatment with trypsin (0.05%)/EDTA (0.5 mM). The viability of cells was determined by trypan blue exclusion. For this purpose, the cell suspensions were incubated with trypan blue for 5 min and the per cent of coloured cells was counted using a haemocytometer.

[^{3}H]-Thymidine incorporation

SMC were seeded into 24 well plates (5×10^4 cells per well) and cultured until confluency was reached. To obtain growth arrest, cells were maintained in FCS-free medium for 24 h. During the following 24 h, SMC were stimulated by the indicated agents. Four hours prior to the end of the stimulation period, SMC were pulse-labelled with [^{3}H]-thymidine (2 μCi ml^{-1}). Medium was removed and SMC were washed sequentially with cold phosphate buffered saline and $HClO_4$ (0.3 M). The cells were solubilized in NaOH (0.1 M) at 37°C for 60 min. [^{3}H]-Thymidine incorporation into the DNA was determined by liquid scintillation spectrometry.

Cytosolic Ca^{2+} measurements

Mobilization of cytosolic Ca^{2+} was measured with minor modification as described elsewhere (Kaufmann *et al*., 1998). Briefly, SMC grown on Lab Tek chambered borosilicate coverglass were washed twice with washing buffer, containing (mM): HEPES 10, NaCl 145, Na_2HPO_4 0.5, glucose 6, $MgSO_4$ 1, $CaCl_2$ 1.5 at pH 7.4. Cells were incubated for 15 min at 37°C in the same buffer supplemented with 1.0 μM fluo-4 acetoxymethylester. Loaded SMC were washed twice, reincubated in washing buffer and stimulated by the agents indicated. For single-cell fluorescence measurements of cytosolic Ca^{2+} an inverted confocal laser scanning microscope (LSM 410, Carl Zeiss Göttingen, Germany) was used. Fluorescence images were collected by using the 488 nm argon ion laser line. The intracellular Ca^{2+} concentration was calculated according to Grynkiewicz *et al*. (1985). F_{max} was obtained by the addition of 10 mM ionomycin + 6 mM $CaCl_2$, F_{min} by the addition of 20 mM EGTA.

Detection of NFκB activation

Nuclear extracts were prepared according to Dignam *et al*. (1983) with minor modifications. Briefly, cells were lysed in buffer A, containing (mM): HEPES 10, $MgCl_2$ 1.5, KCl 10, phenylmethylsulphonyl fluoride 0.2, dithiothreitol 0.5 at pH 7.9 for 30 min on ice. After centrifugation at $13{,}000 \times g$ for 10 min, the nuclei were resuspended in buffer C, containing (mM): HEPES 20, $MgCl_2$ 1.5, KCl 1.2, EDTA 0.2, phenylmethylsulphonyl fluoride 0.2, dithiothreitol 0.5 and 25% glycerol at pH 7.9 and subjected to a brief sonification. Afterwards the samples were centrifuged at $13{,}000 \times g$ for 30 min and protein concentration in the supernatants was measured according to Bradford (1976). Detection of NFκB in nuclear extracts was performed by Western blot after separation of 20 μg of nuclear proteins on a 8% sodium dodecyl polyamide gel. Separated proteins were transferred onto PVDF membranes (Immobilon-P polyvinylidene difluoride, Sigma-Aldrich Chemie, Steinheim, Germany). The p65 subunit of NFκB was detected by a polyclonal antibody after blocking the membrane for 45 min in Blotto, containing TBST (mM): Tris-HCl 10 at pH 8.0, NaCl 150 and 0.05% Tween-20 and 5% dry milk. Afterwards, the membrane was washed three times in TBST (5 min each). The first antibody was detected by a horseradish peroxidase-coupled secondary antibody. Enzymatic activity of the horseradish peroxidase was visualized by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, U.K.).

Drugs and solutions

Trypsin (bovine pancreatic trypsin, 42 U mg^{-1}; Serva, Heidelberg, Germany); Mouse PAR-2-activating peptide (SLIGRL; Institut für Molekulare Biotechnologie, Jena, Germany); soybean trypsin inhibitor; amastatin (Sigma, Deisenhofen, Germany); bovine thrombin receptor activating peptide (SFFLRN, Biogenes, Berlin, Germany); α-actin antibody (Boehringer, Mannheim, Germany); fluo-4 acetoxymethylester (Molecular Probes Europe B.V., Leiden, The Netherlands); polyclonal antibody against p65 (c-20, 1 : 10,000) and secondary antibody; anti-rabbit IgG-HRP (sc-2004, 1 : 5,000, Santa Cruz, Heidelberg, Germany). Media and supplements for cell culture were from Life Technologies (Eggenstein, Germany). The following compounds were gifts: purified bovine α-thrombin (2067 U ml^{-1}, Dr J. Stürzebecher, Zentrum für Vaskuläre Biologie und Medizin Erfurt der FSU Jena, Germany); human PAR-2 activating peptide (SLIGKV; Dr J. Storck, Institut für Physiologie, Westfälische Wilhelms-Universität Münster, Germany).

Statistics

The data on [^{3}H]-thymidine incorporation are mean (s.e.mean) of n independent measurements performed in triplicate. Statistical analysis was performed by one way ANOVA, followed by Bonferroni's multiple comparison test. P levels of <0.05 were considered significant.

Results

Stimulation of [^{3}H]-thymidine incorporation by thrombin and trypsin

Stimulation of SMC by trypsin (0.2–2 nM) caused a concentration-dependent increase in [^{3}H]-thymidine incorpora-

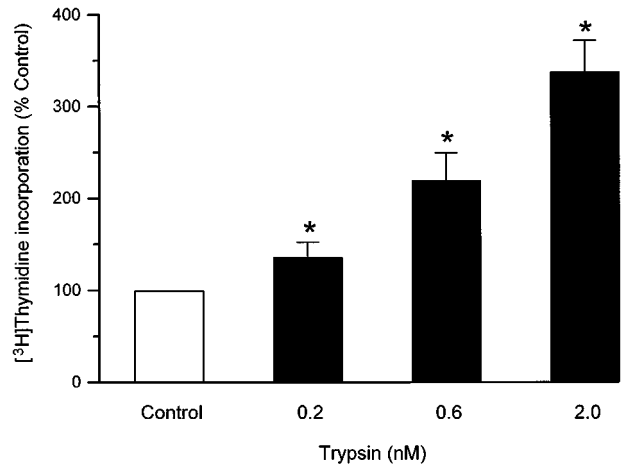

Figure 1 [^{3}H]-Thymidine incorporation in SMC stimulated by trypsin. Quiescent SMC were incubated with trypsin at the concentrations indicated for 24 h. After pulse-labelling with [^{3}H]-thymidine (2 μCi ml^{-1}) during the last 4 h of the incubation period [^{3}H]-thymidine incorporation was determined. Means$\pm$s.e.mean from seven separate experiments; *$P<0.05$ (treatment vs control).

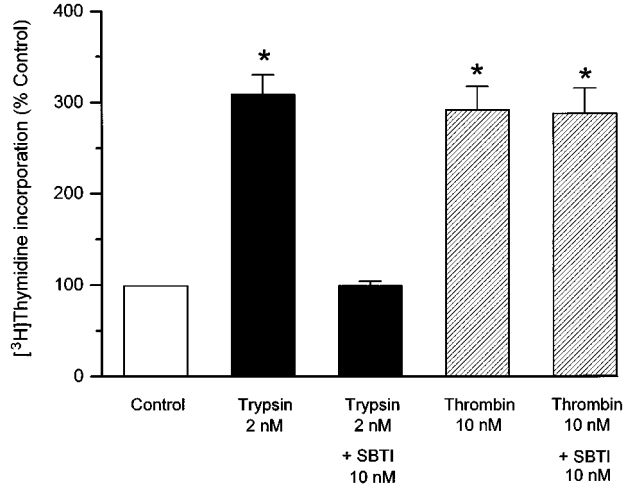

Figure 2 Effect of soybean trypsin inhibitor (SBTI) on trypsin- and thrombin-induced [^{3}H]-thymidine incorporation into SMC. Cells were incubated with SBTI 10 min prior to stimulation by trypsin or thrombin. Means$\pm$s.e.mean from seven separate experiments. *$P<0.05$ (treatment vs control).

tion (Figure 1). A maximum stimulatory effect was obtained at 2 nM and was equivalent to a 3 fold increase in DNA-synthesis. Higher concentrations of trypsin, i.e., 20 nM, elicited toxic effects as seen by a reduction in cell number and viability by 14 and 18% of control, respectively (not shown) and a decrease in [^{3}H]-thymidine incorporation to only 17$\pm$3% of control ($n=5$). To clarify whether the proteolytic activity of trypsin was a prerequisite for its mitogenic effect, SMC were incubated with soybean trypsin inhibitor prior to stimulation by trypsin. At a concentration of 10 nM, the protease inhibitor completely prevented trypsin-induced mitogenesis, whereas the mitogenic effect of thrombin was not affected (Figure 2). Soybean trypsin inhibitor did not influence [^{3}H]-thymidine incorporation on its own: 94$\pm$4% of control ($n=6$, $P>0.05$). The mitogenic response to 2 nM trypsin was comparable to that of 10 nM thrombin (Figure 3).

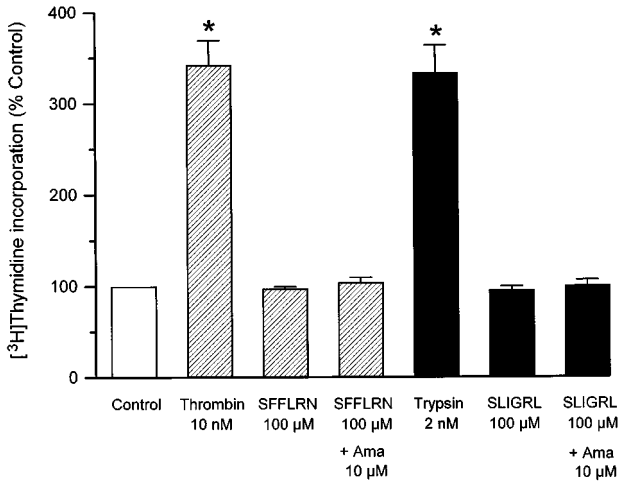

Figure 3 [^{3}H]-Thymidine incorporation into SMC. Quiescent SMC were stimulated by thrombin, trypsin and SFFLRN or SLIGRL in the absence or presence of amastatin (Ama) for 24 h. After pulse-labelling with [^{3}H]-thymidine (2 μCi ml^{-1}) during the last 4 h of the incubation period [^{3}H]-thymidine incorporation was determined. Means$\pm$s.e.mean from 5–7 separate experiments; *$P<0.05$ (treatment vs control).

Effects of SFFLRN and SLIGRL on [^{3}H]-thymidine incorporation

Even at a high concentration (100 μM), neither SFFLRN, derived from the bovine thrombin receptor, nor SLIGRL increased [^{3}H]-thymidine incorporation into SMC (Figure 3). Identical results were obtained when SMC were stimulated with 100 μM SLIGKV: 100$\pm$7% of control ($n=7$; $P>0.05$). To exclude a possible proteolytic degradation of the peptides by cell-derived peptidases during the 24 h incubation period, additional experiments were carried out in the presence of the aminopeptidase inhibitor amastatin (10 μM). Again, no mitogenic effect was observed (Figure 3). Amastatin did not influence [^{3}H]-thymidine incorporation on its own: 102$\pm$5% of control ($n=8$; $P>0.05$).

Mobilization of cytosolic Ca^{2+}

Stimulation of SMC with trypsin (40 nM) resulted in a transient rise in cytosolic Ca^{2+} (Figure 4, upper panel). A second exposure of the cells to the enzyme did not evoke a further Ca^{2+} signal (not shown). When thrombin (15 nM) was added after a prior challenge with trypsin, a significant Ca^{2+} response was produced (Figure 4, upper panel) which was only slightly attenuated compared to the Ca^{2+} response obtained after the first challenge with thrombin (not shown). Similar, after a preceding stimulation with SLIGRL (100 μM) SMC were still responsive to thrombin (Figure 4, middle panel). In contrast, when the SMC were stimulated with SLIGRL the Ca^{2+} response to subsequent addition of trypsin was completely abolished (Figure 4, lower panel).

Activation of NFκB

Stimulation of SMC by trypsin (2 nM) caused a significant, time-dependent activation of NFκB. Maximum activation was obtained after 1–2 h according to immunoblotting of the p65 NFκB subunit. The specific PAR-2 agonist, SLIGRL (100 μM), induced the translocation of NFκB into the nucleus with a comparable time course. Thrombin (10 nM) also

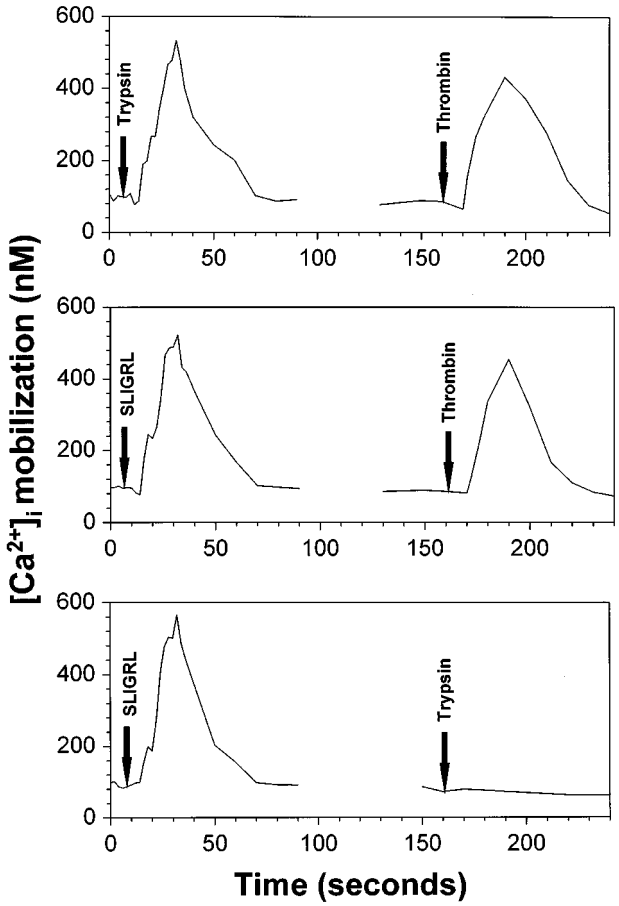

Figure 4 Mobilization of intracellular Ca^{2+} in SMC after stimulation with PAR-1 and PAR-2 agonists. SMC were loaded with fluo-4-acetoxymethylester and stimulated with SLIGRL (100 μM), trypsin (40 nM) or thrombin (15 nM) for the times indicated. Before SMC were challenged with the next stimulant a short washout was performed. Similar data were obtained in at least three independent experiments.

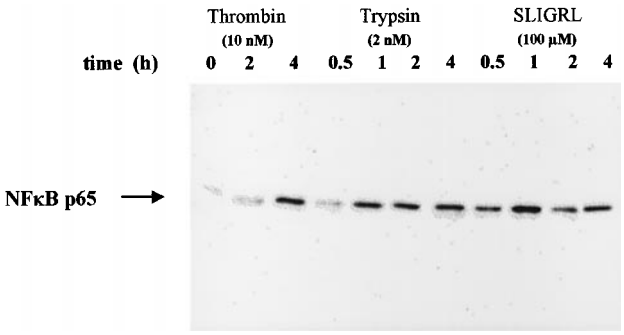

Figure 5 Time-dependent activation of NFκB by thrombin, trypsin and SLIGRL. Activation of NFκB was measured by detection of the p65 subunit in nuclear extracts of SMC by immunoblotting. The presented experiment is representative for three independent experiments with similar results.

activated NFκB; however, maximum activation was detected only after 4–5 h (Figure 5). Densitometric analysis (Scion Image, Scion Corporation, Frederick, U.S.A.) of three different experiments revealed a similar degree of NFκB activation by trypsin and SLIGRL after 2 h (3.2 ± 1.7 and 4.2 ± 1.6 fold increase) and for thrombin after 4 h (3.6 ± 1.5 fold increase) of stimulation.

Discussion

Apart from the thrombin receptors (PAR-1, PAR-3, PAR-4) until now PAR-2 is the only other known receptor which is activated by a proteolytic mechanism. PAR-2 is stimulated both by low concentrations of trypsin and the peptide SLIGRL, representing the sequence of the new N-terminus of the receptor. Thrombin is unable to activate PAR-2. Likewise, SLIGRL specifically activates PAR-2 but fails to activate PAR-1 (Nystedt *et al*., 1994; Blackhart *et al*., 1996). Thus, these compounds provide suitable tools to study the existence of PAR-1 and PAR-2 and their coupling to intracellular signalling. The endogenous ligand(s) for PAR-2 in the vasculature is (are) still under discussion. Human endothelial cells were found to express trypsinogen-2 mRNA and trypsin *in vitro* and *in vivo* (Koshikawa *et al*., 1997). Thus, it is possible that endothelial cell-derived trypsin activates PAR-2 in SMC and perhaps other cells. Another candidate is tryptase, released from activated mast cells (Fox *et al*., 1997; Molino *et al*., 1997a).

This study demonstrates that PAR-2 is functionally active in coronary artery SMC and mediates a mitogenic response. Trypsin increased [^{3}H]-thymidine incorporation into the SMC at low nanomolar concentrations. Trypsin (2 nM) caused a mitogenic effect comparable to that of 10 nM thrombin. This action was not further increased at higher trypsin concentrations. Trypsin-induced mitogenesis was inhibited by soybean trypsin inhibitor indicating that the proteolytic activity of the enzyme was required for its mitogenic effect.

Bono *et al*. (1997) have recently shown that trypsin and thrombin at 10 nM stimulated proliferation of human aortic SMC about 2 fold. This action was seen after 24 h. Similar effects were found with 1 μM SFLLRN or SLIGRL, respectively (Bono *et al*., 1997). In contrast, we did not observe cell proliferation when bovine coronary SMC were stimulated with thrombin (10 nM) or trypsin (2 nM) for the same period of time, i.e. 24 h. In our previous studies stimulation of these SMC by thrombin (10 nM) for 48 h increased cell number by 20% (Zucker *et al*., 1998). In the present experiments, neither SLIGRL nor SFFLRN (each 100 μM) increased [^{3}H]-thymidine incorporation. McNamara *et al*. (1995) suggested that human thrombin receptor-activating peptide-induced proliferation of SMC may be species-specific. In the present experiments, bovine SMC were stimulated by SFFLRN derived from the bovine thrombin receptor. Therefore, species-specific differences can be excluded as a possible cause for the missing mitogenic response to the peptide.

In the literature conflicting results concerning the ability of trypsin to activate PAR-1 and PAR-2 are reported. Trypsin did not activate the thrombin receptor expressed in *Xenopus* oocytes (Blackhart *et al*., 1996). In endothelial cells or COS-1 cells transfected with PAR-1 or PAR-2, trypsin was found to activate both receptors (Molino *et al*., 1997b). To clarify this issue for coronary SMC, we have studied desensitization of cytosolic Ca^{2+} mobilization after the activation of PAR-1 and PAR-2. Both receptors are coupled to activation of phospholipase C, the generation of inositol 1,4,5-trisphosphate and the release of Ca^{2+} from intracellular stores and both receptors are desensitized by similar mechanisms (Böhm *et al*., 1996a; Molino *et al*., 1997b). Stimulation of the SMC with trypsin or SLIGRL resulted in a transient rise in cytosolic Ca^{2+}. The Ca^{2+} response to thrombin in SMC preexposed to trypsin or SLIGRL was only slightly attenuated. In contrast, after the stimulation of SMC by SLIGRL the Ca^{2+} response to trypsin was completely abolished indicating homologous

desensitization. These data provide clear evidence that trypsin and SLIGRL activated a common receptor, PAR-2.

It has been shown that activation of the thrombin receptor in vascular SMC activates the transcription factor, NFκB (Nakajima *et al*., 1994; Bretschneider *et al*., 1997). Trypsin also caused a significant time-dependent activation of NFκB. A similar time-course of NFκB activation was observed after stimulation of SMC by SLIGRL, suggesting that both agonists acted *via* PAR-2. This hypothesis is additionally supported by the finding that activation of NFκB by thrombin and thrombin receptor activating peptide (Bretschneider *et al*., 1997) was detected significantly later. In agreement with these data, SLIGRL stimulated MAP kinase in rat aortic SMC with a time course that closely resembled activation by trypsin. Moreover, when SMC were exposed to SLIGRL a desensitization of MAP kinase activation was observed after subsequent stimulation with trypsin, but not with thrombin (Belham *et al*., 1996).

In conclusion, the present study suggests that, in addition to PAR-1, PAR-2 is present in bovine coronary SMC and mediates a mitogenic response. As shown for thrombin receptor activating peptide (Bretschneider *et al*., 1997), SLIGRL activated NFκB but did not induce a mitogenic effect. This confirms the previous finding that activation of the transcription factor NFκB does not predict a mitogenic response (Bretschneider *et al*., 1997). It is suggested that the induction of mitogenesis *via* both PAR-1 and PAR-2 requires activation of additional receptors and/or signal transduction pathways. PAR-2 mRNA was also detected in human coronary SMC (Molino *et al*., 1996). Thus, activation of PAR-2 may contribute to proliferation of SMC in coronary vessels subsequent to vascular injury in man.

The sequence of the bovine thrombin receptor-activating peptide (SFFLRN) was generously provided prior to publication of the complete bovine thrombin receptor sequence by Dr Ute Reuning (München, Germany). The authors are grateful to Marlies Laube and Christine Machunsky for competent technical assistance. This study was supported by the Verbund für Klinische Forschung of the Friedrich-Schiller-Universität Jena (FS 5, Projekt 4).

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