British Journal of Pharmacology (2001) 134, 815–826 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00

www.nature.com/bjp

Mechanism of trypsin-induced endothelium-dependent vasorelaxation in the porcine coronary artery

[1]Tetsuzo Nakayama, [1]Katsuya Hirano, [1]Junji Nishimura, [2]Shosuke Takahashi & *,[1]Hideo Kanaide

[1]Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and [2]Department of Anesthesiology and Critical Care Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

1 To investigate the mechanism underlying the trypsin-induced endothelium-dependent relaxation, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension development of smooth muscle were simultaneously monitored in the porcine coronary artery, and $[Ca^{2+}]_i$ of *in situ* endothelial cells were monitored in the porcine aortic valvular strips, using fura-2 fluorometry.

2 During the contraction induced by 30 nM U46619, a thromboxane A_2 analogue, 100 nM trypsin induced a rapid transient significant decrease in both $[Ca^{2+}]_i$ (from 67.9 ± 5.1 to $15.7 \pm 4.4\%$) and tension (from 97.5 ± 9.2 to $16.8 \pm 3.5\%$) of smooth muscle only in the presence of endothelium (100% level was assigned to the level obtained with the 118 mM K^+-induced contraction). $[Ca^{2+}]_i$ and the tension thus returned to the levels prior to the application of trypsin by 5 and 10 min, respectively.

3 The initial phase of this relaxation was partly inhibited by 100 μM N^{ω}-nitro-L-arginine (L-NOARG), and was completely inhibited by L-NOARG plus 40 mM K^+ or L-NOARG plus 100 nM charybdotoxin and 100 nM apamin, while the late phase of the relaxation was inhibited by L-NOARG alone.

4 Trypsin induced a transient $[Ca^{2+}]_i$ elevation in the endothelial cells mainly due to the Ca^{2+} release from the intracellular stores, at the concentrations (1–100 nM) similar to those required to induce relaxation.

5 In conclusion, trypsin induced an elevation in $[Ca^{2+}]_i$ mainly due to Ca^{2+} release in endothelial cells, and thereby caused endothelium-dependent relaxation. The early phase of relaxation was due to nitric oxide and hyperpolarizing factors, while the late phase was mainly due to nitric oxide in the porcine coronary artery.

British Journal of Pharmacology (2001) **134,** 815–826

Keywords: Trypsin; porcine coronary artery; vasorelaxation; endothelial cells; protease-activated receptor; nitric oxide; endothelium-derived hyperpolarizing factor

Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle medium; EDHF, endothelium-derived hyperpolarizing factor; fura-2/AM, acetoxymethyl ester form of fura-2; L-NOARG, N^{ω}-nitro-L-arginine; NO, nitric oxide; p-APMSF, 4-amidino-phenylmethane-sulphonyl fluoride; PAR, protease-activated receptor; PAR2-AP, PAR2-activating peptide; PSS, physiological salt solution

Introduction

Trypsin is a protease and one of the digestive enzymes excreted from the pancreas. Trypsinogen is a major component of pancreatic juice and, once activated in the intestinal lumen by enterokinase, trypsin digests dietary proteins and activates other zymogens (Kong *et al*., 1997). In addition to its role as a digestive enzyme, trypsin has also been shown to exert various cellular effects including endothelium-dependent relaxation and myometrial contraction (Hwa *et al*., 1996; Kawabata *et al*., 1999). The cellular effects of trypsin have recently been shown to be mediated by protease-activated receptor (PAR) which is a unique family of G-protein coupled receptor (Dery *et al*., 1998). The activation of PAR is initiated by the proteolytic cleavage of its extracellular domain to create the new N-terminus that functions as a tethered ligand (Dery *et al*., 1998). Four members of PAR, PAR-1, PAR-2, PAR-3 and PAR-4, have so far been isolated and cloned (Ishihara *et al*., 1997; Kahn *et al*., 1998; Nystedt *et al*., 1994; Vu *et al*., 1991; Xu *et al*., 1998). PAR-1, PAR-3 and PAR-4 serve as the receptor for thrombin, while PAR-2 and PAR-4 serve as the receptor for trypsin (Nystedt *et al*., 1994; Xu *et al*., 1998). The synthetic peptides corresponding to the tethered ligand regions have been shown to activate PARs without cleavage, and have also been successfully used as a useful tool to investigate the functional role of PARs in the cellular effects of thrombin and trypsin (Dery *et al*., 1998).

Trypsin has been reported to cause endothelium-dependent vasorelaxation in various types of blood vessels including the porcine coronary artery (Hamilton & Cocks, 2000; Hamilton *et al*., 1998; Hwa *et al*., 1996; Sobey *et al*., 1999). Hamilton & Cocks (2000) have suggested that both NO and EDHF involved in trypsin-induced relaxation in the porcine coronary artery. However, the temporal relative contribution

*Author for correspondence at: Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; E-mail: kanaide@molcar.med.kyushu-u.ac.jp

of NO and EDHF to the trypsin-induced relaxation and the intracellular signal transduction in endothelial cells that regulate the trypsin-induced relaxation remains to be elucidated. In the present study, using front-surface fluorometry, we investigated the mechanism of trypsin-induced endothelium-dependent vasorelaxation, especially with respect to Ca^{2+} signalling in both smooth muscle and endothelial cells. We simultaneously determined the effect of trypsin on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of smooth muscle and tension development in the fura-2-loaded strips of the porcine coronary artery. The temporal change in the contribution of possible mediators of the endothelium-dependent relaxation, nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin, were examined using their specific inhibitors. We also determined the effect of trypsin on $[Ca^{2+}]_i$ of *in situ* endothelial cells in strips of the porcine aortic valve.

Methods

Tissue preparation

Coronary arterial strips: The segments of the left circumflex arteries (2–3 cm from the origin) were excised from the porcine hearts at a local slaughterhouse immediately after the animals had been slaughtered. The segments were brought back to the laboratory in ice-cold normal physiological salt solution (PSS). After removing the adventitia, the segments were opened longitudinally, and cut into circular strips (approximately 1 mm wide, 5 mm long, and 0.1 mm thick). Care was taken to avoid damaging the endothelial cells. The strips without endothelium were prepared by rubbing off the inner surface with a cotton swab.

Aortic valvular strips: Porcine aortic valves were obtained at a local slaughterhouse. The valve leaflets were cut into strips in an axial direction (approximately 3 mm wide, 5 mm long, and 0.18 mm thick). Care was taken to avoid damaging the endothelial cells of the strips.

Simultaneous measurement of $[Ca^{2+}]_i$ and tension in the coronary arterial strips

The arterial strips with and without endothelium were loaded with fura-2 by incubating for 3 h at 37°C in gassed (5% CO_2 and 95% O_2) Dulbecco's modified Eagle medium (DMEM) containing 5% foetal bovine serum and 25 μM fura-2/AM (acetoxymethyl ester form of fura-2) as previously described (Kuroiwa *et al*., 1993). The strips were then washed to remove fluorescent dye remaining in the extracellular space, and equilibrated in normal PSS for 1 h at room temperature. The strips were mounted vertically to a force transducer, TB-612T (Nihon Koden, Japan), in a quartz organ bath (37°C) filled with normal PSS gassed with 5% CO_2 and 95% O_2. The strips were stimulated with 118 mM K^+ four times during the equilibration period, and resting tension was adjusted to 200 mg in normal PSS (5.9 mM K^+). Two hundred mg is minimum resting tension to obtain the maximal tension development with 118 mM K^+. The response of each strip to 118 mM K^+-depolarization was then recorded as a control response before starting the experimental protocols. The change in $[Ca^{2+}]_i$ of smooth muscle in the vascular strips monitored with a front-surface fluorometer, CAM-OF-3, (JASCO, Tokyo, Japan), as previously described (Kanaide, 1999). The fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and their ratio (F340/F380) were continuously monitored. The fluorescence ratio and tension development were expressed as a percentage, assigning the values in normal PSS (5.9 mM K^+) and those in 118 mM K^+-PSS to be 0 and 100%, respectively. When the effects of N^{ω}-nitro-L-arginine (L-NOARG), indomethacin, apamin or charybdotoxin on the trypsin-induced relaxation were examined, the control response to 118 mM K^+-depolarization were recorded in the presence of these regents, except for Figure 5, where the levels of $[Ca^{2+}]_i$ and tension at rest and those obtained just prior to the application of trypsin during the precontraction under the each protocol were designated as 0 and 100%, respectively. L-NOARG, indomethacin, apamin and charybdotoxin, either separately or in combination, were applied 30 min before precontractions were induced by U46619 or 118 mM K^+. Trypsin was applied 10 min after the initiation of the precontraction. We previously showed that fura-2 fluorescence of the vascular strips recorded as above exclusively originated from smooth muscle cells despite the presence of endothelium, and the fluorescence from endothelial cells was, if any, negligible (Kuroiwa *et al*., 1993). All simultaneous measurement of $[Ca^{2+}]_i$ and force were carried out at 37°C.

Measurement of $[Ca^{2+}]_i$ in endothelial cells in situ

The aortic valvular strips were loaded with fura-2 by incubating in DMEM containing 50 μM fura-2/AM, 1 mM probenecid, 5% foetal bovine serum for 90 min at 37°C. Probenecid was added to prevent the leakage of fluorescent dye (Di Virgilio *et al*., 1989). After loading with fura-2, strips were washed and equilibrated in normal PSS for 1 h at room temperature. The strips were mounted vertically in a quartz organ bath filled with normal PSS. Changes in $[Ca^{2+}]_i$ in endothelial cells of the valvular strips were monitored as described above. We previously reported that endothelial cells actively excrete fura-2 at 37°C (Kuroiwa *et al*., 1995). Fluorometry was thus performed at 25°C to prevent any leakage of fluorescent dye in the aortic valvular endothelial cells. The fluorescence intensities (500 nm) at 340 and 380 nm excitation and their ratio were continuously monitored. The control response was obtained by exposing the strip to 10 μM ATP for 1 min at the beginning of the experimental protocol. The fluorescence ratio was expressed as a percentage, assigning the value obtained in normal PSS and that obtained with 10 μM ATP to be 0 and 100%, respectively.

Drugs and solutions

The normal PSS was composed of (mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5, and aerated with a mixture of 5% CO_2 and 95% O_2, with the resulting pH determined to be 7.4. High K^+-PSS was prepared by equimolar substitution of KCl for NaCl. Fetal bovine serum was obtained from Sanko Junyaku (Tokyo, Japan). DMEM was purchased from Life Technologies (Rockville, MD, U.S.A.). Trypsin (10,900 units mg^{-1}, bovine pancreas), probenecid, p-APMSF, charybdotoxin and

apamin were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium salt of ATP was purchased from Boehringer Mannheim (Germany). Fura-2/AM was obtained from Dojindo (Kumamoto, Japan). L-NOARG and indomethacin were purchased from Aldrich Chemical (U.S.A.) and Wako (Osaka, Japan), respectively. PAR2-AP (SLIGRL) were purchased from BACHEM (Bubendorf, Switzerland). $NiCl_2$ were obtained from Katayama Chemical (Osaka, Japan). Substance P was purchased from Peptide Inc. (Osaka, Japan).

Data analysis

The data are the mean ± s.e.mean of the number of experiments as indicated. Significant differences were statistically analysed by the unpaired Student's *t*-test and by an analysis of variance (ANOVA). A P value of less than 0.05 was considered to be statistically significant. The concentrations for a half maximal response (EC_{50} or IC_{50}) were determined by fitting the concentration-response curves to a four-parameter logistic model (Delean *et al*., 1978). All data were collected at a sampling rate of 17 Hz using a computerized data acquisition system (MacLab, Analog Digital Instruments, Australia: Macintosh, Apple computer, U.S.A.)

Results

Trypsin-induced endothelium-dependent relaxation of the porcine coronary arterial strips

U46619, a thromboxane A_2 analogue, induced sustained contractions in the porcine coronary arterial strips with an endothelium (Figure 1). Upon exposure to 30 nM U46619, the $[Ca^{2+}]_i$ and tension of smooth muscle rapidly increased reaching steady state within 10 min ($[Ca^{2+}]_i = 59.5 \pm 7.8\%$, tension = $102.5 \pm 12.5\%$; $n = 8$), and remained at this level for more than 15 min. When 100 nM trypsin was applied during the steady state of U46619-induced contraction, a rapid and transient decrease in $[Ca^{2+}]_i$ and tension was observed (Figure 1A). $[Ca^{2+}]_i$ decreased significantly from 67.9 ± 5.1 to $15.7 \pm 4.4\%$ ($P < 0.05$) (Figure 1E), and the tension decreased significantly from 97.5 ± 9.2 to $16.8 \pm 3.5\%$ ($P < 0.05$) (Figure 1E). After the maximal decrease, $[Ca^{2+}]_i$ returned to the level seen during the U46619-induced precontraction within 5 min (Figure 1E), while tension required slightly longer period for recovery. The level of tension obtained at 10 min after application did not significantly differ from that obtained just prior to the application of trypsin (Figure 1E). As a result, the late phase of the relaxation seen at 5 min and thereafter was not accompanied by a decrease in $[Ca^{2+}]_i$ (Figure 1E). Trypsin pretreated with 30 μM 4-amidino-phenylmethane-sulfonyl fluoride (p-APMSF) failed to induce a relaxation in the strips with endothelium, while the subsequent application of the untreated trypsin caused relaxation (Figure 1B). The removal of the endothelium had no significant ($P < 0.05$) effect on the U46619-induced precontraction ($[Ca^{2+}]_i = 62 \pm 8.0\%$, tension = $105.5 \pm 18.5\%$, $n = 5$), but it completely abolished the trypsin-induced relaxation (Figure 1C). Trypsin had no effect on $[Ca^{2+}]_i$ and tension during the U46619-induced precontraction.

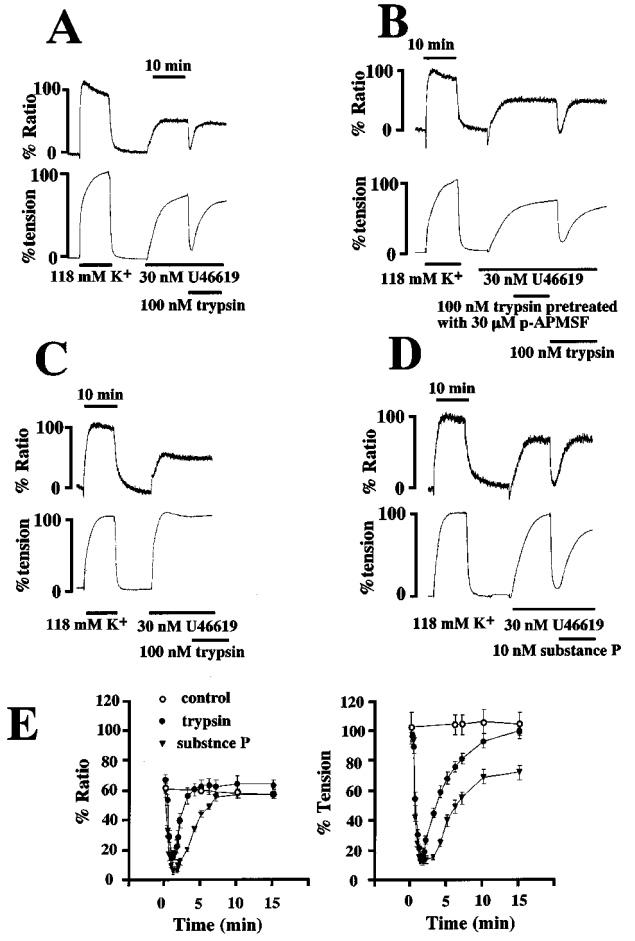

Figure 1 Endothelium-dependent relaxation induced by trypsin in the porcine coronary arteries. (A–D) Representative recordings of $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) of smooth muscle showing the trypsin-induced relaxation in the strips with endothelium (A), the effect of trypsin pretreated with 30 μM 4-amidino-phenylmethane-sulfonyl fluoride (p-APMSF) in the strips with endothelium (B), the abolishment of the trypsin-induced relaxation in the strips without endothelium (C) and the Substance P-induced relaxation in the strips with endothelium (D). In (B), the p-APMSF-pretreated trypsin failed to induce a relaxation, while the subsequent application of the un-treated trypsin caused a relaxation. (E) Summary of time course of the endothelium-dependent changes in $[Ca^{2+}]_i$ and tension induced by 100 nM trypsin ($n = 5$) and 10 nM Substance P ($n = 5$). Time course of the control contraction induced by U46619 without trypsin treatment was also shown ($n = 5$). The data represent the mean ± s.e.mean. The level of $[Ca^{2+}]_i$ and tension at rest and during the sustained phase of the contraction induced by 118 mM K^+-depolarization were assigned values of 0 and 100%, respectively.

Substance P induced an initial rapid relaxation and a subsequence sustained relaxation in the porcine coronary artery (Figure 1D). The $[Ca^{2+}]_i$ level decreased rapidly reaching the maximal level in 1 min, and then returned within 10 min to the level seen just prior to the application of substance P. The tension also rapidly decreased and reached a maximal level at 1 min, and then increased to the level lower than that seen just prior to the application of substance P. The initial phase of the relaxation was thus associated with the decrease in $[Ca^{2+}]_i$, while the sustained phase was not associated with the decrease in $[Ca^{2+}]_i$. The maximal decreases in $[Ca^{2+}]_i$ and tension obtained with 10 nM

substance P was comparable to those obtained with 100 nM trypsin, while the sustained relaxation obtained with substance P was significantly greater than that obtained with trypsin (Figure 1E). We previously reported that the initial phase of the substance P-induced relaxation was mediated by NO and EDHF, while the sustained phase of the relaxation was mainly mediated by NO (Kuroiwa *et al*., 1995).

The endothelium-dependent decreases in $[Ca^{2+}]_i$ and tension induced by trypsin were concentration-dependent (Figure 2A,B). A significant decrease in $[Ca^{2+}]_i$ was induced by 3 nM and higher concentrations, while a significant decrease in tension was induced by 1 nM and higher concentrations. The maximal decreases (E_{max}) in $[Ca^{2+}]_i$ ($15.7 \pm 4.4\%$, $n=4$) and tension ($16.8 \pm 4.4\%$, $n=4$) were both obtained at 100 nM. A further increase in the trypsin concentration did not cause further any decrease. The concentrations of trypsin required to induced a 50% maximal decrease (EC_{50}) in $[Ca^{2+}]_i$ and tension were 4.2 ± 1.3 and 2.7 ± 0.6 nM ($n=4$), respectively. On the other hand, in the absence of an endothelium, trypsin (up to 10 μM) had no effect on $[Ca^{2+}]_i$ and tension of smooth muscle during the U46619-induced precontraction (Figure 2A,B) and at a resting state (data not shown). We also determined the concentration-response curve for the trypsin-induced relaxation in the presence of 100 nM L-NOARG (Figure 2A,B). In the presence of L-NOARG, the significant decrease in both $[Ca^{2+}]_i$ and tension were observed at 10 nM and higher concentrations, and the maximal effect obtained with 100 nM trypsin was attenuated. E_{max} for the decrease in $[Ca^{2+}]_i$ and tension were 28.2 ± 5.2 and $53.1 \pm 4.9\%$ ($n=4$), and EC_{50} for the decrease in $[Ca^{2+}]_i$ and tension were 4.3 ± 1.1 and 3.3 ± 0.9 nM ($n=4$), respectively.

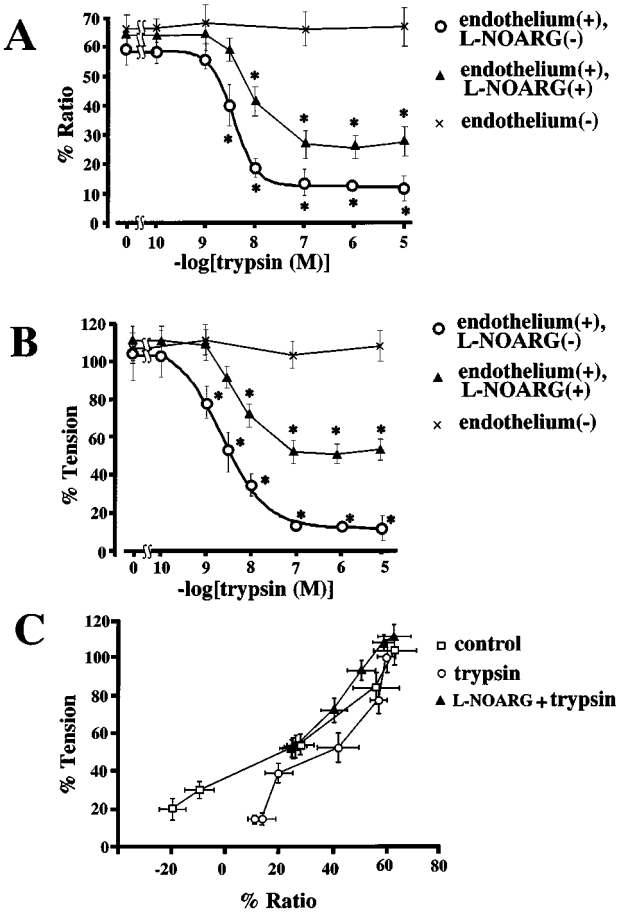

Figure 2 The concentration-response curves of $[Ca^{2+}]_i$ and tension, and the $[Ca^{2+}]_i$-tension relationship during the trypsin-induced relaxations in porcine coronary artery. (A,B) Concentration-dependent effects of trypsin were evaluated at the maximal decrease in $[Ca^{2+}]_i$ (A) and tension (B) during the U46619-induced contraction in arterial strips with and without endothelium. In the presence of endothelium, the effect of trypsin was examined both in the presence and absence of L-NOARG. The level of $[Ca^{2+}]_i$ and tension at rest and during the sustained phase of the contraction induced by 118 mM K^+-depolarization were assigned to be 0 and 100%, respectively. Data are the mean ± s.e.mean ($n=4$). *$P<0.05$ compared with the control value. (C) The $[Ca^{2+}]_i$-tension relationship during the trypsin-induced relaxation, reconstructed from the data shown in (A) and (B). The control $[Ca^{2+}]_i$-tension relationship was obtained based on the contraction induced by the cumulative application of extracellular Ca^{2+} (0.0125, 0.25, 0.5, 1.25 and 2.5 mM) during stimulation with 30 nM U46619 in the absence of trypsin. The data are the mean ± s.e.mean ($n=4$).

The $[Ca^{2+}]_i$-tension relationship of the trypsin-induced relaxation was evaluated by plotting the levels of tension as a function of $[Ca^{2+}]_i$ (Figure 2C). The $[Ca^{2+}]_i$-tension relation curve for the maximal relaxation induced by trypsin was reconstructed from data shown in Figure 2A,B. The control $[Ca^{2+}]_i$-tension relationship of the contraction induced by U46619 was constructed from the levels of $[Ca^{2+}]_i$ and tension obtained during the contraction induced by the cumulative and stepwise applications of extracellular Ca^{2+} (0.0125, 0.25, 0.5, 1.25 and 2.5 mM) under the stimulation with 30 nM U46619 (Figure 2C). This procedure caused stepwise increase in $[Ca^{2+}]_i$ and tension when the extracellular Ca^{2+} concentration was increased in a step wise manner (data not shown). The $[Ca^{2+}]_i$-tension relationship of the maximal relaxation overlapped with the control relation curve at 0.1 and 1 nM trypsin, and shifted slightly downward at 3 and 10 nM and markedly 100 nM and 1 μM (Figure 2C). On the other hand, the $[Ca^{2+}]_i$-tension relationship of the maximal relaxation obtained in the presence of L-NOARG overlapped with the control relation curve at all concentrations examined.

Effects of L-NOARG, indomethacin and depolarization on the trypsin-induced relaxation in the coronary arterial smooth muscle

The relative contribution of NO, prostacyclin and EDHF in the trypsin-induced relaxation, was evaluated by examining the effects of 100 μM L-NOARG, 10 μM indomethacin and high K^+-depolarization, respectively, as previously reported (Mizuno *et al*., 1998). Representative recordings are shown in Figure 3. A summary of the time course of changes in $[Ca^{2+}]_i$ and tension of five independent experiments is shown in Figure 4. In the presence of L-NOARG, 100 nM trypsin decreased $[Ca^{2+}]_i$ and tension of the 30 nM U46619-induced contraction from 55.8 ± 1.5 to $30.5 \pm 2.0\%$ (at the maximal decrease) and from 98.1 ± 4.6 to $55.0 \pm 5.7\%$ (at the maximal decrease), respectively ($n=5$) (Figures 3A and 4). The maximal decrease in $[Ca^{2+}]_i$ and tension in the presence of L-NOARG was significantly smaller than that in the absence of L-NOARG ($P<0.05$). After the maximal decrease, $[Ca^{2+}]_i$ returned to the precontraction level within 5 min (Figure 4), as observed in the absence of L-NOARG (Figure 1E). However, tension returned to the

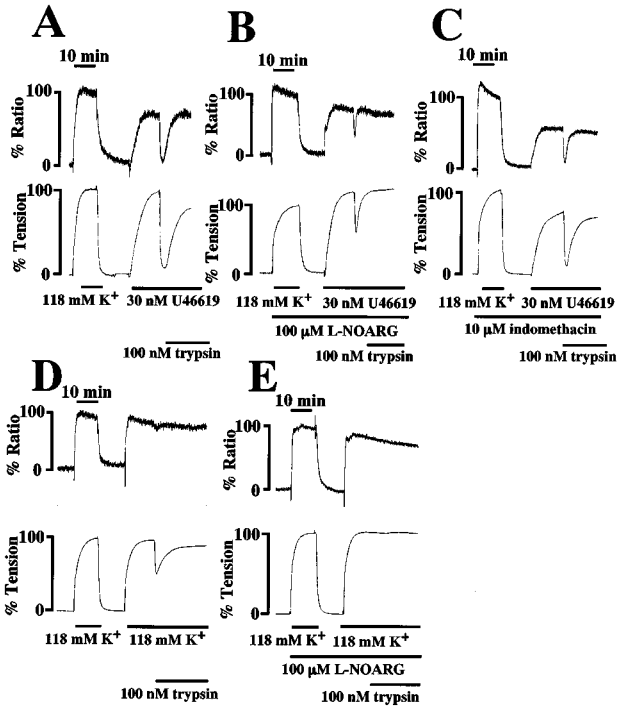

Figure 3 The effects of L-NOARG, indomethacin and depolarization on the trypsin-induced relaxation in the porcine coronary artery. Representative recordings of $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) of smooth muscle showing the effects of trypsin during the contractions induced by U46619 in the absence (A), and in the presence of 100 μM L-NOARG (B) and 10 μM indomethacin (C), and during the contractions induced by 118 mM K^+-depolarization in the absence (D) and the presence of 100 μM L-NOARG (E). The control response to 118 mM K^+-depolarization was recorded at the beginning of each experimental protocol to obtain the 100% level of $[Ca^{2+}]_i$ and tension. Similar results were obtained in five independent experiments.

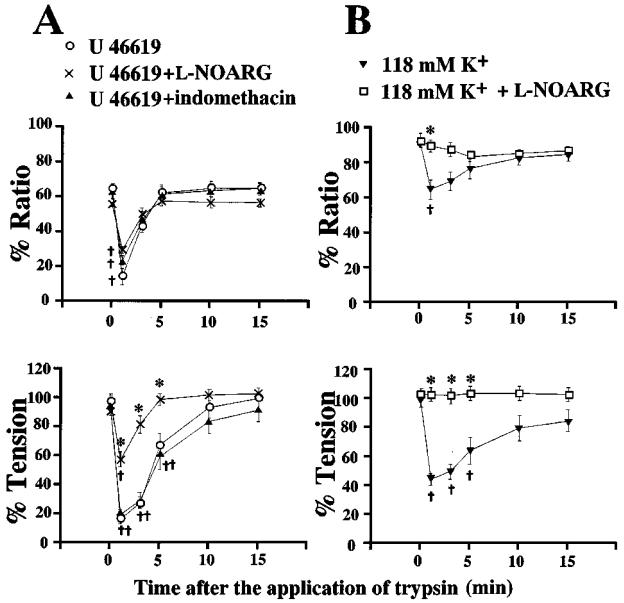

Figure 4 Summary of the time course of the effects of L-NOARG, indomethacin and depolarization on the $[Ca^{2+}]_i$ (A) and tension (B) during the trypsin-induced relaxations. The trypsin-induced relaxation was evaluated in the time course of the experimental protocols (0, 1, 3, 5, 10, 15 min) as shown in Figure 3. The levels of $[Ca^{2+}]_i$ and tension at the rest and those obtained during the 118 mM K^+-depolarization in each experimental protocol were designated as 0 and 100%, respectively. Precontractions were induced by 30 nM U46619 or 118 mM K^+-depolarization in the absence or presence of 100 μM L-NOARG or 10 μM indomethacin. When the effects of L-NOARG and indomethacin were examined, the reference contraction was induced in their presence. $*P<0.05$ compared to the value obtained with the trypsin-induced control relaxation in the absence of L-NOARG and indomethacin; $\dagger P<0.05$ compared to the levels obtained during precontraction. The data are the mean$\pm$s.e.mean ($n=5$).

precontraction level by 5 min in the presence of L-NOARG, which was significantly earlier than in its absence ($P<0.05$), and the level of tension obtained at 5 min after the application of trypsin (99.0$\pm$4.0%) did not significantly ($P>0.05$) differ from the precontraction level (Figure 4). On the other hand, pretreatment with 10 μM indomethacin had no effect on the decrease in $[Ca^{2+}]_i$ and tension induced by 100 nM trypsin during 30 nM U46619-induced contraction (Figures 3C and 4).

The trypsin-induced relaxation was next examined during the contraction induced by 118 mM K^+-depolarization (Figure 3D). The tension development induced by 118 mM K^+ was similar to that obtained with 30 nM U46619, while $[Ca^{2+}]_i$ elevation was significantly greater. During the steady state of contraction induced by 118 mM K^+-depolarization, 100 nM trypsin decreased $[Ca^{2+}]_i$ and tension from 87.0$\pm$2.8 to 65.2$\pm$5.8% and from 99.2$\pm$4.7 to 41.8$\pm$3.8%, respectively ($n=5$) (Figure 4). As a result, the extent of the decrease in tension induced by trypsin during the 118 mM K^+-induced contraction was significantly ($P<0.05$) smaller than that during 30 nM U46619-induced contraction. After the maximal decrease, $[Ca^{2+}]_i$ returned to the level just prior to the application of trypsin within 5 min (Figure 4), while tension returned by 10 min. The level of tension (85.0$\pm$7.0%, $n=5$) obtained at 10 min after the application of trypsin did not significantly ($P>0.05$) differ from the level just prior to the application of trypsin (Figure 4). This trypsin-induced transient relaxation during the contraction induced by 118 mM K^+ was completely abolished by treatment with 100 μM L-NOARG (Figures 3E and 4).

The contribution of NO and EDHF to the trypsin-induced relaxation was further investigated as shown in Figure 5. In this experiment, the effects of L-NOARG and elevation of external K^+ concentration on the trypsin-induced relaxation was examined during the U46619-induced sustained contraction in the presence of 10 μM indomethacin. The levels of $[Ca^{2+}]_i$ and tension during the U46619-induced precontraction were augmented by elevating the external K^+ concentration. In this case, therefore the 100% level of $[Ca^{2+}]_i$ and tension was assigned to the level obtained just before the application of trypsin in these experiments. Indomethacin had no significant effect on the trypsin-induced relaxation as shown in Figures 3C and 4. Figure 5A shows the control trypsin-induced relaxation observed in the presence of indomethacin, and the level of $[Ca^{2+}]_i$ and tension obtained at the maximal decrease was 24.0$\pm$2.8 and 20.0$\pm$3.3% ($n=5$), respectively. Addition of 100 μM L-NOARG to indomethacin partially inhibited this relaxation similarly to that observed with L-NOARG alone as shown in Figures 3B and 4 (data not shown). However, the relaxation observed in

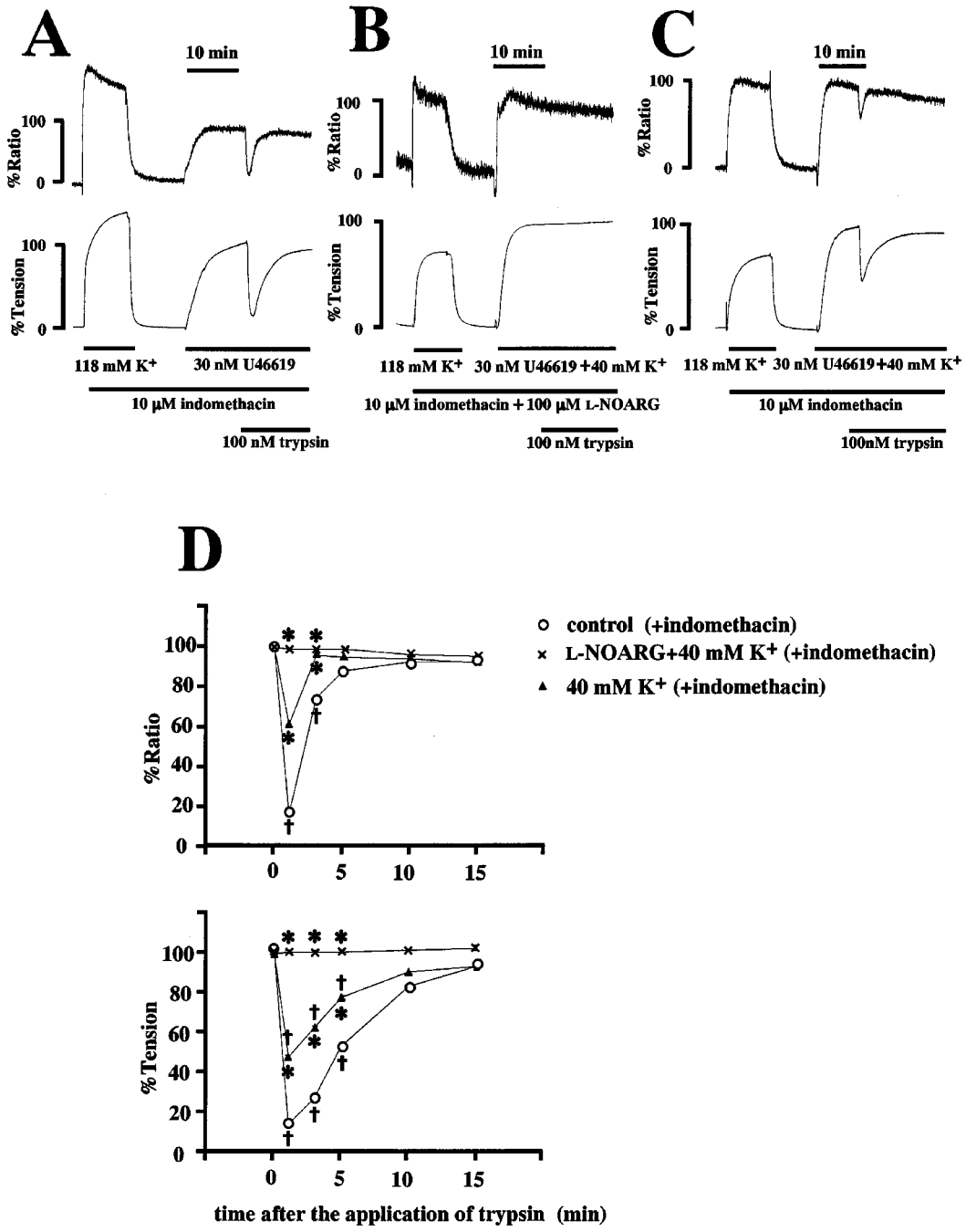

Figure 5 Involvement of NO and EDHF in the trypsin-induced relaxation during the U46619-induced contraction in the porcine coronary artery. (A–C) Representative recordings of the trypsin-induced changes in $[Ca^{2+}]_i$ and tension during the contraction induced by 30 nM U46619 (A) or U46619 plus 40 mM K^+ in the presence (B) and absence (C) of 100 μM L-NOARG. All experiments were performed in the presence of 10 μM indomethacin. (D) Summary of the level of $[Ca^{2+}]_i$ and tension obtained at 1, 3, 5, 10 and 15 min after the application of trypsin as shown in A–C. The levels of $[Ca^{2+}]_i$ and tension at rest and those obtained just prior to the application of trypsin during the precontraction under the each protocol were designated as 0 and 100%, respectively. $^*P<0.05$ compared to the value obtained with the trypsin-induced control relaxation during U46619-induced contraction only in the presence of indomethacin; $\dagger P<0.05$ compared to the levels obtained during precontraction. The data are the mean $\pm$ s.e.mean ($n=5$).

the presence of indomethacin and L-NOARG became smaller as the external K^+ concentration was elevated. When the external K^+ concentration reached 25 mM, trypsin induced significant ($P<0.05$) but attenuated decrease in $[Ca^{2+}]_i$ ($80.0\pm6.0\%$, $n=3$) and tension ($78.2\pm5.0\%$, $n=3$). At 30 mM K^+, trypsin still induced significant ($P<0.05$) but attenuated decrease in $[Ca^{2+}]_i$ ($82.5\pm3.0\%$, $n=3$) and tension ($87.2\pm3.8\%$, $n=3$). However, when the external K^+ concentration reached 40 mM, the relaxation was completely abolished as shown in Figure 5B,D. The removal of 100 μM L-NOARG from this condition caused a re-appearance of a transient relaxation (Figure 5C). However, the maximal

decrease in tension (47.9±1.1%, $n=5$) during this relaxation was significantly ($P<0.05$) smaller than that seen in the control relaxation, while the tension returned to a level just prior to the application of trypsin by 10 min, and the level of tension obtained at 10 min (91.0±0.5%, $n=5$) did not significantly ($P>0.05$) differ from the control relaxation (84.5±1.2%, $n=5$) (Figure 5D).

Effect of K^+ channel blockers on the trypsin-induced L-NOARG-resistant relaxations

The contribution of EDHF to the trypsin-induced L-NOARG-resistant relaxation was further investigated by using K^+ channel blockers as shown in Figure 6. In this experiment, the effects of charybdotoxin and apamin on the trypsin-induced L-NOARG-resistant relaxation were examined during the U46619-induced sustained contraction in the presence of 10 μM indomethacin and 100 μM L-NOARG. Figure 6A shows the control trypsin-induced L-NOARG-resistant relaxation observed in the presence of indomethacin and L-NOARG. The addition of 100 nM charybdotoxin significantly ($P<0.05$) but partially attenuated the reduction of $[Ca^{2+}]_i$ induced by trypsin, while it had no significant ($P>0.05$) effect on the decrease in tension (Figure 6B,E). Similarly, 100 nM apamin significantly ($P<0.05$) attenuated the reduction of $[Ca^{2+}]_i$ induced by trypsin, while having no significant ($P>0.05$) effect on the decrease in tension (Figure 6C,E). However, the combination of 100 nM charybdotoxin and 100 nM apamin completely abolished the trypsin-induced L-NOARG-resistant decrease in $[Ca^{2+}]_i$ and tension during the U46619-induced contraction (Figure 6D,E). During the 118 mM K^+-induced contraction, the combination of charybdotoxin and apamin had no effect on the trypsin-induced relaxation (data not shown). We previously reported that the L-NOARG-resistant component of the substance P-induced relaxation was not inhibited by 1 μM iberiotoxin or 1 mM (d)-tubocurarine (Kuroiwa *et al*., 1995). However, a combination of 100 nM charybdotoxin and 100 nM apamin completely abolished the substance P-induced L-NOARG-resistant relaxation, although charybdotoxin or apamin alone had no significant effect. As a result, the sensitivity of the L-NOARG-resistant component of the trypsin-induced relaxation was similar to that seen with the substance P-induced L-NOARG-resistant relaxation.

Effects of L-NOARG, high K^+ depolarization, and K^+ channel blockers on the concentration-response curves of the trypsin-induced relaxations

We examined the effects of L-NOARG, high K^+ depolarization, and K^+ channel blockers on the relaxations induced by different concentrations of trypsin (Figure 7). In this experiment, we cumulatively applied trypsin from 1 nM to 1 μM, and evaluated the effects of the same treatments as performed in Figures 3–6 on the concentration-response curves of the trypsin-induced decreases in $[Ca^{2+}]_i$ and tension. During the 118 mM K^+-depolarization, trypsin induced a significant ($P<0.05$) decrease in $[Ca^{2+}]_i$ and tension at 3 nM and higher concentrations (Figure 7A). The addition of 100 μM L-NOARG completely abolished the trypsin-induced relaxation in an entire concentration range (Figure 7A). During the 30 nM U46619-induced contraction in the presence of 10 μM indomethacin, trypsin induced a significant ($P<0.05$) decrease in $[Ca^{2+}]_i$ and tension at 3 nM and higher concentrations (Figure 7B). The increase in the external K^+ concentration to 40 mM partly inhibited these trypsin-induced relaxations in an entire concentration range, and the remaining relaxations were completely abolished by the addition of 100 μM L-NOARG (Figure 7B). On the other hand, during the 30 nM U46619-induced contraction in the presence of 10 μM indomethacin and 100 μM L-NOARG, trypsin induced a significant decrease in $[Ca^{2+}]_i$ and tension at 10 nM and higher concentrations (Figure 7C). The addition of either 100 nM charybdotoxin or 100 nM apamin alone had no significant ($P>0.05$) effect on the concentration-response curves (Figure 7C), but their combination completely inhibited the trypsin-induced relaxation in an entire concentration range. As a consequence, these observations obtained with various concentrations of trypsin were consistent with those observed with single dose experiments shown in the Figures 3–6. However, there is one slight discrepancy between Figures 6 and 7. We observed the slight but significant inhibition of the decrease in $[Ca^{2+}]_i$ induced by 100 nM trypsin with either charybdotoxin or apamin alone in Figure 6. On the other hand, we did not see any significant effect of either charybdotoxin or apamin alone on the concentration-response curves of the trypsin-induced decrease in $[Ca^{2+}]_i$. This apparent discrepancy may be due to the variation of the control relaxation or the difference in the method of application of trypsin (single dose application vs cumulative application). Despite this discrepancy, the most important finding common to Figures 6 and 7 is that the combination of charybdotoxin and apamin completely inhibited trypsin-induced relaxation.

Effect of trypsin on $[Ca^{2+}]_i$ in endothelial cells in situ *of the porcine aortic valve*

After recording a reference response to 10 μM ATP in normal PSS, trypsin was applied, which induced a rapid and transient increase in $[Ca^{2+}]_i$ in the valvular strips (Figure 8). A significant $[Ca^{2+}]_i$ elevation was observed at 1 nM and higher concentrations, and the maximal $[Ca^{2+}]_i$ elevation (56.5±7.5%, $n=5$) was obtained at 100 nM. The concentration of trypsin required to induce half the maximal $[Ca^{2+}]_i$ elevation was 10.2±2.5 nM ($n=5$). The concentration required to induce $[Ca^{2+}]_i$ elevation in endothelial cells (Figure 8) was thus similar to that required to induce the endothelium-dependent relaxation in the coronary artery (Figure 2). To rule out the possibility that the inhibition of trypsin-induced relaxation by L-NOARG and 40 mM K^+ was due to the inhibition of $[Ca^{2+}]_i$ transient in endothelial cells, we examined the effect of L-NOARG and 40 mM K^+ on the trypsin-induced $[Ca^{2+}]_i$ transient in endothelial cells. The concentration-response curve for the trypsin-induced $[Ca^{2+}]_i$ elevation obtained in the presence of 100 μM L-NOARG or 40 mM K^+ did not differ from that obtained in the absence of both (Figure 8C), thus indicating that L-NOARG or 40 mM K^+ had no effect on the trypsin-induced $[Ca^{2+}]_i$ elevation in endothelial cells of aortic valve. In the presence of 3 mM $NiCl_2$, trypsin induced a transient elevation of $[Ca^{2+}]_i$ similar to that observed in the absence of $NiCl_2$ (Figure 8B). The peak $[Ca^{2+}]_i$ level induced by 100 nM trypsin in the presence of 3 mM $NiCl_2$ (51.5±7.1%, $n=4$) did not differ from that obtained in its absence (52.5±5.2%, $n=4$) (Figure 8D).

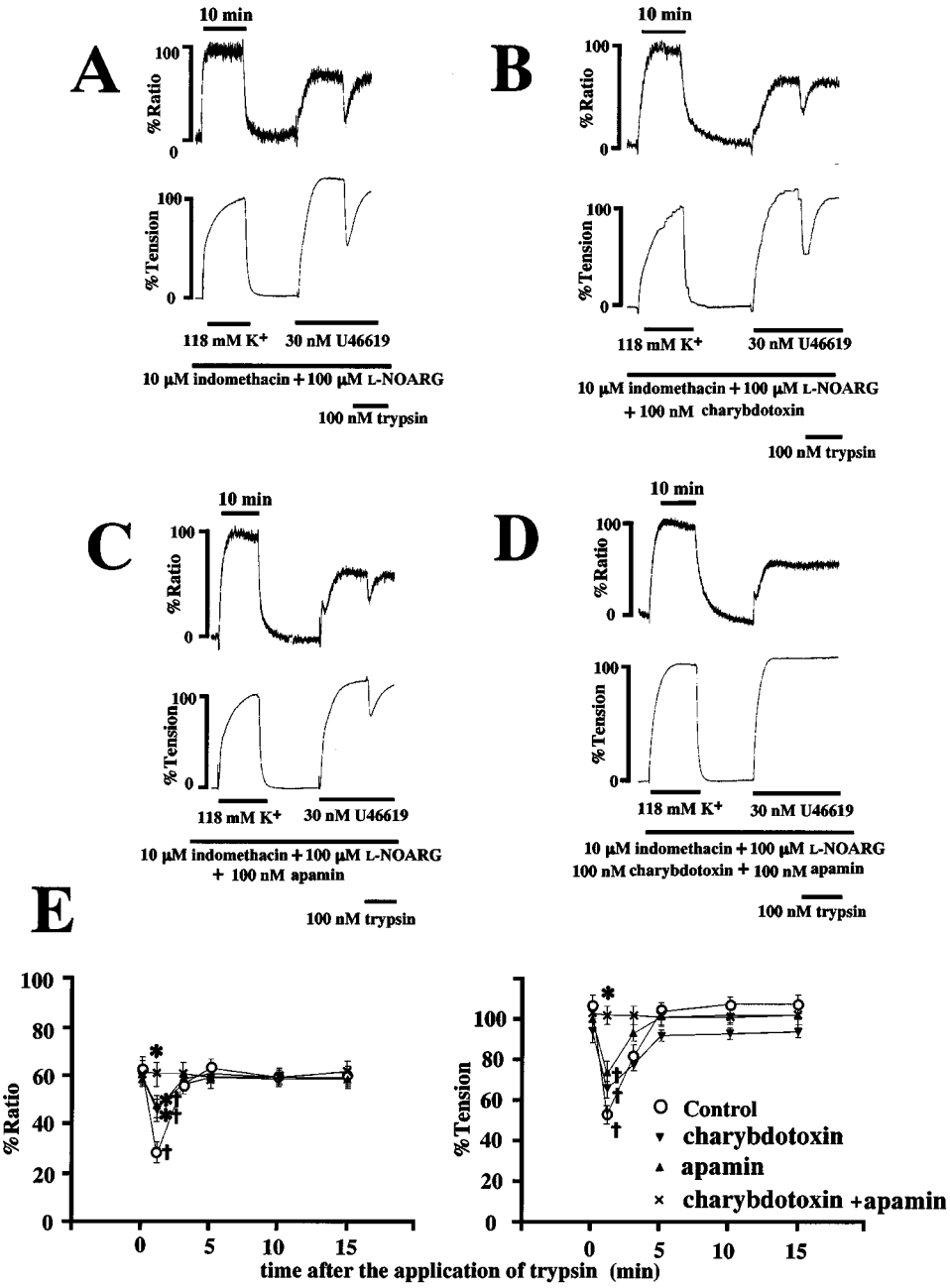

Figure 6 Effect of K^+ channel blockers on the L-NOARG-resistant component of the trypsin-induced relaxation during the U46619-induced contraction in the porcine coronary artery. (A–D) Representative recordings of the trypsin-induced L-NOARG-resistant changes in $[Ca^{2+}]_i$ and tension during the contraction induced by 30 nM U46619 in the absence (A) and the presence of 100 nM charybdotoxin (B), 100 nM apamin (C) and 100 nM charybdotoxin plus 100 nM apamin (D). All measurements were performed in the presence of 10 μM indomethacin and 100 μM L-NOARG. (E) A summary of the effects of charybdotoxin and apamin on the trypsin-induced L-NOARG-resistant decreases in $[Ca^{2+}]_i$ and tension obtained at 0, 1, 3, 5, 10 and 15 min after the application of trypsin. The levels of $[Ca^{2+}]_i$ and tension at the rest and those obtained during the 118 mM K^+-depolarization in each experimental protocol were designated as 0 and 100%, respectively. $^*P<0.05$ compared to the value obtained with the control relaxation induced by trypsin in the presence of L-NOARG and indomethacin; $\dagger P<0.05$ compared to the levels obtained during precontraction just prior to the application of trypsin. The data represent the mean ± s.e.mean ($n=5$).

Effects of pretreatment of PAR2-AP on the subsequent response to trypsin in endothelial cells of the porcine aortic valve

To determin the involvement of PAR2 in the trypsin-induced responses, we examined the effect of pre-treatment of PAR2-AP on the subsequent response to trypsin in endothelial cells of valvular strips. PAR2-AP induced a transient $[Ca^{2+}]_i$ elevation at the concentrations higher than 1 μM, while causing the maximal elevation at 30 μM. As shown in Figure 9B, 30 μM PAR2-AP induced a transient elevation of $[Ca^{2+}]_i$, which declined to the resting level within 5 min. The second stimulation with PAR2-AP did not induce any elevation of $[Ca^{2+}]_i$, suggesting the desensitization of PAR2. Under this

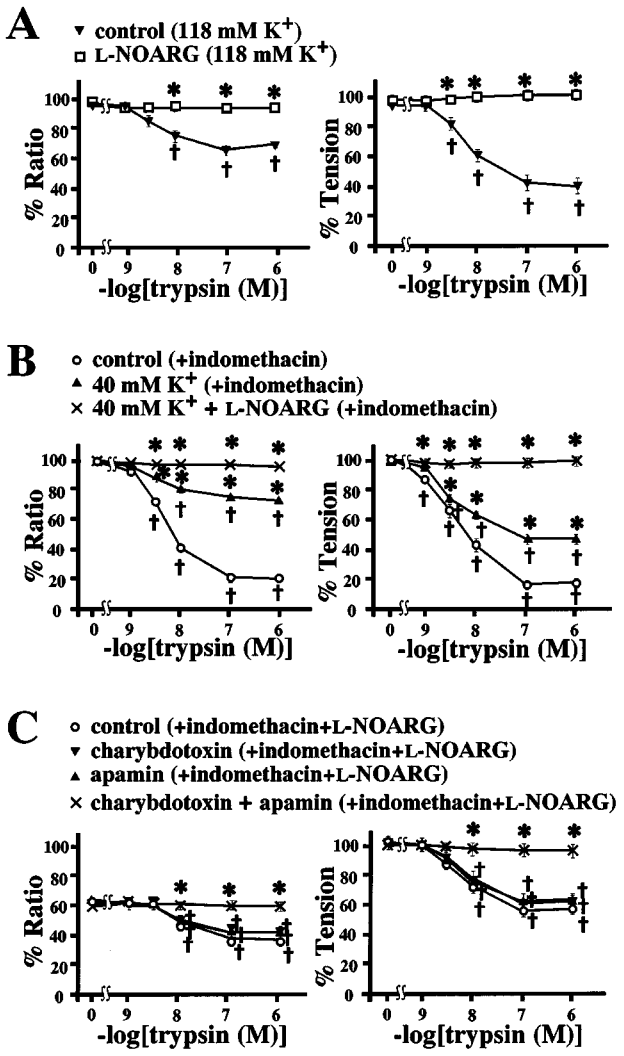

Figure 7 Effects of L-NOARG, high K^+ depolarization and K^+ channel blockers on the concentration-response curves of the trypsin-induced relaxation in the porcine coronary artery. (A) Trypsin was cumulatively applied during the 118 mM K^+-induced contraction in the absence or presence of 100 μM L-NOARG, and the concentration-response curves of the relaxation were constructed. The experimental protocol was the same as Figure 3E, except for that trypsin was applied in a cumulative manner. (B) Trypsin was cumulatively applied in the absence or presence of either 40 mM K^+ or 100 μM L-NOARG during the 30 nM U46619-induced contractions in the presence 10 μM indomethacin. The experimental protocol was the same as Figure 5, except for that trypsin was applied in a cumulative manner. (C) Trypsin was cumulatively applied in the absence or presence of 100 nM charybdotoxin or 100 nM apamin during the 30 nM U46619-induced contraction in the presence of 10 μM indomethacin and 100 μM L-NOARG. The experimental protocol was the same as Figure 6, except for that trypsin was applied in a cumulative manner. In A and C, the levels of $[Ca^{2+}]_i$ and tension at the rest and those obtained during the 118 mM K^+-depolarization in each experimental protocol were designated as 0 and 100%, respectively. In B, the levels of $[Ca^{2+}]_i$ and tension at rest and those obtained just prior to the application of trypsin during the precontraction under the each protocol were designated as 0 and 100%, respectively. *Significantly different ($P<0.05$) from the control relaxation; †Significantly different ($P<0.05$) from the levels obtained during precontraction just prior to the application of trypsin. The data are the mean$\pm$s.e.mean ($n=4$).

situation, trypsin-induced $[Ca^{2+}]_i$ elevation was greatly attenuated (Figure 9B) compared to that obtained without

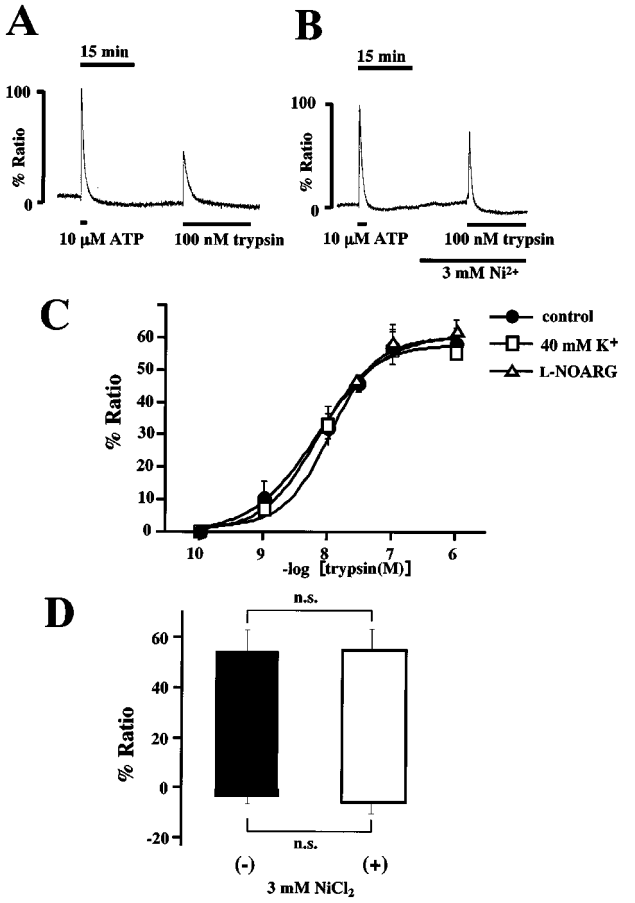

Figure 8 $[Ca^{2+}]_i$ transient induced by trypsin in the *in situ* endothelial cells of the porcine aortic valve. (A,B) Representative recordings of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and 100 nM trypsin in the normal PSS (A) and in the presence of 3 mM $NiCl_2$ (B). (C) The concentration-response curves for the trypsin-induced $[Ca^{2+}]_i$ transients in the absence and presence of 40 mM K^+ or 100 μM L-NOARG in the endothelial cells. The data are the mean$\pm$s.e.mean ($n=4$). The fluorescence ratio was expressed as a percentage, assigning the level obtained in normal PSS and that at the maximum elevation induced by 10 μM ATP to be 0 and 100%, respectively. (D) The effect of 3 mM $NiCl_2$ on the trypsin-induced $[Ca^{2+}]_i$ elevation. The bottom and top of the columns indicate the $[Ca^{2+}]_i$ level obtained just before the application and at the peak response to trypsin, respectively. The data are the mean$\pm$s.e.mean ($n=4$). n.s., not significant ($P>0.05$).

pre-treatment with PAR2-AP (Figure 9A). The level of $[Ca^{2+}]_i$ elevation obtained with the pre-treatment with PAR2-AP (8.5 ± 4.6, $n=4$) was much lower than that obtained without pretreatment (48.2 ± 3.8, $n=4$). The most of the trypsin-induced response was thus inhibited when the response to PAR2-AP was desensitized.

Discussion

It has recently been reported that trypsin activates protease-activated receptor and also exerts various cellular effects (Dery *et al*., 1998). Trypsin has been reported to cause an endothelium-dependent vasorelaxation in various types of blood vessels including the porcine coronary artery (Hamilton & Cocks, 2000; Hamilton *et al*., 1998; Hwa *et al*., 1996;

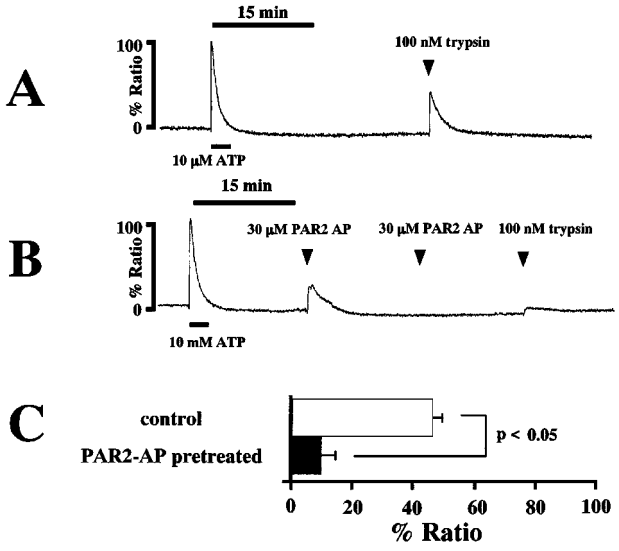

Figure 9 Effects of pretreatment of PAR2-AP on the subsequent response to trypsin in endothelial cells of the porcine aortic valve. (A, B) Representative recordings of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and 100 nM trypsin without (A) and with (B) the preceding stimulation by PAR2-AP. (C) The summary of the level of $[Ca^{2+}]_i$ obtained with 100 nM trypsin without (control) and with the preceding stimulation by PAR2-AP. The fluorescence ratio was expressed as a percentage, assigning the level obtained in normal PSS and that at the maximum elevation induced by 10 μM ATP to be 0 and 100%, respectively. The data are the mean $\pm$ s.e.mean ($n=4$).

Sobey *et al*., 1999). An endothelium-independent direct contractile effect on the rabbit aorta (Komuro *et al*., 1997) and the endothelium-dependent contraction in the human umbilical vein (Saifeddine *et al*., 1998) were also reported. However, there has so far been no report on direct relaxation. The present study elucidated the mechanism of the trypsin-induced relaxation in the porcine coronary artery, especially in terms of Ca^{2+} signalling in both smooth muscle and endothelial cells. The major novel findings of the present study are as follows. (1) We clarified the temporal change in the contribution of NO and EDHF to the trypsin-induced relaxation. The initial phase of the relaxation was due to both NO and EDHF, while the late phase of the relaxation was mainly due to NO. The mechanism of the trypsin-induced relaxation was similar with different concentrations of trypsin. (2) We demonstrated the correlation between the trypsin-induced Ca^{2+} signalling in endothelial cells and the endothelium-dependent relaxation. Furthermore, we found that the Ca^{2+}-sensitivity of the contractile apparatus of smooth muscle decreased during trypsin-induced relaxation, especially in the late phase of the relaxation.

Previous reports have demonstrated the contribution of NO to the trypsin-induced relaxation by using L-arginine analogues such as L-NOARG (Hamilton *et al*., 1998; Hwa *et al*., 1996). However, their inhibition was only partial, and therefore relaxing factors other than NO have been suggested to be involved in trypsin-induced relaxation. Prostacyclin and EDHF are candidates for such additional factors. Hamilton & Cocks (2000) reported that that elevating external K^+ concentration abolished the L-NOARG-resistant component of the trypsin-induced relaxations, and suggested involvement of EDHF. In the present study, we also observed that the inhibition of NO production by L-NOARG was not sufficient for the complete inhibition of the trypsin-induced relaxation during the U46619-induced contraction, which thus suggested the involvement of additional relaxing factors. We thus clearly demonstrated the contribution of EDHF to the trypsin-induced relaxation in the porcine coronary artery. First, a combination of charybdotoxin and apamin completely abolished the L-NOARG-resistant component of the trypsin-induced relaxation. Second, the L-NOARG-resistant relaxation was also inhibited by elevating the external K^+ concentration. The observation that the trypsin-induced relaxation during the 118 mM K^+-induced contraction was significantly smaller than that seen during the U46619-induced contraction also supports the involvement of EDHF. As a result, we concluded that NO and EDHF account for most, if not all, of the trypsin-induced relaxation. Furthermore, the close examination of the effect of these inhibitors on the duration of the relaxation revealed the temporal change in the relative contribution of NO and EDHF to the trypsin-induced relaxation. It was a consistent observation that the trypsin-induced relaxation returned to the precontraction level faster in the presence of L-NOARG than that seen in its absence or in the presence of K^+-depolarization. These observations suggested that the initial relaxation was mediated by both NO and EDHF, while the later phase of the relaxation was mediated by NO.

The present study demonstrated, for the first time, the decrease in the Ca^{2+}-sensitivity of the contractile apparatus during the trypsin-induced relaxations. Most of the relaxation, especially at maximal relaxation, was accompanied by a decrease in $[Ca^{2+}]_i$, however, a dissociation between the decreases in $[Ca^{2+}]_i$ and tension was also noticed in the following situations: (1) In the time course of the trypsin-induced relaxation (Figure 1D), $[Ca^{2+}]_i$ returned to the precontraction level faster than tension, and therefore the late phase of the relaxation was not associated with decrease in $[Ca^{2+}]_i$. (2) In the concentration-response curves (Figure 2A,B), trypsin at 1 nM induced the relaxation without any significant reduction of $[Ca^{2+}]_i$. (3) The $[Ca^{2+}]_i$-tension relation curve obtained at the maximal relaxation induced by 100 nM and 1 μM trypsin (Figure 2C) apparently was placed below the control relationship of the U46619-induced contraction. These results suggested that the Ca^{2+}-sensitivity of the contractile apparatus apparently decreased during the trypsin-induced relaxation under these situations. Therefore, the decrease in the Ca^{2+}-sensitivity as well as the decrease in $[Ca^{2+}]_i$ contributes to the trypsin-induced relaxation, especially in the late phase. NO activates guanlylate cyclase and increases cellular cyclic GMP level (Ignarro *et al*., 1987). The increase in cyclic GMP was reported to be associated with the decrease in the Ca^{2+}-sensititivy (Abe *et al*., 1990; Nishimura & van Breemen, 1989). It is thus conceivable that the decrease in the Ca^{2+}-sensitivity was mediated by NO. In fact, the $[Ca^{2+}]_i$-tension relationship obtained in the presence of L-NOARG overlapped with the control relationship, thus supporting the contribution of NO to decrease in the Ca^{2+}-sensitivity. The decrease in the Ca^{2+}-sensitivity in the late phase of relaxation is thus consistent with the contribution of NO in this phase.

The Ca^{2+} signal in the endothelial cells is a primary determinant of production of NO and EDHF (Fleming *et al*., 1997; Fukao *et al*., 1997). We demonstrated that trypsin induced a transient elevation of $[Ca^{2+}]_i$ in *in situ* endothelial

cells of the porcine aortic valve, and found that the concentrations of trypsin required to induce the endothelium-dependent relaxation was similar to those required to induce $[Ca^{2+}]_i$ elevation in endothelial cells. This finding suggested that the Ca^{2+} signal induced by trypsin related to the production of NO and EDHF in the endothelial cells. In the present study, the trypsin-induced transient $[Ca^{2+}]_i$ elevation seen in the presence of Ni^{2+} did not significantly differ from that seen in its absence. The level of Ca^{2+} transient seen in the Ca^{2+}-free media was also similar to that seen in the normal media containing 1.25 mM Ca^{2+} (data not shown). These observations suggest that the trypsin-induced $[Ca^{2+}]_i$ elevation was mediated mainly by the Ca^{2+} release. In the non-excitable cells including endothelial cells, depolarization of the membrane potential is considered to decrease a driving force of Ca^{2+} and thereby inhibit the Ca^{2+} influx (Adams *et al*., 1989). The observation in the present study that 40 mM K^+ depolarization had no effect on the trypsin-induced $[Ca^{2+}]_i$ transient in the endothelial cells supports the negligible contribution of the Ca^{2+} influx in the trypsin-induced transient $[Ca^{2+}]_i$ elevation. Hamilton & Cocks (2000) showed that trypsin-induced relaxation of porcine coronary artery was not affected by L-type-voltage-operated calcium inhibitor nifedipine. As a consequence, we concluded that the trypsin-induced $[Ca^{2+}]_i$ elevation was mainly due to Ca^{2+} release in the endothelial cells of the porcine aortic valve, and that the Ca^{2+} influx played, if any, a negligible role. An important implication from these conclusions is that the Ca^{2+} release maybe sufficient to induce the production of relaxing factors in endothelial cells.

The present study suggested that PAR2 played a major role in the trypsin-induced $[Ca^{2+}]_i$ elevation in endothelial cells. The physiological role of the PAR2 (trypsin receptor)-mediated vasorelaxation remains to be determined as trypsin does not exist in plasma under physiological conditions, although human umbilical vein endothelial cells in culture have been shown to produce trypsin in response to enterokinase (Koshikawa *et al*., 1997). Acute pancreatitis is one of pathological conditions where trypsin is found in plasma (Temler & Felber, 1976). The plasma level of trypsin increased to a level (0.87 μM) high enough to induce endothelium-dependent relaxation according to the concentration-dependent curve obtained in the present study (Temler & Felber, 1976). Severe pancreatitis causes circulatory shock, and therefore the trypsin-induced vasorelaxation may play an important role in the pathogenesis of such shock. The PAR2 activation may also contribute to the pathogenesis of the septic shock (Cicala *et al*., 1999). Lipopolysaccharide was shown to upregulate the expression of PAR2 mRNA up to 5–10 fold in human umbilical vein endothelial cells (Nystedt *et al*., 1996), and to augment vasorelaxation induced by trypsin and PAR2-activating peptide (Cicala *et al*., 1999).

In conclusion, trypsin was shown to induce an endothelial-dependent relaxation in the porcine coronary artery, and a transient elevation of $[Ca^{2+}]_i$ *in situ* endothelial cells of the porcine aortic valves. Trypsin had no direct effect on $[Ca^{2+}]_i$ and tension of smooth muscle. The initial phase of the relaxation was due to both NO and EDHF, while the late phase of the relaxation was mainly due to NO. The relaxation was linked to a decrease in the $[Ca^{2+}]_i$ of smooth muscle and a decrease in the Ca^{2+}-sensitivity of the contractile apparatus. The contribution of the decrease in the Ca^{2+}-sensitivity was apparent in the late phase of relaxation. The trypsin-induced production of relaxing factors closely correlated with the $[Ca^{2+}]_i$ elevation in the endothelial cells. Since the $[Ca^{2+}]_i$ transient in the endothelial cells was considered to be mainly due to the Ca^{2+} release from the intracellular stores, these findings suggested that the Ca^{2+} release was sufficient to induce the production of relaxing factors in endothelial cells.

We thank Mr Brian Quinn for comments and help with the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (Nds. 10557072, 11838013, 11670687) and for Scientific Research on Priority Area (No. 12213103) from the Ministry of Education, Science, Sports and Culture, Japan, and by a Research Grant for Cardiovascular Diseases (11C-1, 12C-2) from the Ministry of Health and Welfare, Japan, and by grants from the Foundation for the Promotion of Clinical Medicine, the Suzuken Memorial Foundation and the KANZAWA Medical Research Foundation.

References

ABE, S., KANAIDE, H. & NAKAMURA, M. (1990). Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and on tension in the coronary artery of the pig. *Br. J. Pharmacol.,* **101,** 545–552.

ADAMS, D.J., BARAKEH, J., LASKEY, R. & VAN BREEMEN, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.,* **3,** 2389–2400.

CICALA, C., PINTO, A., BUCCI, M., SORRENTINO, R., WALKER, B., HARRIOT, P., CRUCHLEY, A., KAPAS, S., HOWELLS, G.L. & CIRINO, G. (1999). Protease-activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. *Circulation,* **99,** 2590–2597.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.,* **235,** E97–E102.

DERY, O., CORVERA, C.U., STEINHOFF, M. & BUNNETT, N.W. (1998). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am. J. Physiol.,* **274,** C1429–C1452.

DI VIRGILIO, F., STEINBERG, T.H. & SILVERSTEIN, S.C. (1989). Organic-anion transport inhibitors to facilitate measurement of cytosolic free Ca^{2+} with fura-2. *Methods Cell Biol.,* **31,** 453–462.

FLEMING, I., BAUERSACHS, J. & BUSSE, R. (1997). Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J. Vasc. Res.,* **34,** 165–174.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1997). Sources of Ca^{2+} in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *Br. J. Pharmacol.,* **120,** 1328–1334.

HAMILTON, J.R. & COCKS, T.M. (2000). Heterogeneous mechanisms of endothelium-dependent relaxation for thrombin and peptide activators of protease-activated receptor-1 in porcine isolated coronary artery. *Br. J. Pharmacol.,* **130,** 181–188.

HAMILTON, J.R., NGUYEN, P.B. & COCKS, T.M. (1998). Atypical protease-activated receptor mediates endothelium-dependent relaxation of human coronary arteries. *Circ. Res.,* **82,** 1306–1311.

HWA, J.J., GHIBAUDI, L., WILLIAMS, P., CHINTALA, M., ZHANG, R., CHATTERJEE, M. & SYBERTZ, E. (1996). Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells. *Circ. Res.,* **78,** 581 – 588.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 9265 – 9269.

ISHIHARA, H., CONNOLLY, A.J., ZENG, D., KAHN, M.L., ZHENG, Y.W., TIMMONS, C., TRAM, T. & COUGHLIN, S.R. (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature,* **386,** 502 – 506.

KAHN, M.L., ZHENG, Y.W., HUANG, W., BIGORNIA, V., ZENG, D., MOFF, S., FARESE, JR., R.V., TAM, C. & COUGHLIN, S.R. (1998). A dual thrombin receptor system for platelet activation. *Nature,* **394,** 690 – 694.

KANAIDE, H. (1999). Measurement of $[Ca^{2+}]_i$ in smooth muscle strips using front-surface fluorimetry. In *Methods in Molecular Biology*, ed. Lambert, D.G. pp. 269 – 277. Totowa, NJ: Humana Press Inc.

KAWABATA, A., KURODA, R., NISHIKAWA, H. & KAWAI, K. (1999). Modulation by protease-activated receptors of the rat duodenal motility in vitro: possible mechanisms underlying the evoked contraction and relaxation. *Br. J. Pharmacol.,* **128,** 865 – 872.

KOMURO, T., MIWA, S., MINOWA, T., OKAMOTO, Y., ENOKI, T., NINOMIYA, H., ZHANG, X.F., UEMURA, Y., KIKUCHI, H. & MASAKI, T. (1997). The involvement of a novel mechanism distinct from the thrombin receptor in the vasocontraction induced by trypsin. *Br. J. Pharmacol.,* **120,** 851 – 856.

KONG, W., MCCONALOGUE, K., KHITIN, L.M., HOLLENBERG, M.D., PAYAN, D.G., BOHM, S.K. & BUNNETT, N.W. (1997). Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 8884 – 8889.

KOSHIKAWA, N., NAGASHIMA, Y., MIYAGI, Y., MIZUSHIMA, H., YANOMA, S., YASUMITSU, H. & MIYAZAKI, K. (1997). Expression of trypsin in vascular endothelial cells. *FEBS Lett.,* **409,** 442 – 448.

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1993). Role of GTP-protein and endothelium in contraction induced by ethanol in pig coronary artery. *J. Physiol.,* **470,** 521 – 537.

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1995). Mechanism of endothelium-dependent relaxation induced by substance P in the coronary artery of the pig. *Br. J. Pharmacol.,* **116,** 2040 – 2047.

MIZUNO, O., HIRANO, K., NISHIMURA, J., KUBO, C. & KANAIDE, H. (1998). Mechanism of endothelium-dependent relaxation induced by thrombin in the pig coronary artery. *Eur. J. Pharmacol.,* **351,** 67 – 77.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochem. Biophys. Res. Commun.,* **163,** 929 – 935.

NYSTEDT, S., EMILSSON, K., WAHLESTEDT, C. & SUNDELIN, J. (1994). Molecular cloning of a potential proteinase activated receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 9208 – 9212.

NYSTEDT, S., RAMAKRISHNAN, V. & SUNDELIN, J. (1996). The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. *J. Biol. Chem.,* **271,** 14910 – 14915.

SAIFEDDINE, M., ROY, S.S., AL-ANI, B., TRIGGLE, C.R. & HOLLENBERG, M.D. (1998). Endothelium-dependent contractile actions of proteinase-activated receptor-2-activating peptides in human umbilical vein: release of a contracting factor via a novel receptor. *Br. J. Pharmacol.,* **125,** 1445 – 1454.

SOBEY, C.G., MOFFATT, J.D. & COCKS, T.M. (1999). Evidence for selective effects of chronic hypertension on cerebral artery vasodilatation to protease-activated receptor-2 activation. *Stroke,* **30,** 1933 – 1941.

TEMLER, R.S. & FELBER, J.P. (1976). Radioimmunoassay of human plasma trypsin. *Biochim. Biophys. Acta,* **445,** 720 – 728.

VU, T.K., HUNG, D.T., WHEATON, V.I. & COUGHLIN, S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell,* **64,** 1057 – 1068.

XU, W.F., ANDERSEN, H., WHITMORE, T.E., PRESNELL, S.R., YEE, D.P., CHING, A., GILBERT, T., DAVIE, E.W. & FOSTER, D.C. (1998). Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 6642 – 6646.

(Received April 5, 2001
Revised July 31, 2001
Accepted August 3, 2001)