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The involvement of a novel mechanism distinct from the thrombin receptor in the vasocontraction induced by trypsin

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1 The vasocontracting effect of a serine protease trypsin and its mechanisms were investigated by monitoring the isometric tension in endothelium-denuded rings of rabbit thoracic aortae and its effects on intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in dispersed rabbit vascular smooth muscle cells with a Ca^{2+} indicator fura-2. The actions of trypsin were compared with those of thrombin.

2 Both thrombin and trypsin reversibly contracted aortic rings without endothelium in a concentration-dependent manner. The vasocontraction induced by trypsin was well correlated with the protease activity of trypsin actually added to the tissue baths containing the aortic rings and was completely blocked by soybean trypsin inhibitor and phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor.

3 The trypsin-induced contractions of the aortic rings were not the result of irreversible damage to vascular smooth muscle cells, since the contractile responses induced by noradrenaline or 30 mM KCl were unaffected by pretreatment with trypsin.

4 The contractions induced by either thrombin or trypsin were reduced to about 30% of control responses after removal of extracellular Ca^{2+}, indicating that most of the contraction is dependent on extracellular Ca^{2+}. By contrast, the contractions induced by either of the proteases were reduced by an antagonist of L-type voltage-operated Ca^{2+} channels, nifedipine, to about 70% of control responses, indicating that both nifedipine-sensitive and -resistant Ca^{2+} channels are involved in these contractions.

5 In the aortic rings precontracted by a maximally effective concentration of thrombin, the second application of thrombin virtually failed to induce contractions but trypsin could still induce contractions amounting to 10% of control values by it's protease activity.

6 After the first application of a maximal concentration of thrombin, the second application of thrombin could not induce an increase in $[Ca^{2+}]_i$, but an application of trypsin could still induce an increase in $[Ca^{2+}]_i$ in dispersed rabbit vascular smooth muscle cells.

7 These data suggest that in addition to activation of a thrombin receptor, trypsin can contract rabbit aortae by a proteinase-activated receptor 2 or a novel mechanism.

Keywords: Trypsin; thrombin; vasocontraction; intracellular free Ca^{2+} concentration; Ca^{2+} channel; rabbit thoracic aorta; vascular smooth muscle cells; soybean trypsin inhibitor; phenylmethylsulphonyl fluoride

Introduction

Apart from its classic role in the coagulation pathway, the serine protease thrombin stimulates a number of physiological responses (Bar-Sharvit *et al*., 1986; Ku, 1986; Shuman, 1986; Walz *et al*., 1986; Jaffe *et al*., 1987; Fuse & Tai, 1988; Emori *et al*., 1992; Suidan *et al*., 1992). Thrombin available in the blood stream may interact with the constituents of the vessel wall. The interaction with endothelial cells results in the release of vasodilating substances such as prostacyclin and endothelium-derived relaxing factor (EDRF) whereas the interaction with smooth muscle cells leads to a contractile response (DeMey *et al*., 1982; Ku, 1986). The recent cloning and characterization of the thrombin receptor from human sources have elucidated a unique mechanism by which thrombin activates target cells (Vu *et al*., 1991; Nanevicz *et al*., 1995; Woolkalis *et al*, 1995; Grand *et al*., 1996). Thrombin binds to its receptor via its anionic binding exosite, which it cleaves after arginine-41 in the NH_2-terminal portion of the receptor. The exposed new NH_2 terminus has been proposed to function as a 'tethered peptide ligand' that binds to the thrombin receptor to cause receptor activation (Vu *et al*., 1991; Nanevicz *et al*., 1996).

Trypsin is a prototype of a family of serine protease and can mimic several actions of thrombin including vasorelaxation (DeMey *et al*., 1982; Rapoport *et al*., 1984) and platelet activation (Ruggiero & Lapetina, 1985; Jakobs & Grandt, 1988; Brass *et al*., 1992; Finotti, 1992). In fact, trypsin has been found to possess a partial agonist activity for the cloned thrombin receptor (Vu *et al*., 1991). In this regard, tryspin has been used mainly as a tool for analysis of mechanisms of actions of thrombin.

Recently, a DNA encoding another proteinase-activated receptor 2 (PAR-2) has been isolated from a mouse and human genomic library (Nystedt *et al*., 1994; 1995). The deduced amino acid sequence of the receptor shows an overall 30% identity to the human thrombin receptor, although it has a similar activation mechanism (Nystedt *et al*., 1994).

Notably, the receptor is activated by low concentrations of trypsin but not thrombin, when expressed in *Xenopus laevis* oocytes (Nystedt *et al*., 1994). Thus it is now conceivable that the actions of trypsin are mediated by a specific receptor like PAR-2. Indeed, we have found that trypsin can induce contractions of rabbit thoracic aortic rings without endothelium and an increase in the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in A7r5 cells, a cell line derived from foetal rat aortic vascular smooth muscle cells (unpublished observations).

Quite recently, to detect functional PAR-2, the actions of PAR-2 activating polypeptide (SLIGRL; an amino acid se-

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quence of PAR-2 which is thought to act as a 'tethered peptide ligand') were evaluated in the rat aorta (Al-Ani *et al*., 1995). This peptide induced endothelium-dependent vasorelaxation, but in the endothelium-denuded aorta, it produced neither relaxation nor contraction (Al-Ani *et al*., 1995). Based on these results, it is unlikely that the direct contracting action of trypsin is mediated by activation of PAR-2. Thus we attempted to determine whether the direct contractile effect of trypsin is mediated totally by the thrombin receptor or not. To clarify this point, we pharmacologically characterized vasocontracting actions of trypsin in rabbit aortic ring specimens without endothelium, and also trypsin-induced changes in $[Ca^{2+}]_i$ in a single rabbit vascular smooth muscle cell. Finally we compared these actions of trypsin with those of thrombin.

Methods

Preparation of rabbit thoracic aortic rings and tension recording

Japanese white rabbits of either sex weighing 2.4 to 2.6 kg were euthanized by i.v. injection of pentobarbitone (60 mg kg^{-1}). The thoracic aorta was removed quickly, and placed into Krebs solution which contained (in mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25, and glucose 5.6. Blood was rinsed from the lumen, adherent connective tissue was removed carefully and rings, approximately 3 to 4 mm in width, were cut from each aorta. Endothelial cells were removed from ring specimens by gently rubbing the intimal surface with a cotton bud moistened with Krebs solution. Successful removal of endothelial cells was confirmed by the inability of acetylcholine (1 μM) to induce relaxation. The aortic rings were horizontally mounted by use of a pair of stainless steel hooks under a resting tension of 1.5 g in organ baths containing 5 ml of Krebs solution which was maintained at 37°C and bubbled with a 95% O_2 and 5% CO_2 mixture. Tension was measured isometrically with a force displacement transducer (Orientec, Tokyo, Japan) and was displayed on a Nihon Kohden (Tokyo, Japan) RJG4128 polygraph.

During the whole experiment, the bath fluid was changed every 20 min. Initially, resting force was readjusted after each wash-out until a stable baseline was attained (usually after about 60 min). Then the preparations were challenged at hourly intervals with 0.3 μM noradrenaline (NA). When two NA contractions had given reproducible results, the actual experiment was started. At the beginning of each experiment, each preparation was challenged with 10 μM NA which caused a maximal contraction, and the contractile responses to trypsin or thrombin were represented as percentages of the tension induced by 10 μM NA. The concentration-response relationship for contractions induced by trypsin or thrombin was determined noncumulatively by adding single concentrations of the protease to individual aortic strips, to avoid desensitization.

For experiments in Ca^{2+}-free solution, Ca^{2+} was omitted from Krebs solution and 1 mM EGTA was added. In some experiments, the aortic ring specimens were stimulated by 30 mM KCl-containing Krebs solution, in which the concentration of KCl was elevated to 30 mM with the concentration of NaCl being reduced to maintain isotonicity.

Measurement of $[Ca^{2+}]_i$ in a single dispersed thoracic aortic smooth muscle cell of the rabbit

A single dispersed smooth muscle cell was prepared from rabbit thoracic aortae as described previously (Inoue & Kuriyama, 1993; Enoki *et al*., 1995). The isolated thoracic aorta was dissected into thin strips (3 mm x 10 mm) and kept in Ca^{2+}-free Krebs-HEPES solution containing (in mM): NaCl 140, KCl 3, $MgCl_2$ 1, glucose 11 and HEPES 10 (pH 7.45, adjusted with NaOH). The strips were enzymatically digested in Ca^{2+}-free Krebs-HEPES solution containing papain (0.2–0.3 mg ml^{-1}) and dithiothreitol (1.25 mM) for 12 h at 4°C. After washing with Ca^{2+}-free Krebs-HEPES solution, the strips were incubated in the same solution containing collagenase (250 to 300 uml^{-1}) for 10 to 12 min at 37°C. The gelatinous tissues were cut into pieces, seeded in glass-bottomed dishes containing Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Hyclone) and cultured at 37°C in a humidified 5% CO_2/95% air atmosphere. The medium was changed every 48 h, and 5 to 7 days later, the cells were used for $[Ca^{2+}]_i$ measurement.

For loading the cells with a Ca^{2+} indicator fura-2, the cells were incubated in Ca^{2+}-free Krebs-HEPES solution containing 4 μM fura-2 acetoxymethyl ester for 60 min at 37°C (Grynkiewicz *et al*., 1985; Itoh *et al*., 1994). The fura-2-loaded cells were washed once with normal Krebs-HEPES solution containing 2 mM $CaCl_2$ and maintained in the same solution at room temperature for 30–60 min. Fura-2 microfluorometry was done with an Attofluor real-time digital fluorescence analyser (Atto Instruments) with excitation wavelengths at 334 nm and 380 nm and an emission wavelength at 520 nm.

Measurement of protease activity of trypsin

Protease activity of trypsin was measured with benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPA) as a substrate (Erlanger *et al*., 1961). The reaction mixture (final volume, 3.0 ml) contained 1 mM DL-BAPA in dimethylsulphoxide, varying amounts of trypsin and 50 mM Tris-HCl (pH 8.2). The mixture was incubated for 10 min at 25°C and 1 ml of 30% acetic acid was added to terminate the reaction. The amount of *p*-nitroaniline formed from DL-BAPA was quantified by measuring an increase in the absorbance at 410 mm in a Beckman DU-50 spectrophotometer.

Chemicals

Chemicals were obtained from the following sources: trypsin (type I, from bovine pancreas, 12,900 BAEE units mg^{-1} protein), soybean trypsin inhibitor (type I-S) and nifedipine, from Sigma (St. Louis, MO, U.S.A.); fura-2 acetoxymethyl ester, −NA bitartrate, acetylcholine bromide and phenylmethylsulphonyl fluoride (PMSF), thrombin (from bovine plasma, >1500 units mg^{-1} protein), from Wako Pure Chemical Industries (Osaka, Japan); DL-BAPA, from Nacalai Tesque Inc. (Kyoto, Japan).

Statistical analysis

Data are presented as mean ± s.e. mean. Results from control and treated groups were expressed as percentages of the 10 μM NA-induced contraction of each ring. Comparisons were made by Student's *t* test for paired values. A probability level of $P<0.05$ was considered to be statistically significant.

Results

As shown in Figure 1, both trypsin and thrombin concentration-dependently induced contractile responses in ring specimens of rabbit thoracic aortae without endothelium. The contractile responses to trypsin reached a maximum at 6.7 μM; the EC_{50} value was around 600 nM. On the other hand, the responses to thrombin reached a maximum at 3.6 μM with an EC_{50} value of around 300 nM.

To rule out the possibility that the trypsin-induced contraction was the result of irreversible damage to vascular smooth muscle cells, we compared the contractile responses of the ring specimens to stimulations by NA or 30 mM KCl before and after treatment with trypsin. As shown in Figure 2, contractile responses to stimulation by NA (Figure 2a) and 30 mM KCl (Figure 2b) after treatment with trypsin and its wash-out were as large as those before treatment with trypsin.

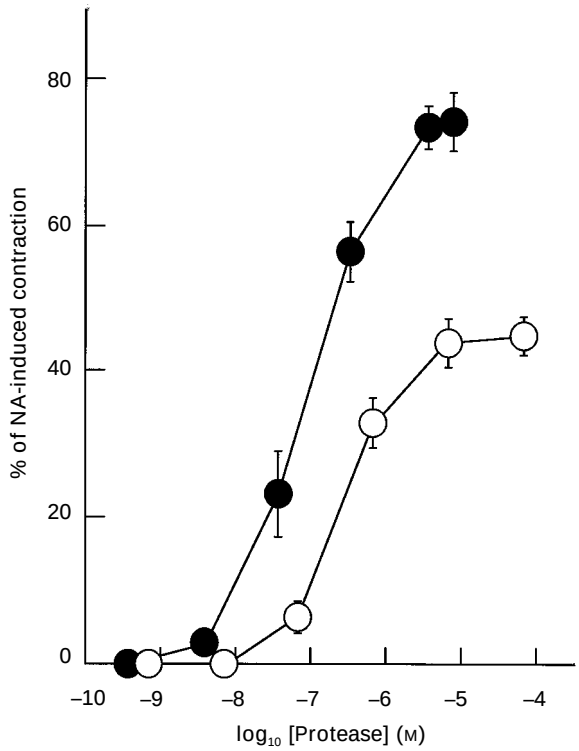

Figure 1 Noncumulative concentration-response curves for trypsin- (○) and thrombin- (●) induced contractions of rabbit thoracic aortic rings without endothelium. The contractile responses to trypsin or thrombin were represented as percentages of the tension induced by 10 μM noradrenaline (NA) in each preparation. Each point represents the mean of 6 experiments with s.e.means shown by vertical lines.

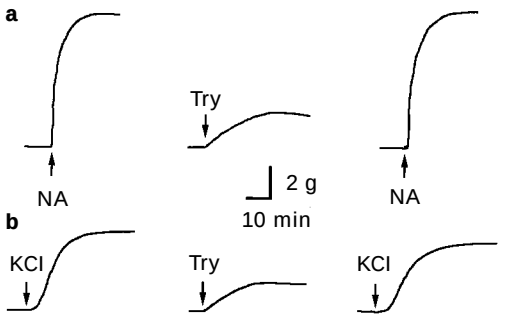

Figure 2 Typical tracings showing reproducibility of noradrenaline (NA)- and 30 mM KCl-induced contractions after treatment with trypsin. Rabbit thoracic aortic rings without endothelium were stimulated by 10 μM NA (a) or 30 mM KCl (b). After washout of NA or 30 mM KCl solution, the rings were stimulated with 670 nM tryspin (Try). After washing, the rings were challenged again with 10 μM NA or 30 mM KCl.

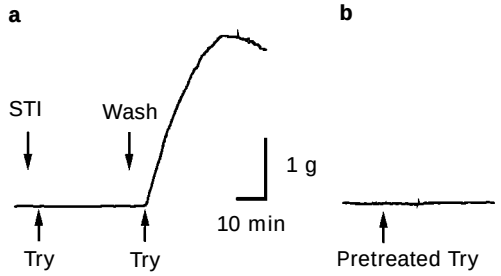

Figure 3 Typical tracings showing inhibition of trypsin-induced contractions of rabbit thoracic aortic rings without endothelium by soybean trypsin inhibitor (STI) or phenylmethylsulphonyl fluoride (PMSF). (a) Immediately after administration of 1 μM soybean trypsin inhibitor, 670 nM trypsin (Try) was added. After washout of trypsin and STI, 670 nM trypsin was added. (b) Trypsin was incubated *in vitro* with 10 mM PMSF for 10 min at 25°C and an aliquot of the reaction mixture was added (final concentration of trypsin, 670 nM).

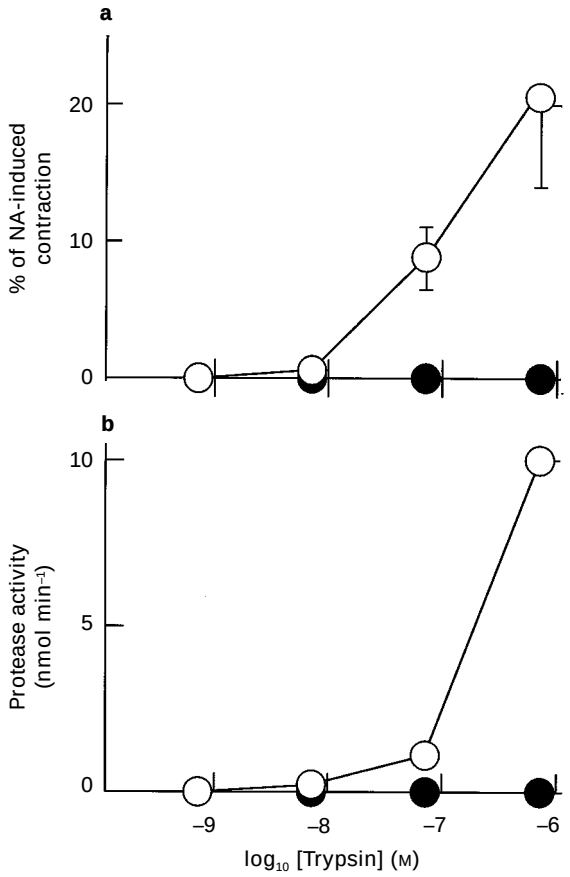

Figure 4 The relationship between the trypsin-induced contractile responses of rabbit thoracic aortic rings without endothelium (a) and the protease activities of trypsin actually added to the bath solution (b). The protease activity of trypsin was expressed as nmol of *p*-nitroaniline formed per minute. When PMSF was used, trypsin was first preincubated *in vitro* with 10 mM PMSF (an irreversible inhibitor of trypsin) for 10 min at 25°C. (○) Untreated trypsin; (●) trypsin pretreated with PMSF.

To clarify whether the vasocontracting action of trypsin is mediated by protease activity of trypsin itself, we examined the effects of inhibitors of trypsin such as soybean trypsin inhibitor and PMSF on trypsin-induced vasocontraction, and also the relationship between the protease activities actually added to the bath solution and the resulting contractile responses (Figures 3 and 4). When trypsin was added to the bath solution immediately after administration of soybean trypsin inhibitor (a reversible inhibitor of trypsin), no contractile responses were observed (Figure 3a). In another experiment, trypsin was first preincubated *in vitro* with 10 mM PMSF (an irreversible inhibitor of trypsin) for 10 min at 25°C and an aliquot of the reaction mixture was added to the bath solution containing aortic ring specimens. After the preincubation with PMSF, trypsin could not induce contractile responses (Figures 3b and 4).

In separate experiments, we actually determined the protease activity of trypsin *in vitro* with DL-BAPA as a substrate and compared the protease activities of trypsin actually added to the bath solution with the resulting contractile responses. We found a correlation between the

contractile responses (Figure 4a) and the protease activities (Figure 4b). After trypsin had been preincubated with PMSF, both contractile responses and protease activities were completely suppressed.

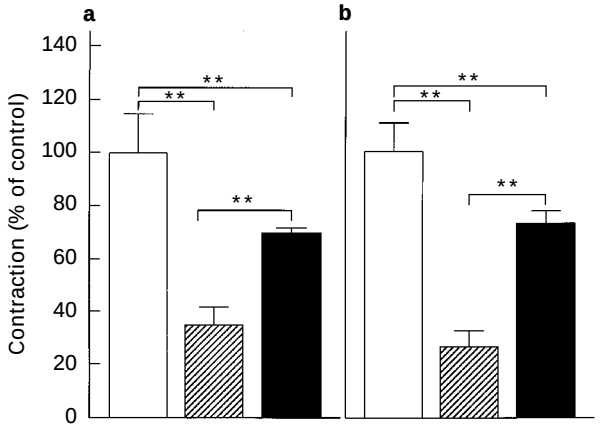

Figure 5 The effects of removal of Ca^{2+} from the bath solution and a blocker of L-type voltage-operated Ca^{2+} channels nifedipine on trypsin-(a) and thrombin-(b) induced contractions of rabbit thoracic aortic rings without endothelium. The rings were stimulated by 670 nM trypsin or 36 nM thrombin in normal Krebs solution (open columns), in Ca^{2+}-free Krebs solution with 1 mM EGTA (hatched columns) or in the presence of 10 μM nifedipine (solid columns). Contractions were represented as percentages of the tension induced by 10 μM NA. Each point represents the mean of 6 experiments together with s.e.means. $^{**}P<0.01$: significantly different from each other.

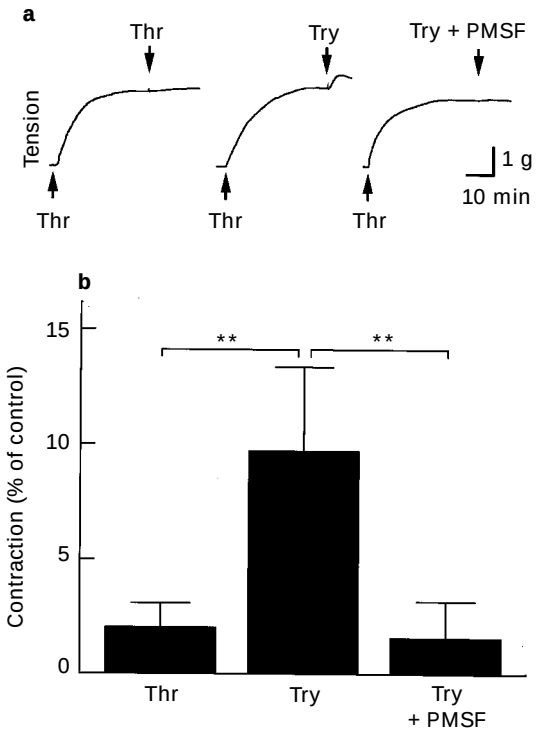

Figure 6 Contractile responses to thrombin (Thr) and trypsin (Try) of rabbit thoracic aortic rings without endothelium precontracted with a maximally effective concentration of thrombin. Rabbit thoracic aortic rings without endothelium were exposed initially to a maximally effective concentration of thrombin (7.2 μM). After the developed tension reached a plateau, either 7.2 μM thrombin, 6.7 μM trypsin or trypsin pretreated with PMSF was added to the tissue bath (a). The contractile responses to the second application of thrombin or trypsin were represented as the percentages of the first application of thrombin (b). Each column represents the mean of 6 experiments together with

Figure 5 shows the effects of removal of Ca^{2+} from the bath solution and a blocker of L-type voltage-operated Ca^{2+} channels nifedipine on trypsin- and thrombin-induced contractile responses of aortic rings without endothelium. In the Ca^{2+}-free buffer, the trypsin-induced contractile responses were reduced to about 30% of the control responses (Figure 5a). Pretreatment with 10 μM nifedipine, which completely inhibited vasocontraction induced by 30 mM KCl (data not shown), suppressed the trypsin-induced contraction to about 70% of the control responses (Figure 5a). Essentially similar results were obtained for the thrombin-induced contraction (Figure 5b).

To test whether the action of trypsin is mediated by thrombin receptors or not, we examined the effects of trypsin and thrombin on contractile responses of deendothelialized aortic rings after a prior challenge with a maximally effective concentration of thrombin (Figure 6). In ring specimens precontracted with a maximally effective concentration of thrombin, the second application of thrombin had little effect ($2.1\pm1.2\%$ of the contraction by the first application of thrombin, $n=6$). In contrast, a maximally effective concentration of trypsin induced small but significant responses ($9.8\pm3.7\%$ of the contraction by the first application of thrombin, $n=6$; significantly different from the response induced by the second challenge with thrombin, $P<0.01$). When trypsin pretreated with PMSF was used, it no longer induced a contraction in the rings precontracted with a maximally effective concentration of thrombin ($1.6\pm1.5\%$ of the contraction by the first application of thrombin, $n=6$; significantly different from the response induced by the second challenge with trypsin, $P<0.01$).

For the same purpose, we examined the second application of thrombin or trypsin on $[Ca^{2+}]_i$ in a single dispersed aortic smooth muscle cell from the rabbit which had been stimulated by a maximally effective concentration of thrombin (Figure 7). The first application of 3.6 μM thrombin evoked a transient increase in $[Ca^{2+}]_i$ but the second application of thrombin produced no increase. In contrast, application of 67 nM trypsin evoked a transient increase in $[Ca^{2+}]_i$ even after application of 3.6 μM thrombin.

Discussion

In the present study, thrombin induced contractions of rabbit thoracic aortic rings without endothelium. This result is consistent with previous findings on rabbit thoracic aorta (Haber & Namm, 1984; Walz *et al*., 1985; Sakiyama *et al*., 1991), guinea-pig aorta (Muramatsu *et al*., 1992), canine coronary artery (Tesfamariam, 1994) and porcine pulmonary artery (Glusa *et al*., 1994). However, the result is in disagreement with data on rat thoracic aorta where thrombin has been shown to induce only endothelium-dependent relaxation but no contraction (Rapoport *et al*., 1984; Muramatsu *et al*., 1992; Antonaccio *et al*., 1993). Notably, trypsin was found to induce contractions

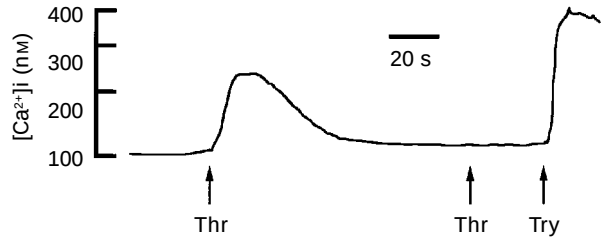

Figure 7 Effects of sequential administrations of proteases on $[Ca^{2+}]_i$ in a single rabbit aortic smooth muscle cell. The cell which had been loaded with a Ca^{2+} indicator fura-2 was stimulated initially with 3.6 μM thrombin (Thr). After $[Ca^{2+}]_i$ reached a plateau, the cell was challenged again with 3.6 μM thrombin. Then the cell was challenged with 67 nM trypsin (Try). Similar results were obtained in 10 other cells.

in rabbit aortic rings without endothelium, which were comparable to those of thrombin.

The trypsin-induced contraction does not seem to be the result of irreversible damage to vascular smooth muscle cells, judging from the data that NA and 30 mN KCl-evoked contractions were unaffected by pretreatment with trypsin.

The vasocontracting action of trypsin seems to depend on its protease activity, as there was a correlation between the protease activities actually added to the tissue baths and the resulting contractile responses and the trypsin-induced contraction was abolished by protease inhibitors such as soybean trypsin inhibitor or PMSF.

The major portion of the contraction induced by trypsin or thrombin depends on influx of extracellular Ca^{2+}, based on the effect of removal of extracellular Ca^{2+}. Furthermore, the contractions by trypsin and thrombin are equally sensitive to nifedipine, indicating that both nifedipine-sensitive and -resistant Ca^{2+} channels are involved in the contractions. The result on the thrombin-induced contraction is essentially consistent with previous findings (Glusa *et al*., 1994) that the thrombin-induced contraction of porcine pulmonary arteries is partially sensitive to verapamil, a blocker of L-type voltage-operated Ca^{2+} channel.

However, the following data strongly indicate that a part of the vasocontraction induced by trypsin is mediated by a mechanism distinct from activation of thrombin receptor, although the remaining portion of the action is due to activation of thrombin receptor. Firstly, in the deendothelialized aortic rings precontracted with a maximally effective concentration of thrombin, trypsin induced small but significant contractions via its protease activity, whereas the second application of thrombin virtually failed to induce contractions. Secondly, trypsin but not thrombin induced an increased in $[Ca^{2+}]_i$ in a single vascular smooth muscle cell (VSMC) even after application of a maximally effective concentration of thrombin. This result is in contrast with that in 1321N1 astrocytoma cells where trypsin did not induce an effect on $[Ca^{2+}]_i$ following challenge with thrombin and *vice versa* (Jones *et al*., 1989).

It is noteworthy that dispersed VSMCs of rabbit aorta were more sensitive to trypsin than the isolated aortic strips. The precise mechanism for this is at present unknown, but several are considered. Firstly, in isolated strips of rabbit thoracic aorta, a barrier to infiltration of trypsin might be present. Alternatively, in the interstititial fluid of isolated strips of rabbit thoracic aorta, there might be some kind of trypsin inhibitor or of substrate for trypsin which competes for the same active site of trypsin.

Al-Ani *et al*. (1995) showed that in rat endothelium-free aortic ring preparations, PAR-2-activating polypeptide (SLIGRL) caused neither relaxation nor contraction at a concentration which induced a robust relaxation in endothelium-intact preparations. From these results, functional PAR-2 seems to be absent from rat aortic smooth muscle cells. If this is the case for rabbit aorta, the present findings strongly indicate that in addition to activation of the thrombin receptor, trypsin causes an increase in $[Ca^{2+}]_i$ and vasocontraction in rabbit aortic smooth muscle via a novel mechanism differing from PAR-2. Alternatively, trypsin might act on PAR-2 which is present on rabbit aortic smooth muscle cells but absent on rat aortic smooth muscle cells.

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