Effects of Trypsin on Cytosolic Calcium Levels in the Rat Aortic Endothelium

Daijiro KANEKO¹⁾, Hiroyuki KOMATSU¹⁾, Takashi OHAMA¹⁾ and Koichi SATO^{1)*}

¹⁾Department of Veterinary Pharmacology, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.

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ABSTRACT. The effect of trypsin on vascular tone and the cytosolic calcium concentration ($[Ca^{2+}]_i$) of endothelial and smooth muscle cells were examined in the rat aorta. A calcium indicator, fura-PE3, was used to measure $[Ca^{2+}]_i$ simultaneously with vascular tone. In the endothelium-intact rat aorta, carbachol and trypsin increased $[Ca^{2+}]_i$ in a dose-dependent manner. In the endothelium-denuded rat aorta, carbachol did not change $[Ca^{2+}]_i$, but trypsin slightly increased it. Addition of trypsin to the norepinephrine-stimulated rat aorta relaxed the muscle with an additional increase in $[Ca^{2+}]_i$. Under calcium-free conditions, trypsin induced a transient increase in $[Ca^{2+}]_i$. Trypsin-induced endothelium-dependent relaxation was inhibited by preincubation with L-NMMA, an endothelial NO synthase inhibitor, U-73122, a phospholipase C inhibitor, cyclopiazonic acid, a sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase blocker, and lanthanum, a nonselective Ca^{2+} channel blocker. However, indomethacin, a nonselective cyclooxygenase inhibitor, and SKF-96365, a store-operated Ca^{2+} -channel blocker, had no effect on the trypsin-induced relaxation. These results suggest that trypsin increases $[Ca^{2+}]_i$ in the endothelial cells through SKF-96365-insensitive Ca^{2+} channels and regulates the release of NO, which results in relaxation of the rat aorta.

KEY WORDS: cytosolic calcium level, endothelium derived relaxation, trypsin.

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Trypsin is one of the proteases excreted from the pancreas. It has many physiological and pathophysiological roles such as a digestive enzyme and for vascular relaxation and myometrial contraction [6, 10, 14]. Trypsin causes endothelium-dependent vasorelaxation in many types of blood vessels including the rat aorta, rat superior mesenteric artery and porcine coronary artery [1, 9, 14]. It has also been reported that the relaxation is due to nitric oxide and hyperpolarizing factors [4, 14].

It is widely known that trypsin activates protease-activated receptor (PAR) 2 under physiological conditions [16]. The PAR family is an unique family of G-protein-coupled seven-transmembrane receptors that are activated by proteolytic cleavage of the extracellular N-terminal domain [5, 7, 16, 22]. Among the PAR family, the function of PAR2 has been widely studied, and this receptor is known to be involved in hypertension, inflammation, allergies, hemorrhaging, exocrine activities and intestinal ion transport [11, 15, 17].

In the porcine coronary artery, trypsin induced an endothelium-dependent relaxation and a decrease in the cytosolic calcium concentration ($[Ca^{2+}]_i$) in smooth muscle cells [13]. In the porcine arterial valve, trypsin induced a transient elevation of $[Ca^{2+}]_i$ of endothelial cells [14]. However, the authors of that report did not measure the relaxing effect of trypsin simultaneously with the changes in $[Ca^{2+}]_i$ in endothelial cells. In a previous study [19], we measured the endothelium-dependent relaxation induced by carbachol simultaneously with the changes in endothelial $[Ca^{2+}]_i$ in the rat aorta. The present study thus aimed to assess the relationship of trypsin-induced smooth muscle relaxation and

the changes in endothelial $[Ca^{2+}]_i$ by using the simultaneous measurement technique.

MATERIALS AND METHODS

Tissue preparation: The animals were maintained in compliance with the guidelines of the Animal Care and Use Committee of Yamaguchi University. Male Wistar rats weighing 150–200 g were anaesthetized using ether and exsanguinated. The thoracic aorta was isolated and cut into helical strips (2–3 mm wide and 8–10 mm length) in normal physiological solution (in mM: NaCl 121.0, KCl 5.4, MgCl₂ 1.0, CaCl₂ 1.8, glucose 5.5, Tris 25, pH 7.4, with HCl). Ca^{2+} -free solution was made by adding 0.2 mM EGTA instead of CaCl₂ into the normal physiological solution. In some experiments, the endothelial cells were removed by gently rubbing with a cotton swab.

Simultaneous measurement of the $[Ca^{2+}]_i$ with muscle contraction in the rat aorta: A Ca^{2+} indicator, fura-PE3, was used to measure $[Ca^{2+}]_i$ simultaneously with muscle tension as reported previously [19, 20]. In brief, muscle strips were loaded with 5 μ M acetoxymethyl ester of fura-PE3 for 3–5 hr in the presence of 0.02% cremophor EL at room temperature and then placed in a tissue bath containing the normal physiological solution aerated with 100% O₂ at 37°C. The muscle strip was illuminated from the side of the endothelial monolayer alternately with 340 and 380 nm light, and the amount of 500 nm fluorescence induced by 340 nm excitation (F340) or that induced by 380 nm excitation (F380) was detected with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). The ratio of F340/F380 was monitored as the change in $[Ca^{2+}]_i$. The muscle contraction was measured simultaneously with $[Ca^{2+}]_i$. A resting tension of 10 mN was applied to each muscle strip.

Chemicals: The chemicals used were as follows: carba-

* CORRESPONDENCE TO: SATO, K., Department of Veterinary Pharmacology, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.
e-mail: k-sato@yamaguchi-u.ac.jp

chol, norepinephrine and fura-PE3 (Sigma, Tokyo, Japan); EDTA (Dojindo Laboratories, Kumamoto, Japan); and other chemicals (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analyses: Results were expressed as means $\pm$ standard error of the mean (SEM). Statistical evaluations of data were performed using paired or unpaired Student's *t*-tests for comparisons between 2 groups and one-way analysis of variance followed by the Tukey test for comparisons among ≥ 3 groups using the GraphPad Prism software (GraphPad Software, San Diego, CA, U.S.A.). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of carbachol and trypsin on $[Ca^{2+}]_i$ and contractile tension in the resting rat aorta: As shown in Fig. 1, 65.4-mM KCl induced a sustained increase in $[Ca^{2+}]_i$ and muscle tension in the endothelium-intact (A) and endothelium-denuded (B) rat aorta. After washing the muscle with normal physiological solution, addition of 3 μ M carbachol or 0.3 μ M trypsin induced a sustained increase in $[Ca^{2+}]_i$ with a slight decrease in the resting tension of the endothelium-intact aorta (Fig. 1A). In endothelium-denuded vessels, carbachol did not change $[Ca^{2+}]_i$ and muscle contraction, but trypsin induced a small increase in $[Ca^{2+}]_i$ without any change in resting tension (Fig. 1B). Addition of carbachol (0.1–10 μ M) and trypsin (1–100 nM) increased $[Ca^{2+}]_i$ in a dose-dependent manner in the endothelium-intact preparation (Fig. 2A and 2B). In endothelium-denuded vessels, a higher concentration of trypsin (0.1–10 μ M) induced a more apparent increase in $[Ca^{2+}]_i$ (Fig. 2C), but carbachol (10 μ M) did not have any effect on $[Ca^{2+}]_i$ (data not shown).

Effect of trypsin on $[Ca^{2+}]_i$ and contraction in the rat aorta precontracted with norepinephrine: Norepinephrine (0.3 μ M) induced a sustained increase in $[Ca^{2+}]_i$ and muscle tension in both of the endothelium-intact and endothelium-denuded rat aorta (Fig. 3A and 3B). Addition of 0.3 μ M trypsin in the presence of norepinephrine caused a rapid and sustained increase in $[Ca^{2+}]_i$ and rapid relaxation in the endothelium-intact preparation (Fig. 3A). By contrast, trypsin had a small effect on $[Ca^{2+}]_i$ without any changes in contraction in endothelium-denuded vessels (Fig. 3B).

Effect of trypsin on $[Ca^{2+}]_i$ and contraction in Ca^{2+} -free solution: After observing 65.4-mM KCl-induced changes, the external solution was replaced with a Ca^{2+} -free solution, which resulted in a rapid decrease in $[Ca^{2+}]_i$ and a slight decrease in resting tension (Fig. 3C). Addition of 0.3 μ M trypsin induced a transient increase in $[Ca^{2+}]_i$ without any changes in muscle tension in the endothelium-intact preparation. However, trypsin showed no effect on $[Ca^{2+}]_i$ in the endothelium-denuded preparation (data not shown).

Effects of inhibitors on endothelium-dependent relaxation induced by trypsin: In the presence of 0.3 μ M norepinephrine, trypsin (0.01–100 nM) induced dose-dependent relaxation of the endothelium-intact rat aorta (Fig. 4). In the vessels preincubated with a NO synthase inhibitor, l-

Fig. 1. Effects of carbachol and trypsin on $[Ca^{2+}]_i$ (upper trace) and contractile tension (lower trace) in the endothelium-intact (A) and endothelium-denuded (B) rat aorta. After the effects of 65.4-mM KCl were examined, carbachol (3 μ M) or trypsin (0.3 μ M) was added. The tonic level of KCl-induced $[Ca^{2+}]_i$ was defined as 100%.

Fig. 2. Dose-dependent effects of carbachol and trypsin on $[Ca^{2+}]_i$ in the endothelium-intact and endothelium-denuded rat aorta. Typical results are shown in left panel. After the effects of 65.4-mM KCl were examined, carbachol (0.1–10 μ M; A) or trypsin (1–100 nM; B) was added cumulatively to the endothelium-intact preparations. Higher concentrations of trypsin (0.1–10 μ M; C) were applied to the endothelium-denuded preparation. Analytical results are shown in the right panels. The tonic level of KCl-induced $[Ca^{2+}]_i$ was defined as 100% ($n=4-13$).

Fig. 3. Effect of trypsin on $[Ca^{2+}]_i$ (upper trace) and contractile tension (lower trace) of the rat aorta. In the presence of norepinephrine (NE, $0.3 \mu M$), trypsin ($0.3 \mu M$) was applied to the endothelium-intact (A) and endothelium-denuded (B) rat aorta. Trypsin ($0.3 \mu M$) was applied in Ca^{2+} -free solution (0Ca) (C). The tonic level of 65.4-mM-KCl-induced $[Ca^{2+}]_i$ was defined as 100%.

NMMA ($200 \mu M$), for 20 min, trypsin-induced relaxation was strongly inhibited (Fig. 4A). Pretreatment with indomethacin ($10 \mu M$) for 20 min, however, had no effect on trypsin-induced relaxation (Fig. 4B). Preincubation with a specific inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase, cyclopiazonic acid ($10 \mu M$), or an inhibitor of phospholipase C, U-73122 ($5 \mu M$), for 10 min inhibited trypsin-induced relaxation (Fig. 4C and 4D). Finally, we examined the effects of calcium channel blockers on trypsin-induced relaxation. The addition of lanthanum (0.1 and 0.3 mM for 10 min), a nonselective Ca^{2+} channel inhibitor, inhibited trypsin-induced relaxation in a dose-dependent manner (Fig. 4E). However, treatment with an inhibitor of store-operated Ca^{2+} -channel, SKF-96365 ($10 \mu M$), for 10 min did not change the dose-dependent relaxation induced by trypsin (Fig. 4F).

DISCUSSION

In the present study, the trypsin-induced changes in $[Ca^{2+}]_i$ of endothelial cells were simultaneously measured with the changes in the mechanical responses of smooth muscle in the rat aorta.

We have previously reported a method of measuring the endothelial and/or smooth muscle $[Ca^{2+}]_i$ simultaneously with muscle contraction by using Ca^{2+} -indicator fura-2 [18–20]. It has been reported that, in endothelium-intact vessels, the detected fluorescent intensity of a calcium indicator is attributed to that from endothelial cells and also from smooth muscle cells [20]. In our method, it is possible to

Fig. 4. Effects of inhibitors on trypsin-induced relaxation. Norepinephrine ($0.3 \mu M$) was added 20 min after application of I-NMMA ($200 \mu M$, A) or indomethacin ($10 \mu M$, B). Cyclopiazonic acid (CPA; $10 \mu M$, C), U-73122 ($5 \mu M$, D), $LaCl_3$ (0.1 or 0.3 mM, E) or SKF-96365 ($10 \mu M$, F) was applied 10 min before addition of norepinephrine. A norepinephrine-induced contraction just before the addition of trypsin ($0.1 \mu M$) was defined as 100%. *, **: statistical significance at the level of $P < 0.05$ and $P < 0.01$ vs. the control, respectively ($n = 6-11$).

detect $[Ca^{2+}]_i$ only from the endothelial cells by using selective agonists for the endothelial cells such as carbachol and histamine in the rat aorta [19]. In this study, we observed that addition of trypsin and carbachol to the endothelium-intact preparation in a resting state induced a dose-dependent increase in $[Ca^{2+}]_i$ (Figs. 1A, 2A and 2B). In the endothelium-denuded preparations, carbachol had no effect on $[Ca^{2+}]_i$, but a higher concentration of trypsin induced a small but apparent increase in $[Ca^{2+}]_i$ without muscle contraction. Trypsin activates PAR2 [16], which was expressed in both endothelial cells and smooth muscle cells of rat blood vessels [3]. These findings suggest that trypsin increases $[Ca^{2+}]_i$ not only in endothelial cells but also in smooth muscle cells in the rat aorta. In endothelium-denuded preparations, $0.1 \mu M$ trypsin induced a small increase in $[Ca^{2+}]_i$ ($8.2 \pm 0.7\%$), but the same concentration of trypsin induced a marked increase in $[Ca^{2+}]_i$ ($183.5 \pm 11.3\%$) in endothelium-intact preparations. These results suggested that the sensitivity of smooth muscle cells for trypsin is less than that of endothelial cells. Because, to elicit smooth muscle contraction, $[Ca^{2+}]_i$ should be increased to more than the threshold level [19], in the present experiments, the level of trypsin-induced $[Ca^{2+}]_i$ in rat aortic smooth muscle cells may not have been

enough to cause contraction. Therefore, it seems likely that the trypsin-induced effects are mainly mediated by the endothelial cells of the rat aorta.

We have previously shown that carbachol and histamine induce an additional increase in $[Ca^{2+}]_i$ in the endothelium-intact rat aorta precontracted with norepinephrine [20]. Furthermore, it was also demonstrated that there was a positive correlation between the increase in $[Ca^{2+}]_i$ in endothelial cells and muscle relaxation [19]. In the present experiments, trypsin induced a marked increase in $[Ca^{2+}]_i$ with relaxation of the rat aorta precontracted with norepinephrine (Fig. 3A). It was reported that NO decreased $[Ca^{2+}]_i$ of smooth muscle cells to induce relaxation [2, 20]. In the endothelium-denuded preparations precontracted with norepinephrine, trypsin induced a small increase in $[Ca^{2+}]_i$ without any changes in muscle contraction (Fig. 3B). This result suggests that the trypsin-induced additional increase in $[Ca^{2+}]_i$ is mainly due to the increase in endothelial $[Ca^{2+}]_i$, which regulates the endothelium-dependent relaxation.

In the porcine arterial valve, it has been reported that trypsin induced a transient increase in $[Ca^{2+}]_i$ either in the presence or absence of extracellular Ca^{2+} [14]. The authors concluded that the trypsin-induced $[Ca^{2+}]_i$ elevation was mediated mainly by the Ca^{2+} release [14]. Consistent with these findings, we observed that trypsin induced a transient increase in $[Ca^{2+}]_i$ under Ca^{2+} -free conditions (Fig. 3C). In normal physiological solution, however, trypsin induced two-phase changes of $[Ca^{2+}]_i$, a first transient phase and second tonic phase (Fig. 1A). In a previous study, carbachol or histamine induced a transient increase in $[Ca^{2+}]_i$ only under Ca^{2+} -free conditions [19]. These results suggest that the trypsin-induced $[Ca^{2+}]_i$ elevation is mediated by the Ca^{2+} release and Ca^{2+} influx. In the present study, trypsin-induced relaxation was attenuated by L-NMMA, but not by indomethacin (Fig. 4A and 4B). In porcine coronary artery, L-NORG, another NOS inhibitor, attenuated trypsin-induced relaxation [14]. These results suggest that trypsin-induced endothelium-dependent relaxation is mainly mediated by NO.

It is well known that the agonist-induced Ca^{2+} influx in endothelial cells is mediated by the store-operated cation channels (SOCs) that are activated by inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} -store depletion [21]. It was also reported that trypsin-induced Cl^- secretion from the colonic epithelium depends on intracellular Ca^{2+} signaling mediated by Ca^{2+} release from intracellular stores and extracellular Ca^{2+} entry [13]. We revealed that trypsin-induced endothelium-dependent relaxation was inhibited by cyclopiazonic acid, U-73122, and lanthanum (Fig. 4C–E). These results suggest that the increase in $[Ca^{2+}]_i$ induced by trypsin is achieved by generation of IP_3 , release of stored intracellular Ca^{2+} and Ca^{2+} entry through plasma membrane channels in endothelial cells. On the other hand, SKF-96365 did not change the trypsin-induced relaxation (Fig. 4F). Although SKF-96365 is widely used as a SOC-inhibitor [23, 24], it was also reported that agonist-induced store-operated Ca^{2+} entry in several types of cells was not inhibited by treatment

with SKF-96365 [8, 12], suggesting the existence of SKF-96365-insensitive store-operated Ca^{2+} channels. These results suggest that trypsin likely induces Ca^{2+} entry through SKF-96365-insensitive store-operated Ca^{2+} channels.

In conclusion, we revealed that trypsin increases $[Ca^{2+}]_i$ in endothelial cells through the store-operated Ca^{2+} channel that is insensitive to SKF-96365 and that synthesis of NO, which results in relaxation of the rat aorta, is regulated by $[Ca^{2+}]_i$ in endothelial cells of the rat aorta.

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