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Mechanism of Human Urotensin II-Induced Contraction in Rat Aorta

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Abstract. Urotensin II induced sustained contraction with an EC_{50} value of 2.29 ± 0.12 nM in rat aorta. Urotensin II (100 nM) transiently increased cytosolic Ca^{2+} level ($[Ca^{2+}]_i$), followed by a small sustained phase superimposed with rhythmic oscillatory change. In the presence of verapamil and La^{3+}, the $[Ca^{2+}]_i$ oscillation was completely inhibited, although a small transient increase in $[Ca^{2+}]_i$ remained. The urotensin II-induced contraction was also partially inhibited by verapamil and La^{3+}. Combined application of verapamil, La^{3+}, and thapsigargin completely inhibited the increase in $[Ca^{2+}]_i$ with only partial inhibition of the contraction elicited by urotensin II. Urotensin II increased myosin light chain (MLC) phosphorylation to a level greater than that induced by 72.7 mM KCl (high K^+). Pretreatment with Go6983 (PKC inhibitor), U0126 (MEK inhibitor), or SB203580 (p38MARK inhibitor) partially inhibited the urotensin II-induced contraction with no effects on the high K^+-induced contractions. Wortmannin (MLC kinase inhibitor) only partially inhibited urotensin II-induced contraction, although it completely inhibited the high K^+-induced contraction. These results suggest that urotensin II-induced contraction is mediated by the Ca^{2+}/calmodulin/MLC kinase system and modulated by the Ca^{2+} sensitization mechanisms to increase MLC phosphorylation. In addition, activations of PKC, p38MAPK, and ERK1/2 modulate the contractility mediated by urotensin II in rat aorta.

Keywords: urotensin II, rat aorta, Ca^{2+}, myosin phosphorylation, Ca^{2+} sensitization

Introduction

Human urotensin II, the precursor of which has recently been cloned (1), is composed of eleven amino acid residues retaining a conserved cyclic hexapeptide and N-terminal region consisting of four amino acid residues (Glu-Thr-Pro-Asp). A high affinity rat receptor for urotensin II bearing a similarity to the somatostatin and opioid receptor family has been identified as GPR14 orphan G protein-coupled receptor and designated as UII-R1a (2). Simultaneously, a human G-protein-coupled receptor having a 75% sequence similarity to the orphan rat receptor GPR14 and exhibiting pharmacological characteristics of human urotensin II receptor was also cloned (3). Human urotensin II binds with high affinity to this receptor, and the binding is functionally coupled to intracellular calcium mobilization. Human urotensin II and its prepro-mRNA have been found to be localized primarily in the central nervous system and cardiovascular system and also found in smaller amounts in other organs (1, 3 – 5).

Urotensin II was found to be a highly potent vasoactive peptide, comparable to endothelin-1 (3, 6). In rabbit aorta, human urotensin II increased the levels of [³H]-inositol phosphates in a concentration-dependent manner, with the same potency with which it causes vasoconstriction (7). It was recently reported that human urotensin II-induced contractions in rat aorta are mediated by the activation of Rho A and the Rho-kinase-dependent pathway (8). Human urotensin II was also shown to induce actin stress fiber formation and arterial smooth muscle cell proliferation (8). It was also found that goby-urotensin II reduced blood pressure in anesthetized rats (9); and in the isolated rat heart, it elicited a biphasic response on coronary flow consisting

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of initial decreases followed by sustained increase due to vasodilation (10). In contrast, human urotensin II was found to be a potent endothelium-dependent relaxant in intact rat mesenteric arteries pre-contracted with methoxamine (11). These reports indicate that urotensin II plays an important role in the cardiovascular regulatory system.

Smooth muscle contraction is regulated by the Ca^{2+} /calmodulin/myosin light chain (MLC) kinase system (12). In addition, Ca^{2+} sensitization mechanism modulates this system (13 – 15) to change the level of MLC-phosphorylation. In contrast, actin binding proteins, such as calponin and caldesmon, may also regulate tension generation in a MLC-phosphorylation-independent manner (14, 16, 17). In fact, it was reported that extracellular signal-regulated kinase (ERK) and/or p38 mitogen-activated protein kinase (p38MAPK) phosphorylates caldesmon, which in turn induces actin-myosin interaction without changing MLC-phosphorylation in vascular smooth muscles (17, 18). The most potent vasoconstrictors, endothein-1 and angiotensin II, also activate MAPK to modulate tension generation (19).

It has not yet been clarified whether urotensin II mobilizes cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) or how $[Ca^{2+}]_i$ regulates the urotensin II-induced contraction. Therefore, in the present study, we measured the effects of cytosolic Ca^{2+}, MLC phosphorylation, and muscle tension on the urotensin II-induced contraction in rat aorta and examined the effects of p38MAPK and MAPK/ERK kinase (MEK) inhibitors on the contraction.

Materials and Methods

Muscle preparation and measurement of muscle force

The thoracic aorta was isolated from male Wistar rats (200 – 300 g), cut into helical strips (2 mm in width and 8 mm in length) or rings and placed in a normal physiological salt solution (PSS) that contained: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 0.01 mM ethylenediamine-tetraacetic acid, and 5.5 mM glucose. All procedures to measure muscle force were accordance with the method described previously (20). Briefly, endothelium was removed by gently rubbing the intimal surface with a finger moistened with the above solution. The developed tension was recorded with an isometric force transducer. The preparation was stretched to a resting tension of 10 mN. After an equilibration period of 1 h. A high concentration of KCl solution (high K^+) was made by substituting NaCl with equimolar KCl in the PSS. These solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4.

Simultaneously measurement of tension and $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured simultaneously with muscle tension by use of the fluorescent Ca^{2+} indicator fura-2, as reported previously (21). The muscle strips were loaded with 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 4 – 5 h at room temperature. The noncytotoxic detergent cremophor EL (0.02%) was also added to increase the solubility of fura-2/AM. The muscle strip was held horizontally in a temperature-controlled organ bath. One end of the strip was connected to the tension displacement transducer to monitor muscle tension. Experiments were performed with a fluorimeter (CAF-100; Jasco, Tokyo). The ratio of 500 nm fluorescence excited at 340 nm and 380 nm (F340/F380) was used as an indicator of $[Ca^{2+}]_i$, and the quantitative comparison of $[Ca^{2+}]_i$ was made by taking resting and high K^+-stimulated $[Ca^{2+}]_i$ as 0% and 100%, respectively. Absolute Ca^{2+} concentration was not calculated because it was considered that the dissociation constant of fura-2 in the cytoplasm might be different from that obtained in vitro (22). Muscle tension was recorded isometrically under a resting tension of 10 mN.

Measurement of MLC phosphorylation

The extent of MLC phosphorylation was measured according to the method of Word et al. (23). Strips of rat aorta were quickly frozen in liquid nitrogen and then homogenized with 10% trichloroacetic acid and 10 mM dithiothreitol (DTT). The homogenate was centrifuged at $10,000 \times g$ for 1 min, and the pellet was washed with diethyl ether and then suspended in urea-glycerol buffer for electrophoretic analysis of light chain phosphorylation by immunoblotting. Polyacrylamide gels (10%) containing glycerol (40%, vol/vol) were pre-electrophoresed for 30 min at 300 V at room temperature. The reservoir buffer contained Tris-base (20 mM) and glycine (23 mM), pH 6.8, and triglycolate (2.3 mM) and DTT (2.3 mM) were also included in the upper reservoir buffer. Samples (5 – 10 μg protein) were electrophoresed at 300 V for 90 min at room temperature. Protein was transferred to nitrocellulose at 2 mA /cm for 60 min at room temperature. Nonphosphorylated and phosphorylated forms of MLC were localized on nitrocellulose paper with antibody against bovine tracheal MLC, alkaline phosphatase-conjugated goat anti-rabbit IgG, and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as the substrate for the alkaline phosphatase. Relative amounts of non-phosphorylated and phosphorylated MLC, respectively, were measured using NIH-imaging.

Drugs and chemicals

The following drugs and chemicals were used: human

urotensin II (Peptide Institute, Osaka); thapsigargin and verapamil hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); cremophor EL and DTT (Nacalai Tesque); fura-2/AM (Dojindo Laboratories, Kumamoto); Go6983 (2-[1-(3-dimethylaminopropyl)-5-methoxy-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide), wortmannin, and U0126 (1,4-diamino-2,3-dicyano-1,4*bis*(2-amino-phenylthio)-butadiene) (Calbiochem, San Diego, CA, USA); and SB203580 (4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) (Wako, Tokyo).

Statistics

Results of the experiments are expressed as the mean ± S.E.M. The unpaired or paired Student's *t*-test was used where appropriate for comparing the responses of two groups, and ANOVA followed by Scheffe's post hoc test was used when comparing the responses of three or more groups. *P*<0.05 was considered statistically significant.

Results

The concentration-dependent effect of human urotensin II on rat aorta

When the rat aorta denuded endothelium was stimulated with a low concentration of urotensin II (1 nM), the muscle tension began to increase within a 10 – 20-s latency period and gradually increased to reach a peak within 60 min. The maximum tension was $17.3 \pm 2.1\%$

of the 72.7 mM KCl-induced maximum contraction, and tension stayed at this level for at least 90 min (n = 9). When the muscle strips were stimulated with the higher concentrations of urotensin II (10 and 100 nM), the tension began to increase within a latency period of a few seconds, and reached a peak within 30 min ($77.6 \pm 9.5\%$ and $94.6 \pm 4.3\%$ of 72.7 mM KCl-induced contraction, n = 9 each). These contractions gradually decreased after 30-min application of urotensin II (Fig. 1A). In our preliminary experiments, urotensin II-induced contraction did not change in the endothelium intact rat aorta compared with the contractions in the endothelium-denuded strips (unpublished observation).

Figure 1B shows the concentration response curve of urotensin II in rat aorta. Each plot shows maximum responses induced by each concentration. From these results, the EC_{50} value was calculated to be 2.29 ± 0.12 nM.

The effects of urotensin II on tension and $[Ca^{2+}]_i$

We next simultaneously measured muscle tension and $[Ca^{2+}]_i$ using the fura-2-loaded muscle strips. Application of high K^+ rapidly increased $[Ca^{2+}]_i$ followed by a large sustained phase. Muscle tension gradually increased and reached a steady level after 15 min (Fig. 2A). Urotensin II (100 nM) also increased $[Ca^{2+}]_i$ and muscle tension, as shown in Fig. 2A. In contrast to the high K^+-induced responses, however, urotensin II-induced increase in $[Ca^{2+}]_i$ was transient, followed by a small sustained phase of superimposed large sponta-
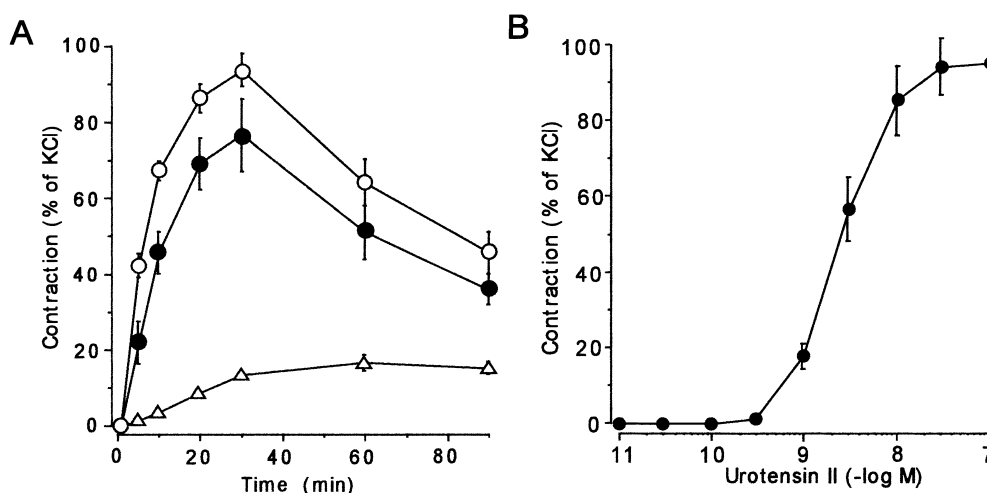

Fig. 1. Time course of the changes in tension induced by human urotensin II (A) and the concentration response curve of urotensin II (B) in the endothelium-denuded rat aorta. 100% represents the maximum contractile tension in response to 72.7 mM high K^+. Each concentration of uroteinsin II was applied to the tissue and then we measured muscle tension during 90 min (A). Open circle, closed circle, and open triangle in panel A show results for 100 nM, 10 nM, and 1 nM urotensin II, respectively. Maximum responses to various concentrations of urotensin II are plotted in panel B. Each concentration of uroteinsin II was cumulatively added in panel B. Each data represents the mean ± S.E.M. (n = 9 in A and n = 10 in B).

neous oscillation. $[Ca^{2+}]_i$ level after 10 min stimulation with urotensin II (100 nM) was significantly smaller than that induced by high K^+. However, muscle tension measured 10 min after the stimulation was greater than that after high K^+-induced contraction (Fig. 2B).

We next examined the effects of Ca^{2+} channel blockers and sarcoplasmic reticulum Ca^{2+} pump inhibitor on urotensin II-induced responses. We used combined

application of 10 μM verapamil and 30 μM La^{3+} to completely inhibit Ca^{2+} influx. Combined application of 10 μM verapamil and 30 μM La^{3+} completely inhibited the sustained increase in $[Ca^{2+}]_i$ and also the oscillatory changes in $[Ca^{2+}]_i$. However, the initial increase in $[Ca^{2+}]_i$ remained ($12.5 \pm 4.7\%$ at 3 min of high K^+-induced maximum response, n = 4). Urotensin II-induced muscle tension was also inhibited to
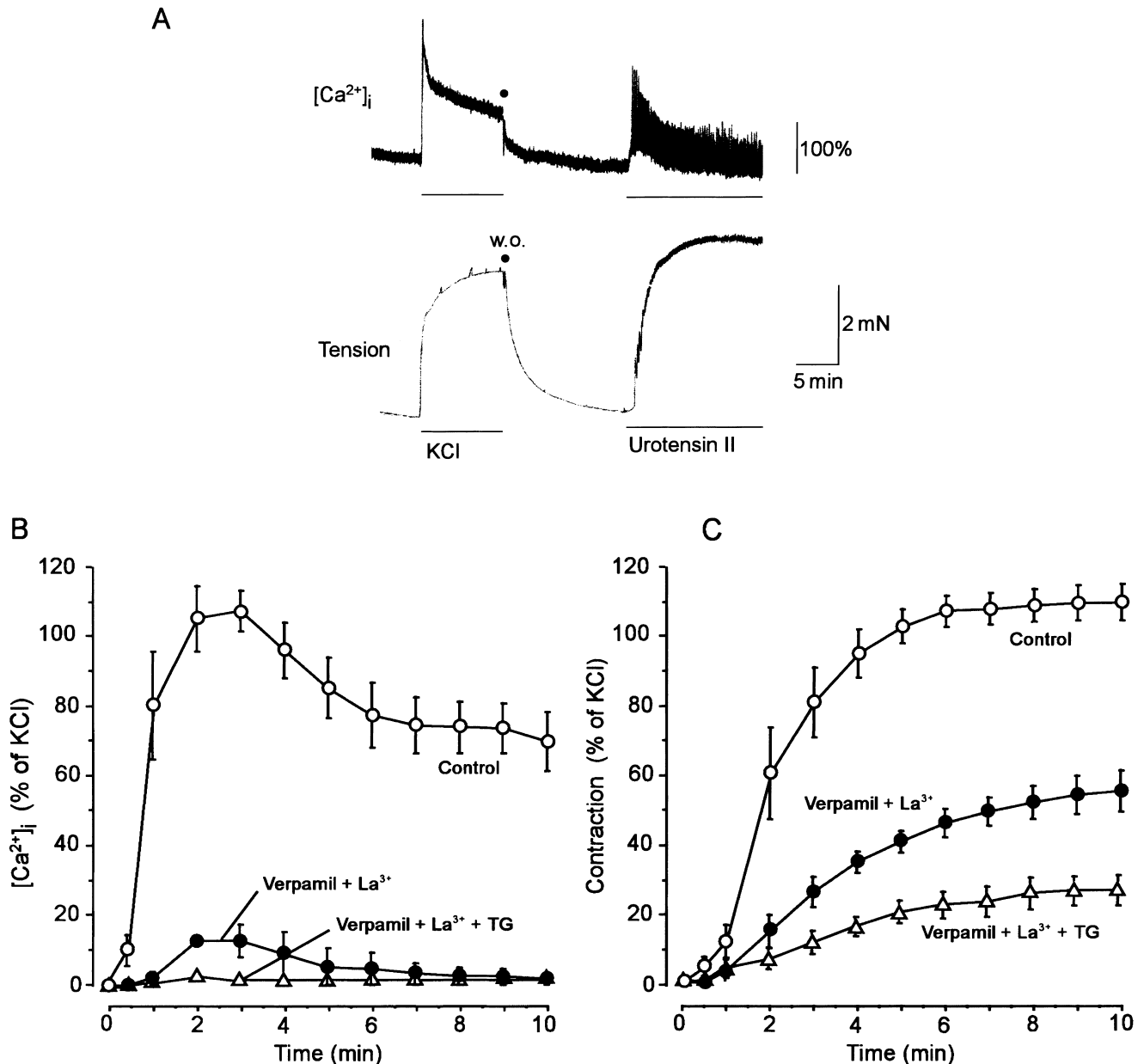

Fig. 2. Effects of urotensin II on $[Ca^{2+}]_i$ and tension. A: Typical trace of simultaneous measurements of muscle tension and $[Ca^{2+}]_i$ stimulated with 100 nM urotensin II in rat aorta. B and C: The time course of the changes in $[Ca^{2+}]_i$ (B) and tension (C) in response to 100 nM urotensin II alone or in response to urotensin II in the presence of 10 μM verapamil and 30 μM La^{3+} or the presence of verapamil, La^{3+}, and 1 μM thapsigargin (TG). Verapamil and La^{3+} were applied 5 min before addition of uroteinsin II. TG was applied 20 min before addition of uroteinsin II. Each data point was calculated from 6 (Control) or 4 (Verapamil + La^{3+}, Verapamil + La^{3+} + TG) experiments, and the maximum response to 72.7 mM high K^+ was taken as 100%.

$54.7 \pm 5.8\%$ (at 10 min, n = 4) of the high K$^+$-induced maximum response. Combined addition of thapsigargin (1 μM) with verapamil and La^{3+} completely inhibited the $[Ca^{2+}]_i$ increase elicited by urotensin II (100 nM). However, muscle tension due to urotensin II was only partially inhibited, to $25.4 \pm 4.2\%$ (at 10 min, n = 4) that of the high K$^+$-induced maximum response (Fig. 2: B and C).

Effects of urotensin II on MLC phosphorylation

The level of MLC phosphorylation at the resting condition was 10 – 16% of total MLC. Addition of high K$^+$ transiently increased MLC phosphorylation to $55.6 \pm 5.8\%$ at 10 s after stimulation (n = 4), and it was gradually decreased to $41.1 \pm 3.7\%$ at 5 min after stimulation (n = 4). Human urotensin II (100 nM) induced a rapid increase in MLC phosphorylation that reached a maximum level of $71.6 \pm 2.0\%$ of total MLC in 30 s, followed by a slight decrease to $62.3 \pm 1.4\%$ and $54.4 \pm 1.3\%$ at 3 and 5 min, respectively (n = 4 each). In the presence of verapamil and La^{3+}, MLC-phosphorylation induced by high K$^+$ was completely inhibited, to $9.2 \pm 4.5\%$ at 5 min after stimulation (n = 4). In contrast, verapamil and La^{3+} only partially decreased MLC-phosphorylation elicited with urotensin II to $31.6 \pm 2.5\%$ and $38.9 \pm 2.4\%$ at 3 and 5 min, respectively (n = 4 each) (Fig. 3).
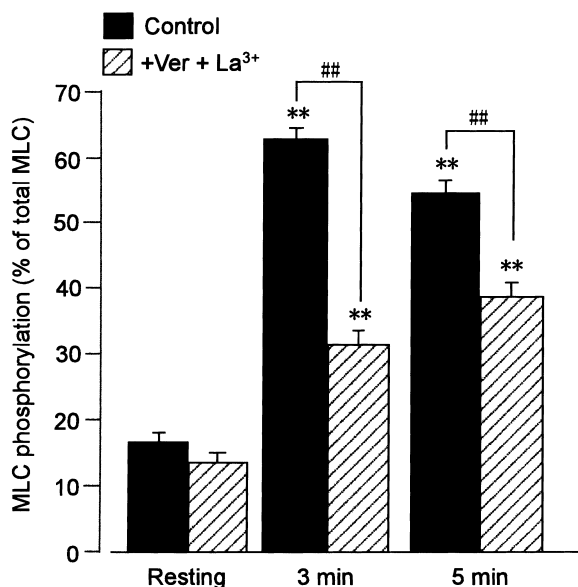
Effects of the PKC inhibitor Go6983 and the MLC kinase inhibitor wortmannin on urotensin II-induced contraction

Figure 4 shows the effects of the protein kinase C (PKC) inhibitor Go6983 (10 μM) and the MLC kinase inhibitor wortmannin (10 μM) on urotensin II-induced contractions. We reported that 1 μM Go6983 significantly inhibited phorbol ester-induced contraction in uterus smooth muscle (24). In the present study, we used a higher concentration (10 μM) of Go6983 to eliminate the PKC-mediated contractile component of uroteinsin II-induced contraction. Pretreatment with Go6983 significantly inhibited urotensin II-induced contraction.

In rat aorta, we reported that wortmannin completely inhibited high K$^+$-induced contraction without changing $[Ca^{2+}]_i$ in rat aorta (25). In the present study, we used a higher concentration of wortmannin to eliminate MLC kinase activity. In fact, wortmannin (10 μM) completely inhibited high K$^+$-induced contraction when muscle strips were pre-treated for 30 min (n = 6, data not shown). On the contrary, urotensin II still induced sustained contractions after such treatment (Fig. 4).
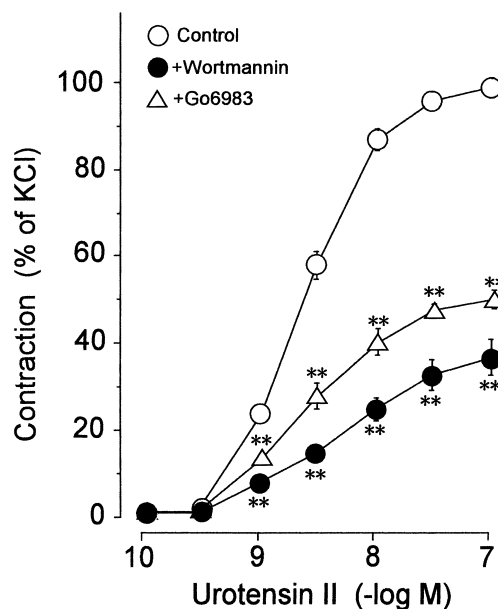

Fig. 3. Myosin light chain (MLC) phosphorylation in response to stimulation with 100 nM urotensin II in the absence or presence of 10 μM verapamil combined with 30 μM La^{3+}. MLC-phosphorylation is shown as % of phosphorylated MLC versus total MLC. Each column shows the mean ± S.E.M. (n = 4). **$P<0.01$, *vs* control at the Resting; ##$P<0.01$.

Fig. 4. Effects of Go6983 (10 μM) or wortmannin (10 μM) on 100 nM urotensin II-induced contractions in the endothelium-denuded rat aorta. Go6983 and wortmannin were applied at 15 min and 30 min before addition of uroteinsin II. Each concentration of uroteinsin II was cumulatively added, and the muscle contraction induced by 72.7 mM high K$^+$ obtained just before starting the experiments was taken as 100%. Each plot shows the mean ± S.E.M. (n = 4 each). **$P<0.01$, *vs* control.
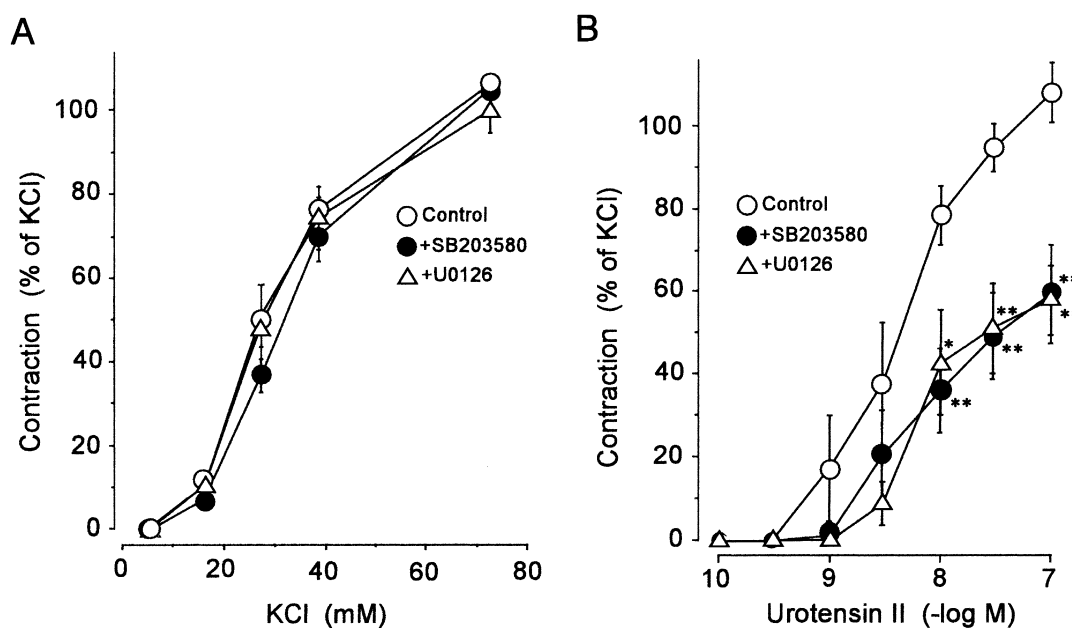
Fig. 5. Effects of SB203580 or U0126 on high K$^+$ (A)- or 100 nM urotensin II (B)-induced contractions in the endothelium-denuded rat aorta. SB203580 (10 μM) and U0126 (10 μM) were added 15 min before addition of high K$^+$ or uroteinsin II. Each concentration of High K$^+$ or uroteinsin II was cumulatively added, and the muscle contraction induced by 72.7 mM high K$^+$ obtained just before starting the experiments was taken as 100%. Each plot shows the mean ± S.E.M. (n = 4 each). *$P<0.05$, **$P<0.01$, vs control.

Effects of the MEK inhibitor U0126 and the p38MAPK inhibitor SB203580 on urotensin II-induced contraction

Figure 5 shows the effects of the MEK inhibitor U0126 (10 μM) and the p38MAPK inhibitor SB203580 (10 μM) on high K$^+$- or urotensin II-induced contractions. The used concentrations (10 μM) of U0126 and SB203580 in this study were reported as selective concentrations in rat aorta (19, 26). In the present study, in fact, 10 μM U0126 and 10 μM SB203580 had no effect on the high K$^+$-induced contraction. In contrast, pretreatment of the artery for 15 min with U0126 or SB203580 significantly reduced urotensin II-induced contractions.

Discussion

In rat aorta denuded with endothelium, in the present study, uroteinsin II induced strong vasoconstriction. Bottrill et al. reported that uroteinsin II did not induce endothelium-dependent relaxation mediated by nitric oxide and endothelium-dependent hyperpolarizing factor in rat aorta (11). Our preliminary experiments also indicated the same results (data not shown). In contrast, they indicated that in rat small resistant arteries such as left anterior descending coronary arteries and small mesenteric arteries, uroteinsin II induced endothelium-dependent relaxation. Taken together, in the present

study, we examined effects of uroteinsin II on rat aorta denuded with endothelium.

It has been reported that the Ca^{2+}-channel blockers, verapamil and nitrendipine, can partially inhibit the urotensin II-induced contraction (27). In the present study, we, for the first time, measured [Ca^{2+}]$_i$ movement elicited with uroteinsin II using fura-2. Urotensin II induced a transient increase in [Ca^{2+}]$_i$ followed by small sustained phase superimposed with large rhythmic oscillation. Combined application of verapamil and La^{3+} completely inhibited both the rhythmic oscillatory response and the small sustained increase in [Ca^{2+}]$_i$. These results suggest that Ca^{2+} influx elicited by urotensin II is mediated through the activation of both voltage-dependent Ca^{2+} channels and La^{3+}-sensitive non-selective cation channels. The small transient increase in [Ca^{2+}]$_i$ remaining in the presence of verapamil and La^{3+} was completely inhibited by thapsigargin, suggesting that Ca^{2+} release from sarcoplasmic reticulum is induced by urotensin II. In biochemical analysis, in fact, urotensin II has been shown to stimulate phospho-inositide hydrolysis (7). In contrast, verapamil and La^{3+} only partly inhibited the urotensin II-induced contraction, although the sarcoplasmic reticulum was almost depleted 5 min after stimulation with urotensin II. This phenomenon indicates that Ca^{2+} sensitization of contractile mechanisms is activated by urotensin II, supporting

the findings in previous reports (8, 27).

We next measured the MLC phosphorylation level induced by high K^+ or 100 nM urotensin II. Although the $[Ca^{2+}]_i$ transient increase induced by urotensin II was smaller than that induced by high K^+, the MLC phosphorylation level at 5 min of stimulation with urotensin II (100 nM) was significantly greater than that in response to stimulation with high K^+. These results indicate that urotensin II induces Ca^{2+} sensitization of contractile elements, which is accompanied by the increase in MLC phosphorylation. The Ca^{2+} sensitization is considered to be mainly mediated by inhibition of myosin phosphatase activity via the RhoA/Rho-kinase system and/or the PKC/CPI-17 system (14, 28). Sauzeau et al. reported that urotensin II induced vaso-constriction and cell proliferation through the RhoA/Rho-kinase system (8). Rossowski et al. (27) indicated that not only Rho-kinase but also PKC regulates urotensin II-induced contraction in rat aorta. In the present study, as shown in Fig. 4, urotensin II-induced contraction was partly inhibited by the PKC inhibitor Go6983. Taken together, the findings suggest that urotensin II-induced Ca^{2+} sensitization may be mediated by both the PKC/CPI-17 system and the RhoA/Rho-kinase system.

Urotensin II is a dodecapeptide that has similar characteristics with somatostatin and endothelin-1 (see introduction). Recently, it was found that urotensin II has physiological functions similar to those of endo-thelin-1 and angiotensin II in blood vessels (such as vasoconstriction, cell proliferation, and endothelium-dependent vasodilation) (see Introduction). Endothelin-1- and angiotensin II-mediated vasoconstrictions are, at least in part, modulated by the activation of genistein-sensitive tyrosine kinases and p44/42 MAP kinases (19, 29). In the present study, we found that urotensin II-induced contraction was only partly inhibited, although high K^+-induced contraction was completely inhibited, by the MLC kinase inhibitor wortmannin (10 μM). In addition, urotensin II-induced contraction but not high K^+-induced contraction was partly inhibited by the MEK inhibitor U0126 or by the p38MAPK inhibitor SB203580. These results suggest that both ERK1/2 and p38MAPK may modulate urotensin II-induced vaso-constriction in rat aorta. ERK1/2 and p38MAPK may phosphorylate target proteins such as caldesmon, which in turn modulates tension generation without affecting MLC-phosphorylation (17, 18). Further investigations are necessary to clarify this point.

In conclusion, our findings suggest that urotensin II induced contraction is mediated by the Ca^{2+}/calmodulin /MLC kinase system and modulated by Ca^{2+} sensitization mechanisms to increase MLC phosphorylation. In addition, PKC, p38MAPK, and ERK1/2 activations may modulate the urotensin II-induced contraction in rat aorta.

Acknowledgment

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