

## Difference in Signal Transduction Mechanisms Involved in 5-Hydroxytryptamine- and U46619-Induced Vasoconstrictions

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### Abstract

In order to elucidate the signal transduction pathways of vascular smooth muscle contractions induced by stimulation of receptors for 5-hydroxytryptamine (5-HT) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>), both of which are released from activated platelets, we examined whether protein kinases, such as tyrosine kinase, p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), are involved in the contraction produced by either 5-HT or U46619 (an analog of TXA<sub>2</sub>) in the rat aorta. Both 5-HT and U46619 induced sustained contractions, which were markedly reduced in the absence of extracellular Ca<sup>2+</sup>. Verapamil (a L-type Ca<sup>2+</sup> channel blocker) markedly inhibited the contractile response to 5-HT, while the U46619-induced contraction was only slightly inhibited by verapamil. Both contractile responses to 5-HT and U46619 were significantly inhibited by calphostin C (a PKC inhibitor). On the other hand, both genistein (5 μM, a tyrosine kinase inhibitor) and SB203580 (a p38 MAPK inhibitor) significantly inhibited 5-HT-induced contractions but had little effects on the contractions induced by U46619. These results suggest that the signal transduction mechanisms involved in the contractions mediated via 5-HT and TXA<sub>2</sub> receptors are different as follows. Both the tyrosine kinase and p38 MAPK pathways are involved in 5-HT contraction but not in TXA<sub>2</sub> contraction, while both contractions are strongly dependent on transplasmalemmal Ca<sup>2+</sup> entry. The contractile responses to both 5-HT and TXA<sub>2</sub> involve voltage-dependent Ca<sup>2+</sup> channels and PKC.

Key words: vasoconstriction, prostaglandins, signal transduction, protein kinases, calcium channels

## Introduction

Both 5-hydroxytryptamine (5-HT) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) have been shown to be potent vasoconstrictors released from activated platelets (Vanhoutte, 1988) and both are involved in ischemia of a variety of thrombotic diseases including ischemic heart disease and stroke. The main mechanism involved in vascular smooth muscle contraction is through an increase in cytoplasmic calcium and phosphorylation of the regulatory light chains of myosin (Karaki *et al.*, 1997). But there has been considerable evidence more recently to indicate that vasoconstrictive agonists activate multiple ancillary pathways that modulate the contractile response: protein kinase C (PKC) (Horowitz *et al.*, 1996), Rho family G proteins (Somlyo and Somlyo, 2000), nonreceptor tyrosine kinases (Hughes and Wijetunge, 1998), and extracellular signal-regulated kinases (ERK1/2) (Ishihata *et al.*, 2002) have been shown to play roles in smooth muscle contraction. Moreover, stress-activated protein kinases have also been implicated in sustained contraction through regulation of the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) (Yamboliev *et al.*, 2000). Activation of phospholipase C, Ca<sup>2+</sup> channels, tyrosine kinase and ERK MAPK have also been reported to be involved in 5-HT-induced contraction (Watts, 1998). However, the role played by p38 MAPK in 5-HT induced-contraction has not been clarified. Moreover, the mechanisms involved in TXA<sub>2</sub>-induced contraction have not been fully explored.

In order to elucidate the contractile mechanisms induced by vasoconstrictive substances released from activated platelets, we examined and compared the effects of inhibitors of voltage-dependent Ca<sup>2+</sup> channels, protein kinase C, tyrosine kinase and p38 MAPK on the contractile responses of the rat thoracic aorta to both 5-HT and U46619 (an analogue of thromboxane A<sub>2</sub>).

## Materials and Methods

### *Tissue preparation*

The experimental protocols regarding treatment of animals used in this study were in accordance with the Guidelines for Experiments Using Laboratory Animals adopted by the Yamagata University School of Medicine. Male Wistar rats (320–380 g) were killed by cervical dislocation under anesthesia with ether. The thoracic aorta was immediately dissected from each animal and excess fat and connective tissue removed. The vessel was cut into rings 3 mm long, which were then cut open and the endothelium removed from the aortic strip by gentle rubbing of the intimal surface with a moistened cotton swab.

### *Tension measurement*

Each aortic strip was suspended in an organ bath containing 10 ml of a physiological salt solution. The components of the solution were as follows (in millimolar): NaCl 118, KCl 4.7, NaHCO<sub>3</sub> 24.9, MgSO<sub>4</sub> 1.18, KH<sub>2</sub>PO<sub>4</sub> 1.18, CaCl<sub>2</sub> 1.8, glucose 11.1, and ascorbic acid 0.057. A solution containing a high concentration of K<sup>+</sup> was made by substituting NaCl with equimolar KCl. These solutions were saturated with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C (pH 7.4). The tension developed was recorded with an isometric force transducer (7T-15-240, Orientec, Tokyo,

Japan). After an equilibration period of 1 hr with a resting tension of 7.84 mN, each strip was contracted with 66.7 mM KCl repeatedly until a reproducible contraction was obtained. Removal of the endothelium was verified by the disappearance of relaxation induced by acetylcholine (1  $\mu$ M) in strips precontracted with 100 nM phenylephrine. Then, each strip was washed and either an inhibitor (verapamil, genistein, calphostin C or SB203580) or control solution (vehicle only) was added to the organ bath 15 min before the start of stimulation with either 5-HT or U46619. The concentration of each inhibitor used in the present study was that determined to inhibit each pathway in previous studies (Florian and Watts, 1998; Banes *et al.*, 1999; Okoro, 1999; Meloche *et al.*, 2000). In the preliminary experiments, we confirmed that genistein, calphostin C and SB203580 at the concentrations used in the present study did not affect the contraction induced by KCl. This implies that the effects of these inhibitors were not due to nonspecific actions on the contractility of the vascular smooth muscle.

For each agent, the EC<sub>50</sub> (the concentration required to induce a half-maximal contractile response) was determined using the Microplate Manager Computer software (Bio-Rad laboratories, CA, USA), and the sensitivity of the contractile response was evaluated using pD<sub>2</sub>, the negative log of the EC<sub>50</sub> (M).

### Drugs

The drugs used were as follows: U46619 (Nacalai Tesque, Kyoto, Japan), 5-hydroxytryptamine creatinine sulfate (5-HT), verapamil hydrochloride, genistein, calphostin C (Sigma Chemical, St. Louis, MO, U.S.A.) and SB203580 (Wako Chemical, Tokyo, Japan). 5-HT and verapamil were dissolved in distilled water to make stock solutions of 10 mM and 1 mM, respectively, and diluted with 0.9% NaCl before use. Genistein, calphostin C and SB203580 were dissolved in dimethylsulfoxide (DMSO) to make each stock solution of 10 mM and diluted with 0.9% NaCl before use.

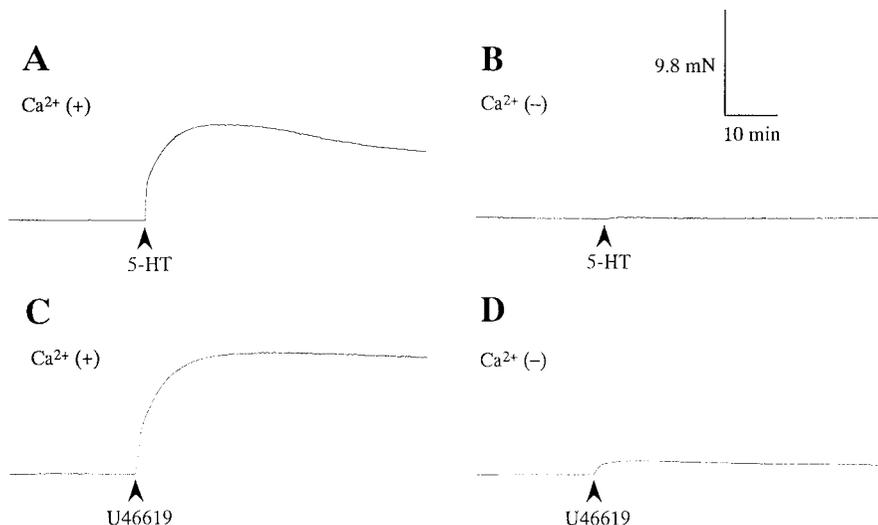
### Statistics

Data are expressed as the mean  $\pm$  S.E.M. The unpaired Student's *t*-test was used in comparing two group responses, and one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test was used when comparing the data of concentration-dependent contractile responses.  $P < 0.05$  was considered to be statistically significant.

## Results

### *Effects of removal of extracellular Ca<sup>2+</sup> and of verapamil on 5-HT- and U46619-induced contractions*

Figure 1 shows representative recordings of contractile responses of rat aortic strips to both 5-HT and U46619. In the absence of Ca<sup>2+</sup> in the medium, both 5-HT- and U46619-induced contractions were strongly inhibited [5-HT contraction (% of 66.7 mM KCl contraction), 104.9  $\pm$  5.0% (in Ca<sup>2+</sup>-containing medium) v.s. 3.8  $\pm$  0.4% (in Ca<sup>2+</sup>-free medium),  $P < 0.01$  (n=3); U46619 contraction (% of 66.7 mM KCl contraction), 123.6  $\pm$  12.5% (in Ca<sup>2+</sup>-containing medium) v.s. 23.8  $\pm$  6.2% (in Ca<sup>2+</sup>-free medium),  $P < 0.01$  (n=3)]. Pretreatment with verapamil (1  $\mu$ M), an L-type Ca<sup>2+</sup>



**Fig. 1.** Representative tension recordings from rat aortic strips. Contractile responses were induced by 100  $\mu\text{M}$  5-hydroxytryptamine (5-HT) (A, B) or 1  $\mu\text{M}$  U46619 (C, D) in the presence (A,C) or absence (B,D) of extracellular  $\text{Ca}^{2+}$ . In the experiments in the absence of extracellular  $\text{Ca}^{2+}$ , the aortic strips were washed three times with  $\text{Ca}^{2+}$ -free medium.

channel inhibitor, markedly inhibited 5-HT-induced contractions of aortic strips, while U46619-induced contractions were only slightly inhibited by verapamil (Fig. 2). The  $\text{pD}_2$  values of both contractile responses to 5-HT and U46619 were significantly decreased by verapamil (Tables 1 and 2).

#### *Effects of calphostin C on 5-HT- and U46619-induced contractions*

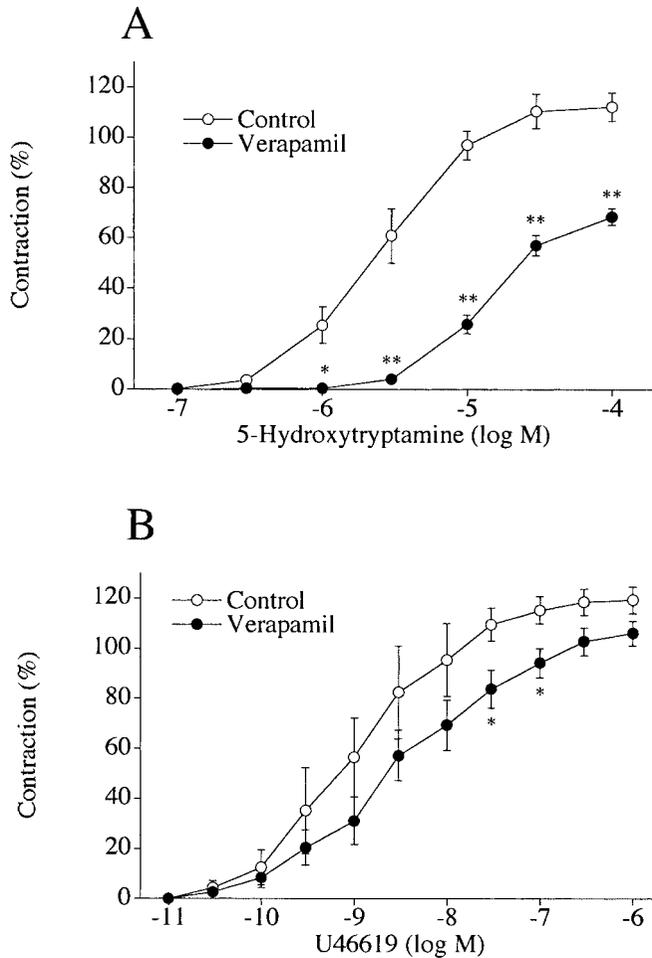
Figure 3 displays the effects of calphostin C (a PKC inhibitor) on both 5-HT- and U46619-induced contractions in aortic strips. Pretreatment with calphostin C (1  $\mu\text{M}$ ) significantly inhibited both 5-HT- and U46619-induced contractions of aortic strips (Fig. 3). Calphostin C treatment resulted in a significant decrease in the  $\text{pD}_2$  value of the U46619-induced contractions, while that of the 5-HT induced-contractions was not significantly affected (Tables 1 and 2).

#### *Effects of genistein on 5-HT- and U46619-induced contractions*

Figure 4 displays the effects of genistein (5  $\mu\text{M}$ ), a tyrosine kinase inhibitor, on both 5-HT- and U46619-induced contractions in the endothelium-denuded aortic strips. Preincubation of the aortic strips with genistein (5  $\mu\text{M}$ ) for 15 minutes significantly reduced the 5-HT-induced contractions. On the other hand, genistein had little effect on contractile responses to U46619. The  $\text{pD}_2$  value of 5-HT-induced contractions was significantly decreased by genistein, while genistein did not affect the  $\text{pD}_2$  value of U46619-induced contractions (Tables 1 and 2).

#### *Effects of SB203580 on 5-HT- and U46619-induced contractions*

Preincubation with SB203580 (10  $\mu\text{M}$ ), a p38 MAPK inhibitor, significantly reduced the



**Fig. 2.** Effects of verapamil on the contractile responses to 5-hydroxytryptamine (A) and U46619 (B) in rat aortic strips. The aortic strips were pretreated with verapamil (1  $\mu$ M) or a vehicle for 15 min. Then, each vasoconstrictor was added in a cumulative manner. Asterisks denote significant differences compared with the control treated with a vehicle (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).  $n = 4-5$ .

contractile responses to 5-HT but did not affect the responses to U46619 (Fig. 5). The  $pD_2$  value of 5-HT-induced contractions was significantly decreased by SB203580, while the  $pD_2$  value of U46619-induced contractions was not affected (Tables 1 and 2).

### Discussion

The contractions induced by both 5-HT and U46619 were strongly attenuated by the absence of  $Ca^{2+}$  in the medium. However, the effects of verapamil on these two contractions were different: verapamil significantly inhibited 5-HT contractions but only slightly inhibited U46619 contractions. Thus, 5-HT induces contraction mainly via voltage-dependent  $Ca^{2+}$

**Table 1** pD<sub>2</sub> values for the contractions of rat aortic strips induced by 5-hydroxytryptamine in the presence and absence of each inhibitor

	Control	Inhibitor
Verapamil	5.58 ± 0.08	4.89 ± 0.04**
Calphostin C	5.88 ± 0.07	5.73 ± 0.03
Genistein	5.60 ± 0.05	5.25 ± 0.03**
SB203580	5.75 ± 0.07	5.40 ± 0.07*

Asterisks denote significant differences compared with the control treated with a vehicle (\*, P<0.05; \*\*, P<0.01). n=4.

**Table 2** pD<sub>2</sub> values for the contractions of rat aortic strips induced by U46619 in the presence and absence of each inhibitor

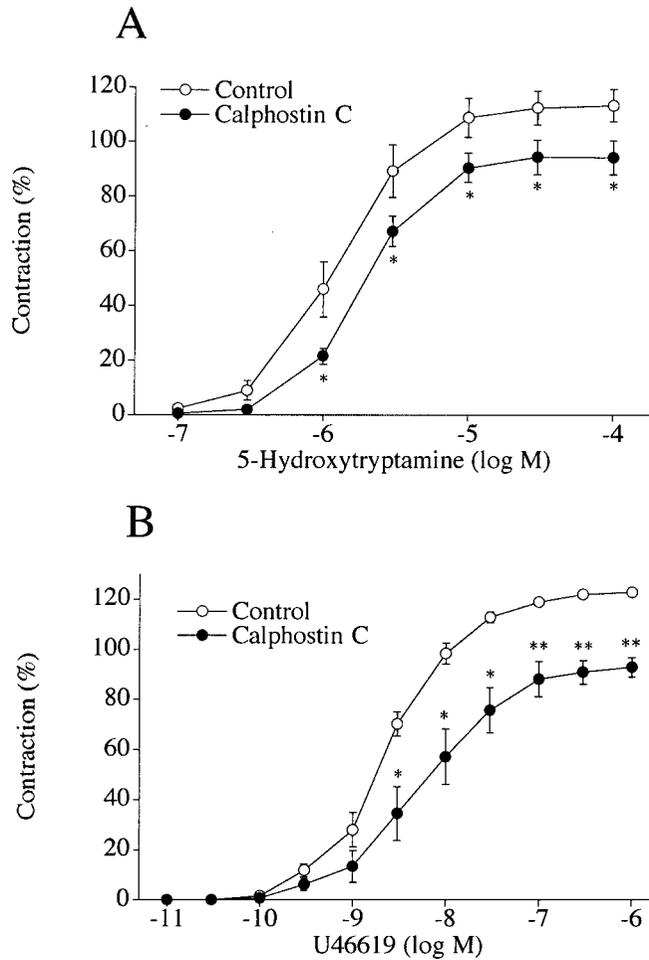
	Control	Inhibitor
Verapamil	9.21 ± 0.16	8.50 ± 0.21*
Calphostin C	8.58 ± 0.07	7.91 ± 0.23*
Genistein	9.11 ± 0.16	9.02 ± 0.30
SB203580	9.04 ± 0.20	8.87 ± 0.24

Asterisks denote significant differences compared with the control treated with a vehicle (\*, P<0.05). n=5.

channels, whereas U46619 induces contraction via the voltage-dependent and -independent Ca<sup>2+</sup> entry pathways.

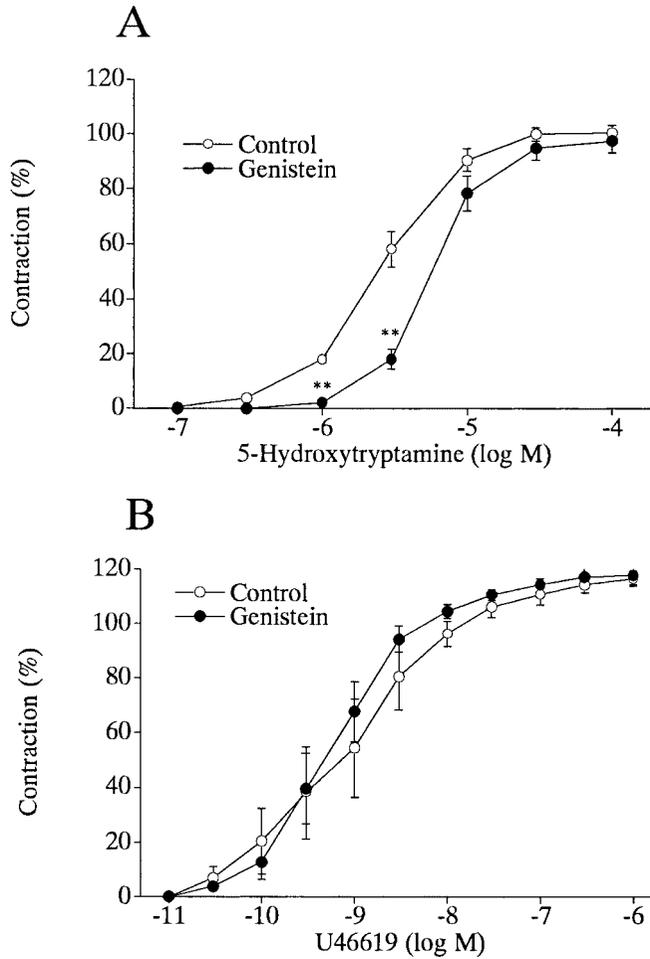
PKC, which is activated by diacylglycerol (a metabolite of phosphatidylinositol), is known to increase the Ca<sup>2+</sup> sensitivity of the contractile proteins of vascular smooth muscle (Takuwa, 1996). Calphostin C significantly inhibited both the 5-HT- and U46619-induced contractions, suggesting that these contractions were mediated via the protein kinase C-dependent pathway. On the other hand, while genistein did not affect U46619-induced contractions, it significantly inhibited 5-HT-induced contractions. This latter finding agrees with the results of recent studies demonstrating that genistein inhibited the 5-HT<sub>2</sub> receptor-mediated contraction of several kinds of rat arteries including the rat aorta (Watts, 1996). Inhibitors of tyrosine kinase have also been reported to attenuate TXA<sub>2</sub> receptor-mediated contraction in a variety of arteries such as bovine cerebral arteries (Watanabe *et al.*, 1998), human small omental arteries (Martinez *et al.*, 2000), canine pulmonary vasculature (Janssen *et al.*, 2001) and rabbit aortae (Sakurada *et al.*, 2001). However, a recent study has shown that tyrphostin A25, a tyrosine kinase inhibitor, did not affect U46619-induced contractions in isolated rat mesenteric resistance arteries (Bolla *et al.*, 2002). This finding agrees with the present result that genistein treatment was ineffective on U46619 induced-contractions. Thus, the involvement of the tyrosine kinase-dependent pathway in arterial contraction varies according to the loci of arteries and the animal species.

The p38 pathway in vascular smooth muscle has been shown to be activated by reactive oxygen species (Kyaw *et al.*, 2001; Yoshizumi *et al.*, 2000), mechanical strain (Li *et al.*, 1999; Li *et al.*, 2000), hypoxia (Lin *et al.*, 2000), and cytokines (Jung *et al.*, 2001). Recent studies have also shown that arterial contraction induced by agonists such as endothelin-1, angiotensin II and



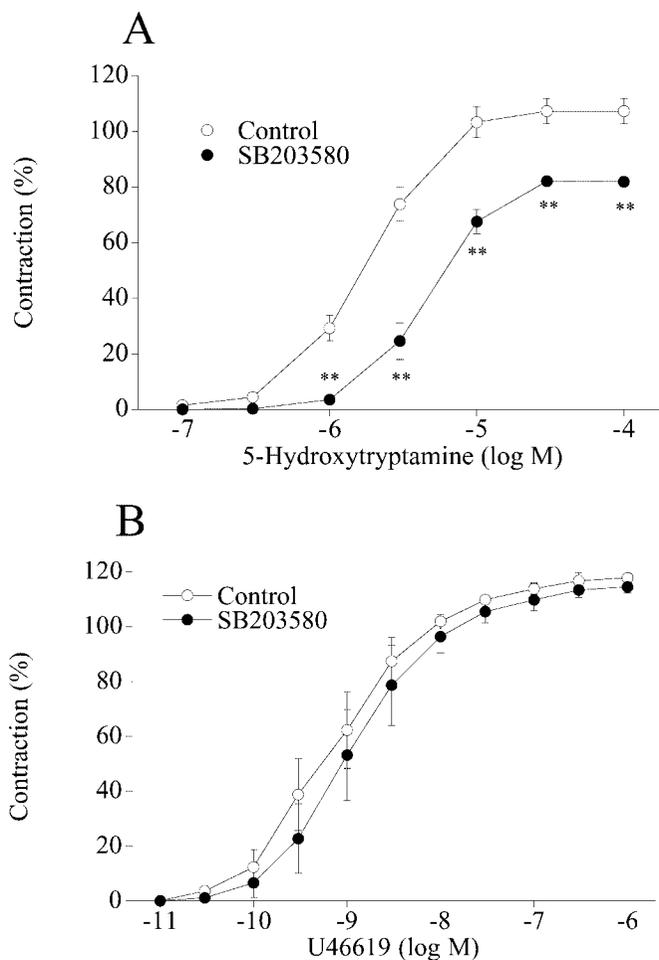
**Fig. 3.** Effects of calphostin C on the contractile responses to 5-hydroxytryptamine (A) and U46619 (B) in rat aortic strips. The aortic strips were pretreated with calphostin C (1  $\mu$ M) or a vehicle for 15 min. Then, each vasoconstrictor was added in a cumulative manner. Asterisks denote significant differences compared with the control treated with a vehicle (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).  $n = 4$ .

noradrenaline were attenuated by p38 inhibition (Yamboliev *et al.*, 2000; Ohanian *et al.*, 2001; Meloche *et al.*, 2000). However, there have been only a few reports of p38 inhibition on the vasoconstriction induced by both 5-HT and  $\text{TXA}_2$ . In cultured smooth muscle cells isolated from the rat aorta, 5-HT has recently been shown not to activate the p38 pathway (Banes *et al.*, 2001). On the other hand, the present study clearly showed that 5-HT-induced contraction of the rat aorta was significantly inhibited by SB203580, a known p38 inhibitor. This discrepancy of the effects of 5-HT on the p38 pathway may be the result of different experimental conditions, e.g., cultured vascular smooth muscle cells *v.s.* isolated aortic tissue. A possible explanation for this discrepancy might be that the coupling of 5-HT receptors to the p38 pathway is down-regulated in the differentiated rat aortic smooth muscle cells. Further experiments including a study of



**Fig. 4.** Effects of genistein on contractile responses to 5-hydroxytryptamine (A) and U46619 (B) in rat aortic strips. The aortic strips were pretreated with genistein (5  $\mu$ M) or a vehicle for 15 min. Then, each vasoconstrictor was added in a cumulative manner. Asterisks denote significant differences compared with the control treated with a vehicle (\*\*,  $P < 0.01$ ).  $n = 4-5$ .

the p38 expression in aortic tissues would be needed to test this possibility. On the other hand, the U46619-induced contractions of the rat aorta were not affected by SB203580. This result is discrepant to that reported in a recent study, in which SB203580 was shown to significantly attenuate U46619-induced contractions of rat mesenteric resistance arteries (Bolla *et al.*, 2002). This discrepancy of the effects of p38 inhibition on U46619 contractions might also be due to the difference in artery type. Thus, the involvement of intracellular signaling via tyrosine kinase and p38 on both 5-HT- and  $\text{TXA}_2$ -induced contractions appears to vary under different experimental conditions. In the present study, activation of both tyrosine kinase and p38 was involved in the 5-HT-induced contractions but not in the U46619-induced contractions and the degree of involvement of voltage-dependent  $\text{Ca}^{2+}$  entry was much higher in the 5-HT contractions



**Fig. 5.** Effects of SB203580 on contractile responses to 5-hydroxytryptamine (A) and U46619 (B) in rat aortic strips. The aortic strips were pretreated with SB203580 (10  $\mu$ M) or a vehicle for 15 min. Asterisks denote significant differences compared with the control treated with a vehicle (\*\*,  $P < 0.01$ ).  $n = 4-5$ .

**Table 3** Involvement of each signaling factor in the rat aortic contractions induced by stimulation of 5-HT and thromboxane  $A_2$  receptors

	5-HT	Thromboxane $A_2$
Ca <sup>2+</sup> entry	+++	++
VDCC	+++	+
PKC	++	++
TK	++	-
p38	++	-

VDCC, voltage-dependent Ca<sup>2+</sup> channels; PKC, protein kinase C; TK, tyrosine kinase; p38, p38 MAPK. +, involved; -, not involved.

compared with the U46619 contractions. These results suggest that there is cross-talk between activation of the tyrosine kinase and p38 pathways and voltage-dependent  $\text{Ca}^{2+}$  entry. A recent study has shown that a part of the  $\text{Ca}^{2+}$  influx pathway involving an L-type  $\text{Ca}^{2+}$  channel is regulated by tyrosine kinase activity in the A10 rat aortic cell line (Nelson *et al.*, 1997). On the contrary, the augmentation by orthovanadate, an activator of tyrosine kinase, of the KCl-induced contraction of the guinea pig aorta, is not mediated by facilitation of transplasmalemmal  $\text{Ca}^{2+}$  entry (Masui and Wakabayashi, 2000). However, it has not been determined whether p38 activation is involved in the  $\text{Ca}^{2+}$  entry mechanism of vascular smooth muscle. Further studies are thus needed to clarify the relationship between the tyrosine kinase and p38 pathways and the activation of voltage-dependent  $\text{Ca}^{2+}$  channels.

Table 3 summarizes the results of the present study. Tyrosine kinase and p38 MAPK pathways are involved in the 5-HT contractions but not in the  $\text{TXA}_2$  contractions, while both contractions are strongly dependent on transplasmalemmal  $\text{Ca}^{2+}$  entry. Voltage-dependent  $\text{Ca}^{2+}$  channels and PKC are also involved in both contractile responses to  $\text{TXA}_2$  and 5-HT.

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