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Specific attenuation of the pressure-induced contraction of rat cerebral artery by herbimycin A

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Abstract

In order to determine whether protein tyrosine kinase mechanisms are involved in pressure-induced contraction, we compared effects of three structurally unrelated tyrosine kinase inhibitors and orthovanadate, a tyrosine phosphatase inhibitor, on the pressure-induced contraction of the posterior cerebral artery isolated from rats. The change in vessel diameter was continuously measured with a width analyzer. Herbimycin A inhibited the pressure-induced contraction, while it only slightly inhibited contractions produced by potassium chloride or 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619). Genistein inhibited not only the pressure-induced contraction but also the U46619-induced one. Tyrphostin 23 significantly attenuated contractions in response to three different stimuli, i.e., pressure, potassium chloride and U46619. Orthovanadate potentiated the pressure-induced contraction. These results suggest that herbimycin A is a specific and potent inhibitor of the pressure-induced contraction and that a protein tyrosine kinase mechanism may play an important role in the genesis of the pressure-induced contraction of the rat cerebral artery. © 1997 Elsevier Science B.V.

Keywords: Myogenic response; Pressure-induced contraction; Cerebral artery, rat; Tyrosine kinase inhibitor; Herbimycin A; Genistein

1. Introduction

Protein tyrosyl phosphorylation has been considered to play an important role in the diverse cellular signaling pathways that are involved in cellular proliferation and transformation. More recent functional and biochemical studies have suggested that protein tyrosine kinases are also involved in contractions of smooth muscles, including those in blood vessels, in response to various stimuli, ranging from growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Yang et al., 1993), to angiotensin II (Yang et al., 1993), norepinephrine (Di Salvo et al., 1993; Abebe and Agrawal, 1995), 5-hydroxytryptamine (Watts et al., 1996) and a depolarizing stimulus such as high potassium chloride (KCl) (Toma et al., 1995). As sodium orthovanadate, an inhibitor of protein tyrosine phosphatase (Laniyonu et al., 1994), itself induced contractions of smooth muscles and enhanced protein tyrosyl phosphorylation, the phosphorylation has been considered to regulate the contraction of smooth muscles.

In the studies mentioned above, tyrosine kinase inhibitors such as genistein, tyrphostin analogues, quercetin and geldanamycin have been often used as pharmacological probes to assess the involvement of protein tyrosine kinase in various cellular functions. However, the results reported so far on the effect of tyrosine kinase inhibitors on the contraction of vascular tissues produced by various agonistic and depolarizing stimuli have been inconsistent. For instance, norepinephrine- or phenylephrine-induced contractions of canine carotid (Di Salvo et al., 1993), rat mesenteric (Toma et al., 1995) and porcine coronary (Saifeddine et al., 1992) arteries, and of rat aorta (Abebe and Agrawal, 1995; Filipeanu et al., 1995), were inhibited by tyrosine kinase inhibitors. However, others reported that norepinephrine- or phenylephrine-induced contractions of rat (Saifeddine et al., 1992; Saturo and Thomas, 1993) or of guinea pig aorta (Saifeddine et al., 1992) were insensitive to them. The contraction of rat mesenteric artery in response to high KCl was inhibited by such inhibitors (Toma et al., 1995), but the contractions of guinea pig mesenteric artery (Di Salvo et al., 1993) and of

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rat aorta (Abebe and Agrawal, 1995) induced by the same means were insensitive to the inhibitors.

These inconsistent results could be possibly attributable to several reasons, including the use of various kinds of tyrosine kinase inhibitors, regional differences in vascular tissues obtained from different animal species, different experimental conditions and/or the non-specificity of the drugs in the preparations used.

We previously reported that cerebral arteries of various animal species, including rats, rabbits, dogs, cats and pigs, were particularly sensitive to mechanical stimulation such as quick stretch (Nakayama, 1982; Nakayama et al., 1986, 1989; Tanaka and Nakayama, 1991; Nakayama and Tanaka, 1993; Tanaka et al., 1994a,b), or pressure (Oyabe et al., 1994), and produced contractions that were apparently myogenic in nature. Our previous studies also showed that the stretch-induced contraction was always preceded by an increase in not only the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) (Nakayama and Tanaka, 1989), but also the production of 1,3,5-inositol trisphosphate (Tanaka et al., 1994a,b).

As to the effects of tyrosine kinase inhibitors on mechanically (pressure) induced contraction of the rat cerebral artery, Osol et al. (1993) suggested a non-specific vasodilator action of the tyrosine kinase inhibitor that they employed, i.e., they proposed that genistein could act through a mechanism other than inhibition of protein tyrosine kinases, such as a direct inhibitory interaction with the voltage-dependent Ca^{2+} channel.

The present experiments were undertaken to elucidate the involvement of protein tyrosine kinase mechanisms in the pressure-induced contraction. In this study, we strictly compared the effect of three structurally unrelated tyrosine kinase inhibitors, each possessing a different mode of inhibitory action on the enzyme activity, i.e., herbimycin A, genistein and tyrphostin 23, as well as the effect of sodium orthovanadate, on contractions of rat cerebral artery produced by mechanical, agonistic and depolarizing stimuli. Our results indicate that herbimycin A specifically and potently inhibits the pressure-induced contraction of the rat cerebral artery.

2. Materials and methods

2.1. Preparation of vessels

Male Sprague-Dawley rats, 9–13 weeks old and weighing 280–400 g (SLC, Hamamatsu, Japan) were anaesthetized with ether. They were then killed by decapitation, and the entire brain was removed immediately and immersed in a dissection dish containing normal Tyrode's solution.

Posterior cerebral artery in a cylindrical form was prepared and mounted on an arteriograph following procedures described by Osol and Halpern (1985). Briefly, the surrounding connective tissues were removed with an extra fine-point No. 5 micro forceps and a secondary branch of the posterior cerebral artery (outer diameter of 173.6 ± 11.3 μm at 10 mmHg, mean ± S.E.M., $n = 92$), approximately 2 mm long, was carefully dissected from each hemisphere under a dissection microscope, and transferred to an experimental chamber of the arteriograph (A-2, Living Systems Instrumentation, Burlington, VT, USA), which was filled with Hepes-buffered physiological salt solution (PSS) of the following composition (in mM): 158.3 NaCl; 4.0 KCl; 2.0 $CaCl_2$; 1.05 $MgCl_2$; 10.42 Hepes; 5.6 glucose. High KCl (80 or 40 mM) PSS was prepared by replacing the NaCl with equimolar KCl. The PSS was bubbled with 100% oxygen, and the pH was adjusted to 7.40 at 37°C with 1 M NaOH.

The arteriograph consisted of a 20-ml vessel chamber, an inlet port for pressurizing with a pressure-servo system, a wire clamp for occlusion, and an optical window in the bottom to facilitate vessel transillumination and visualization with an inverted microscope (IMT-2, Olympus, Tokyo, Japan). The tapered tip of a microcannula made of glass and the wire clamp were suspended within the chamber, and the microcannula was mounted on the bulkhead attached to a digital micrometer to permit an adjustment in axial length. The cannula had a tip diameter of approximately 90 μm, and was heat-polished and siliconized. The cannula was filled with PSS, which was used to suffuse the lumen of the artery and to transmit the intraluminal pressure.

Using the extra fine-point No. 5 micro forceps, the proximal end of the vessel segment was cannulated, and the cannula was then secured with a single strand (60 μm in outer diameter) of silk obtained from an approximately 10-cm length surgical silk thread (No. 0-11, Mastuyoshi Ikakikai, Tokyo, Japan). The contents of the artery were thereafter flushed out by applying a low pressure of less than 10 mmHg to the cannula. The distal end of the vessel segment was held with the wire clamp to prevent it from changing in length and occluded with the same silk thread. The length of the artery was set to its in situ length, as determined with an eyepiece micrometer attached to the microscope.

The intravascular pressure was controlled with a pressure-servo system (PS-5, Living Systems Instrumentation, Burlington, VT, USA) consisting of a controller, a motorizer and a pressure transducer. The intraluminal pressure was adjustable by turning the threaded shaft of a reversible DC linear motor coupled to a syringe. Vessel diameters were monitored with a monochrome video camera (FCD-10, Olympus) attached to a viewing tube. The outer diameter and the wall thickness of vessels were continuously measured with a width analyzer (C3160, Hamamatsu Photonics, Hamamatsu, Japan). The diameter and the intraluminal pressure were recorded with a two-channels strip chart recorder (R-62, Rikadenki Kogyo, Japan) or digitally stored in a computer (Power PC 8500/120, Tokyo Apple,

Japan). Video images were also recorded on videotape for subsequent analysis.

The PSS in the vessel chamber was continuously recirculated with a peristaltic pump (AC-2110, Atto, Tokyo, Japan) at a constant flow of 4 ml/min through a 100-ml external reservoir containing PSS. To exclude effects of nitric oxide (NO) and cyclooxygenase products mainly derived from endothelial cell layer, nitro-L-arginine (L-NNA, 100 μM), an endothelium-derived relaxing factor (EDRF)/NO synthetase inhibitor (Ishii et al., 1990) and indomethacin (10 μM), a cyclooxygenase inhibitor, were added to the reservoir prior to starting the actual experiments. A heat pump connected to a heat exchanger (Jr-80, Taiyo Kagaku Kogyo, Tokyo, Japan) maintained the PSS and the vessel chamber at 37 ± 0.1°C.

2.2. Experimental protocols

The arterial segment mounted in the arteriograph was equilibrated for 1 h at a steady pressure of 50 mmHg. The myogenic tone of the segment developed spontaneously and consistently during the equilibration period, resulting in a significantly reduced luminal diameter. After equilibration, the intraluminal pressure was raised from 0 mmHg to 120 mmHg in stepwise increments of 30 mmHg and lowered in a similar way to attenuate mechanical hysteresis. These maneuvers were conducted three times at a 5-min interval. At the end of the third cycle, the artery was left at a pressure of 10 mmHg, under which condition no apparent pressure-induced contraction was observed, and then subjected to a series of pressure steps.

When we examined the effect of tyrosine kinase inhibitors and a tyrosine phosphatase inhibitor on the pressure-induced contraction, the following protocols were used. The intraluminal pressure was increased from 10 mmHg to 60 mmHg, and the change in diameter of the artery segment was observed for 5 min. The pressure-induced contraction usually reached a maximum within 5 min as reported by Ngai and Winn (1995). Afterwards, the intraluminal pressure was returned to 10 mmHg, and the artery was equilibrated for 5 min. The pressurization was cycled at 10-min intervals three times to secure the stable pressure-induced contraction, and the third response was taken as the control. The pressure-induced contractions were reproducible at least 15 times during the observation period of over 2 h.

Each inhibitor was added cumulatively between successive pressure stimuli. The pressurization was repeated until the maximal drug effect was obtained. The stable contractile response was ordinarily obtained within about 20 min. When two successive responses were stable after application of the inhibitor, the second response was taken as the maximal response to the drug.

When the interaction of herbimycin A with sodium orthovanadate was assessed, herbimycin A (100 nM) was applied for 20 min before or after application of sodium orthovanadate (100 μM).

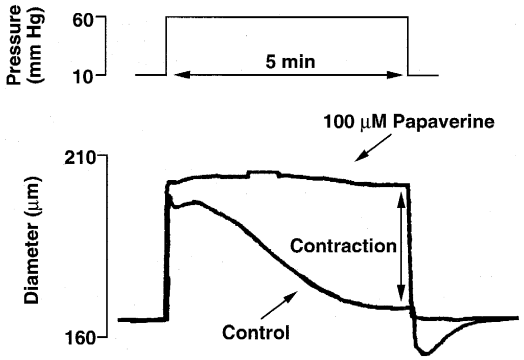

Fig. 1. Typical tracings depict changes in diameter of a rat cerebral artery when intraluminal pressure was increased to 60 mmHg from 10 mmHg with a duration of 5 min. In control, the pressure elevation produced a transient increase in arterial diameter which was subsequently followed by a contraction. In the presence of papaverine (100 μM), the arterial diameter was passively increased with elevating the intraluminal pressure. The vertical line with arrows shows the difference in diameter between maximal contraction that is superimposed on the passive inflation of a pressurized vessel.

In the case of the assessment of the drug action on the contraction produced by either depolarizing or agonistic stimuli, the intraluminal pressure was kept at 10 mmHg to prevent the development of a pressure-induced contraction. The cerebral artery was contracted with 80 mM KCl-PSS or by 100 nM 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), a stable analogue of thromboxane A_2, at a concentration that produced a large and stable contraction as reported previously by us (Tanaka et al., 1995). In some experiments to test the effect of sodium orthovanadate, the contraction of the artery was produced by 40 mM KCl or 30 nM, a submaximal concentration, of U46619. After a steady contraction had been obtained, within 20 min, each drug was added cumulatively.

At the end of each experiment, papaverine (100 μM) was administered to totally eliminate the contractions produced by the various stimuli. For quantitative assessment of the drug action, the diameter of the artery segment was measured before and after administration of the drug, and was subtracted from that in the presence of papaverine (see Figs. 1 and 2). The arterial diameter in the absence of each inhibitor was taken as 100%.

2.3. Chemicals

The following drugs were used. Herbimycin A, genistein, daidzein, tyrphostin 1, tyrphostin 23, sodium orthovanadate, indomethacin and papaverine hydrochloride were purchased from Sigma (St. Louis, MO, USA). 9,11-Dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619) was obtained from Cayman (Ann Arbor, MI, USA) and

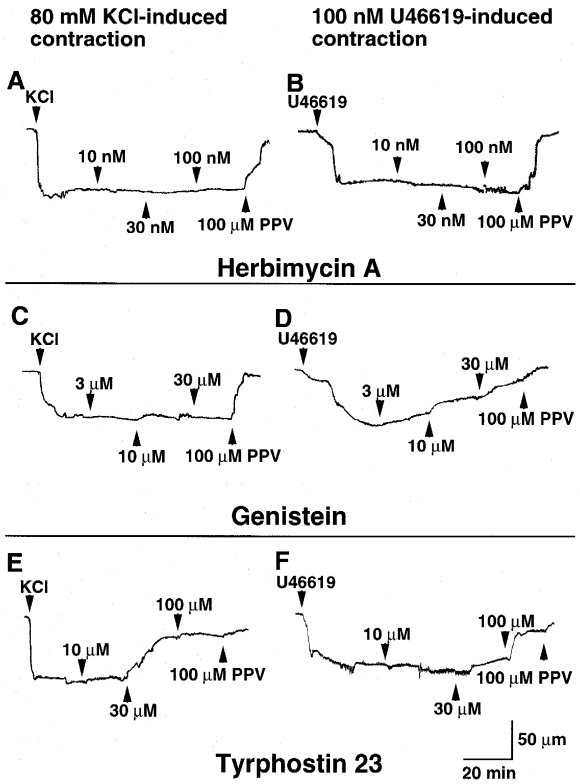

Fig. 2. Responses of rat cerebral arteries to 80 mM KCl (A, C and E) or 100 nM U46619 (B, D and F). Traces illustrating effects of herbimycin A (10–100 nM) (A and B), genistein (3–30 μM) (C and D) and tyrphostin 23 (10–100 μM) (E and F) on KCl- and U46619-induced contractions of rat cerebral arteries are shown. Note that 100 μM papaverine (PPV) totally relaxed the artery. A pair of arterial segments isolated from the same animal were used in experiments with each inhibitor.

nitro-L-arginine (L-NNA) from Aldrich (Milwaukee, WI, USA). Other drugs used were of reagent quality. U46619 was dissolved in ethanol at 100 μM. Indomethacin was dissolved in 0.1 M $NaHCO_3$ solution at 10 mM. Herbimycin A, genistein, daidzein, tyrphostin 1 and tyrphostin 23 were dissolved in dimethyl sulfoxide (DMSO). All drugs were further diluted in distilled water. L-NNA and papaverine hydrochloride were dissolved in distilled water. All drugs were added to the reservoir, and final concentrations were expressed as a molar concentration (M).

2.4. Data analysis

All of the data were expressed as means ± S.E.M. The value of EC_{50} (50% effective concentration, M) or IC_{50} (50% inhibitory concentration, M) was determined geometrically from logarithmic concentration–response curves. The significance of statistical analyses was established according to Tukey's test after analysis of variance (ANOVA). A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Characteristic nature of the pressure-, KCl- and U46619-induced contractions

Fig. 1 shows a typical mechanical response of a rat cerebral artery when the intraluminal pressure was increased from 10 mmHg to 60 mmHg, which was superimposed on the passive dilatation of the vessel in3 response to the same pressurization after administration of 100 μM papaverine. A passive inflation of the vessel wall occurred initially with the increase in intraluminal pressure, and was followed by a delayed contraction that reached a maximum within 5 min after application of the pressure. Our previous studies indicated that the magnitude of the pressure-induced contraction was dependent on the amount of pressure applied, and that the largest contractile response occurred at 60 mmHg. Any further increase in pressure decreased the contraction. The pressure-induced contraction was reproducible during an observation period of over 2 h when the artery was pressurized from 10 mmHg to 60 mmHg with a 5-min stimulus period with a 10-min interval between pressurizations (Oyabe et al., 1994). The pressure-induced contraction was completely prevented in

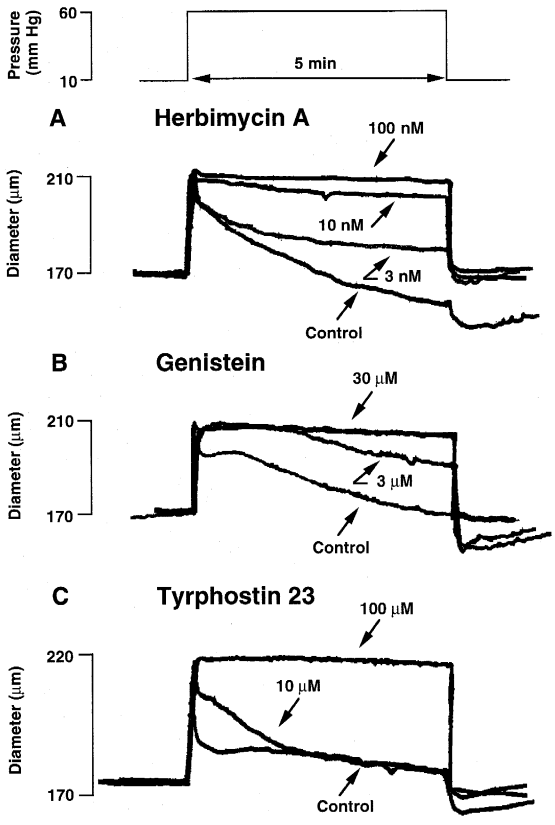

Fig. 3. Inhibitory effects of herbimycin A (3, 10 and 100 nM) (A), genistein (3 and 30 μM) (B) and tyrphostin 23 (10 and 100 μM) (C) on the contraction of rat cerebral artery segments in response to a rise in intraluminal pressure. Typical traces are shown for the inhibitory effects of herbimycin A, genistein or tyrphostin 23 on contraction elicited by an increase in intraluminal pressure up to 60 mmHg from 10 mmHg with a duration of 5 min.

the presence of papaverine (100 μM). An active contractile response was also observed in the cerebral artery when the pressure was returned to its original pressure of 10 mmHg. However, the latency, magnitude and duration of the contraction following depressurization were usually unstable and could not be evaluated adequately, unlike the pressure-induced contraction.

Likewise, a depolarizing stimulus such as high KCl and an agonistic stimulus such as U46619 elicited contraction of the rat cerebral artery. The concentration–response curves for KCl (4–100 mM) indicated that the artery relaxed slightly when KCl in the medium was increased from 4 mM to 20 mM and that the contraction occurred at over 20 mM up to 80 mM in a concentration-dependent manner (data not shown). The contraction reached a maximum at 80 mM KCl and the EC_{50} value was 45.6 ± 8.2 mM ($n = 12$). A further increase in the KCl concentration (100 mM) depressed the contraction of the artery. Therefore, in the present study, we tested the effect of tyrosine kinase inhibitors on the contraction produced by 80 mM KCl (Fig. 2A, C and E).

U46619 (1–100 nM) produced a concentration-dependent contraction of the rat cerebral artery with a EC_{50} value of 31.7 ± 10.3 nM ($n = 13$). U46619 at 100 nM produced a large and long-lasting contraction of the artery, which was suitable for the evaluation of the vasodilator action of the inhibitors (Fig. 2B, D and F).

3.2. Effects of herbimycin A, genistein and tyrphostin 23 on the pressure-induced contraction

Fig. 3 depicts typical tracings as to concentration-dependent inhibitory effects of the three tyrosine kinase inhibitors on the pressure-induced contraction and the results are summarized in Fig. 4. Furthermore, Table 1 summarizes effects of inhibitors on contractions produced by all three different types of stimuli.

Herbimycin A (3–100 nM) caused a concentration-dependent reduction in the pressure-induced contraction; and at 100 nM, the drug inhibited the pressure-induced contraction up to about 90%, with an IC_{50} value of 5.7 ± 4.2 nM (Table 1).

Genistein (3–30 μM) caused a concentration-dependent decrement of the pressure-induced contraction, which was at 10 and 30 μM significantly different from the mean value of the group treated with the vehicle (Fig. 4B). Genistein at 30 μM inhibited the pressure-induced contraction by about 90% and the IC_{50} value was 7.0 ± 5.8 μM (Table 1). On the other hand, 30 μM daidzein, a negative control drug for genistein, had no apparent effect on the pressure-induced contraction ($97.3 \pm 5.6\%$, control = 100%, $n = 4$, $P > 0.05$ vs. corresponding values with the vehicle).

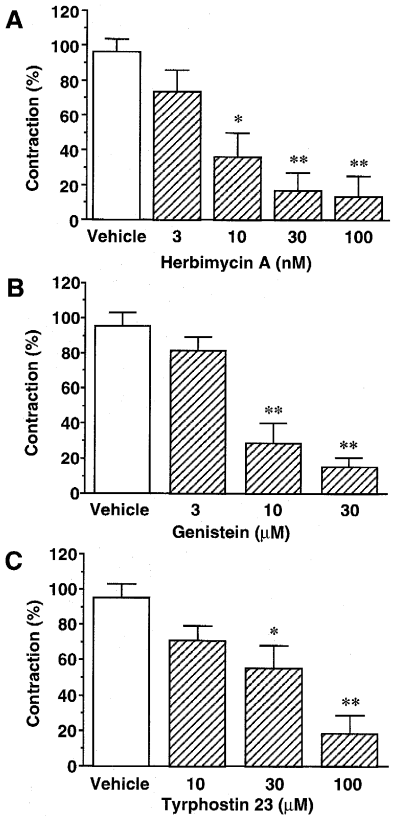

Fig. 4. Concentration–response relationships for the pressure-induced contraction of rat cerebral artery. Effects of herbimycin A (3–100 nM) (A), genistein (3–30 μM) (B) and tyrphostin 23 (10–100 μM) (C) are shown. The change in the diameter in the absence of inhibitors is taken as 100% on the ordinate. Each column represents the mean ± S.E.M. of 4–6 preparations. A significant difference from the vehicle-treated group ($n = 6$) is indicated (* $P < 0.05$, ** $P < 0.01$).

Table 1
Inhibitory effects of herbimycin A, genistein and tyrphostin 23 on contractions produced by three different types of stimuli

Inhibitors		Stimulus		
		Pressure	KCl	U46619
Herbimycin A	IC_{50} (nM)	5.7 ± 4.2	> 100	> 100
	% Maximal inhibition	87.5 ± 11.7 [b]	10.1 ± 2.1	9.8 ± 4.7
Genistein	IC_{50} (μM)	7.0 ± 5.8	> 30	13.6 ± 9.8
	% Maximal inhibition	88.3 ± 5.7 [b]	13.3 ± 4.5	66.9 ± 10.9 [a]
Tyrphostin 23	IC_{50} (μM)	43.6 ± 14.2	25.6 ± 6.8	51.6 ± 11.9
	% Maximal inhibition	81.5 ± 10.0 [b]	70.4 ± 4.7 [b]	73.7 ± 12.8 [b]

Each value represents the mean ± S.E.M. of 4–6 separate experiments. A significant difference from the vehicle-treated group is shown ([a] $P < 0.05$, [b] $P < 0.01$).

Likewise, tyrphostin 23 (10–100 μM) also inhibited significantly the pressure-induced contractions in a concentration-dependent manner with an IC_{50} value of 43.6 ± 14.2 μM (Table 1). The maximum inhibition of the pressure-induced contraction produced by tyrphostin 23 at 100 μM was about 80%. Tyrphostin 1, a negative control drug for tyrphostins, at 100 μM did not show any appreciable effect on the contractile response to the pressure stimulus (98.6 ± 4.2%, control = 100%, $n = 4$, $P > 0.05$ vs. corresponding values with the vehicle).

Therefore, herbimycin A is about 1200 times and 7600 times more potent than genistein and tyrphostin 23, respectively, for the inhibition of the pressure-induced contraction.

3.3. Effects of herbimycin A, genistein and tyrphostin 23 on 80 mM KCl- and 100 nM U46619-induced contractions

Herbimycin A (10–100 nM) had only a slight inhibitory effect on contractions produced by 80 mM KCl or 100 nM U46619 (See Fig. 2A and B). Maximum inhibition of contractions in response to 80 mM KCl and 100 nM U46619 produced by 100 nM herbimycin A was about 10% (Table 1), indicating that herbimycin A is highly specific for inhibition of the pressure-induced contraction.

Genistein at 30 μM, the highest concentration used in the present study, inhibited 80 mM KCl-induced contraction only about 10% (Fig. 2C and Table 1). On the other hand, genistein (3–30 μM) caused a concentration-dependent reduction in the amount of 100 nM U46619-induced contraction. The maximum inhibition of the U46619-induced contraction, up to about 70%, was produced by genistein at 30 μM (Fig. 2D and Table 1). Daidzein (30 μM) had no apparent effect on U46619- and KCl-induced contractions (96.3 ± 7.2% and 98.5 ± 3.5%, respectively, control = 100%, each $n = 4$, each $P > 0.05$ vs. corresponding values with the vehicle). Furthermore, there was no significant difference in the percent maximal inhibition of KCl-induced contraction by herbimycin A or genistein (Table 1).

Tyrphostin 23 (30 and 100 μM) caused a significant reduction in the magnitude of contractions produced by either high KCl at 30 and 100 μM (Fig. 2E) or U46619 at 100 μM (Fig. 2F). IC_{50} values were about 30–50 μM for KCl- and U46619-induced contractions. Tyrphostin 23 (100 μM) inhibited KCl- and U46619-induced contractions about 70% (Table 1). On the other hand, 100 μM tyrphostin 1 had no apparent effect on KCl- and U46619-induced contractions (97.1 ± 0.9% and 96.7 ± 5.3%, respectively, control = 100%, each $n = 4$, each $P > 0.05$ vs. corresponding values with the vehicle).

3.4. Effect of sodium orthovanadate on the pressure-induced contraction

Sodium orthovanadate (10–100 μM) caused a concentration-dependent augmentation of the pressure-induced contraction, which was significantly different at 100 μM from the value of the group treated with vehicle (Fig. 5). Sodium orthovanadate (100 μM) enhanced the pressure-induced contraction by 181.7 ± 16.2% ($n = 4$, control = 100%). The vessel diameter was slowly restored within 5 min to the basal level with reduction of the pressure from 60 mmHg to 10 mmHg, and the potentiated response was completely eliminated by 100 μM papaverine.

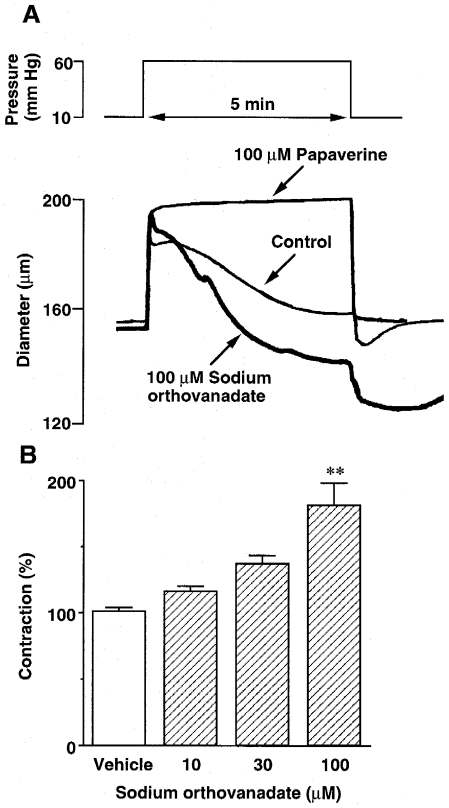

Fig. 5. Effect of sodium orthovanadate on the pressure-induced contraction of rat cerebral artery. (A) Typical traces showing the potentiating effect of sodium orthovanadate (100 μM) on the contraction elicited by an increase in intraluminal pressure up to 60 mmHg from 10 mmHg with a duration of 5 min. (B) Concentration–response relationship for sodium orthovanadate on the change in the diameter of rat cerebral artery in response to pressure increasing up to 60 mmHg from 10 mmHg. The change in the arterial diameter in the absence of sodium orthovanadate was taken as 100% on the ordinate. Each column represents the mean ± S.E.M. of 4 preparations. A significant difference from the vehicle-treated group ($n = 4$) is indicated (** $P < 0.01$).

Interaction of herbimycin A with sodium orthovanadate on the pressure-induced contraction was also assessed. The pretreatment with herbimycin A (100 nM) for 20 min effectively eliminated the potentiating action of sodium orthovanadate (100 μM), i.e., before (183.7 ± 9.3%) and after (114.6 ± 12.9%, $P < 0.01$ vs. corresponding values without herbimycin A, each $n = 4$) application of herbimycin A when the contractile response to the pressure change under control condition was taken as 100%. Sodium orthovanadate (100 μM) slightly restored the pressure-induced contraction (25.3 ± 15.2%, $P > 0.05$ vs. corre-

sponding values in the only presence of herbimycin A) which had been suppressed beforehand by 100 nM herbimycin A up to $10.0 \pm 5.6\%$ (each $n = 4$).

Sodium orthovanadate (100 μM) also enhanced the contractile response to a submaximal concentration of U46619 (30 nM) by $153.6 \pm 10.2\%$ ($n = 4$, control = 100%), and the potentiation was effectively inhibited by genistein (30 μM) ($90.4 \pm 6.8\%$, $P < 0.05$ vs. corresponding values without genistein, $n = 4$). Sodium orthovanadate (100 μM) had no apparent effect on the contraction produced by 40 mM KCl.

4. Discussion

In the present study, we demonstrated that of the three tyrosine kinase inhibitors examined, herbimycin A most specifically and potently attenuated the pressure-induced contraction of rat cerebral artery segments, whereas the other two drugs, genistein and tyrphostin 23, inhibited the contraction in response not only to pressure but also to agonistic and/or depolarizing stimuli. Furthermore, we found that sodium orthovanadate, an inhibitor of protein tyrosine phosphatase (Laniyonu et al., 1994), potentiated the pressure-induced contraction of the arterial segment.

Herbimycin A, a benzoquinone ansamycin, was first reported by Uehara et al. (1985) to inhibit specifically tyrosine kinase(s) of the *src* family (Li et al., 1993). Studies on the mechanism of the inhibitory action of herbimycin A toward $pp60^{v\text{-}src}$ activity revealed that the inhibitor caused an irreversible inactivation of the enzyme due to the reaction of a sulfhydryl group of the enzyme with the quinone moiety of the inhibitor (Uehara et al., 1989). Genistein, an isoflavonoid isolated from a strain of *Pseudomonas* (Ogawara et al., 1986), was reported to inhibit the EGF receptor activity and $pp60^{v\text{-}src}$ activity by competing with ATP (Akiyama et al., 1987). Tyrphostin derivatives, on the other hand, are competitive inhibitors of protein tyrosine kinases with protein/peptide substrates and inhibit the EGF receptor activity strongly (Yaish et al., 1988). The inhibition of the pressure-induced contraction of rat cerebral artery by these three structurally unrelated tyrosine kinase inhibitors possessing different mechanisms of action on the protein tyrosine kinase may confirm that the inhibitions are attributable to an action on protein tyrosine kinases. Additional support for the involvement of protein tyrosine kinases in the pressure-induced contraction of rat cerebral artery is possibly provided by the ability of sodium orthovanadate to potentiate the contraction. Sodium orthovanadate, by inhibiting the activity of protein tyrosine phosphatases, was shown to result in enhanced expression of protein tyrosine kinase-mediated responses (Laniyonu et al., 1994).

We confirmed the previous finding by Osol et al. (1993) that genistein at a high concentration, i.e., micromolar, completely and immediately attenuated the myogenic response of rat cerebral artery. We also found that genistein had inhibitory effects on pressure- and U46619-induced contractions, but no apparent effect on the KCl-induced contraction. Daidzein, a negative control drug for genistein, had no apparent effect on these three kinds of contractions. These results suggest that genistein-sensitive protein tyrosine kinase(s) is involved in the mechanism of pressure- and U46619-induced contractions of the rat cerebral artery.

It is well known that contractions of vascular tissues, including rat cerebral artery in response to high KCl or addition of $CaCl_2$ in Ca^{2+}-free depolarizing medium mainly depend on the transmembrane influx of Ca^{2+} through activation of voltage-dependent L-type Ca^{2+} channels. Furthermore, we found that sodium orthovanadate had no apparent effect on the contractile response of the rat cerebral artery to high KCl, indicating that the involvement of protein tyrosine kinase in the contraction due to a depolarizing stimulus such as high KCl may be unlikely. Tyrphostin 23 inhibited pressure-, KCl- and U46619-induced contractions with a similar IC_{50} value of about 30–50 μM. On the other hand, tyrphostin 1, a negative control drug for tyrphostins, had no apparent effect on contractions in response to these three kinds of stimuli. The relaxing action of tyrphostin 23 on these contractions could be explained by the inhibition of Ca^{2+} channels potentially sensitive to tyrphostin 23, since it has been shown that Ca^{2+} current in rabbit ear artery smooth muscle cells can be inhibited by tyrosine kinase inhibitors, including tyrphostin 23 (Wijetunge et al., 1992). However, the results of the present study may not also exclude the possibility that tyrphostin 23 but not tyrphostin 1 acts through a mechanism other than inhibition of tyrosine kinases, such as a direct inhibitory interaction with the voltage-dependent L-type Ca^{2+} channel (Toma et al., 1995; Watts et al., 1996) and/or other unidentified mechanisms in the rat cerebral artery.

The potent and specific inhibitory action of herbimycin A on the pressure-induced contraction indicates that herbimycin A-sensitive protein tyrosine kinases may be exclusively involved in the regulatory mechanism of the pressure-induced contraction, but not in that of KCl- and U46619-induced contractions of the artery.

The contraction in response to mechanical stimuli is a kind of physical response requiring cellular signal transduction, which may be mediated through a receptive site specific for a mechanical stimulus and the common pathway of Ca^{2+} signaling for agonistic and depolarizing stimuli (Nakayama and Tanaka, 1993).

As to the main vascular wall components implicated in mechanotransduction, it is generally considered that extracellular matrix components including collagen, elastin and other proteoglycans, integrins and other adhesion molecules, and ion channels, in particular, mechanogated membrane ion channels, are important (Osol, 1995). Langton (1993) was able to measure Ca^{2+} channel currents in

response to positive and negative pressure in vascular smooth muscle cells from rat cerebral (basilar) artery. However, the author suggested that the effects of membrane stretch appear to be due to the activation of a dihydropyridine-sensitive voltage-gated Ca^{2+} channel, and not to a separate so-called stretch-activated channel as reported by Kirber et al. (1988). Our preliminary observations also indicate that Gd^{3+}, which is known as an inhibitor of mechanogated ion channels (Hamill and McBride, 1996), inhibited the pressure-induced contraction of rat cerebral artery (Oyabe et al., 1994). However, Gd^{3+} attenuated the high KCl-induced contraction of the artery (our unpublished observations). Therefore, the presence of mechanogated/stretch-activated ion channels in rat cerebral artery remains to be confirmed.

Of these possible receptive sites for the mechanotransduction, the involvement of integrins – a class of heterodimeric transmembrane glycoproteins – in vascular smooth muscle mechanotransduction is particularly appealing for the following reason: adhesion molecules and cytoskeletal proteins support a force-dependent stiffening response in which cytoskeletal stiffness is increased in direct proportion to the applied stress, making them likely candidates for mechanoreceptors (Ingber, 1991). It is noteworthy that adhesion plaques are rich in tyrosine kinases of the *src* family (Rohrschneider, 1980) and that these kinases are common targets of herbimycin A (Uehara et al., 1985) and genistein (Akiyama et al., 1987). It has been reported that mechanical stimuli such as flow and osmotic stress induce tyrosine phosphorylation in cultured bovine arterial endothelial cells (Harada et al., 1995) and in porcine coronary arteriolar endothelium (Muller et al., 1996). In these studies, the authors suggest that tyrosine phosphorylation of cell surface transmembrane glycoprotein in endothelial cells is a critical step in the signaling pathways activated by various mechanical stimuli. Therefore, protein tyrosine kinase activities of adhesion molecules such as *src* may also play an important role as a possible mechano-sensor in the genesis of the pressure-induced contraction.

In the present study, genistein effectively inhibited both pressure- and U46619-induced contractions. We previously reported that U46619 increased not only the influx of Ca^{2+} but also release of Ca^{2+} from intracellular storage sites via augmentation of phosphoinositide hydrolysis (Tanaka et al., 1995). Furthermore, we showed that U46619 increased the Ca^{2+} sensitivity of the contractile elements in the canine basilar artery, which is dependent on GTP-binding protein (Tanaka et al., 1995). As to the vasoconstrictory action of thromboxane A_2, U46619 was reported to inhibit directly Ca^{2+}-activated K^+ channels (K_{Ca}) from pig coronary artery smooth muscle incorporated into lipid bilayers (Scornik and Toro, 1992). Inhibition of K^+ channels would lead to depolarization of the plasma membrane and contraction of the coronary artery. It seems possible that a common signaling pathway involving cytosolic protein tyrosine kinase(s) sensitive to genistein may play a role in the mechanism of contractions produced by pressure and U46619.

Finally, physiological and pathological implications should be mentioned briefly. The contractile reaction of vascular tissue in response to mechanical stimulation such as stretch, first observed in isolated segments of canine carotid artery by Bayliss (1902), has been often postulated to be a mechanism for the control of blood flow. Mechanical factors have been also implicated as a stimulus for the long-term regulation of cardiovascular structures, including remodeling and redistribution of muscle tissue under physiological and pathological conditions (Seidel and Schildmeyer, 1987). The present study demonstrates that herbimycin A is the specific and potent inhibitor for the pressure-induced contraction of rat cerebral artery. It seems possible that this drug would be a good pharmacological alternative to elucidate whether protein tyrosine kinase is involved in the genesis of the vascular tone in response to mechanical stimuli, including pressure. The present study also allows the possibility that the effect of hemodynamic overload, such as high pressure on the vascular wall, may be controlled by tyrosine kinase inhibitors, including herbimycin A.

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