



Attenuation of pressure-induced myogenic contraction and tyrosine phosphorylation by fasudil, a cerebral vasodilator, in rat cerebral artery

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1 The mechanism by which fasudil inhibits pressure-induced myogenic contraction was studied with regard to tyrosine phosphorylation in rat cerebral artery. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and vessel diameter were simultaneously measured. Total tyrosine phosphorylation level and phosphorylation of tyrosine 419 on pp60^{src} required for its full catalytic activity were immunocytochemically detected *in situ*.

2 Fasudil (1–100 μM) partially suppressed the increase in $[\text{Ca}^{2+}]_i$ and totally attenuated contraction elicited by pressurization from 10 to 60 mmHg. Furthermore, fasudil (100 μM) significantly attenuated tyrosine phosphorylation and the activity of pp60^{src} augmented *in situ* by pressure.

3 Herbimycin A (1–100 nM) and genistein (3–30 μM), tyrosine kinase inhibitors, effectively attenuated the pressure-induced increase in $[\text{Ca}^{2+}]_i$, contraction, tyrosine phosphorylation, and activation of pp60^{src}. Both fasudil and herbimycin A directly inhibited the pp60^{src} activity in a cell free system.

4 Orthovanadate (100 μM), a tyrosine phosphatase inhibitor, significantly potentiated the pressure-induced increase in $[\text{Ca}^{2+}]_i$ and contraction.

5 Nicardipine (100 nM), a Ca^{2+} antagonist, completely inhibited pressure-induced increase in $[\text{Ca}^{2+}]_i$ and contraction, but affected neither tyrosine phosphorylation nor activity of pp60^{src} in the pressurized arteries.

6 Arginine-glycine-aspartic acid-serine peptide (1–100 μM) concentration-dependently reduced the pressure-induced contraction.

7 In addition to the hitherto reported vasodilatory actions of fasudil, the present results suggest the inhibition by fasudil of pressure-induced tyrosine phosphorylation and pp60^{src} activation. The wide spectrum of inhibitory actions of fasudil may contribute to the effective attenuation of the pressure-induced contraction in the cerebral artery.

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Abbreviations: DMSO, dimethyl sulphoxide; EDRF, endothelium-derived relaxing factor; EGTA, ethylene glycol-bis(β -aminoethylether) N,N,N',N'-tetra-acetic acid; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; indo-1/AM, indol acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; L-NNA, N^o-nitro-L-arginine; MLC, myosin light chain; NO, nitric oxide; PBS, phosphate-buffered saline; PE, phycoerythrin; PSS, physiological salt solution; R_{405/485}, ratio of fluorescence intensity; SAH, subarachnoidal haemorrhage

Introduction

Fasudil [1-(5-isoquinolinesulphonyl)-homopiperazine: HA1077], a new type of cerebral vasodilator, is different from currently used so-called Ca^{2+} antagonists/channel blockers, such as verapamil, nifedipine, and diltiazem, in that the drug possesses not only blocking action against Ca^{2+} entry (Asano *et al.*, 1987; Takizawa *et al.*, 1993) but also inhibitory effects on a variety of serine/threonine protein kinases, including myosin light chain kinase (Asano *et al.*, 1989; Katsumata *et al.*, 1997; Seto *et al.*, 1991; 1995; Uchata *et al.*, 1997), protein kinase C (Asano *et al.*, 1989; Uchata *et al.*, 1997), and Rho-associated kinase (Uchata *et al.*, 1997). Furthermore, fasudil reversed experimental chronic cerebral vasospasm induced in a two-haemorrhage canine model (Asano *et al.*, 1987; Takayasu *et*

al., 1986) and effectively inhibited proliferation of bovine vascular smooth muscle cells in culture (Shirotani *et al.*, 1991). Fasudil has been reported to be effective in patients with subarachnoidal haemorrhage (SAH), and was first permitted to be used in 1995 in Japan as a remedy for ischemic insults due to chronic cerebral vasospasm.

We previously reported that cerebral arteries isolated from various animal species including rats were particularly sensitive to a variety type of mechanical stimuli, such as increasing intraluminal pressure (Masumoto *et al.*, 1997) and quick stretch (Nakayama, 1982; Nakayama *et al.*, 1989; Nakayama & Tanaka, 1988), and that these stimuli produced a contraction that was myogenic in nature. Furthermore, we reported that herbimycin A, a *src*-family tyrosine kinase inhibitor, specifically attenuated the pressure-induced myogenic contraction of the rat cerebral artery (Masumoto *et al.*, 1997).

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It was previously reported by us that a small amount of erythrocyte lysate (haemolysate) containing $0.01\text{--}0.2\text{ mg ml}^{-1}$ as oxyhaemoglobin potentiated the stretch-induced contraction of the canine basilar artery and that the enhancement was suppressed by Ca^{2+} antagonists (Nakayama *et al.*, 1989). The components of erythrocyte lysate, such as oxyhaemoglobin and other molecules, have been considered to be causal agents in the genesis of cerebral vasospasm after SAH. More recently, it was reported that haemolysate-induced cerebral vasoconstriction was effectively attenuated by genistein, a tyrosine kinase inhibitor (Kim *et al.*, 1998). Furthermore, tyrosine phosphorylations were induced by haemolysate in endothelial cells of bovine cerebral artery (Marton *et al.*, 1996) and in smooth muscle cells of rat cerebral artery (Iwabuchi *et al.*, 1999). As tyrosine phosphorylation has been considered to be involved in the transduction of mechanical forces in vascular reactions, including contraction and remodelling under pathological conditions such as hypertension, arteriosclerosis, and restenosis after angioplasty (Lehoux & Tedgui, 1998), a drug to control tyrosine phosphorylation seems to be a good alternative to prevent vasospastic episodes and hypertrophic events in the vasculature.

The present experiments were thus undertaken to clarify how fasudil affects the myogenic contractile reactivity, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), tyrosine phosphorylation, and pp60^{src} activity assessed by phosphorylation of tyrosine 419 on the kinase required for its full catalytic activity (Piwnicka-Worms *et al.*, 1987), in the rat cerebral artery in response to a mechanical stimulus such as pressure. In addition, a direct effect of fasudil on pp60^{src} activity was also investigated in a cell free system. Our present results suggest that fasudil effectively inhibits the pressure-induced tyrosine phosphorylation and pp60^{src} activation of rat cerebral artery, which may in part contribute to the inhibition of the contractile response to mechanical stimuli.

Methods

Preparation of blood vessels

Rat cerebral arteries were prepared according to the procedures reported previously (Masumoto *et al.*, 1997). Briefly, male Sprague-Dawley rats, 9–13 weeks old and weighing 300–400 g (SLC, Hamamatsu, Japan), were anaesthetized with ether, and killed by decapitation. The secondary branch of the posterior cerebral artery (outer diameter of $142.8 \pm 13.1\text{ }\mu\text{m}$ at intraluminal pressure of 10 mmHg, $n = 243$) was carefully dissected from each hemisphere under a dissection microscope, and mounted on an experimental chamber of an arteriograph (A-2. Living Systems Instrumentation, Burlington, VT, U.S.A.), which was filled with HEPES-buffered physiological salt solution (PSS) of the following composition (in mM): NaCl 158.3; KCl 4.0; CaCl_2 2.0; MgCl_2 1.05; HEPES 10.42; glucose 5.6 High KCl (40 or 80 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 N NaOH.

The intravascular pressure was controlled with a pressure-servo system (PS-5, Living Systems Instrumentation, Burlington, VT, U.S.A.). The PSS in the vessel chamber was kept at $37 \pm 0.1^\circ\text{C}$. In order to exclude effects of the endothelial cell layer, in the experiments on simultaneous measurement of $[\text{Ca}^{2+}]_i$ and arterial diameter, and in those on immunocytochemical *in situ* detections of phosphorylated tyrosine residues and pp60^{src} activity, a heat-polished microcannula was passed

back and forth through the lumen several times. Observation of light and scanning electron photomicrographs of the cerebral artery prepared repeatedly as described previously (Nakayama, 1988), indicated the absence of the endothelium. Unless otherwise stated in the text, to exclude effects of nitric oxide (NO) and cyclo-oxygenase products mainly derived from endothelial cell layer, *N*^ω-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 cyclo-oxygenase inhibitor, were added to the chamber prior to the start of actual experiments.

Measurement of $[\text{Ca}^{2+}]_i$ and arterial diameter

The arterial segment was equilibrated for 1 h at a steady pressure of 50 mmHg. After equilibration, the intraluminal pressure was raised from 0 to 120 mmHg in stepwise increments of 30 mmHg and lowered in a similar way to attenuate mechanical hysteresis (Masumoto *et al.*, 1997). These manoeuvres 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 (Masumoto *et al.*, 1997), and then subjected to a series of subsequent pressure steps.

When we examined the effects of drugs, including fasudil, nicardipine, and arginine-glycine-aspartic acid-serine (RGDS) on the pressure-induced contraction, the following protocols were used. The intraluminal pressure was increased from 10 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. Afterwards, the intraluminal pressure was returned to 10 mmHg. The pressurization was cycled at 10-min intervals three times to secure a stable pressure-induced contraction, and the third response was taken as the control. The pressure-induced contractions were reproducible at least 15 times during an observation period of over 2 h (Masumoto *et al.*, 1997). Each drug was added cumulatively between successive pressure-stimuli. The pressurization was repeated until the maximal drug effect was obtained. When two successive responses were stable after application of the inhibitor, the second response was taken as the maximal response to the drug.

In a separate series of experiments, the $[\text{Ca}^{2+}]_i$ and arterial diameter were simultaneously measured according to the procedures used in previous studies (D'Angelo *et al.*, 1997a, b; Himpens *et al.*, 1992; Meininger *et al.*, 1991). Briefly, arteries were loaded with indo-1, a fluorescent Ca^{2+} -indicator, by a 1-h incubation at room temperature (25°C) in PSS containing acetoxymethyl ester of indo-1 (indo-1/AM, 20 μM , dissolved in dimethyl sulphoxide as a stock solution of 1 mM) and 0.025% cremophor EL^R, a nontoxic detergent. After the loading, the vessel segment was washed with PSS at 37°C to remove uncleaved indo-1/AM. The arteriograph was placed on the ACAS Ultima 575 UVC System interactive with a confocal laser microscope (Meridian, Okemos, MI, U.S.A.). Dual monitors simultaneously provided fluorescence and video-image displays. This allowed us to compare the light optical and fluorescence images. The artery was illuminated by the excitation light of $357 \pm 6\text{ nm}$, and the indo-1 fluorescence was recorded at 405 ± 5 and $485 \pm 5\text{ nm}$ wavelengths. In our experiments, the laser scan strength was set at 25% of 33-mW output of a tunable 5-W Ar ultraviolet-visible laser (1-90, Coherent, U.S.A.), and a 7.5% neutral density filter was used. The pinhole was set at 800 μm . Image scans ($\times 10$ objective, Zewiss, NA 0.3) of $400 \times 400\text{ }\mu\text{m}$ (1- μm step size between respective XY points) were performed every 29 s. When

estimated $[Ca^{2+}]_i$ levels were to be reported, Ca^{2+} -EGTA buffer mixtures were used to yield ionized $[Ca^{2+}]$ between 50 and 1000 nM measured with 40 μ M indo-1 free acid at pH 7.4. The $[Ca^{2+}]_i$ was calculated by computer from a standard curve of the fluorescence ratio vs free Ca^{2+} in the Ca^{2+} -EGTA-HEPES buffer (Himpens *et al.*, 1992). However, these values should be taken with caution, since the calculated $[Ca^{2+}]_i$ values are only an estimate that depends on the calibration conditions used. Therefore, we expressed $[Ca^{2+}]_i$ in the figures as ratio units ($R_{405/485}$) unless otherwise stated in the text.

When the effect of each drug on the $[Ca^{2+}]_i$ and mechanical activity in response to pressure was tested, the following protocols were used: Each measurement was conducted at 1-min intervals. Each drug was added after the diameter had become stable. The maximal drug effect was ordinarily obtained within 15 min.

In the case of the assessment of each drug action on the contraction produced by either depolarizing or agonistic stimuli, the intraluminal pressure was kept at 10 mmHg so as not to develop the pressure-induced myogenic contraction (Masumoto *et al.*, 1997). The cerebral artery was contracted with 80 mM KCl PSS or by 100 nM 9,11-dideoxy-11 α , 9 α -epoxymethano prostaglandin $F_{2\alpha}$ (U46619), a stable analogue of thromboxane A_2 , at the concentration that produced a large and stable contraction over 2 h.

At the end of each experiment, papaverine (100 μ M) was administered to totally eliminate the contractions produced by the three different kinds of stimuli (Masumoto *et al.*, 1997). 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. The arterial diameter in the presence of papaverine was taken as 100%.

Immunocytochemical in situ detections of phosphorylated tyrosine residues and pp60^{src} activity

Immunocytochemical *in situ* detections of total phosphorylated tyrosine residues (Muller *et al.*, 1996; 1997), and pp60^{src} activity assessed by phosphorylation of tyrosine 419 on the kinase required for its full catalytic activity (Pwnica-Worms *et al.*, 1987), were carried out. Briefly, rat cerebral arteries were isolated, mounted on the arteriograph, and kept from developing myogenic tone by maintaining a constant intraluminal pressure of 10 mmHg. The arteries were then pressurized from 10 to 60 mmHg in the absence or the presence of each drug such as fasudil (100 μ M). For each experimental condition (pressurization alone or pressurization in the presence of each drug), basal tyrosine phosphorylation was determined in the control cerebral artery from the same brain, which was cannulated and not allowed to develop myogenic tone over the same time course. At the end of this protocol, 100 μ M sodium orthovanadate, a tyrosine phosphatase inhibitor, was added. After removal of the arteries from the cannulating pipettes, the arteries were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). The fixation was followed by two washes with PBS containing 0.1 mM glycine and a rinse with PBS. The vessels were then permeabilized with 0.1% Triton X-100 in PBS, rinsed three times with PBS, and incubated with 1 μ g ml⁻¹ monoclonal anti- α -smooth muscle actin antibody (diluted in PBS containing 0.9% sodium citrate, 2% horse serum, 1% albumin, 0.05% Triton X-100, and 0.025% NaN₃) for 45 min. The antibody solution was removed by three washes with PBS containing 0.9% sodium citrate and 0.05% Triton X-100. The arteries were next incubated with 1 μ g ml⁻¹ phycoerythrin (PE)-

conjugated monoclonal anti-mouse immunoglobulin G (IgG) antibody for 45 min. This antibody solution was removed by three washes with PBS containing 0.9% sodium citrate and 0.05% Triton X-100. The vessels were finally incubated with 1 μ g ml⁻¹ fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-phosphotyrosine antibody for 45 min, with subsequent antibody removal by three washes with PBS containing 0.9% sodium citrate and 0.05% Triton X-100.

In a separate series of experiments, immunocytochemical *in situ* detection of pp60^{src} activity was carried out with an antibody against a chemically synthesized phosphopeptide derived from the region of pp60^{src} that contains the tyrosine residue positively regulating its activity (rabbit anti-src [pY⁴¹⁸] phosphospecific antibody, 1 μ g ml⁻¹, BioSource International, Camarillo, CA, U.S.A.) for the primary antibody and FITC-conjugated goat anti-rabbit IgG (1 μ g ml⁻¹) for the secondary antibody.

In order to perform the immunocytochemical *in situ* detections, the arteries were then transferred to a culture dish that was thereafter placed on the ACAS Ultima 575 UVC System. In the present experiments, the laser scan strength was set at 50% of 33-mW output, and a 7.5% neutral density filter was used. The pinhole was set at 800 μ m. Image scans ($\times 40$ water objective, Zewiss, NA 1.2) of $200 \times 200 \mu$ m (0.5- μ m step size between respective XY points) were performed every 61 s. A clear focus of the smooth muscle cells of each vessel was obtained under bright-field illumination. Four or five fluorescent images from each vessel were acquired using an excitation wavelength of 480 ± 5 nm and an emission wavelength of 580 ± 5 nm for PE and 530 ± 5 nm for FITC with photomultipliers (R2949, Hamamatsu Photonics, Hamamatsu, Japan). Background fluorescence was subtracted, and the resultant images were captured and stored on a computer within the system.

Measurement of pp60^{src} activity in cell free system

Effects of fasudil and herbimycin A on pp60^{src} activity were investigated by use of protein tyrosine kinase assay kit (QIA28, Calbiochem, San Diego, CA, U.S.A.), based on the previous report (Schraag *et al.*, 1993). Briefly, pp60^{src} (4U) and either fasudil (1–100 μ M) or herbimycin A (1–100 nM) were added to each well of a 96-well microtiter plate which was precoated with a random polymer substrate containing multiple tyrosine residues. The kinase reaction was started by addition of kinase reaction buffer (90 μ l) including 0.1 mM ATP to each well, and the plate was incubated at room temperature for 30 min. The wells were washed six times with wash buffer making sure each well was filled completely. The horseradish peroxidase conjugated PY20, an anti-phosphotyrosine antibody, diluted with kinase reaction buffer (without ATP) was added to each well, and the plate was incubated at room temperature for 30 min. The substrate solution including tetra-methylbenzidine, a chromogenic substrate, was added to each well, and the plate was incubated in the dark at room temperature for 6 min. The stop solution containing 2.5 N H₂SO₄ was added to each well. The absorbance in each well was measured by use of a spectrophotometric plate reader (MTP-32, Corona Electric, Tokyo, Japan) at a wavelength of 450 nm. Each value represents the mean \pm s.e.mean of five determinations.

Chemicals

The following drugs were used: Fasudil hydrochloride was donated by Asahi Chemical Industry (Tokyo, Japan). Herbimycin A, genistein, daidzein, sodium orthovanadate,

nicardipine hydrochloride, indomethacin, papaverine hydrochloride, EGTA (ethylene glycolbis(β -aminoethyl-ether)N,N,N',N'-tetra-acetic acid), cremophor EL^R, PE-conjugated monoclonal anti-mouse IgG antibody, and monoclonal anti- α -smooth muscle actin antibody were purchased from Sigma (St. Louis, MO, U.S.A.). 9,11-Dideoxy-11 α , 9 α -epoxymethano prostaglandin F_{2 α} (U46619) was obtained from Cayman (Ann Arbor, MI, U.S.A.); *N*^ω-nitro-L-arginine (L-NNA), from Aldrich Chemical Company (Milwaukee, WI, U.S.A.); acetoxymethyl ester of indo-1 (indo-1/AM), from Dojindo Laboratories (Kumamoto, Japan); FITC-conjugated monoclonal anti-phosphotyrosine antibody, from Up State Biochemistry (New York, NY, U.S.A.); arginine-glycine-aspartic acid-serine (RGDS), from Peptide Institute, Inc. (Osaka, Japan); protein tyrosine kinase assay kit (QIA28), from Calbiochem (San Diego, CA, U.S.A.); rabbit anti-*src* [pY⁴¹⁸] phosphospecific antibody, from BioSource International (Camarillo, CA, U.S.A.); and FITC-conjugated goat anti-rabbit IgG, from Molecular Probes (Eugene, OR, U.S.A.). Other drugs used were of reagent quality. U46619 was dissolved in ethanol at 100 μ M. Indomethacin was dissolved in 0.1 M NaHCO₃ solution at 10 mM. Herbimycin A, genistein, and daidzein were dissolved in dimethyl sulphoxide (DMSO). The final concentrations of DMSO and ethanol did not exceed 0.1% in the organ bath. In a preliminary study, this concentration (0.1%) of DMSO or ethanol had no significant effect on the pressure-induced contraction, and did not result in any detectable change in the [Ca²⁺]_i or mechanical activity. Fasudil hydrochloride, nicardipine hydrochloride, sodium orthovanadate, L-NNA, and papaverine hydrochloride were dissolved in distilled water. RGDS was solubilized in fresh PSS. All drugs were added to the chamber, and final concentrations were expressed as a molar concentration (M).

Statistical analysis

All data were expressed as mean \pm s.e.mean. The value of EC₅₀ or IC₅₀ was determined geometrically from logarithmic concentration-response curves. Student's *t*-test for paired data was used to determine the significance of differences between means. Tukey's test for multiple comparison was used after analysis of variance (ANOVA) to determine the significance of differences among means. A value of *P* < 0.05 was considered significant.

Results

Characteristic nature of [Ca²⁺]_i and mechanical response to pressurization

Figure 1 shows simultaneous recordings of [Ca²⁺]_i and mechanical responses of rat cerebral artery when the intraluminal pressure was increased from 10 to 60 mmHg. The ratio of fluorescence intensity, $R_{405/485}$, a measure of [Ca²⁺]_i, at 10 mmHg was 0.58 ± 0.06 , arbitrarily corresponding to a [Ca²⁺]_i of 87.9 ± 11.6 nM (*n* = 5). The intracellular Ca²⁺ signal was transiently increased with pressurization. Afterwards, it reached a stable level within 5 min and lasted for at least 90 min. The vessel was passively inflated with an increase in intraluminal pressure from 10 to 60 mmHg, and was followed by a delayed contraction that reached a maximum within 5 min after application of the pressure. The contraction reached stable and lasted for at least 90 min. The effective pressure producing 50% of maximal contraction was 40.7 ± 4.0 mmHg (*n* = 5). In order to decrease [Ca²⁺]_i to resting

level and to eliminate totally the vascular tone, the artery was treated with papaverine (100 μ M) (Masumoto *et al.*, 1997; Tanaka *et al.*, 1995). The arterial diameter in the presence of papaverine was taken as 100% on the ordinate of the figure. The relationship between [Ca²⁺]_i and the change in diameter in response to pressurization from 10 to 20, 40 or 60 mmHg was approximately parallel, as shown in Figure 1b.

In the next step, we compared the effect of depolarizing and agonistic stimuli on the cerebral artery. The artery relaxed slightly when KCl in the medium was increased from 4 to 20 mM, and contraction occurred from over 20 mM up to 80 mM in a concentration-dependent manner. The contraction reached a maximum at 80 mM KCl, and an EC₅₀ 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 subsequent study, we tested each drug on the contraction produced by 80 mM KCl. A separate series of experiments indicated that $R_{405/485}$, a measure of

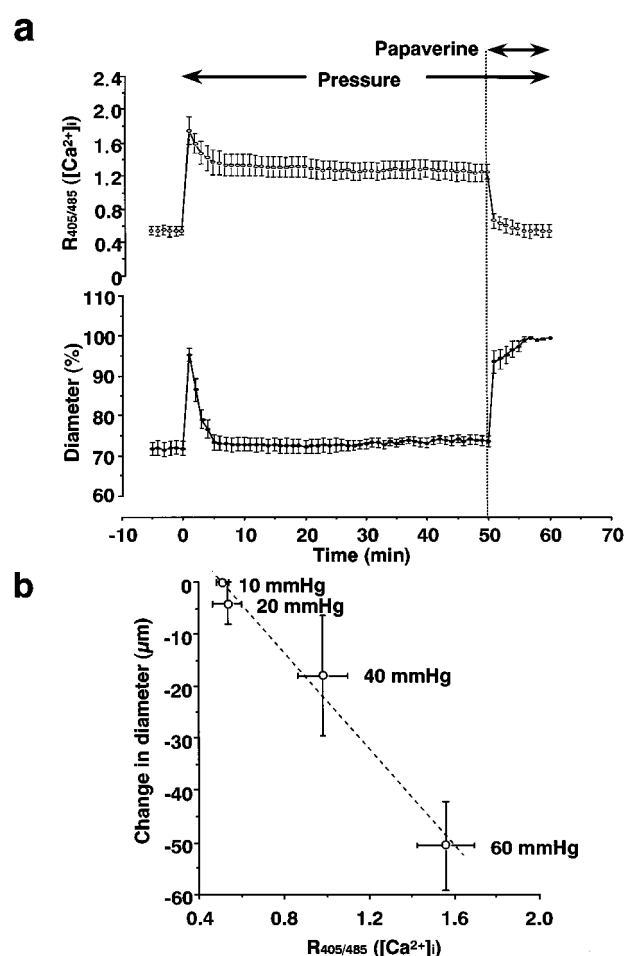


Figure 1 Pressure-induced increase in [Ca²⁺]_i and myogenic contraction of rat cerebral artery loaded with indo-1. (a) Time course of [Ca²⁺]_i and arterial diameter in response to a pressure stimulus from 10 to 60 mmHg. [Ca²⁺]_i (open circle; upper part of panel A) is expressed as the ratio of fluorescence intensity at 405 to that at 485 nm ($R_{405/485}$) in the upper panel. Arterial diameter (closed circle; lower part of panel A) at 60 mmHg in the presence of 100 μ M papaverine (197.6 ± 13.5 μ m, *n* = 5) was taken as 100% in the lower panel, and that at 10 mmHg was 143.5 ± 12.0 μ m (per cent diameter = 72.4 ± 1.7). The [Ca²⁺]_i and contractile response lasted stable for at least 60 to 90 min (outer diameter = 146.8 ± 10.8 μ m, per cent diameter = 74.2 ± 1.4). (b) The relationship between [Ca²⁺]_i and the change in diameter (μ m) in response to pressurization from 10 to 20, 40 or 60 mmHg. Each point represents the mean \pm s.e.mean of five preparations.

$[Ca^{2+}]_i$, and the arterial diameter before the depolarizing stimulation were 0.50 ± 0.01 ($[Ca^{2+}]_i = 82.6 \pm 4.6$ nM) and 149.2 ± 5.7 μ m, respectively. With application of 80 mM KCl, the rapid increase in $[Ca^{2+}]_i$ within 1–2 min was followed by contraction of the artery; and then the $[Ca^{2+}]_i$ and the diameter reached a steady state level ($R_{405/485} = 1.51 \pm 0.05$, $[Ca^{2+}]_i = 342.6 \pm 13.6$ nM; diameter = 101.0 ± 6.4 μ m, per cent diameter = 64.4 ± 2.5 based on 100 μ M papaverine = 100%, each $n = 5$).

U46619 (1–100 nM) produced a concentration-dependent contraction of the rat cerebral artery with an EC_{50} value of 31.7 ± 10.3 nM ($n = 13$). U46619 at 100 nM produced a large and long-lasting contraction, which was suitable for the evaluation of the vasodilator action of the inhibitors (Masumoto *et al.*, 1997; Tanaka *et al.*, 1995). $R_{405/485}$ and the outer diameter before the U46619 stimulus were 0.46 ± 0.05 ($[Ca^{2+}]_i = 79.5 \pm 9.8$ nM) and 151.0 ± 10.4 μ m (per cent diameter = 95.5 ± 1.4 based on 100 μ M papaverine = 100%), respectively. U46619 (100 nM) elicited at the steady-state level an increase in $[Ca^{2+}]_i$ ($R_{405/485} = 0.98 \pm 0.14$, $[Ca^{2+}]_i = 201.9 \pm 28.2$ nM) and contraction (outer diameter = 105.2 ± 5.1 μ m, $68.2 \pm 3.4\%$, each $n = 5$). In the case of the U46619-induced contraction, the steady-state level in $[Ca^{2+}]_i$ was relatively lower than that induced by either pressurization or depolarizing stimulus.

Effect of fasudil on $[Ca^{2+}]_i$ and mechanical responses to pressurization

Figure 2 shows the comparative effects of fasudil and nicardipine on the increased $[Ca^{2+}]_i$ and narrowed vessel diameter in response to pressure. Interestingly, the simultaneous recordings of $[Ca^{2+}]_i$ and mechanical responses to the three different stimuli indicated that fasudil and nicardipine acted differentially on both parameters. Typical tracings showed that the cumulative application of fasudil decreased both $[Ca^{2+}]_i$ and the contraction in a concentration-dependent manner (Figure 2a). At the highest concentrations used in the present study, fasudil (100 μ M) inhibited almost completely the myogenic contraction in spite of causing only a partial suppression of the increased $[Ca^{2+}]_i$, whereas nicardipine (100 nM) abolished both responses (Figure 2b). As summarized in Table 1, fasudil inhibited the concentrations produced by the three different kinds of stimulation in a similar manner with a 50% inhibitory concentration (IC_{50}) of 5–7 μ M, and the maximal inhibition, over 90%, occurred at 100 μ M. Nicardipine mostly inhibited the contractile responses produced by pressurization or depolarizing stimulus, whereas the drug inhibited the U46619-induced contraction less than half (Table 1).

Fasudil (100 μ M) almost completely inhibited the mechanical events, but partially suppressed the increased values of $R_{405/485}$, i.e., from 1.51 ± 0.03 to 1.08 ± 0.08 ($P < 0.05$, $n = 5$) for a pressure stimulus (see Figure 2a), from 1.63 ± 0.10 to 1.16 ± 0.06 ($P < 0.05$, $n = 5$) for 80 mM KCl, and from 1.07 ± 0.06 to 0.83 ± 0.05 ($P > 0.05$, $n = 5$) for U46619. Nicardipine (100 μ M) reduced the increased values of $R_{405/485}$ in response to all three different stimuli mostly to the resting level, i.e., from 1.47 ± 0.04 to 0.52 ± 0.06 ($P < 0.01$, $n = 5$) for pressure, 1.70 ± 0.08 to 0.62 ± 0.06 ($P < 0.01$, $n = 5$) for 80 mM KCl, and 0.92 ± 0.08 to 0.53 ± 0.02 ($P < 0.05$, $n = 5$) for U46619.

In order to clarify whether tyrosine kinase regulates intracellular Ca^{2+} signalling in the genesis of the pressure-induced contraction, we examined the effects of herbimycin A and genistein on the $[Ca^{2+}]_i$ and mechanical activity. Herbimycin A (100 nM) significantly reduced the $R_{405/485}$ that

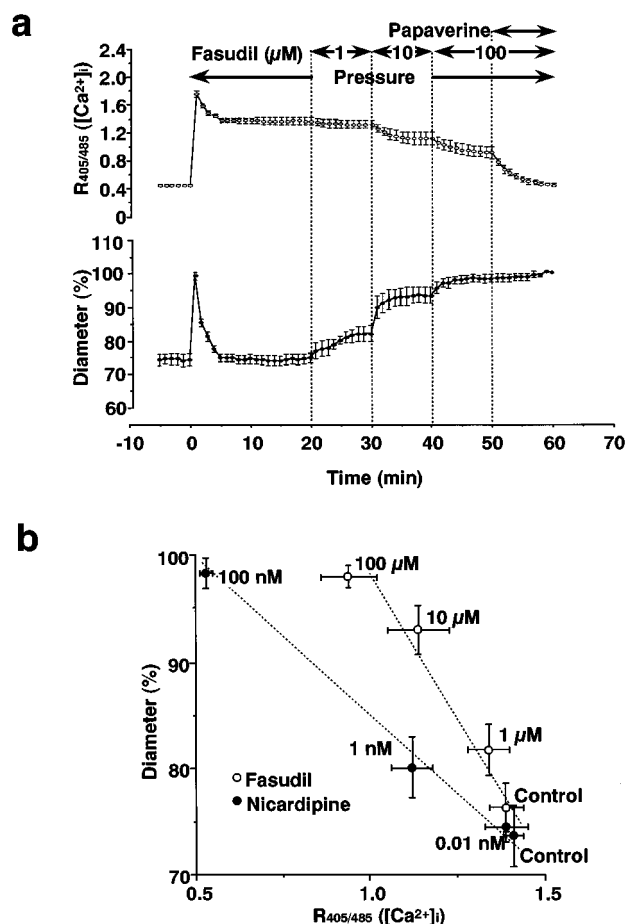


Figure 2 Effects of fasudil and nicardipine on the pressure-induced changes in $[Ca^{2+}]_i$ and diameter of rat cerebral artery. (a) Simultaneous measurements of $[Ca^{2+}]_i$ and diameter changed by a pressure stimulus from 10 to 60 mmHg and the effect of fasudil. (b) Summarized data representing the concentration-response relation as to the effects of fasudil (open circle; upper part of panel a) and nicardipine (closed circle; lower part of panel a) on the $[Ca^{2+}]_i$ and arterial diameter. Each point represents the mean \pm s.e. mean of four preparations. Corresponding control values in the absence of fasudil are not significantly different from those in the absence of nicardipine.

Table 1. Inhibitory effects of fasudil and nicardipine on contractions produced by three different types of stimuli

Drugs		Stimulus		
		Pressure	KCl (80 nM)	U46619
Fasudil	IC_{50} (μ M)	4.8 ± 0.8	4.9 ± 2.1	6.9 ± 1.8
	% Maximal inhibition	99.3 ± 0.5	93.1 ± 1.9	95.6 ± 1.7
Nicardipine	IC_{50} (μ M)	2.3 ± 0.9	5.4 ± 3.8	> 100
	% Maximal inhibition	91.0 ± 2.4	90.0 ± 3.7	42.6 ± 1.5

Each value represents the mean \pm s.e. mean of five separate experiments.

was increased by pressure, and the resultant contraction was also almost totally suppressed by the drug (Figure 3a). Summarized data indicated that $[Ca^{2+}]_i$ and contraction in response to pressure were inhibited by herbimycin A in a concentration-dependent manner (Figure 3b). Herbimycin A (100 nM) at the maximal concentration used reduced the maximal contraction in the pressure-contraction relationship, and the effective pressure producing 50% of maximal

contraction was 42.9 ± 7.3 mmHg in the control and 46.8 ± 6.9 mmHg in the presence of 100 nM herbimycin A (each $n=4$, $P>0.05$ vs control), respectively, indicating that the inhibition by herbimycin A of the pressure-induced contraction was noncompetitive. However, herbimycin A (100 nM) neither significantly changed the 80 mM KCl-induced increase in $R_{405/485}$ (1.38 ± 0.10 to 1.39 ± 0.08 , $P>0.05$, $n=5$) nor altered the narrowed arterial diameter (64.2 ± 0.7 to $65.8 \pm 1.6\%$, based on 100 μ M papaverine = 100%, $P>0.05$). Herbimycin A (100 nM) slightly changed the U46619-induced increase in $R_{405/485}$ (1.13 ± 0.03 to 1.07 ± 0.03 , $P>0.05$, $n=5$), and the resultant contraction (61.5 ± 4.1 to $60.6 \pm 3.8\%$, $P>0.05$).

Genistein (3–30 μ M) concentration-dependently inhibited the increase in $R_{405/485}$ induced by pressure and totally suppressed the resultant contraction in response to pressure (Figure 3b). Genistein (30 μ M) at the maximal concentration used reduced the maximal contraction in the pressure-contraction relationship, and the effective pressure producing 50% of maximal contraction was 43.3 ± 5.8 mmHg in the control and 45.6 ± 7.3 mmHg in the presence of 30 μ M genistein (each $n=4$, $P>0.05$ vs control), indicating that the

inhibition by genistein of the pressure-induced contraction was also noncompetitive in nature. Genistein (30 μ M) did not significantly affect the 80 mM KCl-induced increase in $R_{405/485}$ (1.32 ± 0.11 to 1.33 ± 0.07 , $P>0.05$, $n=5$) or contraction (62.0 ± 3.0 to $61.8 \pm 2.0\%$, based on 100 μ M papaverine = 100%, $P>0.05$). In contrast to herbimycin A, genistein (30 μ M) significantly attenuated the increased $R_{405/485}$ elicited by U46619 (100 nM) (1.14 ± 0.10 to 0.55 ± 0.06 , $P<0.05$, $n=5$), and abolished the resultant contraction (64.4 ± 3.4 to $99.2 \pm 0.7\%$, $P<0.01$). Daidzein (30 μ M), a negative control drug for genistein, affected neither the $R_{405/485}$ values nor the resultant contractions in response to pressure, high KCl, or U46619 (data not shown).

Orthovanadate (100 μ M), a tyrosine phosphatase inhibitor, significantly potentiated the pressure-induced increase in $R_{405/485}$ (1.48 ± 0.05 to 1.78 ± 0.06 , $P<0.05$, $n=4$) and contraction (78.4 ± 1.8 to $59.9 \pm 3.5\%$, based on 100 μ M papaverine = 100%, $P<0.05$). However, orthovanadate (100 μ M) did not apparently affect the 40 mM KCl-induced increase in $R_{405/485}$ (1.00 ± 0.04 to 0.99 ± 0.04 , $P>0.05$, $n=4$) or contraction (79.3 ± 2.5 to $79.7 \pm 2.4\%$, $P>0.05$). On the other hand, orthovanadate (100 μ M) significantly augmented the increased $R_{405/485}$ elicited by U46619 (30 nM) (0.83 ± 0.07 to 1.12 ± 0.08 , $P<0.05$, $n=4$), and potentiated the resultant contraction (78.8 ± 2.5 to $61.1 \pm 3.5\%$, $P<0.05$).

In order to clarify whether integrin is involved in the genesis of pressure-induced contraction, we examined an effect of arginine-glycine-aspartic acid-serine (RGDS) on the contractile response. RGDS peptide (1–100 μ M) concentration-dependently reduced the pressure-induced myogenic contraction with an IC_{50} value of 12.6 ± 4.5 μ M ($n=4$). The maximal inhibition of the pressure-induced contraction by RGDS (100 μ M) was $81.7 \pm 3.9\%$, based on 100 μ M papaverine = 100% ($n=4$). The peptide (1–100 μ M) had no apparent effect on high KCl- or U46619-induced contractions (data not shown).

Effect of fasudil on tyrosine phosphorylation

Typical images of rat cerebral artery subjected to pressure before and after application of fasudil and stained immuno-fluorescently for phosphotyrosine and the summarized data are shown in Figures 4 and 5, respectively. The image of a vessel under the control condition (no pressurization) is also shown in Figure 4a. Fluorescence intensity (arbitrary units) with antiphosphotyrosine staining was significantly increased in vessels pressurized from 10 to 60 mmHg. The image of the artery stained by anti-phosphotyrosine antibody (Figure 4b) was identical to that of the one stained by anti- α -smooth muscle actin antibody (Figure 4d), indicating that tyrosine phosphorylation occurred in medial smooth muscle cells of the same artery. Furthermore, the stained preparation showed that individual smooth muscle cells were oriented perpendicular to the longitudinal axis of the artery (Figure 4).

Pretreatments with fasudil (100 μ M), herbimycin A (100 nM), and genistein (30 μ M), at which concentrations each drug totally inhibited the contraction in response to pressure, significantly reduced the pressure-induced increase in tyrosine phosphorylation (Figures 4b,c and 5a, respectively), whereas nicardipine (100 nM) and daidzein (30 μ M, data not shown) only slightly suppressed the pressure-induced tyrosine phosphorylation (Figure 5a).

A depolarizing stimulus such as high KCl (80 mM) did not significantly affect the fluorescence intensity with antiphosphotyrosine staining in the artery (10.0 ± 4.2 vs 22.8 ± 9.6 , $P>0.05$, control arteries vs high KCl-treated arteries, each $n=5$).

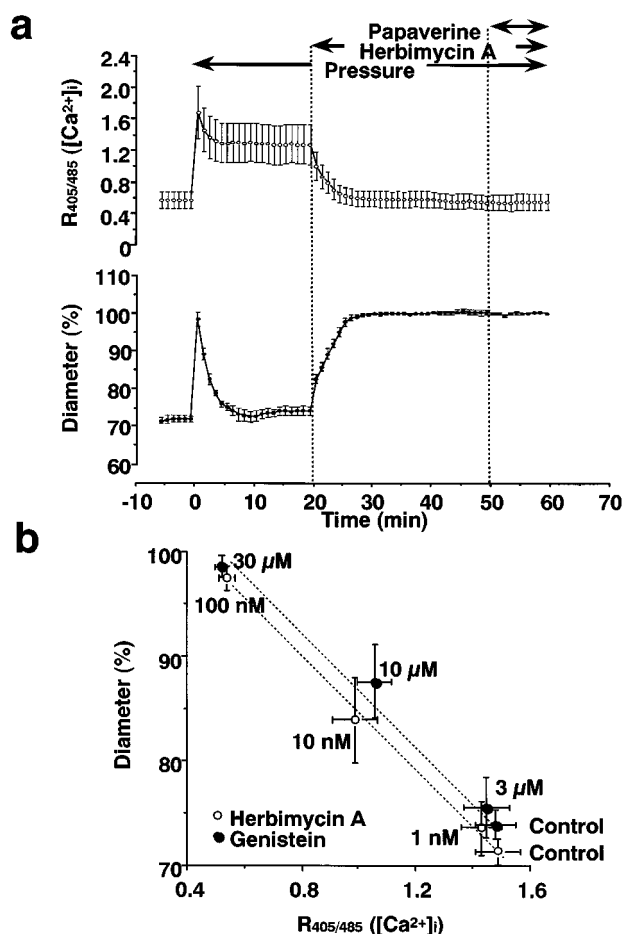


Figure 3 Effects of tyrosine kinase inhibitors on the $[Ca^{2+}]_i$ and arterial diameter of rat cerebral arteries in response to a pressure stimulus. (a) Herbimycin A (100 nM) significantly inhibited the $[Ca^{2+}]_i$ (open circle; upper part of panel a) and the resultant contraction (closed circle; lower part of panel a) increased by a pressure stimulus from 10 to 60 mmHg. (b) Summarized data representing the concentration-response relation as to the effects of herbimycin A (open circle) and genistein (closed circle) on the $[Ca^{2+}]_i$ and arterial diameter. Each point represents the mean \pm s.e. mean of four to five preparations. Corresponding control values in the absence of herbimycin A are not significantly different from those in the absence of genistein.

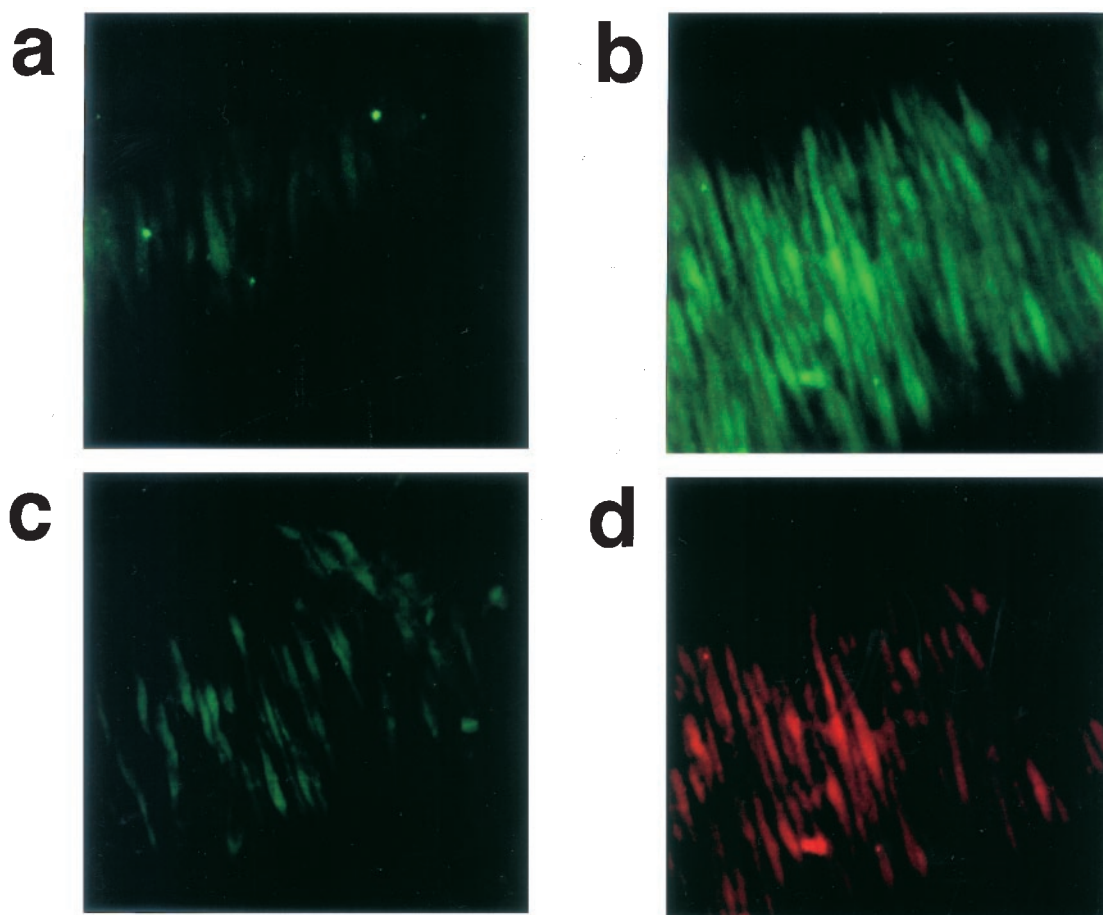


Figure 4 Effect of fasudil on pressure-induced tyrosine phosphorylation. Staining of phosphorylated tyrosine residues in rat cerebral arteries under control conditions (a) and following exposure to pressurization from 10 to 60 mmHg for 5 min in the absence (b) or the presence of fasudil (100 μ M) (c). The image stained by anti-phosphotyrosine antibody (b) was identical to that stained by anti- α -smooth muscle actin antibody (d), indicating that tyrosine phosphorylation occurred in the medial smooth muscle cells of the same artery.

U46619 (100 nM) significantly increased the fluorescence intensity in the artery (Figure 5b). Pretreatment with fasudil (100 μ M) significantly reduced the tyrosine phosphorylation (Figure 5b). It is noteworthy that herbimycin A and genistein acted differently on the tyrosine phosphorylation. Herbimycin A (100 nM) did not significantly affect the tyrosine phosphorylation, whereas genistein (30 μ M) significantly attenuated the tyrosine phosphorylation (Figure 5b). Neither nicardipine (100 nM) nor daidzein (30 μ M, data not shown) pretreatment, on the other hand, significantly inhibited the tyrosine phosphorylation (Figure 5b).

Effect of fasudil on pp60^{src} activity in situ

The pp60^{src} has two tyrosine residues that can be phosphorylated, and their phosphorylation states affect its kinase activity (Piwnicka-Worms *et al.*, 1987). The phosphorylation of tyrosine 419 positively regulates the kinase activity. Therefore, immunocytochemical *in situ* detection of the phosphorylated tyrosine 419, a measure of the kinase activity, was carried out with an antibody against a chemically synthesized phosphopeptide derived from the region of pp60^{src} that contains the tyrosine residue positively regulating its activity.

Typical images of rat cerebral arteries stained immunofluorescently for the phosphorylation indicating pp60^{src} activity and the summarized data are shown in Figures 6 and 7, respectively. Figure 6a shows the image of a vessel under the control condition (no pressurization). Fluorescence intensity

(arbitrary units) was significantly increased in vessels pressurized from 10 to 60 mmHg (Figure 6b). The image of the artery stained by anti-*src* [pY⁴¹⁸] phosphospecific antibody was identical to that of the one stained by anti- α -smooth muscle actin antibody (data not shown), indicating that phosphorylation of the tyrosine residue on pp60^{src} occurred in medial smooth muscle cells of the same artery.

Pretreatments with fasudil (100 μ M), herbimycin A (100 nM), and genistein (30 μ M) each for 20 min significantly reduced the pressure-induced increase in the phosphorylation of pp60^{src} (Figures 6c,d and 7), whereas nicardipine (100 nM) and daidzein (30 μ M, data not shown) only slightly suppressed the phosphorylation (Figure 7).

Effect of fasudil on pp60^{src} activity in cell free system

Fasudil significantly inhibited the pp60^{src} activity in a concentration-dependent manner with an IC₅₀ value of 32.5 ± 2.3 μ M (Figure 8). Herbimycin A also significantly reduced the enzyme activity in a concentration-dependent manner with an IC₅₀ value of 8.0 ± 0.5 nM (Figure 8).

Discussion

In the present study, we showed that fasudil (1–100 μ M) concentration-dependently inhibited the pressure-induced myogenic contraction of rat cerebral artery, and the drug at

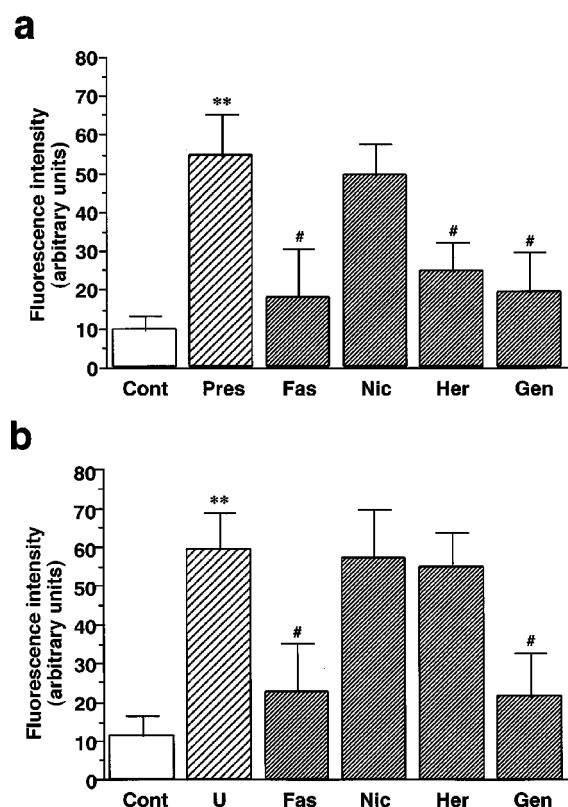


Figure 5 Effects of fasudil, nicardipine, herbimycin A, and genistein on tyrosine phosphorylation of rat cerebral artery. (a) Pressurization from 10 to 60 mmHg significantly increased the fluorescence intensity. Note that fasudil (Fas) (100 μ M), herbimycin A (Her) (100 nM), and genistein (Gen) (30 μ M) except nicardipine (Nic) (100 nM) treatments significantly reduced the fluorescence intensity. (b) U46619 (100 nM) significantly potentiated fluorescence intensity. Note that fasudil (100 μ M) and genistein (30 μ M) except nicardipine (100 nM) and herbimycin A (100 nM) treatments significantly reduced the fluorescence intensity. Each column represents the mean \pm s.e. mean of five preparations. Cont, control; Pres, pressure; U, U46619. ** indicates $P < 0.01$ vs corresponding control values. # indicates $P < 0.05$ vs corresponding values stimulated with either pressure (a) or U46619 (b).

100 μ M abolished the mechanical activity in spite of its ability to only partially suppress the increase in $[Ca^{2+}]_i$ in response to intraluminal pressurization. The pressurization elicited a marked increase in total tyrosine phosphorylation level and pp60^{src} activity assessed by phosphorylation of tyrosine 419 on the kinase required for its full catalytic activity (Piwnicka-Worms *et al.*, 1987), in medial smooth muscle cells of the artery. These augmentations were also inhibited by fasudil. In addition, the pp60^{src} activity was directly inhibited by fasudil in a cell free system.

The contractile reaction of vascular tissue to mechanical stress is a kind of physical response and thus requires cellular signal transduction, which may be mediated through a receptive site specific for a mechanical stimulus and the pathways of Ca^{2+} signalling common to pharmacological agonists. It has been considered that vascular smooth muscle directly responds to mechanical forces in a variety of ways, including changes in membrane ionic conductance, activation/inactivation of a number of second messenger systems, and changes in cytoskeletal structures (Osol, 1995). In the present study, the pressure-induced increase in $[Ca^{2+}]_i$ and myogenic contraction of rat cerebral artery were abolished by nicardipine, a voltage-dependent L-type Ca^{2+} channel blocker.

Furthermore, the change in $[Ca^{2+}]_i$ and arterial diameter in response to varying pressure at the tonic contraction phase occurred in an almost parallel manner (see Figure 1b). Therefore, we consider that the activator Ca^{2+} involved in the pressure-induced contraction of the rat cerebral artery is mainly dependent on the transmembrane influx *via* voltage-dependent L-type Ca^{2+} channels, and that any mechanism involved in Ca^{2+} sensitization plays a minor role in the pressure-induced contraction.

Integrins, a large family of heterodimeric transmembrane glycoproteins, have been considered to play an important role in the mechanotransduction in various tissues including vascular smooth muscle cells and endothelial cells (Ingber, 1991). With the binding of specific ligands or haemodynamic stimuli, including blood pressure and blood flow, integrins have been reported to activate a variety of biochemical signalling pathways. A feature common to many integrin-binding proteins is the presence of the tripeptide sequence, arginine-glycine-aspartic acid (RGD), (Ruoslahti & Piersbacher, 1986; 1987). Mogford *et al.* (1996) reported that Gly-Pencillamine-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala (cyclic RGD) or Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) counteracted the contraction of pressurized rat cremaster arterioles even after removal of the endothelium. Furthermore, the vasodilatory effect of the RGD peptides could be blocked almost completely by a function-blocking monoclonal antibody to β_3 -integrin (F11) (Mogford *et al.*, 1996). D'Angelo *et al.* (1997b) found that the RGD peptide decreased $[Ca^{2+}]_i$ and attenuated the contraction of pressurized rat cremaster arterioles. In the present study, we showed that RGDS peptide, Arg-Gly-Asp-Ser, effectively suppressed the pressure-induced contraction of rat cerebral artery without any appreciable effects on the contractions in response to high KCl or U46619. Taken together with our results and those of others, it seems very likely that the interaction of integrins with extracellular matrix proteins is primarily involved in the genesis of pressure-induced myogenic contraction.

The interaction of integrins with ion channels has not been definitely established. There may be a direct coupling between the integrin and the channel protein, as suggested by Schwartz *et al.* (1993). They found a 50-kD protein, termed integrin-associated protein, in endothelial cells, which has been considered to act as an ion channel or to directly interact with one. On the basis of such an arrangement, with mechanical stimuli and/or ligand, integrins could allosterically modulate channel function. Mechanogated membrane ion channels have been also considered to be another candidate as a mechanosensor (Osol, 1995). Our previous observations indicated that Gd^{3+} , a known inorganic inhibitor of mechanogated channels, inhibited the pressure-induced contraction of rat cerebral artery (Masumoto *et al.*, 1997), as was reported by Langton (1993) to occur in isolated single myocytes of the same artery. However, Gd^{3+} also suppressed high KCl-induced contraction of the artery (Masumoto *et al.*, 1997), indicating a non-specific inhibitory action of Gd^{3+} . Thus, although the presence of mechanogated channel in rat cerebral artery remains to be confirmed, mechanical force could be transferred to integrins and/or mechanogated channels, resulting in the transmembrane ion entry.

It is noteworthy that adhesion plaques are rich in tyrosine kinases such as *src*-family and focal adhesion kinase (FAK) (Rohrschneider *et al.*, 1980). In single rabbit ear artery cells, intracellular application of a *src* tyrosine kinase increased Ca^{2+} channel currents, suggesting that intrinsic *src* regulates voltage-dependent Ca^{2+} channel in vascular smooth muscle cells (Wijetunge & Hughes, 1996). As nicardipine abolished the

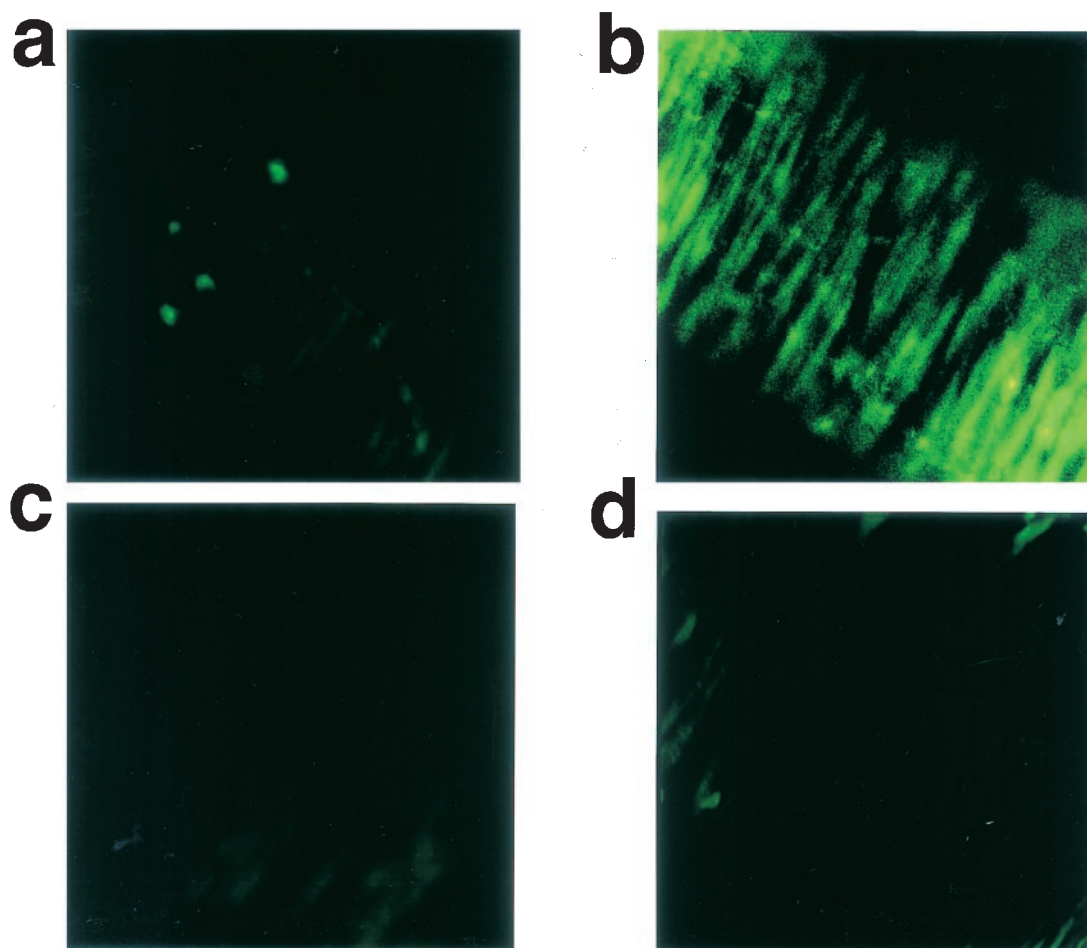


Figure 6 Effects of fasudil and herbimycin A on pressure-induced phosphorylation of tyrosine 419 on pp60^{src} required for its full catalytic activity. Typical images stained by anti-*src* [pY⁴¹⁸] phosphospecific antibody in rat cerebral arteries under control conditions (a) and following exposure to pressurization from 10 to 60 mmHg for 5 min in the absence (b), or the presence of fasudil (100 μ M) (c) were shown. Image of an pressurized artery with herbimycin A (100 nM) was also shown (d). Note that the stained individual smooth muscle cells were oriented perpendicular to the longitudinal axis of the artery.

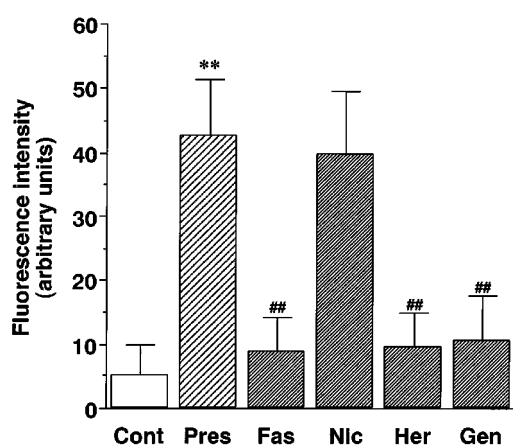


Figure 7 Effects of fasudil, nicardipine, herbimycin A, and genistein on phosphorylation of tyrosine 419 on pp60^{src} in rat cerebral artery. Pressurization from 10 to 60 mmHg significantly increased the fluorescence intensity. Note that fasudil (Fas) (100 μ M), herbimycin A (Her) (100 nM), and genistein (Gen) (30 μ M) except nicardipine (Nic) (100 nM) treatments significantly reduced the fluorescence intensity. Each column represents the mean \pm s.e. mean of five preparations. Cont, control; Pres, pressure. ** indicates $P < 0.01$ vs corresponding control values. ## indicates $P < 0.01$ vs corresponding values stimulated with pressure.

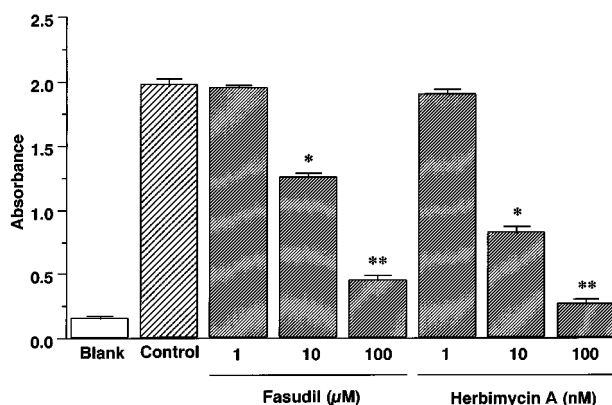


Figure 8 Effects of fasudil and herbimycin A on pp60^{src} activity in cell free system. Note that fasudil significantly inhibited the pp60^{src} activity in a concentration-dependent manner (IC_{50} value = 32.5 ± 2.3 μ M). Herbimycin A also significantly reduced the enzyme activity (IC_{50} value = 8.0 ± 0.5 nM). The absorbance in each well was measured by use of a spectrophotometric plate reader at a wavelength of 450 nm. Each column represents the mean \pm s.e. mean of five determinations. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, vs corresponding control values.

pressure-induced increase in $[Ca^{2+}]_i$ and myogenic contraction, but the drug did not suppress not only tyrosine phosphorylation but also $pp60^{src}$ activity augmented by intraluminal pressurization, we consider that the tyrosine phosphorylation precedes activation of voltage-dependent L-type Ca^{2+} channels in the myogenic contraction. We recently reported that herbimycin A specifically suppressed the pressure-induced myogenic contraction but did not affect high KCl- or U46619-induced contractions of the rat cerebral artery (Masumoto *et al.*, 1997), indicating that herbimycin A possesses neither specific inhibitory actions on Ca^{2+} entry and prostanoid receptors nor non-specific actions.

There are two tyrosine residues that can be phosphorylated on $pp60^{src}$ (Piwnica-Worms *et al.*, 1987). In rat, one is tyrosine 530 in the carboxyl-terminal domain, which negatively regulates the kinase activity. Another is tyrosine 419 in the kinase domain, which positively regulates the $pp60^{src}$ activity. The sequence containing the tyrosine residue able to increase its kinase activity is conserved in a variety of animal species including rat (Rohrschneider *et al.*, 1979). In the present study, we demonstrated that pressure increased $pp60^{src}$ activity in the artery, which was assessed by immunocytochemical *in situ* detection of the phosphorylated tyrosine residue with a phosphospecific antibody. The antibody is against a chemically synthesized phosphopeptide derived from the region of $pp60^{src}$ that contains the tyrosine residue positively regulating its activity. Furthermore, both tyrosine phosphorylation and $pp60^{src}$ activity were suppressed by herbimycin A. We also showed that herbimycin A reduced the increase in $[Ca^{2+}]_i$ and myogenic contraction and that orthovanadate potentiated the responses to a pressure stimulus.

Our preliminary experiments also showed that pressure-induced myogenic contraction of rabbit basilar artery and the enhanced tyrosine phosphorylation of various proteins including FAK were effectively inhibited by herbimycin A (Saito *et al.*, 1999). Therefore, according to our studies and those of others, we consider that integrins, herbimycin A-sensitive tyrosine kinases including $pp60^{src}$, and ion channels eliciting membrane depolarization, may interact with one another to assemble focal adhesion plaque, resulting in the regulation of transmembrane Ca^{2+} entry *via* voltage-dependent L-type Ca^{2+} channel in the genesis of myogenic contraction.

Our present study showed that fasudil (1–100 μ M) effectively inhibited all three kinds of contractions in a similar manner in spite of only partial suppression of the increase in $[Ca^{2+}]_i$. On the other hand, nicardipine abolished the increment of $[Ca^{2+}]_i$ and the resultant contractions in response to mechanical or depolarizing stimuli. Asano *et al.* (1987) indicated that fasudil antagonized competitively Ca^{2+} influx induced by extracellular high Ca^{2+} in the rabbit aorta. Therefore, the $[Ca^{2+}]_i$ decreased by fasudil, which occurred prior to inhibition of the three types of contractions, could be attributable to its common pharmacological action, i.e., direct blockade of L-type Ca^{2+} channel. Our present study indicates that herbimycin A-sensitive tyrosine phosphorylation and activation of $pp60^{src}$, in particular in focal adhesion plaques, seems to be the initial step in the mechanotransduction process of pressure-induced myogenic contraction. In the present study, fasudil effectively suppressed the pressure-induced phosphorylation of tyrosine 419 in the catalytic domain of $pp60^{src}$. Furthermore, fasudil (1–100 μ M) directly inhibited the $pp60^{src}$ activity with the IC_{50} value of about 30 μ M. The IC_{50} value of fasudil for the pressure-induced contraction was about 5 μ M, which was somehow lower than that for the $pp60^{src}$ activity. The difference between the IC_{50} value of fasudil for the

pressure-induced contraction and that for $pp60^{src}$ activity is at present unknown. Alternatively, fasudil can disassemble a cascade involved in the mechanotransduction process by inhibition of herbimycin A-sensitive tyrosine phosphorylation *via src*-family tyrosine kinase(s), which may also partially contribute to decreasing $[Ca^{2+}]_i$ in the case of pressure-induced contraction.

Our present experiments showed that high KCl-stimulation did not elicit tyrosine phosphorylation in the rat cerebral artery. In addition, herbimycin A, genistein, and orthovanadate had no apparent effect on high KCl-induced increase in $[Ca^{2+}]_i$ and contraction. These findings suggest that the inhibitory mechanism of fasudil of the contractile response to a depolarizing stimulus is not due to its elimination of tyrosine phosphorylation but due to other actions, including its direct blockade of the transmembrane Ca^{2+} entry *via* voltage-dependent L-type Ca^{2+} channels.

Recently, it has been reported that protein tyrosine phosphorylation could be also involved in receptor-mediated increase in $[Ca^{2+}]_i$ and subsequent contractions of arterial smooth muscle. In isolated smooth muscle cells from porcine coronary arteries, genistein effectively inhibited the initial transient $[Ca^{2+}]_i$ and sustained $[Ca^{2+}]_i$ responses to endothelin-1, suggesting the involvement of tyrosine kinase in the regulatory mechanism for the peptide-induced $[Ca^{2+}]_i$ responses in the coronary artery (Liu *et al.*, 1996). Endothelin-1 (1–100 nM) produced rapid (20 s) and sustained (up to 20 min) increases in protein tyrosine phosphorylation levels in four main clusters of proteins in rat mesenteric artery, which occurred even in the Ca^{2+} -free medium (Ohanian *et al.*, 1997). Here, we demonstrated that U46619 augmented not only $[Ca^{2+}]_i$ but also tyrosine phosphorylation, as well as the subsequent contraction and that these augmentations were effectively inhibited by genistein but not herbimycin A or daidzein. In addition, orthovanadate augmented U46619-induced increase in $[Ca^{2+}]_i$ and contractile activity. Although genistein has been recently reported to possess non-specific effects such as a direct action on Cl^- channel proteins (Hwang *et al.*, 1997; Zhou *et al.*, 1998), our present study suggests that tyrosine phosphorylation, sensitive to genistein but not to herbimycin A, is involved in the mechanism governing the contractile processes in the U46619-induced contraction of rat cerebral artery. It seems possible that a common signalling pathway involving cytosolic tyrosine kinase(s) sensitive to genistein may play a role in the mechanism of contractions produced by pressure and U46619 (Masumoto *et al.*, 1997).

Although fasudil only partially suppressed the increase in $[Ca^{2+}]_i$, the drug totally attenuated the three different kinds of contractile responses. It is generally considered that the Ca^{2+} -dependent phosphorylation of myosin light chain (MLC) is one of the most important mechanisms involved in the contraction of smooth muscle. We previously reported that ML-9 [1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride], a MLC kinase inhibitor, almost completely depressed the mechanical reactivity elicited by quick stretch, but only slightly attenuated the $[Ca^{2+}]_i$ (Nakayama & Tanaka, 1988). Fasudil possesses an inhibitory effect on Ca^{2+} /calmodulin-dependent MLC kinase (Asano *et al.*, 1989; Katsumata *et al.*, 1997; Seto *et al.*, 1991, 1995; Uehata *et al.*, 1997), which may contribute to the attenuation of the three different kinds of contractile responses. We previously reported that U46619 increased not only the influx of Ca^{2+} but also the release of Ca^{2+} from intracellular storage sites, as well as heightened the Ca^{2+} sensitivity of the contractile elements in the canine basilar artery (Tanaka *et al.*, 1995). Since fasudil also suppresses protein kinases

involved in Ca^{2+} sensitization, such as protein kinase C (Asano *et al.*, 1989; Uehata *et al.*, 1997) and Rho-associated kinase (Uehata *et al.*, 1997), the drug effectively inhibited the U46619-induced contraction.

As to pathophysiological interventions, Katsumata *et al.* (1997) reported an enhancement of MLC phosphorylation as a central mechanism for coronary artery spasm in a swine model with interleukin 1β . Fasudil dose-dependently inhibited the serotonin-induced coronary hyperconstriction due to its suppression of MLC phosphorylation in this model. Cerebral vasospasm, a persistent narrowing of major cerebral arteries, is a major cause of mortality and morbidity following SAH. The components of erythrocyte lysate, such as oxyhaemoglobin and other molecules, have been proposed as causative agents. Recently, it has been reported that haemolysate induced tyrosine phosphorylation in endothelial cells of bovine cerebral artery (Marton *et al.*, 1996) and in smooth muscle cells of rat cerebral artery (Iwabuchi *et al.*, 1999). Furthermore, Kim *et al.* (1998) reported that erythrocyte lysate-induced contraction was inhibited by genistein in the rabbit basilar artery.

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