Effects of Flufenamic Acid on Smooth Muscle of the Carotid Artery Isolated from Spontaneously Hypertensive Rats

Keiichi SHIMAMURA¹, Ming ZHOU¹, Yasuko ITO¹, Shinichi KIMURA¹, L.-B. ZOU², Fumiko SEKIGUCHI², Kenji KITAMURA³, and Satoru SUNANO²

¹Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
²Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka 577-8502, Japan
³Department of Pharmacology, Fukuoka Dental College, Tamaru, Sawara-ku, Fukuoka 814-0103, Japan

Abstract

Endothelium-removed carotid artery strips from stroke-prone spontaneously hypertensive rats spontaneously developed a tonic myogenic contraction. Flufenamic acid reduced the resting tone observed during superfusion with Tyrode's solution, in a concentration-dependent manner. Flufenamic acid also inhibited contractions produced by high-K⁺ solutions in a concentration-dependent manner. The resting membrane potential of smooth muscle cells in the artery was around −32 mV, with occasional oscillatory potentials. Flufenamic acid hyperpolarized the membrane in a concentration-dependent manner. The voltage-dependent outward currents recorded in isolated cells with micropipettes filled with high-K⁺ solution (holding potential, −60 mV) were enhanced by flufenamic acid and inhibited by tetraethylammonium. When the recording micropipette was filled with high Cs⁺ to inhibit the K⁺-current, depolarizing step pulses evoked nifedipine-sensitive inward currents. Flufenamic acid inhibited the inward currents. These results indicate that flufenamic acid inhibits the spontaneous active tone of the carotid artery by inhibiting L-type Ca²⁺-channels and possibly by membrane hyperpolarization through activation of the voltage-dependent K⁺-channels.

Key words: Flufenamic acid, Carotid artery, K⁺ current, Ca²⁺ current, SHR

Introduction

Large arteries of hypertensive rats have been shown to develop spontaneous tonic myogenic contractions (Noon et al., 1978; Sada et al., 1990). We have reported that the endothelium-
removed carotid artery of stroke-prone spontaneously hypertensive rats (SHRSP) contracts spontaneously with an increase in intracellular free Ca\(^{2+}\) concentration (Sekiguchi et al., 1996). This preparation was thought to be suitable for the study of the cellular mechanisms involved in the action of chemicals that may cause relaxation of arteries during hypertension.

Flufenamic acid, a diphenylamine carboxylate derivative, is a cyclooxygenase inhibitor (Flower, 1974), and has been shown to modulate several types of ion channels in different smooth muscle tissues. This chemical potentiates the potassium current (K'-current) in the canine and human jejunum (Farrugia et al., 1993a; 1993b), the rabbit portal vein (Greenwood and Large, 1995), the canine coronary artery (Xu et al., 1994) and the pig coronary artery (Ottolino and Toro, 1994). Flufenamic acid inhibits the L-type Ca\(^{2+}\)-current in the rat basilar artery (Doughty et al., 1998), the Ca\(^{2+}\)-dependent Cl\(^{-}\)-current in the rabbit portal vein (Greenwood and Large, 1995) and the non-selective cationic current in the guinea pig ileum (Chen et al., 1993). Although these effects of flufenamic acid on ion channels can inhibit contraction of smooth muscle, no report is available on the effects of this chemical on the membrane potential or ionic currents of arterial smooth muscle obtained from hypertensive rats.

In the present study, we investigated the effects of flufenamic acid on the spontaneous tonic contractions and on the membrane potential and membrane currents of smooth muscle isolated from the carotid artery of SHRSP. A part of this study was reported as a preliminary report (Shimamura et al., 1999).

**Materials and Methods**

*Animals and preparations*

Sixteen week old male SHRSP (Okamoto et al., 1974), with a systolic blood pressure of 220–260 mmHg, were anesthetized with ethyl ether and exsanguinated. The left or right common carotid artery was dissected from the animals and the endothelial cells were removed by rubbing the lumenal surface. Helically cut preparations were prepared from the artery segments. The hepatic portal vein was also dissected, opened by a longitudinal incision and the lumenal surface rubbed to remove the endothelial cells.

*Mechanical recordings*

Both ends of the tissue segments were tied with a tungsten wire (30 \(\mu\)m diameter) and mounted vertically in a recording organ bath filled with modified Tyrode's solution (warmed to 37\(^\circ\)C). One of the wires was connected to a force-displacement transducer (UL-10, Minebea, Karuizawa, Japan) to record contractile forces isometrically. A stretch of 1.3 times the original length was applied to each preparation and the preparation was subjected to high K\(^{+}\)-induced contraction and was then allowed to incubate until development of spontaneous tonic contractions.

The composition of the modified Tyrode's solution was as follow (in mM); NaCl 137, KCl 5.4, CaCl\(_2\) 2.0, MgCl\(_2\) 1.0, NaH\(_2\)PO\(_4\) 0.04, glucose 5.6. The solution was equilibrated with a gas mixture of 95% O\(_2\) and 5% CO\(_2\), and the pH of the solution maintained at 7.3. High-K\(^{+}\) solution ([K\(^{+}\)]\(_o\) = 80 mM) was prepared by replacing equimolar amounts of NaCl with KCl. Nominally
Ca²⁺-free Tyrode's solution (Ca²⁺-free solution) was prepared by omitting CaCl₂ from the Tyrode's solution.

Permeabilized preparations of circular muscle of the rat portal vein were made, as described previously (Shimamura et al., 1998). Both ends of a thin strip (50 μm thick, 100 μm wide, 2 mm long) of the circular muscle were tied to tungsten wires with silk thread. The preparations were set horizontally in a recording chamber (volume, 1 ml) and filled with Tyrode's solution at 25°C. One of the wires was connected to an arm of the micromanipulator, while the other was connected to a lever of the isometric force-displacement transducer (UL-10 Minebea). After equilibration in the Tyrode's solution for 1 hr, the tissue was stimulated with high-K solution to elicit contraction. The tissues were then permeabilized with β-escin, using the methods reported by Gonzalez et al. (1995). Briefly, the muscle strip was incubated in a relaxing solution containing 10 μM β-escin for 30 min. Permeabilizing treatment was considered successful when the amplitude of contraction induced by a solution containing high-Ca (pCa = 6.0) exceeded that of the high K-solution (Jensen, 1994). After removal of β-escin from the incubating solution, responses of muscle strips to solutions containing different concentrations of Ca²⁺ were tested using Ca²⁺-buffered solutions, according to the methods reported by Kerrick and Hoar (1994). Free Ca²⁺ concentrations in the solution were calculated with the association constants in accordance with the temperature and pH of the solution (Fabriato and Fabriato, 1979). The composition of the incubating solution was as follows (in mM): NaCl 130, KCl 5.6, glucose 11, Tris 20, MgCl₂ 1, and CaCl₂ 2.5. High-K solution ([K⁺]₀ = 80 mM) was prepared by an equimolar replacement of NaCl with KCl. Composition of the relaxing solution was as follows (in mM): K propionate 130, Tris-malate 20, EGTA 10, MgCl₂ 1, ATPMg 3, Creatine phosphate Na₂ 10 and Creatine phosphokinase 10 unit/ml (pH = 6.8).

Membrane potential recording

The membrane potential was recorded intracellularly using a glass capillary microelectrode (tip resistance, 40–80 MΩ) filled with 3M KCl, as previously described (Sekiguchi et al., 1996). The reference electrode was an Ag-AgCl plate. The potential differences between these two electrodes were measured using a microelectrode amplifier (MEZ-8201, Nihon Kohden, Tokyo), and were displayed on a dual beam cathode-ray oscilloscope (VC-10, Nihon Kohden, Tokyo) and also on an ink-writing pen oscillograph (8561 ME-1H Sanei, Tokyo) and a tape recorder (RMG-5304, Nihon Kohden). Arterial strips were mounted on a silicon rubber plate, with the lumenal side up, in a recording organ bath (volume, 1 ml). The strips were superfused continuously with modified Tyrode's solution (37°C) at a flow rate of 4 ml/min. Successful impalements of the recording electrode into cells were characterized by a sudden negative shift in the voltage followed by a stable negative potential for at least 5 min.

Cell dispersion and whole cell recording using the patch clamp method

Preparations were incubated in nominally Ca²⁺-free physiological salt solution (PSS) containing 2 mg/ml collagenase (type I, Wako pure Chemical, Osaka Japan), 1 mg/ml papain (Wako), 1 mg/ml soybean trypsin inhibitor (type II, Wako), 0.5 mg/ml dithiothreitol and 1.5 mg/ml bovine serum albumin (Fraction V, Sigma) at 37°C for 60 min. The digested tissue was
cut into pieces and triturated in Ca$^{2+}$-free PSS. Cells were stored at 4°C until used. Standard whole-cell recording was performed as described by Hamill et al. (1981). Currents were recorded with a patch clamp amplifier (CEZ-2200, Nihon Kohden) and an A/D, D/A converter (Labmaster DMA, Scientific Solutions, INC, Solon, USA) with pClamp v.5.5 programs (Axon Instruments, Foster City USA). The capacitance of the cell membrane was measured using small ramp pulses (10 mV depolarization in 10 msec) and the current amplitudes obtained by the step pulses normalized by the capacitance. All experiments were carried out at room temperature.

In the patch clamp experiments, the bathing solution (= PSS) contained the following ionic concentrations (mM): NaCl 140, KCl 5.4, glucose 12, MgCl$_2$ 1.2, CaCl$_2$ 2, HEPES 10 (titrated to pH 7.35–7.45 by NaOH). The nominally Ca$^{2+}$-free PSS was made by omitting CaCl$_2$ from the PSS. The composition of the high-K$^+$ micropipette solutions was as follows (in mM): KCl 135, glucose 12, MgCl$_2$ 5, ATP Na$_2$ 5, EGTA 5 and HEPES 10 (pH adjusted to 7.3 by KOH). The CsCl micropipette solution contained the following ions (in mM): CsCl 135, glucose 12, MgCl$_2$ 5, ATP2Na 5, EGTA 5 and HEPES 10 (pH adjusted to 7.3 by CsOH).

Flufenamic acid (Wako pure Chemical, Osaka, Japan) was dissolved in a mixture of 10% dimethylsulfoxide (DMSO, Sigma, USA) and 0.1 N NaOH. The maximum final concentration of DMSO in the bath solution was 0.01%. Nifedipine, pinacidil, papaverine, and tetraethylammonium chloride were supplied by Sigma (USA).

**Statistics**

Data were expressed as the mean ± S.E.M., with the number of animals in parentheses. Statistical analysis was performed using the Student's t-test, and probabilities of less than 5% (P<0.05) were considered to be statistically significant.

**Results**

**Characteristics of active tone in arteries from SHRSP**

In Tyrode's solution, endothelium-removed preparations of the carotid artery isolated from SHRSP developed spontaneous tonic contraction or active tone. The mean amplitude of this active tone was 74.9 ± 5.6% (n=14) of the contraction produced by high-K$^+$ solution. The nominally Ca$^{2+}$-free Tyrode's solution caused a complete inhibition of the active tone. Re-administration of Tyrode's solution containing CaCl$_2$ 2 mM restored the tone. Papaverine 10$^{-4}$ M did not produce any further relaxation during the relaxation induced by Ca$^{2+}$-free solution. Application of 10$^{-6}$ M of nifedipine, a L-type Ca$^{2+}$-channel antagonist, or 10$^{-6}$ M pinacidil, an ATP-sensitive K$^+$-channel opener, also inhibited the active tone (data not shown).

Flufenamic acid (10$^{-6}$–10$^{-4}$ M) inhibited the active tone of the carotid artery strips in a concentration-dependent manner (Fig. 1). Decreases of the active tone by flufenamic acid at 10$^{-5}$ M and 10$^{-4}$ M were 36.7 ± 8.1% (n=17) and 97.6 ± 0.9% (n=15), respectively. Indomethacin 10$^{-5}$ M did not alter either the active tone or the inhibition of active tone by flufenamic acid 10$^{-5}$ M (35.6 ± 10.1%, n=6, P>0.05).

Flufenamic acid also inhibited the contraction induced by high-K$^+$ in the carotid artery strips in a concentration-dependent manner (Fig. 1). At 10$^{-4}$ M, flufenamic acid decreased the
Flufenamic acid on SHR carotid artery

![Diagram](image)

Fig. 1 The relationship between the concentration of flufenamic acid and its inhibition of both spontaneously active tone (open circles) and the contractile responses induced by high-K⁺ (filled circles) in endothelium-removed carotid artery strips isolated from stroke-prone spontaneously hypertensive rats (SHRSP). The amplitude of the tension in the presence of flufenamic acid was shown relative to that before application of flufenamic acid (%). Number of animals: 10-17 for data of spontaneously active tone and 9-14 for data of high-K⁺ induced contractions.

contractions induced by high-K⁺ by 65.0 ± 7.3% (n=15). Pinacidil 10⁻⁶ or 10⁻⁵ M did not inhibit the contractions induced by high-K⁺.

The effects of flufenamic acid on contractile proteins were examined in skinned circular muscle of the hepatic portal vein. In the circular muscle of the intact hepatic portal vein, flufenamic acid 10⁻⁴ M relaxed preparations which had been contracted with high-K⁺ solution (Fig. 2A). However, in the β-escin permeabilized preparations, flufenamic acid did not modulate the contraction produced by solutions containing either pCa 6 or pCa5 (Fig. 2B). The amplitude of contractions elicited by 10⁻⁴ M of flufenamic acid was 101.4 ± 2.5% (n=8) of the pCa 6-induced contraction and 98.8 ± 11.1% (n=4) of the pCa 5-induced contraction (P>0.05 for both).

**Effect of flufenamic acid on the membrane potential**

The resting membrane potential of smooth muscle cells in preparations of the endothelium-removed carotid artery of SHRSP was -32 ± 2.3 mV (n=18). In some preparations (6 out of 23), the membrane potential showed spontaneous oscillations with an amplitude of a few mV. Flufenamic acid 10⁻³ M hyperpolarized the membrane and inhibited this spontaneous oscillation of the membrane (Fig. 3A). Figure 3B shows the changes in membrane potential in the presence of different concentrations of flufenamic acid. Flufenamic acid hyperpolarized the membrane in a concentration-dependent manner. The ED₉₀ of the hyperpolarization induced by flufenamic acid was approximately 10⁻⁴ M.

Nifedipine (10⁻⁵ M) did not alter the resting membrane potential of smooth muscle cells in
preparations of the endothelium-removed carotid artery (control, $-36 \pm 1.2$ mV, n=7; in nifedipine, $-37.1 \pm 2.0$ mV, n=7; P>0.05).

**Effect of flufenamic acid on membrane currents**

In enzymatically isolated smooth muscle cells of the carotid artery, held in whole-cell patch clamp mode in PSS solution, voltage-dependent outward currents were recorded. The membrane capacitance ranged between 20 pF and 60 pF. Using high-K\(^+\) solution in the micropipette, depolarizing step pulses with duration of 500 msec evoked outward currents in a voltage-dependent way (Fig. 4A). The outward current was abolished by tetraethylammonium 20 mM (data not shown). Flufenamic acid enhanced the outward current elicited by depolarization (Fig. 4A). Ramp pulses of from $-120$ mV to $+80$ mV (300 msec in pulse duration, with $-60$ mV holding potential) evoked a voltage-dependent current with a reversal potential of approximately $-70$ to $-60$ mV (data not shown). The relationship between the amplitude of the outward current and the membrane potentials obtained from 6 preparations is summarized in Fig. 4B. Flufenamic acid ($10^{-4}$ and $10^{-3}$ M) markedly increased the outward current.

In some preparations, when currents were recorded with high-K\(^+\) solution in the micropipette, a small transient inward current preceded the outward current in response to the depolarizing pulse. When outward potassium currents were inhibited by adding cesium to the micropipette, inward currents were evoked with depolarizing step pulses of 300 msec duration at the holding potential (Vh) of $-60$ mV. The current amplitude peaked within 10 msec of the commencement of the depolarization steps and then decayed exponentially. The threshold depolarization for the inward current was around $-30$ mV, and the current amplitude peaked at around $+10$ mV. These inward currents were abolished in the presence of $10^{-6}$ M nifedipine.
Flufenamic acid on SHR carotid artery

Fig. 3  A: A typical trace of the membrane potential recorded from a smooth muscle cell in a preparation from the endothelium-removed carotid artery isolated from stroke prone spontaneously hypertensive rats. Flufenamic acid $10^{-3}$ M was applied at the arrow. B: Membrane potentials measured in smooth muscle cells in the endothelium-removed carotid artery of stroke prone spontaneously hypertensive rats in the presence of different concentrations of flufenamic acid ($10^{-4}$-$10^{-3}$ M). Control, n=21; in the presence of flufenamic acid, n=4-9.

(data not shown), indicating that they were carried through voltage-gated L-type Ca$^{2+}$-channels. Flufenamic acid ($3 \times 10^{-4}$ M) inhibited the voltage-gated Ca$^{2+}$-current (Fig. 5, A and B). The inhibitory actions of flufenamic acid on the Ca$^{2+}$-currents occurred in a concentration-dependent manner (Fig. 5C).

Discussion

Endothelium-removed preparations of large arteries from spontaneously hypertensive rats have been shown to develop spontaneous active tone (Noon et al., 1978; Sada et al., 1990). The
Fig. 4 Effects of flufenamic acid on outward currents elicited by depolarization of the membrane in isolated single smooth muscle cells of the carotid artery of stroke-prone spontaneously hypertensive rats. A: Outward currents were recorded by applying step pulses of 300 msec duration, from the holding potential of -60 mV to +10 mV, in the absence (control) and presence of 3 \times 10^{-4} \text{M} flufenamic acid. B: The I-V relationships of outward currents evoked by depolarizing step pulses (Vh=-60 mV) in isolated smooth muscle cells of carotid artery strips from stroke-prone spontaneously hypertensive rats, in the absence (control) and presence of flufenamic acid 10^{-3} \text{M} and 10^{-4} \text{M}. Symbols with error bars indicate the mean ± S.E.M. (n=6). Amplitudes of the outward current were expressed with current density, which was calculated by dividing current amplitudes by the cell capacitance.

Present experiments indicated that the tone is sensitive to either extracellular Ca^{2+} or to Ca^{2+} antagonists. These results suggest that the opening of voltage-gated L-type Ca^{2+} channels contributes to the generation of active tone. In the present experiments with carotid artery
Fig. 5 Effects of flufenamic acid on inward currents measured in enzymatically isolated smooth muscle cells of carotid artery strips from stroke-prone spontaneously hypertensive rats. A: I-V relationship of the inward currents measured in the absence (control) and presence of flufenamic acid $3 \times 10^{-4}$ M (Fluf). The currents were evoked by depolarizing step pulses of 300 msec duration from the holding potential of $-60$ mV to $+20$ mV, and the peak amplitudes were measured. Error bars indicate the mean ± S.E.M. (n=22 for control, n=4 for fluf). B: Inward current traces evoked by depolarizing pulses from $-60$ mV to $+20$ mV, in the absence (control) and presence of flufenamic acid $3 \times 10^{-4}$ M. The recording micropipette contained high Cs. C: The concentration-response relationship of the effects of flufenamic acid on the peak amplitude of the inward currents evoked by depolarizing pulses from $-60$ mV to $+10$ mV or $+20$ mV. Symbols with error bars indicate the mean ± S.E.M. (n=4–8).
preparations from SHRSP, the resting membrane potential of smooth muscle cells was approximately ~30 mV, which is less negative than that of arterial smooth muscle cells in other mammals (Kuriyama et al., 1995). Although our patch clamp experiments did not detect inward currents at the membrane potential of ~30 mV, pharmacological evidence provided by the use of a Ca\(^{2+}\) antagonist and a ATP-sensitive K' channel opener, showed that Ca\(^{2+}\) may flow into cells through voltage-gated L-type Ca\(^{2+}\) channels. Thus, it is reasonable to consider that the active tone is produced by a spontaneous increase in Ca\(^{2+}\) influx through voltage-gated channels.

Studies of the response of arterial smooth muscle to chemicals that inhibit spontaneous active tone may help to elucidate the mechanisms involved in its development. They would also facilitate the development of a new therapeutic strategy for hypertension. Nonsteroidal anti-inflammatory drugs, that are derivatives of diphenylamine-2-carboxylic acid, have been shown to block a variety of ion channels, such as nonselective cation channels (Chen et al., 1993; Hescheler and Schultz, 1993) and calcium-activated chloride channels (Greenwood and Large, 1995). These chemicals activate potassium currents in jejunal smooth muscle cells (Farrugia et al., 1993a; 1993b) and release Ca\(^{2+}\) from internal stores in mouse mandibular cell lines (Poronnik et al., 1992). It has been reported that flufenamic acid potentiates large conductance Ca\(^{2+}\)-activated potassium channels (Ottolia and Toro, 1994). The potassium currents recorded in the present study were similar to those reported previously in hypertensive rats (Liu et al., 1995). Although we did not have enough evidence to determine the channel type involved in the outward currents, Ca\(^{2+}\)-activated potassium channels present in blood vessels of SHRSP may be the site of action of flufenamic acid.

Flufenamic acid has been reported to inhibit L-type Ca\(^{2+}\)-channel in both cardiomyocytes (Conforti et al., 1994) and the rat basilar artery (Dougherty et al., 1998). In the present study on carotid artery, high-K' induced contractions were inhibited by flufenamic acid. The present experiments also indicated that the nifedipine-sensitive inward Ca\(^{2+}\) currents were recorded when the outward current was inhibited by cesium in the micropipette. The current amplitude achieved its peak within 10 msec of the depolarization step and decayed exponentially as reported in other arterial smooth muscle cells in hypertensive rats (Ohya et al., 1993). Flufenamic acid inhibited the L-type Ca\(^{2+}\) currents in a concentration-dependent manner. The effects of flufenamic acid on contractile proteins were unlikely to have contributed to the inhibition of contractions produced by the high-K solution, because flufenamic acid did not change the contraction of permeabilized circular smooth muscle cells of the hepatic portal vein.

Comparison of the inhibitory actions of flufenamic acid indicated that the effects on active tone appeared at lower concentrations than those on high-K' induced contraction (Fig. 1). This suggests that, at the resting membrane potential level, unidentified mechanisms may be contributing to the inhibition of active tone by flufenamic acid, in addition to the inhibition of voltage-gated L-type Ca\(^{2+}\) channels. Although we did not examine the effects of flufenamic acid on the membrane potential in the presence of K'-channel blockers, it is speculated that, in the presence of flufenamic acid, the increased activity of K'-channel which induces hyperpolarization of the smooth muscle membrane may close the voltage-gated Ca\(^{2+}\) channels and inhibit Ca\(^{2+}\) influx. This will finally diminish the spontaneous active tension. The reversal potential for the current evoked by ramp pulse was distributed at approximately ~60 mV. The
present results indicate that the Ca\textsuperscript{2+} channels were active at a potential close to the resting membrane potential of approximately –30 mV.

Abnormalities in the membrane mechanisms during arterial hypertension have been reviewed (Sunano et al., 2000). In pulmonary hypertension of the rat, active tone in the pulmonary artery and elevated chloride channel activity have been reported in smooth muscle cells (Nakazawa et al., 2001). Flufenamic acid has been shown to inhibit Ca\textsuperscript{2+}-activated chloride current (Greenwood and Large, 1995) and non-selective cationic current (Chen et al., 1993; Hescheler and Schultz, 1993). The possible contribution of an effect of flufenamic acid on membrane hyperpolarization needs to be further examined.

In conclusion, flufenamic acid inhibits the active tone of smooth muscle in the carotid artery of SHRSP by inhibiting Ca\textsuperscript{2+} influx, by direct inhibition of the voltage-gated Ca\textsuperscript{2+}-channels and indirectly through hyperpolarization of the membrane due to activation of K\textsuperscript{+}-channels.

Acknowledgement

The authors are grateful to T. Tsukahara for assistance in computer system. This work was supported in part by a Grant-in-Aid for High Technology Research Programs from the Ministry of Education, Science, Sports and Culture of Japan.

References


(Received April 7, 2002: Accepted April 14, 2002)