

## Full Paper

Facilitation of L-type  $\text{Ca}^{2+}$  Currents by Fluid Flow in Rabbit Cerebral Artery MyocytesSatoshi Amano<sup>1</sup>, Tomohisa Ishikawa<sup>1,\*</sup>, and Koichi Nakayama<sup>1</sup><sup>1</sup>Department of Cellular and Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka City, Shizuoka 422-8526, Japan

Received April 18, 2005; Accepted June 16, 2005

**Abstract.** Blood vessels are receptive to hemodynamic forces, such as blood pressure and flow, which result in myogenic responses. The present study aimed to investigate the effect of mechanical stresses on L-type voltage-dependent  $\text{Ca}^{2+}$  channels in rabbit cerebral artery myocytes. Cell swelling induced by the exposure to a 16% hypotonic solution increased peak values of whole-cell  $\text{Ba}^{2+}$  currents ( $I_{\text{Ba}}$ ). Similarly, an elevation of bath perfusion rate increased peak values of  $I_{\text{Ba}}$ . However, the response was reduced by the continued fluid flow stimulation and the current amplitude almost returned to the baseline. This reduction of the current was abolished by pretreatment with thapsigargin, implying the contribution of  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum to the response. These results suggest that L-type  $\text{Ca}^{2+}$  currents are facilitated not only by cell swelling but also by fluid flow in cerebral artery myocytes.

**Keywords:** L-type voltage-dependent  $\text{Ca}^{2+}$  channel, rabbit cerebral artery, patch clamp, swelling, flow

## Introduction

Blood vessels constantly receive hemodynamic stresses such as those due to blood pressure and blood flow. It is well established that arteries contract in response to such mechanical stresses (1). Cerebral arteries are particularly sensitive to mechanical stresses, which initiate a myogenic contraction that plays a pivotal role in maintaining constant cerebrovascular circulation (2–5). Myogenic contraction is considered to be produced, at least in part, by the activation of voltage-dependent  $\text{Ca}^{2+}$  channels (VDCC) following membrane depolarization (6). The membrane depolarization seems to be produced by the activation of volume-sensitive  $\text{Cl}^-$  channels and nonselective cation channels, which are sensitive to mechanical stimuli (6). Recently, we proposed the following mechanism for hypotonically induced contraction: Osmotic cell swelling first activates  $\text{Ca}^{2+}$ -permeable cation channels, through which  $\text{Ca}^{2+}$  influx activates  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels ( $\text{Cl}_{\text{Ca}}$  channels). The  $\text{Cl}^-$  efflux through  $\text{Cl}_{\text{Ca}}$  channels results in membrane depolarization, thereby activating L-type

VDCC and causing contraction (7). In addition, several reports have shown that the currents through L-type VDCC are potentiated by mechanical stimuli such as osmotic cell swelling and longitudinal stretch (1, 8–10).

It has also been shown that intraluminal flow causes contraction of endothelium-denuded arterial preparations, which is accompanied with membrane depolarization,  $\text{Ca}^{2+}$  influx through L-type VDCC, or  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR) in smooth muscle cells (11–14). However, the mechanisms responsible for flow-induced contraction have not been defined. In particular, few studies have investigated the sensitivity of L-type VDCC to fluid flow stimulation in vascular smooth muscle cells.

In the present study, we investigated the effects of two different types of mechanical stimulations, that is, hyposmotic cell swelling and fluid flow, on L-type VDCC of rabbit cerebral artery myocytes, and we found that the VDCC is sensitive to these stimulations.

## Materials and Methods

## Cell isolation

Japanese white rabbits of either sex weighing 2.5–

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3.5 kg were housed in separate cages in a temperature-controlled room and treated as approved by the Institutional Animal Care and Use Committee of the University of Shizuoka and the Guidelines of Animal Experiments established by The Japanese Pharmacological Society. The rabbits were anesthetized with an intravenous injection of sodium pentobarbital (50 mg/kg) and exsanguinated by bleeding from the carotid arteries. The basilar, middle cerebral, and posterior cerebral arteries were carefully dissected and placed in cold Krebs-Henseleit solution containing 113 mM NaCl, 4.7 mM KCl, 2.6 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , and 11.1 mM glucose, aerated with 95%  $\text{O}_2$  – 5%  $\text{CO}_2$ .

Single smooth muscle cells were enzymatically isolated from the cerebral arteries as described previously (15, 16). Briefly, the dissected cerebral arteries were cut into small segments (about 1 mm in length) and placed in  $\text{Ca}^{2+}$ -free Hank's solution (125 mM NaCl, 5.4 mM KCl, 0.44 mM  $\text{KH}_2\text{PO}_4$ , 15.5 mM  $\text{NaHCO}_3$ , 0.34 mM  $\text{Na}_2\text{HPO}_4$ , 10 mM glucose, and 2.9 mM sucrose, adjusted to pH 7.4 with HCl) aerated with 95%  $\text{O}_2$  – 5%  $\text{CO}_2$  for 120 min at 37°C. The segments were then placed in the  $\text{Ca}^{2+}$ -free Hank's solution containing 1 mg/ml collagenase (type IA; Sigma, St. Louis, MO, USA), 4 mg/ml bovine serum albumin (BSA), 2 mg/ml trypsin inhibitor (type I-S, Sigma), 0.1 mg/ml protease (type XIV, Sigma), and 0.2 mg/ml ATP-Mg and incubated for about 20 min with gentle agitation at 37°C. After completion of the digestion, the segments were washed three times in the  $\text{Ca}^{2+}$ -free Hanks' solution and transferred in Kraft-Brühe (K-B) solution (110 mM KOH, 70 mM glutamic acid, 10 mM taurine, 25 mM KCl, 10 mM  $\text{KH}_2\text{PO}_4$ , 5 mM HEPES, 0.5 mM EGTA, and 11 mM glucose, adjusted to pH 7.4 with HCl). Single cells were dispersed by gentle agitation with a wide-pore glass pipette in the K-B solution containing 0.5 mg/ml trypsin inhibitor and 1 mg/ml BSA. The dispersed single cells were stored at 4°C and used within 6 h.

#### *Patch-clamp recordings*

The dispersed cells were placed in a chamber (7 mm × 22 mm, about 0.5 ml in volume) on the stage of an inverted microscope, and superfused with the bath solution [10 mM  $\text{BaCl}_2$ , 50 mM NaCl, 10 mM tetraethylammonium chloride (TEA-Cl, Sigma), 3 mM 4-aminopyridine (4-AP, Sigma), 5 mM HEPES, 5.5 mM glucose, 150 mM mannitol, adjusted to pH 7.4 with HCl]. Whole-cell currents were recorded by the amphotericin B-perforated patch-clamp technique as described previously (15, 16). Patch electrodes had a resistance of 1 to 2 M $\Omega$  when filled with the pipette

solution (40 mM CsCl, 100 mM Cs aspartate, 10 mM TEA-Cl, 10 mM HEPES, adjusted to pH 7.2 with CsOH) containing 270  $\mu\text{g/ml}$  of amphotericin B (Sigma). Current recordings were started after an access resistance of 5 – 15 M $\Omega$  was achieved. Whole-cell currents were amplified, digitized, and processed using an Axopatch 1-D amplifier, a TL-1 DMA interface, and pCLAMP 6.0 software (Axon Instruments, Foster City, CA, USA). Data were filtered at 1 kHz, digitized at 2 – 10 kHz, and stored in the computer for subsequent analyses. The experiments were performed at room temperature; the temperature in the room was kept at  $24 \pm 1^\circ\text{C}$ , and the temperature of the bath solution ranged from 20 to 22°C. Membrane capacitance of rabbit cerebral artery myocytes, which was simply estimated from capacitive currents, varied widely from 13.1 to 30.9 pF ( $24.5 \pm 2.0$  pF,  $n = 14$ ). A 16% hypo-osmotic challenge was applied by replacing the superfusing bath solution to the hypotonic solution composed of 10 mM  $\text{BaCl}_2$ , 50 mM NaCl, 10 mM TEA-Cl, 3 mM 4-AP, 5 mM HEPES, 5.5 mM glucose, and 100 mM mannitol (adjusted to pH 7.4 with HCl). The flow rate was regulated by a peristaltic pump (Gilson Minipuls 3, Villiers le Bel, France).

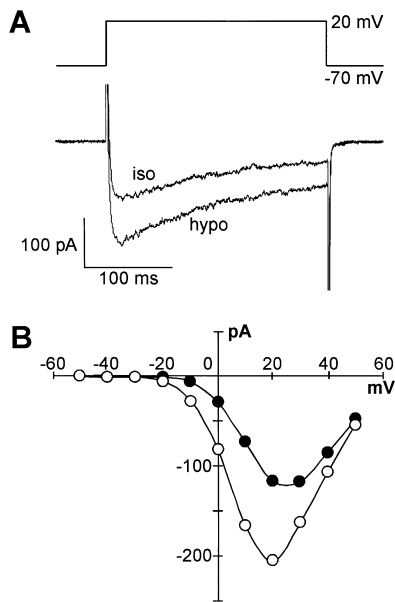
#### *Statistics*

Data are expressed as the mean  $\pm$  S.E.M., and  $n$  indicates the number of cells tested. The effects of treatment were analyzed with the paired Student's  $t$ -test or analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A probability of  $P < 0.05$  was accepted as the level of statistical significance.

## **Results**

#### *Effect of osmotic cell swelling on $I_{\text{Ba}}$*

As shown in Fig. 1A, the inward currents were elicited by 250-ms step pulses to 0 mV from a holding potential of  $-70$  mV and did not exhibit the run-down phenomenon for more than 1 h. Figure 1B shows the current-voltage relationships for the inward currents; the threshold potential for activation was about  $-30$  mV, the maximum current was obtained at  $+10$  or  $+20$  mV, and the reversal potential was about  $+60$  mV. The inward currents were nearly abolished by nicardipine (0.1  $\mu\text{M}$ , data not shown). These results suggest that the inward currents recorded in this study were  $\text{Ba}^{2+}$  currents ( $I_{\text{Ba}}$ ) mostly carried by L-type VDCC. A 16% hypo-osmotic challenge (314 to 264 mOsm/L) significantly increased peak values of  $I_{\text{Ba}}$  elicited by depolarizing steps to 0 mV to  $174 \pm 11\%$  ( $n = 4$ ) of the control at a constant perfusion rate of 0.5 ml/min (Fig. 1A). The facilitation of  $I_{\text{Ba}}$  by the hypotonicity was observed over



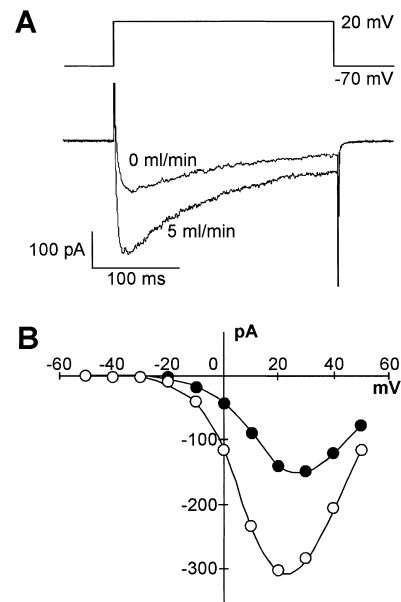
**Fig. 1.** Effect of hyposmotic challenge on whole-cell  $\text{Ba}^{2+}$  currents ( $I_{\text{Ba}}$ ) in rabbit cerebral artery myocytes. A: typical tracings of  $I_{\text{Ba}}$  elicited by voltage steps to 20 mV from a holding potential of  $-70$  mV in isotonic (iso) and hypotonic (hypo) solutions. B: current-voltage relationship for the peak  $I_{\text{Ba}}$  in isotonic (closed circle) and hypotonic (open circle) solutions in the cell shown in panel A. The cells were perfused with the bath solution at a rate of around 0.5 ml/min.

a range of test potentials (Fig. 1B) and was maintained for at least 10 min.

#### Effect of fluid flow on $I_{\text{Ba}}$

We next investigated the influence of fluid flow on the  $I_{\text{Ba}}$  of rabbit cerebral artery myocytes. The control  $I_{\text{Ba}}$  elicited by depolarizing step pulses to 0 mV was recorded without fluid flow. When the cells were perfused with the bath solution at a rate of 5 ml/min, the amplitude of  $I_{\text{Ba}}$  increased (Fig. 2A). The fluid flow stimulation did not shift the current-voltage relationship for  $I_{\text{Ba}}$  along the voltage axis (Fig. 2B). The facilitation of  $I_{\text{Ba}}$  by fluid flow was dependent on flow rate (Fig. 3C).

Interestingly, continued fluid flow stimulation at 3 ml/min caused a decline of  $I_{\text{Ba}}$  (Fig. 3: A and C). This decline of  $I_{\text{Ba}}$  seemed to be induced by the flow stimulation since the amplitude of  $I_{\text{Ba}}$  was increased when the fluid flow was terminated and was again decreased when the fluid flow was returned to 3 ml/min (Fig. 3A). It has been shown that flow stimulation induces the release of  $\text{Ca}^{2+}$  from the SR (13, 14) and that L-type VDCC is inactivated when cytosolic  $\text{Ca}^{2+}$  concentration is elevated (17). Thus, we tested the effect of pretreatment with the SR  $\text{Ca}^{2+}$ -ATPase inhibitor thapsigargin, which depletes SR  $\text{Ca}^{2+}$  stores. The pretreatment with thapsigargin (1  $\mu\text{M}$ ) for 15 min abolished



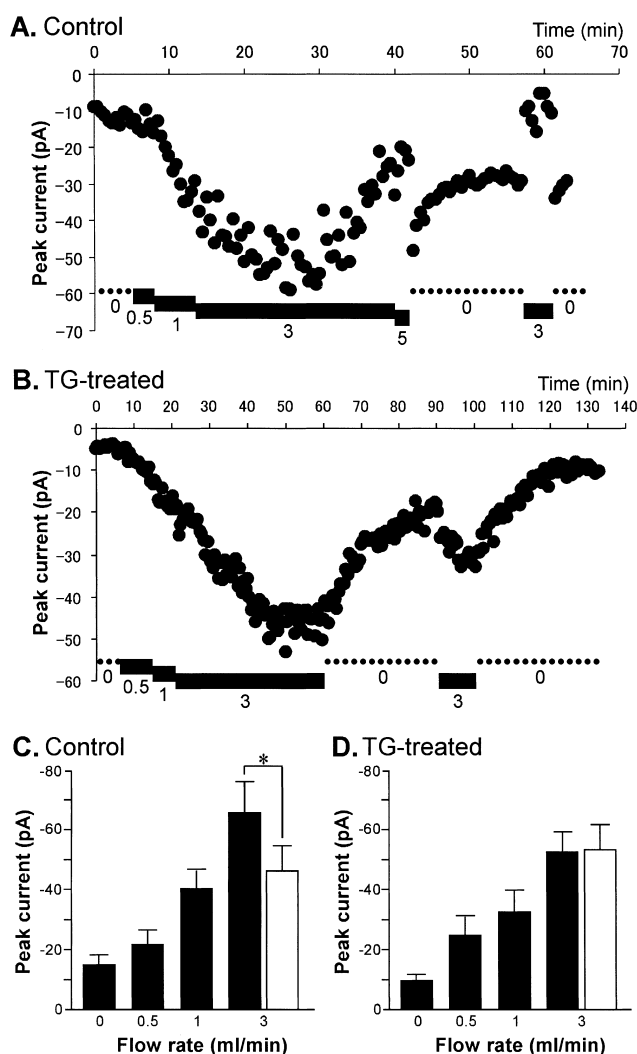
**Fig. 2.** Effect of fluid flow stimulation on whole-cell  $I_{\text{Ba}}$  in rabbit cerebral artery myocytes. A: typical tracings of  $I_{\text{Ba}}$  elicited by voltage steps to 20 mV from a holding potential of  $-70$  mV at flow rates of 0 and 5 ml/min. B: current-voltage relationship for the peak  $I_{\text{Ba}}$  at flow rates of 0 (closed circle) and 5 ml/min (open circle) in the cell shown in panel A.

the decline of  $I_{\text{Ba}}$  induced by the continued fluid flow stimulation at 3 ml/min (Fig. 3: B and D).

#### Discussion

Several studies have reported the enhancement of L-type  $\text{Ca}^{2+}$  currents by mechanical stimuli such as hyposmotic cell swelling and longitudinal stretch in vascular smooth muscle cells (1, 8–10). The present study showed that L-type VDCC of rabbit cerebral artery myocytes is activated not only by hyposmotic cell swelling but also by fluid flow stimulation. This is, to our knowledge, the first report demonstrating the flow dependency of L-type VDCC activity in vascular smooth muscle cells.

In endothelium-intact arteries, an increase in fluid flow induces the release of vasodilator substances from endothelial cells, which relax smooth muscle cells and results in vasodilatation (18–20). In contrast, intraluminal flow has also been shown to produce contraction of endothelium-denuded arteries, which is accompanied with an elevation of intracellular  $\text{Ca}^{2+}$  concentration (11–14). Thus, not only endothelial cells but also vascular smooth muscle cells are sensitive to changes in fluid flow. Since the endothelial layer shields the underlying smooth muscle layer from blood flow,



**Fig. 3.** Time-course of fluid flow-induced changes in whole-cell  $I_{Ba}$  in rabbit cerebral artery myocytes. A, B: changes in peak  $I_{Ba}$  elicited by voltage steps to 0 mV from a holding potential of  $-70$  mV in the control cell (A) and in the cell pretreated with  $1 \mu\text{M}$  thapsigargin (TG) for at least 15 min (B). The currents were recorded every 30 s. The cell was superfused at flow rates of 0.5, 1, 3, and 5 ml/min as indicated by the labeled bars. Dotted line shows a flow rate of 0 ml/min. C, D: Summary of flow-dependent increases in the peak  $I_{Ba}$  in control (C) and TG ( $1 \mu\text{M}$ )-treated cells (D). Filled columns show the maximum responses to each flow rate. Open columns show the amplitude of the peak  $I_{Ba}$  10 min after the maximum response to the flow rate of 3 ml/min was obtained. Data show the mean  $\pm$  S.E.M. of 4 cells from different preparations.  $*P < 0.05$ .

smooth muscle cells are not normally exposed to fluid mechanical shear stress associated with blood flow. However, in the case of endothelial injury and denudation, as may occur at sites of vessel repair by angioplasty, at the anastomoses of vascular grafts, and in other types of cardiovascular interventions, superficial smooth muscle cells would be exposed to flowing blood. It is

also possible that internal elastic lamina has fenestral pores, through which superficial smooth muscle cells are exposed to sufficient interstitial flow under physiological conditions (21). Accordingly, blood flow could be regarded as one of the mechanical stresses that blood vessels are subjected to. The present study clearly demonstrated that L-type VDCC currents are augmented by fluid flow in cerebral artery myocytes. This is in accordance with the studies showing that L-type VDCC in human intestinal smooth muscle cells is sensitive to bath perfusion (22, 23). Thus, it is likely that facilitation of L-type VDCC currents by fluid flow stimulation is one of the mechanisms involved in flow-induced vessel contraction.

In response to fluid flow stimulation, the amplitude of L-type  $\text{Ca}^{2+}$  currents first increased, and then decreased despite of the continuous stimulation. The second descending response seems to be mediated by the release of  $\text{Ca}^{2+}$  from the SR, since the decline was abolished by the pretreatment with thapsigargin, which is an SR  $\text{Ca}^{2+}$ -ATPase inhibitor and depletes SR  $\text{Ca}^{2+}$  stores. In the present study, since the bath solution did not contain  $\text{Ca}^{2+}$ , we could rule out the possibility that  $\text{Ca}^{2+}$  influx through store-operated  $\text{Ca}^{2+}$  channels or  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release is involved in the response. Mechanical stimulations have been shown to activate phospholipase C (PLC) accompanied with the production of inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), which induces  $\text{Ca}^{2+}$  release from the SR (6). L-type VDCC is well known to be inactivated when cytosolic  $\text{Ca}^{2+}$  concentration is elevated (17). It is therefore likely that fluid flow stimulation induces  $\text{Ca}^{2+}$  release from the SR by activating PLC signal cascade, which in turn inactivates L-type VDCC. Interestingly, the second descending phase was not observed in the response to hyposmotic stimulation. Our previous study in canine basilar arteries has shown that thapsigargin does not affect the contraction induced by a hyposmotic challenge (7). Thus, the mechanical stress induced by fluid flow seems to be different from that induced by hyposmotic cell swelling; the former causes  $\text{Ca}^{2+}$  release from the SR, whereas the latter does not.

Myogenic contraction in response to mechanical stimuli such as an elevation of intraluminal pressure plays a pivotal role for the autoregulation of cerebral resistance and blood flow. The following chain of events is now considered to be a principal mechanism for contractions (6): Mechanical stimuli first activate stretch-activated cation channels producing membrane depolarization. The depolarization then activates L-type VDCC facilitating  $\text{Ca}^{2+}$  influx. Subsequently, activation of myosin light chain kinase by  $\text{Ca}^{2+}$  augments myosin light chain phosphorylation, resulting in vascular

contraction. Moreover, blockade of L-type VDCC with dihydropyridine  $\text{Ca}^{2+}$  channel blockers eliminate or dramatically attenuate myogenic contraction (3, 24, 25). Thus, L-type VDCC seems to be essential for myogenic contraction. Consistent with our previous study in canine basilar arteries, the present study demonstrated that hyposmotic cell swelling facilitates L-type  $\text{Ca}^{2+}$  currents of rabbit cerebral arteries. Thus, L-type VDCC is likely to be modulated by the mechanical stretching of the membrane resulting from cell swelling. This idea is supported by the reports showing that the currents through L-type VDCC of vascular smooth muscle cells are potentiated by longitudinal stretch (1) and by application of positive pressure to the patch pipette (10). As to the question about whether hyposmotic cell swelling mimics mechanical stretch under physiological conditions, we have recently shown that a hyposmotic challenge evokes contraction of canine basilar arteries via mechanisms similar to those for myogenic contraction (7).

In summary, the results presented suggest that L-type  $\text{Ca}^{2+}$  currents are facilitated not only by cell swelling but also by fluid flow in cerebral artery myocytes. In addition, we found that the response to fluid flow was transient and the current amplitude almost returned to the baseline despite of the continued fluid flow stimulation. This decline of the current seems to result from the inactivation of L-type VDCC by  $\text{Ca}^{2+}$  released from the SR.

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