L-Arginine-induced current in portal venous smooth muscle cells

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Abstract

In our previous report, we showed that L-arginine induced depolarization of smooth muscle cells of the rat portal vein with an increased contraction. To clarify the ionic mechanism of the membrane depolarization, the effect of L-arginine on the holding current was studied in freshly isolated smooth muscle cells of the rat portal vein. The whole-cell patch-clamp technique was used, with the membrane potential held at -60 mV. In the presence of Na+ in the perfusate, L-arginine 10 mM induced an inward current in about 50% of the cells. In Na+-deficient perfusate, L-arginine 10 mM increased the amplitude of the inward current in a Na+ concentration-dependent manner. BCH, an inhibitor of the Na+-dependent amino acid transporter, ceased the L-arginine-induced current. These results indicate that L-arginine induces an inward current via Na+-dependent mechanisms in rat portal venous smooth muscle cells.

Key words: rat, portal vein, smooth muscle, current, L-arginine, Na+

Introduction

L-Arginine is important not only as a substrate for nitric oxide (NO) (Moncada et al., 1989), but it has also been shown to affect the activity of excitatory cells in a NO-independent manner. For example, in the rat hypothalamus, L-arginine was released by afferent stimulation (Do et al., 1994). L-Arginine as well as N6-monomethyl-L-arginine, N6-nitro-L-arginine and Nω-monomethyl-L-arginine increased intracellular calcium of β-cells in the mouse pancreas with membrane depolarization (Smith et al., 1997). In anesthetized cats, L-arginine enhanced the excitability of spinal cord dorsal horn cells when NO synthesis was inhibited (Wilson et al., 2000). L-Arginine
was found to induce a transient contraction in the circular muscle of the rat portal vein when NO
synthesis was inhibited (Shimamura et al., 2000). L-Arginine also increased the spontaneous
contraction frequency associated with membrane depolarization and resulted in increased action
potential bursts in longitudinal smooth muscle cells of the rat portal vein (Shimamura et al.,
2003).

L-Arginine has been shown to be transported into cells via transporters. L-Arginine was
reported to be transported by system y\(^{-}\) and system B\(^{0+}\) into porcine pulmonary arterial
endothelial cells (Greene et al., 1993), and by system B\(^{0+}\) in Xenopus oocytes (Mackenzie et al.,
1994). Arginine was transported into human B\(^{0+}\)-expressed Xenopus oocytes Na\(^{+}\)/Cl\(^{-}\)-
dependently (Sloan and Mager, 1999). Arginine was transported by system y\(^{-}\) and system B\(^{0+}\) in
rat aortic smooth muscle cells (Escobales et al., 2000). The positive charge of L-arginine may
depolarize the membrane and increase both electrical and mechanical activity of smooth muscle
cells. However, little information is available on the mechanism of the excitatory effect of L-
arginine in portal venous smooth muscle cells. In the present study, we examined the ionic
mechanism of the L-arginine-induced inward current in freshly isolated smooth muscle cells of
the rat portal vein.

**Materials and methods**

Animals used were Wistar rats of either sex at ages of between 4–5 weeks old. They were
anesthetized with CO\(_2\) and treated according to the Guiding principles for the care and use of
laboratory animals approved by the Japanese Pharmacological Society. The hepatic portal vein
was isolated from each animal and dissected under a binocular in nominally Ca\(^{2+}\)-free
physiological salt solution (Ca\(^{2+}\)-free PSS).

Preparations were incubated at 37°C for 90 min in Ca\(^{2+}\)-free PSS containing 2.5 mg/ml
collagenase (type I, Wako Pure Chemical, Osaka Japan). The digested tissue was cut into
pieces and tritiated in Ca\(^{2+}\)-free PSS and stored at 4°C until used.

Whole-cell patch-clamp recording (Hamill et al., 1981) was performed using standard whole
cell recording or perforated patch method with nystatin 250 \(\mu\)g/ml in the pipette (Horn and
Marty, 1988). Currents were recorded with a patch clamp amplifier (Axopatch 200B, Axon
Instruments, Foster City, CA, USA) filtered at 1 kHz and digitized with an A/D, D/A converter
(DigiData 1200, Axon Instruments) with pClamp v.6.0 programs (Axon Instruments). Cell
capacitance was measured by a small ramp pulse (10 mV hyperpolarization in 10 msec) and
current amplitude was expressed as current density. Cells were held at -60 mV and a holding
current was observed before and during the application of L-arginine-HCl or L-arginine. The
current was recorded on a thermal array recorder (Graphtec Handyocorder HC7100, Tokyo, Japan)
and on a video tape through a pulse code modulator (PCM-501ES, Sony, Tokyo, Japan) which was
modified to give a frequency response from DC to 20 kHz. In several cells, a ramp pulse from
-160 to +40 mV was applied for 300 msec. All experiments were carried out at room temperature.

Current was recorded in nominally Ca\(^{2+}\)-free condition to prevent cellular contraction by L-
arginine. Composition of the Ca\(^{2+}\)-free PSS was the following (in mM): 140 NaCl, 5.4 KCl, 1.2
MgCl\(_2\), 12 glucose, 10 HEPES (pH 7.37 titrated by NaOH). In experiments for Na\(^{+}\) deficient
perfusate, part of the Na⁺ was replaced with an equimolar concentration of tetrathylenammonium⁺ (TEA⁺). In experiments with system B0⁺ inhibitor, Cl⁻ in the perfusate was replaced with aspartate⁻. In the experiment using 30 mM L-arginine, Na⁺ in the bath solution was decreased to 110 mM to avoid hypertonicity. When Cl⁻ in the bath solution was replaced with aspartate⁻, agar/AgCl was used in the reference electrode. Cs⁺-containing pipette solution was used to minimize the outward current. The composition of the pipette solution was 135 CsCl, 5 EGTA, 5 Na₂ATP, 5 MgCl₂, 10 HEPES, 12 glucose unless otherwise stated.

L-Arginine·HCl and nystatin were obtained from Wako Pure Chemical (Osaka, Japan). EGTA was from Cica (Tokyo, Japan). 2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) and all other drugs are from Sigma (St. Leuoi, MO, USA). In the experiments for BCH, L-arginine was used instead of L-arginine·HCl, to avoid the contamination of Cl⁻ in perfusate.

Results are expressed as mean ± S.E.M. with the number of cells in parenthesis.

Statistical analysis was performed using Statview software (Abacus Concepts Inc., Berkeley, CA, USA), considering values of P<0.05 to be statistically significant. Curve fitting was performed using Prizm software (GraphPad Software Inc., San Diego, CA, USA).

Results

Isolated cells did not have spines or branches and had rather a smooth spindle to round shape with a membrane capacitance of 12–40 pF, similarly to that previously reported (Loirand et al., 1989). When the cell was voltage clamped with a perforated patch at −60 mV and a stable holding current recorded, 10 mM L-arginine·HCl induced a sustained inward current with an amplitude of 0.33 ± 0.05 pA/pF in 11 of the 18 cells recorded (Fig. 1). L-Arginine·HCl at a concentration of 10 mM did not induce obvious changes in the holding current of remaining 7 cells.

When the concentration-response of L-arginine·HCl on the holding current was examined, the probability of current induction was 33 to 67% at each concentration with no tendency for there to be any concentration-dependence of the inward current between 0.1 and 30 mM L-arginine. The relationship between L-arginine concentration and the inward current density (data obtained from the cells with no L-arginine-induced current was excluded from the calculation) is shown in Fig. 2. L-Arginine at concentrations higher than 0.1 mM induced an inward current, and the current density increased in an L-arginine-concentration-dependent manner to reach a maximum (0.3 pA/pF) at around 10 mM. The EC₅₀ of L-arginine was estimated to be 0.55 mM.

Dependency of the L-arginine-induced inward current on the extracellular Na⁺ concentration was examined by replacing part of the Na⁺ in the perfusate with TEA⁺ and with 140 mM CsCl in the pipette solution in nystatin patch whole-cell recording. L-Arginine·HCl at a concentration of 10 mM induced an inward current in 11 of the 18 recorded cells and the probability of an inward current induction was not dependent on the Na⁺ concentration. The relationship of Na⁺ concentration with inward current density (data obtained from the cells with no L-arginine-induced current was excluded from the calculation) is shown in Fig. 3. The concentration-response (current density) curve was fitted with a Hill equation with an EC₅₀ of 5.1 mM.
Fig. 1. A typical trace showing the L-arginine (10 mM) induced inward current in smooth muscle cells of the rat portal vein. The cell membrane was held at -60 mV. The whole cell clamp mode with nystatin-perforated patch method was used. Vertical lines were induced by ramp pulses.

Fig. 2. Relationship between L-arginine concentration and the amplitude of the inward current in rat portal vein myocytes. Recording conditions are the same as in Fig. 1. The amplitude of the L-arginine-induced current was normalized by cell capacitance. Each point indicates data from 2 to 5 cells. Data points are fitted with Hill’s equation.

To confirm the contribution of extracellular Na⁺ to the inward current, the 10 mM L-arginine-induced current was investigated with a standard whole-cell patch-clamp. With 145 mM TEA-aspartate in the bath and 140 mM Cs-aspartate in the pipette, L-arginine-HCl did not induce an inward current in any of the cells examined (13 cells). Similarly with 145 mM TEA-aspartate in the bath and 140 mM CsCl in the pipette, L-arginine induced no inward current in any of the cells examined (13 cells).

To examine the role of intracellular Cl⁻ in the inward current, the 10 mM L-arginine-induced current was investigated with the standard whole-cell patch-clamp. With 145 mM Na-aspartate in the bath and 140 mM Cs-aspartate in the pipette, L-arginine induced an inward current with an amplitude of 0.41 ± 0.06 pA/pF in 5 of the 9 cells recorded. In the remaining 4 cells, no current was induced. Similarly, with 145 mM Na-aspartate in the bath and 140 CsCl in the pipette, L-arginine induced an inward current with an amplitude of 0.30 ± 0.06 pA/pF in 5 of the 8 cells recorded. In the remaining 3 cells, no current was induced. These results indicate that extracellular Na⁺, but not intracellular Cl⁻, plays a significant role in the L-arginine-induced
Fig. 3. Effects of Na⁺ concentration of the perfusate on the amplitude of the L-arginine (10 mM)-induced inward current in rat portal vein myocytes. The NaCl in the perfusate was replaced with an equimolar concentration of tetraethylammonium-chloride. The pipette solution was 140 mM CsCl with a nystatin perforated patch. Current amplitude is normalized by cell capacitance. Each point indicates data from 2 to 5 cells. The curve is fitted with Hill's equation.

Fig. 4. Effects of BCH, an inhibitor of system B⁰⁺⁺, on the L-arginine-induced current. Typical traces of two cells are demonstrated. A: Control, L-arginine (1 mM) was applied twice with an interval of 5 min. In both cases, inward currents were induced by L-arginine (Aa & Ab). B: Effect of BCH, L-arginine (1 mM) was applied twice with an interval of 5 min. After L-arginine (1 mM) induced the inward current (Ba), the cell was treated with BCH (10 mM) for 5 min. L-Arginine failed to induce an inward current in that cell (Bb).

inward current.

The effect of BCH, a known inhibitor of the B⁰⁺⁺ amino acid transport system, on the L-arginine-induced inward current was examined in extracellular and intracellular Cl⁻-free solution using standard patch recording. Before BCH (10 mM) pretreatment, induction of the inward current was confirmed in each cell by application of 1 mM L-arginine at the holding potential of −60 mV (Figs. 4Aa and 4Ba). In all cells of both control and BCH-pretreated experiments, amplitude of L-arginine (1 mM)-produced inward current was 0.17-0.25 pA/pF (average 0.19 pA/pF; n=3) and 0.16-0.24 pA/pF (average 0.22 pA/pF; n=3), respectively. In control cells (without BCH treatment), L-arginine (1 mM) produced an inward current with an amplitude of 0.13-0.25 pA/pF (average 0.2 pA/pF; n=3, Fig. 4Ab). On the other hand, in BCH-pretreated cells, the same concentration of L-arginine either failed to produce or gave a greatly reduced inward current 0-0.05 pA/pF (average 0.02 pA/pF; n=3, Fig. 4Bb). These results indicate that L-arginine-induced inward current was inhibited by BCH.
Discussion

Our previous report showed that L-arginine depolarized the membrane and increased the action potential burst rate in the rat portal vein (Shimamura et al., 2003). It has been shown that transport of L-arginine evoked an inward current in pancreatic β-cells (Smith et al., 1997), and Xenopus oocytes (Mackenzie et al., 1994). Kavanaugh (1993) also showed that L-arginine induced an inward current in the system y'-expressed Xenopus oocytes.

In the present study, we have shown that L-arginine at a concentration of higher than 0.1 mM induced an inward current in smooth muscle cells of the rat portal vein in the presence of Na⁺ in the extracellular fluid. This L-arginine-induced inward current diminished within 1 min following washout (Figs. 1 and 4). The concentration-response relationship for the current amplitude was similar to that of the contraction frequency in the same tissue (Shimamura et al., 2003). As no inward current was recorded in the absence of Na⁺ in the bath solution, Na⁺ is thought to play an important role in the L-arginine-induced inward current. In the present study, we have observed an L-arginine (1 mM)-induced inward current of 0.2 pA/pF which might depolarize the cell membrane by 2 mV by using values of 0.2 pA/pF current density, 25 pF cell capacitance of the present study and employing input resistance of 400 MΩ from a study of rabbit portal vein smooth muscle cell (Hume and Leblanc, 1989). Thus, in the present study, we could have a few mV depolarization of the membrane by L-arginine, which is slightly smaller than the 5 mV that we found in the previous experiments obtained using intracellular microelectrodes (Shimamura et al., 2003). However, the experimental conditions of the two studies were quite different (e.g. the present experiments were performed at room temperature while the previous experiments were performed at 36°C). If the present values were corrected for temperature for example, they might be close to the amplitude of the depolarization observed in our previous study.

Two types of arginine uptake system were considered: the Na⁺-dependent system B⁰⁺ and the Na⁺-independent system y⁺ (Wagner et al., 2001). A role for the system B⁰⁺ has been shown in Xenopus oocytes (Taylor et al., 1989), blastocytes (van Winkel et al., 1985), porcine pulmonary arterial endothelial cells (Greene et al., 1993) and rat aortic smooth muscle cells (Escobales et al., 2000). Although Kavanaugh (1993) reported that the arginine-induced current depended on both the membrane potential and the intracellular arginine concentration in the y⁺ transport system in the Xenopus oocyte, little information is available on the contribution of the Na⁺-dependent effect of L-arginine on the electrical activity in the rat vascular smooth muscle.

In the present study, the EC₅₀ of Na⁺ in the L-arginine-induced inward current was 5 mM in the rat portal vein, and thus it was considered that Na⁺ is not the rate-limiting factor for arginine transport under physiological conditions. This EC₅₀ value of Na⁺ was lower than that for the Na⁺-dependent glutamate transport system in Xenopus oocytes (50 mM: Mackenzie et al., 1994).

We have also observed that 10 mM BCH inhibited the L-arginine-induced current in smooth muscle cells of the rat portal vein. As BCH has been shown to inhibit the B⁰⁺ transport system (Mackenzie et al., 1994), and as the L-arginine-induced inward current depended on the Na⁺-concentration in the bath, the Na⁺-dependent amino acid transport system, system B⁰⁺, is a possible candidate as the L-arginine transporter in the rat portal vein.
The EC₅₀ for L-arginine in the L-arginine-induced inward current was around 0.5 mM which was about 10 times higher than that of the Na⁺-dependent transport in porcine pulmonary artery endothelial cells (Greene et al., 1993), and about 5 times higher than that of the human amino acid transporter B⁰⁺⁺-expressed in Xenopus oocytes (Sloan and Mager, 1999). Therefore, it might have different characteristics from the B⁰⁺⁺ transport systems in Xenopus oocytes or porcine pulmonary artery endothelial cells, although we did find that the L-arginine-induced inward current was sensitive to BCH.

The presence of cells without a response to L-arginine was unexpected and their origin remained unclear. Several possible reasons for this observation are proposed: (1) different kinds of cells were intermingled such as smooth muscle cells from both the circular and longitudinal layers of the portal vein, (2) enzymatic digestion with collagenase may have destroyed the transport protein, and (3) the degree of cellular arginine depletion may affect arginine transport (Escobales et al., 2000). Although only a proportion of the smooth muscle cells studied showed a response to L-arginine, the depolarization or inward current induced by L-arginine may spread over the tissue via cell-to-cell electrical coupling. In rat portal vein using lucifer yellow, we observed that intercellular communication is present (Shimamura et al., 2005).

Cl⁻ plays important roles in the depolarization of portal vein smooth muscle cells through spontaneous transient inward currents (Wang et al., 1992). In the present study, replacement of Cl⁻ in the pipette solution did not affect the arginine-induced inward current. The fact that the L-arginine-induced inward current is independent of the extracellular Cl⁻ is not compatible with the Na/Cl dependency reported in B⁰⁺⁺-expressed oocytes (Sloan and Mager, 1999). However, we could not exclude a role for Cl⁻ in the bath solution, because it was always contaminated with a several mM concentration of Cl⁻ as we mainly used L-arginine-HCl in the present experiments. Thus, further studies are necessary to elucidate the Cl⁻ involvement.

In conclusion, we have reported the first evidence of an inward current related to Na⁺-dependent L-arginine transport in vascular smooth muscle, which seems to be responsible for membrane depolarization and an increase in contraction frequency in rat portal vein smooth muscle cells.

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References
