Effects of cAMP and cGMP on L-Type Calcium Channel Currents in Rat Mesenteric Artery Cells

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ABSTRACT—L-type Ca^{2+} channel currents in cultured rat mesenteric artery smooth muscle cells were recorded by the cell-attached patch-clamp technique. Depolarizing voltage steps from a holding potential of -40 mV elicited voltage-dependent inward Ba^{2+} currents. The inward currents were inhibited by nifedipine ($10 \mu\text{M}$) but enhanced by Bay K 8644 ($5 \mu\text{M}$), which suggests that the inward currents are carried almost exclusively by L-type Ca^{2+} channels. Application of dibutyryl cAMP (0.1 – $1 \mu\text{M}$) and forskolin (0.01 – $1 \mu\text{M}$) enhanced the activity of these Ca^{2+} channels. The dibutyryl cAMP induced enhancement of Ca^{2+} channels was antagonized by the serine/threonine kinase inhibitor H-8 ($1 \mu\text{M}$). Application of 8-bromo-cGMP (0.01 – $1 \mu\text{M}$) and the cGMP inducer nitroglycerin (0.01 – $1 \mu\text{M}$) inhibited the activity of these Ca^{2+} channels, and the inhibition of channel activity induced by 8-bromo-cGMP was antagonized by the serine/threonine kinase inhibitor H-8 ($1 \mu\text{M}$). These results suggest that in rat mesenteric artery cells, the L-type Ca^{2+} channel current is enhanced by a rise in intracellular cAMP levels and suppressed by a rise in intracellular cGMP levels. Furthermore, cGMP-induced Ca^{2+} channel inhibition may play a role in the expression of the nitric oxide-mediated vasodilating action of drugs such as nitroglycerin and atrial natriuretic peptide.

Keywords: Ca^{2+} channel, cAMP, cGMP, Mesenteric artery, Patch-clamp

Voltage-dependent Ca^{2+} channels are essential for vascular smooth muscle contraction (1) and consist of 2 types, the L-type and T-type (2). L-type Ca^{2+} channels are important as the sites of action of the Ca^{2+} antagonists such as 1,4-dihydropyridines, verapamil and diltiazem, which are the drugs of choice for the treatment of cardiovascular diseases (3, 4).

In cardiac muscle cells, the L-type Ca^{2+} channel is well-known to be enhanced via a phosphorylation process induced by a cAMP-dependent kinase (protein kinase A) (5, 6). Although cAMP is also an important second messenger in vascular smooth muscle cells (7, 8), there is still considerable controversy concerning regulation of Ca^{2+} channels via cAMP in vascular smooth muscle cells. A rise in intracellular cAMP levels is reported to enhance L-type Ca^{2+} channels in porcine coronary artery cells (9) and rabbit portal vein cells (10). In contrast, dibutyryl cAMP has been shown to inhibit the L-type Ca^{2+} channel current in rabbit aorta (11). Isoprenaline has also been shown to suppress the L-type Ca^{2+} channel inward current in rabbit ear artery cells (12).

cGMP is another important second messenger in vas-

cular smooth muscle cells (13, 14). Vasodilating substances like acetylcholine and histamine release nitric oxide from vascular endothelium; this results in the nitric oxide entering vascular smooth muscle cells to produce activation of guanylate cyclase, which in turn produces cGMP, leading to vascular relaxation (15, 16). Nitrate vasodilators like nitroglycerin also release nitric oxide to mediate their blood vessel relaxing effects (17). However, little is known of Ca^{2+} channel regulation by cGMP in vascular smooth muscle cells. In the present study, the effects of membrane permeable cAMP and cGMP analogues and drugs that induce both cyclic nucleotides on the L-type Ca^{2+} channel were examined in rat mesenteric smooth muscle cells using the cell-attached patch technique.

MATERIALS AND METHODS

Preparation

Smooth muscle cells of the mesenteric artery of male Wistar rats (80–120 g) were obtained as previously described (18). Briefly, mesenteric arteries were excised

and cut into small pieces in normal Tyrode's solution following removal of the endothelial tissue. The pieces were then explanted in cultured dishes filled with medium 199 containing 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 100 $\mu\text{g}/\text{ml}$ of penicillin and stored in a CO_2 incubator (5% CO_2 , 37°C). Three or four glass

coverslips were placed on the bottom of each culture dish. Single smooth muscle cells that migrated out of the tissues and adhered to the coverslips during primary culture for 6–10 days were used for the experiments. No proteolytic enzyme was used for isolation of single cells.

Fig. 1. Inward currents elicited by voltage step depolarizations from a holding potential of -40 mV. Depolarizing pulses of 400 msec in duration to various membrane potentials were applied. A: Typical tracings of the currents are shown. B: The current-voltage relationship of the peak current (mean $\pm$ S.E., $n=6$).

Fig. 2. Effects of nifedipine (10 μM) and Bay K 8644 (5 μM) on open probability of inward currents elicited by a voltage step to +20 mV from a holding potential of -40 mV. Values are each the mean $\pm$ S.E. from 5 experiments. * $P < 0.05$, compared to the control.

Fig. 3. Effects of dibutyryl cAMP on the open probability of inward currents elicited by a voltage step to +20 mV from a holding potential of -40 mV. **A:** 5 consecutive cell-attached recordings at times indicated by arrows in **B**. **B:** Time course of dibutyryl cAMP-induced changes in open probability of inward currents by a voltage step every 10 sec. **C:** Maximal dibutyryl cAMP-induced changes in open probability of inward currents. Values are each the mean $\pm$ S.E. from 5 experiments. * $P < 0.05$, compared to the control.

Current measurement

The cells grown on coverslips were placed in an experimental chamber (2 ml) on the stage of an inverted microscope (TMD; Nikon Kogaku, Tokyo). Heat-polished glass patch electrodes with a tip resistance of between 5 and 10 megohms were used. The seal resistance between the pipette tip and the cell membrane was more

than 10 gigaohms. Experiments were conducted on cell-attached membrane patches, as previously described (19).

Single-channel currents were recorded using a patch-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo) and were stored on digital audio tape by a PMC datarecorder (D-180T; TEAC, Tokyo) for later analysis. Currents were low-pass filtered at 1–2 kHz. Actual records were

Fig. 4. Antagonistic effect of H-8 (1 μ M) on dibutyl cAMP (0.1 μ M)-induced enhancement of open probability of inward currents. Values are each the mean $\pm$ S.E. from 6 experiments. * $P < 0.05$ (paired t -test).

recalled from the datarecorder at low speeds and were written out using a conventional thermowriting recorder (RTA-1100, Nihon Kohden). In the figures, the downward direction of the single channel currents indicate the inward current of the membrane. All experiments were performed at 30°C.

Solutions

The pipette solution contained: 110 mM BaCl₂, 10 mM tetraethylammonium chloride, 10 mM *N*'-2-hydroxyethyl-piperazine-*N*'-2-ethanesulphonic acid (HEPES) and was adjusted to pH 7.4 with tris(hydroxymethyl)ethane-OH. The bath solution contained: 140 mM potassium gluconate, 10 mM ethyleneglycol-bis-*N,N,N,N*'-tetraacetic acid (EGTA), 1 mM MgCl₂, and 10 mM HEPES, and was adjusted to pH 7.4 by KOH. The high-K⁺ concentration solution was used as a bath solution in order to produce a smooth muscle cell membrane potential of approximately 0 mV except at the patch membrane. The solution in the chamber was rapidly exchanged by flushing with 5–10 ml of the test solutions, and the over-flow was siphoned off by a water pump. Exchange of solution was carried out within 1 min.

Data analyses

Histograms of open probability were calculated by a microcomputer (Signal processor 7T17; Nihondenki Sanei Instrument Co., Ltd., Tokyo). The probability of the

channel being open (open probability) in each sweep was calculated from the total period of the channel being open divided by the duration (400 msec) of the pulse. In general, when using patch pipettes, simultaneous opening of two or three unitary Ba²⁺ channels is usually observed. However, in the present experiments, patches that contained only one channel were used. Data are reported as the mean $\pm$ S.E., and Student's t -test or the paired t -test was used for statistical analyses of the results.

Drugs

The medium used was Medium 199 (Nissui Chemicals, Tokyo), supplemented with fetal bovine serum (Kanto Reagents, Tokyo), streptomycin and penicillin (Meiji Pharmaceutical Chemicals, Tokyo). Nifedipine and Bay K 8644 were purchased from Research Biochemicals International (Natick, MA, USA), dibutyl cAMP and 8-bromo-cGMP from Sigma (St. Louis, MO, USA), nitroglycerine from Nihonkayaku (Tokyo), forskolin from Wako (Osaka) and H-8 from Seikagaku Corporation (Tokyo). Nifedipine, Bay K 8644 and forskolin were dissolved in dimethylsulfoxide before addition to the bath. The final concentration of organic solvents was less than 1% and thus had no effect on the opening of the Ca²⁺ channel.

RESULTS

Characterization of L-type Ca²⁺ channel currents

Voltage-dependent Ca²⁺ channels in vascular smooth muscle consist of L- and T-types (1). Although both L- and T-type channels are activated by steady holding potentials of -70 mV, only L-type channels are activated by steady holding potentials of -40 to -30 mV (20). Figure 1A shows inward currents elicited by brief depolarizing steps to various potentials from a holding potential of -40 mV. Figure 1B shows the relationship between current and voltage (I - V). The voltage-dependent characteristics of inward currents were very similar to the L-type Ca²⁺ channel I - V curve found by Inoue et al. (21). Application of the L-type Ca²⁺ antagonist nifedipine (10 μ M) to the bathing solution abolished the open probability (Fig. 2A). Application of the L-type Ca²⁺ agonist Bay K 8644 (5 μ M) increased the open probability (Fig. 2B). These results indicate that the inward currents observed in the present study were carried almost exclusively by L-type Ca²⁺ channels.

Effects of dibutyl cAMP and forskolin on inward currents

Application of the membrane permeable dibutyl cAMP (0.01–0.1 μ M) increased the open probability in a concentration-dependent manner (Fig. 3: A and B). Pre-

Fig. 5. Effects of forskolin on open probability of inward currents elicited by a voltage step to +20 mV from a holding potential of -40 mV. **A:** 5 consecutive cell-attached recordings at times indicated by arrows in **B**. **B:** Time course of forskolin-induced changes in open probability of inward currents by a voltage step every 10 sec. **C:** Maximal forskolin-induced changes in open probability of inward currents. Values are each the mean $\pm$ S.E. from 6 experiments. * $P < 0.05$, compared to the control.

application of the serine/threonine kinase inhibitor H-8 (1 μM) inhibited the enhancement of the open probability induced by dibutyl cAMP (0.1 μM) (Fig. 4). Application of forskolin (0.01–1 μM), a direct activator of adenylate cyclase, also increased the open probability in a concentration-dependent manner (Fig. 5: A and B).

Effects of 8-bromo-cGMP and nitroglycerin on inward currents

Application of the membrane permeable 8-bromo-cGMP to the bath (0.01–1 μM) produced a concentration-dependent inhibition of the open probability (Fig. 6A). Pre-application of the serine/threonine kinase inhi-

Fig. 6. Effects of 8-bromo-cGMP on open probability of inward currents elicited by a voltage step to +20 mV from a holding potential of -40 mV (A) and reversal of the 8-bromo-cGMP (1 μM) effects by H-8 (1 μM) (B). Values are each the mean ± S.E. from 6 experiments. * $P < 0.05$.

bitor H-8 (1 μM) antagonized the inhibition of the open probability induced by 8-bromo-cGMP (1 μM) (Fig. 6B). Application of the nitric oxide releaser nitroglycerin (0.01–1 μM) to the bathing solution also inhibited the activity of the Ca^{2+} channel and decreased the open probability in a concentration-dependent manner (Fig. 7). H-8 alone did not affect the activity of the Ca^{2+} channel ($n=5$).

DISCUSSION

The effect of cAMP on vascular smooth muscle Ca^{2+} channels is controversial. In the present study, both the membrane permeable cAMP analogue dibutyryl cAMP and the direct adenylate cyclase activator forskolin produced an increase in the open probability of Ba^{2+} currents in rat mesenteric artery cells. The enhanced effect of dibutyryl cAMP was antagonized by the serine/threonine kinase inhibitor H-8. These results suggest that a rise in intracellular cAMP levels leads to an enhancement of L-type Ca^{2+} channels. These results are in agreement with previous studies of porcine coronary artery cells (9) and rabbit portal vein cells (10).

We also found that the membrane permeable cGMP analogue 8-bromo-cGMP and the nitric oxide releaser nitroglycerin produced a decrease in inward currents in rat mesenteric artery cells. The inhibition effect of 8-bromo-cGMP was antagonized by the serine/threonine

kinase inhibitor H-8. cGMP is widely accepted to be an important intracellular messenger in vascular smooth muscle cells and to mediate the effects of vasodilators such as endothelium-derived relaxing factor (nitric oxide) and nitrovasodilators (14, 15). Sodium nitroprusside has been shown to inhibit both high K^{+} - and norepinephrine-induced ^{45}Ca uptake into tissue strips, suggesting cGMP-mediated suppression of L-type Ca^{2+} channels (22, 23). These results suggest that a rise in intracellular cGMP levels may be due to inhibition of L-type Ca^{2+} channels. These observations are in agreement with a previous study of rabbit pulmonary artery cells using sodium nitroprusside, another nitric oxide releasing vasodilator (24).

In contrast with the results of the present study, in rabbit aorta, dibutyryl cAMP has been shown to inhibit L-type Ca^{2+} channel current (11). Isoprenaline has also been reported to suppress L-type Ca^{2+} channel inward current in rabbit ear artery cells (12). Ohya et al. observed that cAMP (but not ATP) had no effect on the voltage-dependent Ca^{2+} current in rabbit jejunum smooth muscle cells (25). In addition, cAMP did not prevent the run-down phenomena of the Ca^{2+} channel current in single smooth muscle cells of guinea pig urinary bladder (26). Furthermore, internal perfusion of cAMP or dibutyryl cAMP had no effect on potentiation of the Ca^{2+} channel current by β -adrenoreceptor stimulation with isoprenaline (27). Despite the numerous reports suggesting a

Fig. 7. Effects of nitroglycerin on open probability of inward currents elicited by a voltage step to $+20$ mV from a holding potential of -40 mV. **A:** 5 consecutive cell-attached recordings at times indicated by arrows in **B**. **B:** Time course of nitroglycerin-induced changes in open probability of inward currents by voltage step every 10 sec. **C:** Maximal nitroglycerin-induced changes in the open probability of inward currents. Values are each the mean $\pm$ S.E. from 6 experiments. * $P < 0.05$, compared to the control.

regulatory role for cAMP in the voltage-dependent Ca^{2+} current, the action of cAMP has not been completely elucidated. Additionally, tissue differences may be partially responsible for these differences in cAMP-mediated modulation of smooth muscle Ca^{2+} channels.

In cardiac muscles, the phosphorylation of L-type Ca^{2+} channels by cAMP-dependent protein kinase is thought to be the most important in determining the force of contraction induced by β -adrenergic stimulation (6). However, in vascular smooth muscles, the possible phys-

iological significance of cAMP-induced activation of Ca^{2+} channels is unclear because a rise in intracellular cAMP in vascular smooth muscles results in vasodilatation. On the other hand, cGMP-induced inhibition of L-type Ca^{2+} channels in vascular muscles may be involved in the expression of the blood vessel relaxing action of nitric oxide-mediating vasodilators such as atrial natriuretic peptide, nitroglycerin and acetylcholine (15–17).

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