Regulation of Ca\(^{2+}\) Channels by cAMP and cGMP in Vascular Smooth Muscle Cells

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Whole-cell Ca\(^{2+}\) channel currents in rabbit portal vein cells were recorded using the amphotericin B-perforated patch-clamp technique at 35°C. This technique allowed recording of stable inward currents in the absence of run-down for more than 30 minutes. Depolarizing voltage steps from a holding potential of \(-70\) mV elicited voltage-dependent inward currents. The voltage dependence of inward currents measured in either 2.5 mmol/L Ba\(^{2+}\)– or 2.5 mmol/L Ca\(^{2+}\)–containing solution were very similar. However, maximum Ba\(^{2+}\) current (obtained at around +10 mV) was approximately 1.5-fold larger than maximum Ca\(^{2+}\) current. Changing the holding potential from \(-70\) to \(-40\) mV decreased inward currents but did not shift the voltage dependence significantly. Inward currents were also completely blocked by the dihydropyridine Ca\(^{2+}\) channel blocker, nicardipine (10 \(\mu\)mol/L), suggesting the presence of predominantly L-type Ca\(^{2+}\) channels in rabbit portal vein cells. Isoproterenol caused small increases in the amplitude of Ba\(^{2+}\) currents in a concentration-dependent manner (10 \(\mu\)mol/L to 1 mmol/L), which were reversed with propranolol. Forskolin (1 mmol/L) or 8-bromo-cAMP (0.1 mmol/L) also caused small increases in the amplitude of Ba\(^{2+}\) currents, suggesting that the stimulatory actions of isoproterenol are importantly linked to the production of cAMP. Higher concentrations of isoproterenol (10 \(\mu\)mol/L) or forskolin (10 \(\mu\)mol/L) caused a transient increase in Ba\(^{2+}\) currents followed by a decrease in current amplitude. Higher doses of 8-bromo-cAMP (1 mmol/L) and low doses of 8-bromo-cGMP (0.1 mmol/L) inhibited Ba\(^{2+}\) currents, increased the rate of current inactivation, and produced a negative voltage shift in steady-state availability. These results indicate that low concentrations of intracellular cAMP produce modest increases in Ca\(^{2+}\) channel activity, whereas cGMP and higher concentrations of cAMP result in inhibition of Ca\(^{2+}\) channel activity in vascular smooth muscle cells. The observed similarities of cGMP and high concentrations of cAMP on Ba\(^{2+}\) current amplitude, kinetics, and steady-state inactivation suggest mediation by a common mechanism, possibly involving activation of cGMP-dependent protein kinase. (Circ Res. 1993;73:1128-1137.)

**KEYWORDS** • rabbit portal vein • isoproterenol • forskolin • 8-bromo-cAMP • 8-bromo-cGMP

Voltage-dependent Ca\(^{2+}\) channels are involved in excitation-contraction coupling in vascular smooth muscle.1,2 The activity of these channels can be modulated indirectly by changes in membrane potential or more directly through the actions of agonist. Various vasoconstrictor substances such as acetylcholine,3 histamine,4,5 and angiotensin II6 have been reported to enhance L-type Ca\(^{2+}\) channel activity. In cardiac myocytes, it has been well established that \(\beta\)-adrenergic stimulation increases the amplitude of the L-type Ca\(^{2+}\) channel current (\(I_{\text{Ca}}\)) by activating adenylate cyclase to produce cAMP, which in turn stimulates cAMP-dependent phosphorylation of the channel or a channel subunit.1,3,9 In contrast, there is still considerable controversy concerning the effects of \(\beta\)-adrenergic stimulation and cAMP on \(I_{\text{Ca}}\) in vascular smooth muscle cells. Droogmans et al10 have reported that isoproterenol (ISO) inhibits \(I_{\text{Ca}}\) or has no effect in rabbit ear artery cells. In rabbit aorta, dibutylryl analogues of cAMP have been shown to suppress \(I_{\text{Ca}}\).11 Satoh and Sperelakis12 have also reported that ISO inhibits \(I_{\text{Ca}}\) in cultured A7r5 cells. In contrast, Marks et al13 using the same preparation (A7r5 cells), have reported that \(I_{\text{Ca}}\) was augmented by either ISO or forskolin (FSK). ISO and FSK have also been reported to increase \(I_{\text{Ca}}\) in porcine coronary artery cells.14

Although tissue diversity may be partially responsible for some of the reported differences in \(\beta\)-adrenergic modulation of smooth muscle Ca\(^{2+}\) channels, some of these differences may be related to experimental conditions. For example, most studies that have examined adrenergic regulation of Ca\(^{2+}\) channels in vascular smooth muscle have been carried out in dialyzed isolated cells. This potential complication can now be controlled with the recent development of the perforated patch technique. This technique uses polyene antibiotics such as nystatin to form voltage-insensitive ion pores in the membrane patch under the recording pipette.15 Use of the perforated whole-cell current recording technique has been shown to prevent the “run-down” of \(I_{\text{Ca}}\), which typically occurs with time when dialyzing pipettes are used.5,16,17 Furthermore, this technique is useful for maintaining physiological intracellular conditions; eg, cytoplasmic small metabolites and proteins remain in the cell rather than being

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washed out. Use of the perforated patch technique may thus be particularly critical when responses are modulated by intracellular second messengers. For instance, in cardiac muscle cells, the stimulatory effects of ISO on \(I_{Ca}\) decrease drastically with time when conventional dialyzing patch pipettes are used\(^{18}\) but can be maintained nearly constant for up to 60 minutes when the nystatin–perforated patch technique is used.\(^{16}\) In chromaffin cells, chromostatin-induced inhibition of \(I_{Ca}\) can be demonstrated using the perforated patch technique but is absent when conventional dialyzing patch pipettes are used.\(^{19}\)

In the present study, \(Ca^{2+}\) and \(Ba^{2+}\) inward currents were studied in rabbit portal vein smooth muscle cells under physiological conditions using the perforated patch technique at 35°C. ISO, FSK, and 8-bromo-cAMP were all found to stimulate \(L\)-type \(Ca^{2+}\) channel currents at low concentrations, whereas higher concentrations of these drugs mimicked the ability of 8-bromo-cGMP to inhibit \(Ca^{2+}\) channel currents.

### Materials and Methods

Single vascular smooth muscle cells were isolated from rabbit portal veins using a dispersal procedure similar to that described previously.\(^{3}\) Briefly, male albino rabbits (1.5 to 2.0 kg) were killed with an overdose of sodium pentobarbital (50 mg/kg) injected into the ear vein. The portal vein was dissected and cleared of adhering tissue and fat in a Krebs' solution (mmol/L: NaCl, 118.5; KCl, 4.2; CaCl\(_2\), 1.8; MgCl\(_2\), 1.2; NaHCO\(_3\), 23.8; KH\(_2\)PO\(_4\), 1.2; and glucose, 11; aerated with 95% O\(_2\)-5% CO\(_2\)). The vessels were cut into small segments (approximately 2 x 2 mm) and placed in a Ca\(^{2+}\)-free Hanks' solution (mmol/L: NaCl, 125; KCl, 5.4; NaHCO\(_3\), 25.5; Na\(_2\)HPO\(_4\), 0.34; KH\(_2\)PO\(_4\), 0.44; glucose, 10; and sucrose, 2.9; aerated with 95% O\(_2\)-5% CO\(_2\)) for 30 minutes at room temperature. The segments were then placed in the Ca\(^{2+}\)-free Hanks' solution containing 1 mg/mL collagenase (cl 2s, Worthington Biochemical Co., Freehold, NJ), 2 mg/mL bovine serum albumin (BSA), 2 mg/mL trypsin inhibitor (type II-S, Sigma Chemical Co, St Louis, Mo), 0.1 mg/mL Na\(_2\)-ATP, and 0.1 mg/mL protease (type XIV, Sigma) and incubated for approximately 15 minutes with gentle agitation at 37°C. After completion of the digestion, single cells were dispersed by gentle agitation in the Ca\(^{2+}\)-free Hanks' solution. The cells obtained were stored in Hanks' solution (0.1 mmol/L Ca\(^{2+}\)) containing 1 mg/mL BSA and 0.5 mg/mL trypsin inhibitor at 4°C.

The dispersed cells were placed in a small chamber (300 \(\mu\)L) on the stage of an inverted microscope. The bath solution was superfused through the chamber by gravity at a rate of approximately 1 mL/min. Micropipettes had a resistance of 1 to 2 M\(\Omega\) when filled with the internal solution. The perforated patch technique\(^{16}\) with amphotericin B\(^{20}\) was used to record whole-cell inward currents. The channels formed with this antibiotic have twice the channel conductance of nystatin\(^{21}\) and are therefore expected to produce lower access resistance.\(^{20}\) Amphotericin B (Sigma) stock solution (90 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and diluted in the pipette solution to give a final concentration of 270 \(\mu\)g/mL. Approximately 50% of the series resistance (6 to 11 M\(\Omega\)) was compensated. The voltage-dependent \(Ca^{2+}\) channel currents recorded with the perforated technique showed little run-down. The bath temperature was maintained at 35°C using a temperature controller (model TC-1, NBD, Stony Brook, NY). Voltage clamp was performed using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, Calif). Command signals were generated by an IBM AT compatible computer using pCLAMP 5.5 software with TL-1 (Axon Instruments). Data were filtered at 5 kHz (3 dB) and stored in the computer. Leak subtraction was not performed.

The bath solution was of the following composition (in mmol/L): NaCl, 120; NaHCO\(_3\), 10; tetraethylammonium chloride, 10; CsCl, 6; MgCl\(_2\), 0.5; BaCl\(_2\) or CaCl\(_2\), 2.5; glucose, 5.5; and HEPES, 10 (titrated to pH 7.35 with NaOH). The internal solution used to fill the patch pipettes consisted of the following (mmol/L): cesium aspartate, 120; CsCl, 20; tetraethylammonium chloride, 10; and HEPES, 10 (titrated to pH 7.2 with CsOH). ISO, 8-bromo-cAMP, and 8-bromo-cGMP (Sigma) were dissolved in the bath solution. FSK (Sigma) was prepared as a stock solution of 10 mmol/L in DMSO and diluted into the bath solution.

Averaged data are expressed as mean±SEM. Statistical analyses were performed with Student's \(t\) test for paired or unpaired values. Changes were considered significant at \(P<.05\).

### Results

**Characterization of \(Ca^{2+}\) Channel Currents**

Experiments were carried out on relaxed spindle-shaped cells (total cell capacitance \([C_m]\), 25 to 50 pF; large cells \([C_m, >50\) pF] were avoided). Fig 1A shows families of inward currents elicited by brief depolarizing steps to various potentials from a holding potential of −70 mV and recorded in 2.5 mmol/L Ba\(^{2+}\) solution or in 2.5 mmol/L \(Ca^{2+}\) solution. The currents in Ba\(^{2+}\) solution inactivated more slowly than did currents in \(Ca^{2+}\) solution. The current-voltage (I-V) relation shows detectable inward currents at approximately −30 mV and maximum currents between 0 and +10 mV in both Ba\(^{2+}\) - and \(Ca^{2+}\)-containing solutions (Fig 1B). The maximum current in \(Ba^{2+}\) solution was approximately 1.5-fold the amplitude of currents recorded in \(Ca^{2+}\) solution. Both I-V curves had a single peak without a secondary "hump."

The voltage dependence of steady-state inactivation of \(I_{Ca}\) was studied in 2.5 mmol/L Ba\(^{2+}\) solution using a double-pulse protocol. From a holding potential of −70 mV, hyperpolarizing and depolarizing conditioning voltage steps ranging from −100 to +30 mV were first applied for a duration of 2 seconds. After a 20-millisecond interpulse interval at a potential of −70 mV, a 200-millisecond test pulse to 0 mV was applied. Fig 1C shows the average steady-state inactivation data for Ba\(^{2+}\) currents. The curve was fit by a conventional Boltzmann distribution of the following form:

\[I/I_{\text{max}} = 1/1 + \exp[(V-V_{0.5})/k]\]

where \(I\) represents current, \(I_{\text{max}}\) is maximal current, \(V_{0.5}\) is the membrane potential producing 50% inactivation, and \(k\) is the slope factor. In 2.5 mmol/L Ba\(^{2+}\) solution, \(V_{0.5}\) was −25.4±1.4 mV with a k of 8.7±0.8 mV (n=6). Fig 1C also shows the average steady-state activation of Ba\(^{2+}\) currents. This relation was determined from the
peak current amplitudes with steps to various voltages and the maximum conductance value obtained from the slope of the I-V relation extrapolated through the reversal potential using linear regression. The average activation curve for Ba$^{2+}$ currents was also fit by a Boltzmann distribution and exhibited $V_{1/2}$ at $-7.1 \pm 1.4$ mV with a $k$ value of $-5.3 \pm 0.4$ mV ($n=6$).

The existence of two types of voltage-dependent Ca$^{2+}$ currents has been shown in various cells. They can be distinguished on the basis of differences in kinetics, voltage dependence, pharmacology, and single-channel conductance. Typically, one type is activated by small depolarizations and inactivates relatively quickly (T type); in contrast, another requires larger depolarizations for activation and inactivates relatively slowly (L type). These two types of channels are often separated by holding potential: T-type channels are inactivated by steady holding potentials of $-40$ to $-30$ mV. Fig 2A shows Ba$^{2+}$ currents elicited by voltage steps to $-20$, $-10$, and $0$ mV from holding potentials of $-70$ and $-40$ mV. The amplitude of currents from $-70$ mV was larger than those from $-40$ mV, and clear differences in the time course between them was observed; the inactivation of currents from $-70$ mV was faster than that from $-40$ mV. However, the threshold for activation and the maximum current were observed at about the same voltage, independent of the holding potential (Fig 2A, graph c).

It is well known that dihydropyridines such as nicardipine selectively block L-type Ca$^{2+}$ channels. Therefore, the effects of nicardipine on inward currents were investigated. In a concentration-dependent manner, nicardipine diminished the inward currents elicited by a step to $0$ mV from a holding potential of $-70$ mV (Fig 2B, tracing a). Nicardipine at concentrations up to $1 \mu$mol/L not only reduced the peak amplitude of the Ba$^{2+}$ current but also hastened its decay, an effect previously attributed to open-channel blocking properties of dihydropyridines. Higher concentrations of nicardipine (3 or $10 \mu$mol/L) almost completely abolished all inward currents. Fig 2B, graph b, shows the I-V relations of inward currents before and after exposure to nicardipine at concentrations between $0.1$ and $10 \mu$mol/L. The voltage at which the peak of I-V relation curve was obtained was not shifted by any concentration of nicardipine.

The voltage- and time-dependent characteristics of inward currents recorded with the perforated patch technique were very similar to the results of previous studies of portal vein cells that used conventional whole-cell recording techniques. The results shown in Fig 2 support previous conclusions that inward currents in this tissue are carried almost exclusively by L-type Ca$^{2+}$ channels.

**Stimulatory Effects of ISO, FSK, and 8-Bromo-cAMP on Inward Currents**

The effect of ISO on $I_{Ca}$ was investigated with $2.5$ mmol/L Ba$^{2+}$ as a charge carrier in order to eliminate the contamination of other current(s) activated by Ca$^{2+}$ (eg, Cl$^-$ currents). Fig 3A shows the effects of ISO on inward currents. Bath application of ISO ($0.1 \mu$mol/L) increased the peak amplitude of inward currents in 18 of 24 cells tested. In the continued presence of ISO ($0.1$ mmol/L, Fig 3B, tracing a), the peak of the I-V relation curve was not shifted by ISO, but there was a marked diminution of the peak amplitude. In the presence of 8-Bromo-cAMP ($0.1$ mmol/L), the peak amplitude of $I_{Ca}$ could be further diminished (Fig 3B, tracing b).
10 μmol/L nicardipine completely blocked all inward current, suggesting that the actions of ISO were exclusively mediated by activation of L-type Ca\(^{2+}\) channels. The ISO-stimulated inward currents were also completely blocked by the β-adrenoceptor antagonist propranolol (1 μmol/L) (Fig 3B), suggesting that the response to ISO was mediated through β-adrenergic receptors. An increase of inward currents by ISO was observed over the whole range of depolarizing pulses (from -20 to +50 mV), but the level at which currents could be detected (around -30 mV) and the potential at which maximum current was observed (between 0 and +10 mV) remained the same (Fig 3C). The effect of ISO was relatively quick in onset and reversible. Current amplitude began to increase within 30 seconds to 1 minute after ISO-containing solution first entered the bath, and the current returned to the control level within 10 minutes after washout (Fig 3C). Figure 4C shows the concentration-response relation for the stimulatory effects of ISO on Ba\(^{2+}\) currents. The apparent EC\(_{50}\) value for ISO was estimated to be between 0.01 and 0.1 μmol/L.

It is well known that β-adrenergic receptors are coupled to adenylate cyclase and that most of the effects of β-adrenergic agonists are mediated by cAMP and cAMP-dependent protein kinase (A kinase).\(^{27}\) To determine if the actions of ISO on Ba\(^{2+}\) currents can be mimicked by changes in cAMP levels in the cell, we investigated the effects of FSK, a direct activator of adenylate cyclase,\(^{28}\) and 8-bromo-cAMP, a membrane-permeable derivative of cAMP. Both FSK (1 μmol/L) and 8-bromo-cAMP (0.1 mmol/L) increased inward currents (six of eight cells with FSK and five of five cells with 8-bromo-cAMP, Fig 4), although the effects developed more slowly than with ISO. These results suggest the involvement of the cAMP cascade in the stimulatory effect of ISO on Ba\(^{2+}\) currents, similar to that of cardiac Ca\(^{2+}\) channels.

Biphasic Effects of High Concentrations of ISO and FSK on Inward Currents

When ISO was applied cumulatively, we noticed that ISO at concentrations greater than 1 μmol/L caused no greater increase in Ba\(^{2+}\) currents but rather decreased them (data not shown). Therefore, we examined the effects of higher concentrations of ISO (10 μmol/L) on Ba\(^{2+}\) currents. Figure 5B shows the time course of effects of 10 μmol/L ISO on peak inward currents elicited by voltage steps to 0 mV from a holding potential of -70 mV. Switching the superfusing solution to one containing 10 μmol/L ISO led to an initial increase in inward current. However, unlike lower concentrations of ISO (0.01 to 1 μmol/L, Fig 5A), the effect of 10 μmol/L ISO was not sustained; after reaching maximum response, the current gradually declined to levels lower than the control level. In the presence of 10 μmol/L propranolol, 10 μmol/L ISO caused only a slight increase in Ba\(^{2+}\) currents (Fig 5C and 5E), suggesting that the inhibitory action of 10 μmol/L ISO was also mediated through β-adrenergic receptors. The inhibition of inward currents by 10 μmol/L ISO was accompanied by an apparent acceleration of the rate of inactivation (Fig 5F). The time course of inactivation of Ba\(^{2+}\) currents could be fit with two exponential rates of decay. Both the fast time
Fig 3. Effects of isoproterenol (ISO) on Ba\textsuperscript{2+} currents. A, Typical tracings of the current elicited by voltage step to 0 mV from a holding potential of −70 mV under the control condition, in the presence of 0.1 μmol/L ISO, and in the presence of 0.1 μmol/L ISO and 10 μmol/L nicardipine (NIC) were superimposed. All tracings were obtained from the same cell. B, Time course of peak inward currents elicited by voltage step to 0 mV from a holding potential of −70 mV every 30 seconds is shown. The cell was superfused consecutively with 0.1 μmol/L ISO followed by 0.1 μmol/L ISO plus 1 μmol/L propranolol (PROP). C, Graph shows the current-voltage relation for the peak current before (○), after exposure to 1 μmol/L ISO (●), and 10 minutes after washout (▲).

Effects of Cyclic Nucleotide Analogues on Inward Currents

As shown in Fig 4, 0.1 mmol/L 8-bromo-cAMP increased Ba\textsuperscript{2+} currents. However, the biphasic effects constant and the slower time constant were significantly reduced by 10 μmol/L ISO (Table). Similar results were obtained with high concentration of FSK (10 μmol/L) (Fig 5D and 5G, Table).

Fig 4. A and B, Effects of forskolin (FSK) and 8-bromo-cAMP (8-Br-cAMP) on Ba\textsuperscript{2+} currents are shown. Typical tracings of the current were elicited by voltage step to 0 mV from a holding potential of −70 mV in the absence (control) and in the presence of 1 μmol/L FSK (A) or 0.1 mmol/L 8-Br-cAMP (B). C, Effects of various concentrations of isoproterenol (ISO), 1 μmol/L FSK, and 0.1 mmol/L 8-Br-cAMP on the peak current were elicited by voltage step to 0 mV from a holding potential of −70 mV. Graph shows mean values (mean±SEM, n=4 to 9) of normalized amplitude.
Fig 5. Effects of high concentration of isoproterenol (ISO) or forskolin (FSK) on Ba\textsuperscript{2+} currents. A. A through D. Time courses of peak inward currents elicited by voltage step to 0 mV from a holding potential of -70 mV every 30 seconds are shown. The cell was superfused consecutively with 1 \mu mol/L (A) or 10 \mu mol/L (B and C) ISO or 10 \mu mol/L FSK (D). Note that 10 \mu mol/L ISO (B) or 10 \mu mol/L FSK (D) caused a biphasic response: stimulation followed by inhibition. In the presence of 10 \mu mol/L propranolol (PROP), 10 \mu mol/L ISO caused only a slight increase in Ba\textsuperscript{2+} currents (C). E. Bar graph summarizes the effects of 10 \mu mol/L PROP on the biphasic response to 10 \mu mol/L ISO (n=5). Data were determined as percentage increase or decrease in average of five peak currents (2 to 4 minutes or 12 to 14 minutes) from the control value (CONT). Graphs show mean±SEM values from five independent experiments. \*P<.05 vs corresponding CONT value (Student's t test for unpaired values). F and G, Current tracings a through c (F) and d through f (G) were obtained at the time corresponding indicated in B and D, respectively. All experiments with ISO were performed in the presence of 10 \mu mol/L phentolamine.

Table: Changes of the Inactivation Time Constants of Ba\textsuperscript{2+} Currents by Isoproterenol, Forskolin, 8-Bromo-cAMP, or 8-Bromo-cGMP in Portal Vein Cells

<table>
<thead>
<tr>
<th>ISO Studies</th>
<th>FSK Studies</th>
<th>8-Br-cAMP Studies</th>
<th>8-Br-cGMP Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 \mu mol/L ISO</td>
<td>Control 10 \mu mol/L FSK</td>
<td>Control 1 \mu mol/L 8-Br-cAMP</td>
<td>Control 0.1 \mu mol/L 8-Br-cGMP</td>
</tr>
<tr>
<td>(\tau_f)</td>
<td>(\tau_s)</td>
<td>(\tau_f)</td>
<td>(\tau_s)</td>
</tr>
<tr>
<td>19.0±2.0</td>
<td>14.3±0.5*</td>
<td>18.7±1.2</td>
<td>13.3±0.8*</td>
</tr>
<tr>
<td>82.5±8.4</td>
<td>52.7±3.5*</td>
<td>112.1±13.5</td>
<td>51.6±1.4*</td>
</tr>
</tbody>
</table>

ISO indicates isoproterenol; FSK, forskolin; 8-Br-cAMP, 8-bromo-cAMP; 8-Br-cGMP, 8-bromo-cGMP; \(\tau_f\), fast time constant; and \(\tau_s\), slow time constant. The time course of inactivation of currents evoked by steps to 0 mV could be represented by the sum of two exponentials. Values are mean±SEM of five to six experiments. \*P<.05 vs corresponding control value (Student's t test for paired values).
a high concentration of ISO. As shown in the Table, 8-bromo-cAMP (1 mmol/L) decreased both fast and slow time constants of inactivation.

Little is known about the actions of another important cyclic nucleotide, cGMP, on \( I_{Ca} \) in vascular smooth muscle cells. In the present study, we also investigated the effects of 8-bromo-cGMP on \( I_{Ca} \) currents in rabbit portal vein cells. A typical response to 0.1 mmol/L 8-bromo-cGMP is shown in Fig 6B. The effects of 8-bromo-cGMP on inward currents were very similar to the effects of high concentrations of ISO and 8-bromo-cAMP. 8-Bromo-cGMP significantly reduced \( I_{Ca} \) currents in 12 of 15 cells tested, with no detectable shift along the voltage axis of the I-V relation (Fig 6B). This analogue also increased the rate of inactivation of currents (Table). The influence of 8-bromo-cGMP and 8-bromo-cAMP on steady-state inactivation of \( I_{Ca} \) currents was also examined. As shown in Fig 7A, 0.1 mmol/L 8-bromo-cGMP did not affect steady-state inactivation. However, 1 mmol/L 8-bromo-cAMP shifted the steady-state inactivation relation for inward currents toward more negative potentials. 8-Bromo-cGMP (0.1 mmol/L) also shifted the inactivation relation in the same direction (Fig 7B). These results suggest that the inhibitory effects of high concentrations of ISO, FSK, and 8-bromo-cAMP on \( I_{Ca} \) are similar to those produced by 8-bromo-cGMP. These similarities include reduction of \( I_{Ca} \) current amplitude, acceleration of current inactivation, and a negative voltage shift in \( I_{Ca} \) current steady-state availability.

**Discussion**

Activation of adenylyl cyclase has been implicated in the actions of a number of different vasodilatory substances that relax vascular smooth muscles, including adenosine \( A_2 \) receptor stimulants, \( \beta \)-adrenergic receptor stimulants, and calcitonin gene-related peptide. Numerous studies have investigated the relation of the cAMP pathway to \( \text{Ca}^{2+} \) sequestration and changes in myofilament sensitivity to \( \text{Ca}^{2+} \). However, the effects of cAMP on smooth muscle \( \text{Ca}^{2+} \) channels is controversial. The results of the present study suggest that under physiological conditions a moderate rise in intracellular cAMP levels leads to a small enhancement of inward current, whereas higher levels of intracellular cAMP lead to inhibition of inward current. The inhibitory effects of higher concentrations of cAMP were mimicked by cGMP.

Using the perforated patch technique, we found that ISO (0.01 to 1 \( \mu \)mol/L) produced a small increase in the amplitude of \( I_{Ca} \). This effect was mediated through \( \beta \)-adrenergic receptors, since it was blocked by propranolol. Similarly, both FSK (1 \( \mu \)mol/L) and 8-bromo-cAMP (0.1 mmol/L) increased inward currents. Although we cannot discount the contribution of an additional direct G-protein-mediated effect, the ability of FSK and 8-bromo-cAMP to mimic the actions
of ISO suggests that ISO acts predominantly to raise intracellular cAMP levels. These observations are in agreement with previous studies of porcine coronary artery cells and the A7r5 cell line. Regulation of L-type Ca2+ channels by β-adrenoceptors in vascular smooth muscle may therefore be very similar to that previously reported for cardiac muscle. In cardiac muscle cells, cAMP-dependent phosphorylation of L-type Ca2+ channels appears to occur via activation of A kinase.

It is of interest that higher concentrations of ISO (10 μmol/L) caused inhibition of Ba2+ currents after stimulation of the currents. The inhibitory effect of ISO was accompanied by an acceleration of current decay. A similar biphasic response was also produced by a high concentration of FSK (10 μmol/L), suggesting that the inhibitory effect of ISO is also mediated by cAMP. This hypothesis was further supported by experiments in which a higher concentration of 8-bromo-cAMP (1 mmol/L) was also found to cause inhibition of inward current amplitude accompanied by an increase in the rate of current decay. These results suggest that the actions of cAMP on Ba2+ currents are concentration dependent and that cAMP-dependent inhibition of inward currents predominates at higher cAMP levels.

We also found that 8-bromo-cAMP (0.1 mmol/L) reduced the amplitude of inward currents in portal vein myocytes. This result is in agreement with studies of cardiac myocytes. cGMP is widely accepted to be an important second messenger in vascular smooth muscle cells and to mediate the effects of such vasodilators as endothelium-derived relaxing factor (nitric oxide), nitrovasodilators (e.g., sodium nitressoxide), and atrial natriuretic peptide. Sodium nitroprusside has been shown to inhibit both high K+- and norepinephrine-induced 45Ca uptake into tissue strips, suggesting cGMP-mediated suppression of L-type Ca2+ channels. Recent studies in rabbit pulmonary artery cells have provided direct evidence for sodium nitroprusside suppression of voltage-dependent Ca2+ channel currents. Other studies relating cGMP and high Ca2+ levels may be mediated by the same mechanism, i.e., activation of cGMP-dependent protein kinase (G kinase). This hypothesis is supported by several observations. First, although cAMP has a greater affinity for A kinase, it can also activate purified G kinase, with the affinity of cAMP for A kinase being approximately 100-fold greater than for G kinase. Second, ISO (100 μmol/L) and FSK (1 and 10 μmol/L) have been reported to increase the activity of both A kinase and G kinase in intact pig coronary artery strips by elevating cAMP levels without a change in cGMP levels. Third, Lincoln et al have shown that the effects of ISO and FSK on [Ca2+]i in cultured rat aortic smooth muscle cells change with cell passage. In primary cell cultures, both ISO and FSK decreased [Ca2+]i. However, in passaged cells, ISO and FSK increased [Ca2+]i. The change in the actions of ISO and FSK were correlated with a loss of G kinase expression in the passaged cells. cAMP was proposed to enhance the entry of Ca2+ into the cell by an A kinase–mediated mechanism and inhibit the entry of Ca2+ into the cell through a G

Fig 7. Effects of 8-bromo-cAMP (8-Br-cAMP) or 8-bromo-cGMP (8-Br-cGMP) on voltage dependence of Ba2+ current availability. Steady-state inactivation curves were obtained before (○) and after exposure to 0.1 mmol/L (△) or 1 mmol/L (▲) 8-Br-cAMP (a) and before (○) and after exposure to 0.1 mmol/L 8-Br-cGMP (△) (b). Each curve was fitted to a Boltzmann distribution equation. For graph a, the values were as follows: for the control condition, V0.5 = -30.0 mV and k = 7.8 mV, where V0.5 is the membrane potential producing 50% inactivation and k is the slope factor; for 0.1 mmol/L 8-Br-cAMP, V0.5 = -30.2 mV and k = 7.8 mV; and for 1 mmol/L 8-Br-cAMP, V0.5 = -41.1 mV and k = 7.8 mV. For graph b, the values were as follows: for the control condition V0.5 = -29.6 mV and k = 8.3; and for 0.1 mmol/L 8-Br-cGMP, V0.5 = -37.9 mV and k = 8.9 mV.
kinase–mediated mechanism. Thus, in primary cultured cells the G kinase mechanism predominated, and in passaged cells the A kinase–mediated mechanism predominated. All of the above studies therefore implicate an action of cAMP on G kinase and, together with the present study, support the hypothesis that high levels of cAMP in freshly dispersed portal vein cells may activate G kinase, leading to a decrease in Ca\(^{2+}\) channel activity.

The degree of enhancement of Ba\(^{2+}\) currents that was observed with ISO in the present study (ie, 50% for 1 \(\mu\)mol/L ISO) is smaller than that previously reported for cardiac myocytes (sixfold by 0.5 \(\mu\)mol/L ISO\(^55\) and fourfold by 1 \(\mu\)mol/L ISO\(^56\)). The cAMP-dependent enhancement of Ca\(^{2+}\) currents observed in cardiac muscle cells is directly related to the well-known inotropic effects of \(\beta\)-adrenergic stimulation in the heart. In contrast, \(\beta\)-adrenergic stimulation of vascular smooth muscle (or stimulation with other agonists that raise intracellular cAMP levels) leads to vasodilatation\(^57\) with either no change or a small reduction in resting [Ca\(^{2+}\)]\(_i\).\(^32\) The possible physiological role that \(\beta\)-adrenergic stimulation of Ca\(^{2+}\) channels plays in smooth muscle is therefore unclear. It would appear that, although \(\beta\)-adrenergic regulation of Ca\(^{2+}\) channels is similar to that observed in cardiac cells, cAMP activation of G kinase and the direct inhibitory effects of cAMP on contractile proteins may predominate and thus alter the resulting physiological response in smooth muscle. Conceivably, small increases in Ca\(^{2+}\) entry as a result of stimulation of Ca\(^{2+}\) currents by \(\beta\)-adrenergic stimulation may be rapidly sequestered into the superficial sarcoplasmic reticulum, producing no detectable increase in averaged [Ca\(^{2+}\)]\(_i\), measured with fura 2 fluorescence, or may lead to a small local increase in free Ca\(^{2+}\) in a subsarcolemmal region between sarcolemma and superficial sarcoplasmic reticulum.\(^48\) Various membrane conductances can be modified by changes in [Ca\(^{2+}\)]\(_i\).\(^39\) It is possible that one or more of these conductances is modified when Ca\(^{2+}\) current is enhanced by cAMP-dependent mechanisms. Whether this effect would contribute to or limit vasodilatation depends on which conductance changes predominate.

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References


42. Karaki H, Nakagawa H, Urakawa N. Comparative effects of verapamil and sodium nitroprusside on contraction and \(^{45}\text{Ca}\) uptake in the smooth muscle of rabbit aorta, rat aorta and guinea pig taenia coli. *Br J Pharmacol.* 1984;81:393-400.
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