Atrial Natriuretic Factor and Isosorbide Dinitrate Modulate the Gating of ATP-Sensitive K+ Channels in Cultured Vascular Smooth Muscle Cells

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Abstract The effects of atrial natriuretic factor (ANF) and isosorbide dinitrate (ISDN), activators of particulate and soluble guanylate cyclase, respectively, on K+ currents were investigated in patch-clamp recordings of smooth muscle cells cultured from rat thoracic aorta. In the cell-attached patch configuration, ANF enhanced Ca2+-activated K+ (KCa) channel activities as reported previously. When KCa channels were blocked with 1 mmol/L tetraethylammonium or 10-7 mol/L charybdotoxin, ANF and ISDN applied to the bathing solution activated ATP-sensitive K+ (KATP) channels without altering channel conductance. Pretreatment with methylene blue, a guanylate cyclase inhibitor, abolished the effects of ISDN on KATP channels, whereas 8-bromo-cGMP activated these channels, suggesting that the effects of ISDN on KATP channels were mediated by cGMP. Our results suggest that vasorelaxant agents that increase intracellular cGMP concentrations modulate the gating of two major potassium channels, ATP sensitive and Ca2+ activated, that might play an important role in controlling vascular tone by changing the membrane potential. (Circ Res. 1994;74:471-476.)

Key Words • isosorbide dinitrate • vascular smooth muscular cells • 8-bromo-cGMP • ATP-sensitive K+ channels • atrial natriuretic factor

Vascular smooth muscle is relaxed by a number of endogenous and exogenous agents that increase intracellular cGMP.1-3 The nitrovasodilators and endothelium-derived relaxing factor (EDRF) activate the soluble form of guanylate cyclase,4-6 whereas atrial natriuretic factor (ANF) and adenosine act through receptors on the cell surface to activate the particulate form of this enzyme.6,7 Despite extensive studies, the mechanism by which cGMP relaxes smooth muscle remains unknown.

Cyclic nucleotides and their dependent protein kinases are known to act on ion channels. In aortic smooth muscle cells, activation of large conductance Ca2+-activated K+ (KCa) channels by a nitrovasodilator, cGMP, and its primary metabolite, GMP, has been reported.8,9 ATP-sensitive K+ (KATP) channels present in vascular smooth muscle have also been shown to contribute to vascular tone.10-12 To date, however, there have been no published studies on the effects of ANF and nitrovasodilators on this channel. In the present study, we investigated the ability of ANF and isosorbide dinitrate (ISDN) to modulate the activity of KATP channels in vascular smooth muscle cells.

Materials and Methods

Cell Culture

Rats were anesthetized with diethylether and asphyxiated. The thoracic aorta was removed, and after removing the endothelial layer, the remaining tissue was placed in normal Tyrode’s solution and cut into small pieces. The pieces were transferred to culture dishes filled with medium 199 (Nissui Chemical, Japan) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY) and stored in a 37°C incubator in the presence of 5% CO2. Four glass coverslips were placed on the bottom of each culture dish. Single smooth muscle cells that had migrated after 5 to 10 days in primary culture and adhered to the coverslips were used in all experiments. The cultured cells formed cell mounds after growing to confluence, as is characteristic of smooth muscle cells.

Solutions and Chemicals

Normal Tyrode’s solution contained (mmol/L) NaCl 140, KCl 2.7, MOPS buffer (pH 7.2) 7.5, and glucose 5. High K+ solution contained (mmol/L) KCl 140 and K+ MOPS buffer (pH 7.2) 10. Ca2+-EGTA buffer was used to adjust Ca2+ concentrations below 5x10-5 mol/L.

ATP, glibenclamide, methylene blue, and 8-bromo-cGMP (Br-cGMP) were purchased from Sigma Chemical Co, St Louis, Mo. ANF was purchased from Peptide Institute Co, Osaka, Japan, and ISDN was purchased from Eisai Co, Tokyo, Japan.

Electrical Measurements and Analysis

Cells grown on coverslips were placed in an experimental 2-mL chamber on the stage of an inverted microscope (model TMD, Nihon, Tokyo, Japan). All experiments were performed at 35°C to 36°C. Heat-polished glass-patch electrodes with a resistance of 5 to 7 MΩ were used to record one to three single-channel currents. The tips of the patch pipettes were coated with Silgard. Single-channel currents were recorded from cell-attached and inside-out patches with a patch-clamp amplifier (CEZ 2200, Nihon Koden, Tokyo, Japan) and were digitized with a modified digital audio processor (PCM-501 ES, Sony, Tokyo, Japan). The recordings were stored on videotape using a videocassette.
Integumentary System

Results

Two Different Types of K⁺ Channels Activated by ANF

Fig 1A shows the effect of ANF on single-channel currents in cell-attached patches using a pipette voltage of +40 mV (membrane potential, −40 mV). The bathing solution contained 140 mmol/L K⁺, and the pipette solution contained 10⁻⁷ mol/L Ca²⁺ and 140 mmol/L K⁺. A brief channel openings were observed in the control recordings. ANF applied to the bathing solution at a concentration of 10⁻⁶ mol/L increased channel activity. Two types of channels with different conductances and opening kinetics, i.e., one with a large conductance of 120 pico-siemens and a shorter open time and one with a smaller conductance of 15 pico-siemens and a longer time, were observed in three separate experiments.

The K⁺ selectivity of these channels was tested by exposing the cytoplasmic surface of the patch to different concentrations of KCl (NaCl substituting for KCl) in the inside-out patch configuration and measuring the reversal potential in each solution. The reversal potentials of both the 120- and 15-pico-siemens channels obtained from the current-voltage relations were 0 mV with the bathing solution that contained 150 mmol/L K⁺ and −25 and −22 mV, respectively, with the bathing solution that contained 50 mmol/L K⁺, values that were close to the calculated equilibrium potential (−29 mV). Thus, these channels were selective for K⁺ ions. The large-conductance channels were activated when Ca²⁺ was added to the cytosolic side (not shown) and were

Statistical Analysis

Data are expressed as mean±SEM. Intergroup differences were analyzed by the Wilcoxon test. Values of P<.05 were considered statistically significant.
similar to those detected in calf aortic smooth muscle cells. Similar results were obtained in three experiments. Thus, this K⁺ channel was the KCa channel.

The aim of the present study was to test the effects of ANF and ISDN on channels other than KCa channels. Therefore, we performed a series of experiments in which the pipette contained 1 mmol/L tetraethylammonium (TEA) or 10⁻⁷ mol/L charybdotoxin to block KCa channels. By using a pipette filled with a solution of 140 mmol/L K⁺, 10⁻⁷ mol/L Ca²⁺ solution, and 10⁻⁷ mol/L charybdotoxin in cell-attached patches, only the small-conductance (15.0±0.4 picosiemens, n=3) K⁺ channels were recorded. Fig 1B shows the effect of ANF (10⁻⁶ mol/L) on small-conductance K⁺ channels. A few brief channel openings were observed in control recordings by use of a pipette solution containing 10⁻⁷ mol/L Ca²⁺, although this channel was more active with physiological concentrations of extracellular Ca²⁺ (>10⁻⁴ mol/L, data not shown). By using a pipette containing 1 mmol/L TEA or 10⁻⁷ mol/L charybdotoxin, the open probabilities of these channels were determined to be 0.103±0.08 in the absence and 0.493±0.169 in the presence of 10⁻⁴ mol/L ANF (n=4, P<.05). These channels activated by ANF were blocked by 20 μmol/L glibenclamide.

Fig 2 shows the effects of ATP and glibenclamide on the small-conductance K⁺ channels. Recordings were made from inside-out patches by use of a pipette containing 140 mmol/L K⁺, 10⁻⁷ mol/L Ca²⁺, and 1 mmol/L TEA (tracing a). In inside-out patches in the absence of ATP, more channel activities were observed than in cell-attached patches. The recorded channel

![Figure 3](image-url) Fig 3. Recordings of single-channel currents in cell-attached patches and amplitude histograms showing the effects of isosorbide dinitrate (ISDN) on steady-state single ATP-sensitive K⁺ channels at a pipette voltage of +40 mV. The pipette solution contained 140 mmol/L K⁺, 10⁻⁷ mol/L Ca²⁺, and 1 mmol/L tetraethylammonium, and the bath solution contained 140 mmol/L K⁺. The bin width of the amplitude histograms is 0.1 pA.

![Figure 4](image-url) Fig 4. Dose-response curve for isosorbide dinitrate (ISDN). The mean open probabilities of ATP-sensitive K⁺ channels, shown in Fig 3, are plotted against various ISDN concentrations. The data for the dose-response curve are the mean±SEM of four observations. EC₅₀ of ISDN was 1.17x10⁻⁷ mol/L.
activity was suppressed by 1 mmol/L ATP applied to the cytosolic side (tracing b) and was almost completely blocked by 20 μmol/L glibenclamide, a specific blocker of a K\textsubscript{ATP} channel (tracing c). Thus, these channels appeared to be K\textsubscript{ATP} channels.

**Effect of ISDN on K\textsubscript{ATP} Channels**

We also studied the effect of ISDN, which increases intracellular cGMP concentrations, on K\textsubscript{ATP} channels. Fig 3 shows the effect of ISDN on cell-attached patches by use of a pipette containing 10\textsuperscript{-7} mol/L Ca\textsuperscript{2+} and 1 mmol/L TEA at a pipette voltage of +40 mV. In the absence of ISDN, the open probability was 0.08 (panel A). ISDN applied at various concentrations to the bath cumulatively increased the open probabilities, as shown in panels B through D. These channels had small conductances (15.1±0.3, \(n=4\)) and were abolished by 20 μmol/L glibenclamide (not shown). Fig 4 shows the relation between ISDN concentration in the bath and K\textsubscript{ATP} channel open probability. ISDN activated the K\textsubscript{ATP} channels in a concentration-dependent manner but did not alter their individual conductance.

**Effect of Br-cGMP on K\textsubscript{ATP} Channels**

Both ANF and ISDN are known to activate guanylate cyclase and cGMP. Therefore, we next examined the effects of a membrane-permeant analogue of cGMP on the K\textsubscript{ATP} channels. Fig 5 shows single-channel recordings and amplitude histograms of K\textsubscript{ATP} channels before and after application of 0.3 mmol/L Br-cGMP. When applied to the bath, Br-cGMP caused a large increase in channel activity. The open probability was 0.052±0.024 in the absence and 0.32±0.086 with 0.3 mmol/L in the presence of Br-cGMP (\(P<.05, n=4\)).

Fig 6 shows the current-voltage curves of Br-cGMP-induced, ANF-induced, and ISDN-induced channels. The single-channel conductance activated by Br-cGMP was 14.7±0.5 pico siemens (\(n=3\)) and closely resembles conductances activated by ANF and ISDN.

To investigate further the mechanism by which ISDN activated K\textsubscript{ATP} channels, we tested the effects of ISDN on K\textsubscript{ATP} in the presence of methylene blue. After prolonged exposure (10 to 15 minutes) to 10\textsuperscript{-5} mol/L methylene blue, the effects of ISDN on K\textsubscript{ATP} channels were markedly abolished (Fig 7, tracings a and b). However, Br-cGMP applied to the bath, as well as the absence of methylene blue (tracing c), increased the channel activity. The open probabilities were 0.038±0.021 in the control condition, 0.046±0.026 in the presence of 10\textsuperscript{-5} mol/L ISDN, and 0.331±0.098 in the presence of 0.3 mmol/L Br-cGMP (\(n=3\)).

**Discussion**

The aim of our investigation was to assess whether, in addition to their well-known effects on K\textsubscript{Ca} channels,
ANF and ISDN modulate $K_{\text{ATP}}$ channels. We found that both compounds activated $K_{\text{ATP}}$ channels in smooth muscle cells cultured from rat aorta without changing the amplitude of individual channel currents. These results provide a possible explanation of hyperpolarizing effects of ANF and ISDN.

The $K_{\text{Ca}}$ channel is modulated by a number of intracellular second messengers. Williams et al. and Fujino et al. have demonstrated that both nitrocompounds and cGMP activate the large-conductance $K_{\text{Ca}}$ channel in vascular smooth muscle cells. In the present study, we confirmed that bath-applied ANF activates $K_{\text{Ca}}$ channels in cell-attached patches, presumably through the cGMP–protein kinase pathway.

There have been no published studies on the effect of cyclic nucleotides on $K_{\text{ATP}}$ channels in vascular smooth muscle, although $K^+$ channel openers are known to modulate this channel. In the present study, we found that ANF and ISDN activated $K_{\text{ATP}}$ channels. In the present study, the effector concentration for half-maximum response (EC$_{50}$) of ISDN was 1.17 × 10$^{-7}$ mol/L, and the threshold concentration was estimated to be between 10$^{-7}$ and 10$^{-6}$ mol/L. ISDN has been reported to relax coronary arterial rings at similar concentrations (10$^{-4}$ to 10$^{-5}$ mol/L). Br-cGMP activated these channels and had effects similar to those of ANF and ISDN, suggesting that ANF and ISDN modulation of $K^+$ channels is mediated by cGMP. Bkaiy also has reported that ANF opens a cGMP-sensitive delayed outward-rectifying $K^+$ channel with large unitary channel conductance (80 picosiemens), but this author did not study the effects of ATP or glibenclamide on this channel. The $K_{\text{ATP}}$ channels studied in the present report had no inward or outward rectification, suggesting that they are different channels. It has been reported that 5 × 10$^{-7}$ mol/L ANF elevates intracellular cGMP content from 0.5 to 52.7 pmol/mg protein in rat aortic smooth muscle cells, and 2 × 10$^{-7}$ to 10$^{-6}$ mol/L ISDN elevates cGMP content up to 550% to 800% of the control value. It has been reported that only high concentrations of Br-cGMP (>0.11 mmol/L) are effective in causing relaxation and hyperpolarization. Thus, we tested 0.3 mmol/L Br-cGMP in referring to previous reports, although we could not measure intracellular cGMP content after the administration of Br-cGMP.

The vasorelaxant effect of nitrovasodilators is thought to be due in part to hyperpolarization of the membrane, and hyperpolarization by nitroglycerin and nitroprise has been reported. The present study indicates that ISDN-induced hyperpolarization might be caused by the activation of $K_{\text{ATP}}$ channels. Brayden has demonstrated that acetylcholine-induced hyperpolarization of rabbit cerebral middle arteries was reversed by glibenclamide, a specific blocker of $K_{\text{ATP}}$ channels. In his study, exogenous nitric oxide relaxed but did not hyperpolarize vascular smooth muscle cells, and methylene blue did not inhibit acetylcholine-induced hyperpolarization. He concluded that glibenclamide-sensitive hyperpolarization, indicating a role for the $K_{\text{ATP}}$ channel, was not due to nitric oxide. In the present study, however, pretreatment of methylene blue markedly abolished the effects of ISDN on $K_{\text{ATP}}$ channels. Recently, Tare et al. have found that hyperpolarization and relaxation evoked by acetylcholine are reduced by $N^\text{O}$-monomethyl-L-arginine, an inhibitor of nitric oxide biosynthesis from L-arginine, indicating the possibility that nitric oxide derived from the endothelium can cause hyperpolarization of vascular smooth muscle.

In vascular smooth muscle cells, $K_{\text{ATP}}$ channels are active at the resting membrane potential and play an important role in generating the resting potential. Our results suggest that, in the smooth muscle cells of the rat aorta, vasorelaxant agents that increase intracellular cGMP concentrations activate two major classes of $K^+$ channels, ATP sensitive and Ca$^{2+}$ activated. Modulation of these channels contributes not only to the resting membrane potential and vascular tone but also plays an important role in controlling vascular tone under contracted conditions.

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