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Activation of the cGMP-Dependent Protein Kinase Mimics the Stimulatory Effect of Nitric Oxide and cGMP on Calcium-Gated Potassium Channels

V. HAMPL, J.M. HUANG, E.K. WEIR, S.L. ARCHER

Department of Medicine, Medical Center and University of Minnesota, Minneapolis, USA

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Summary

Nitric oxide (NO) is an endogenous vasodilator and inhaled NO is a promising therapeutic agent for the treatment of pulmonary hypertension. However, NO's mechanism of action is not completely understood. Previous studies have shown that NO increases intracellular levels of cyclic guanosine 3',5'-monophosphate (cGMP) and that leads to activation of calcium-gated potassium channels in vascular smooth muscle cells. Resulting cell membrane hyperpolarization causes vasorelaxation. The potassium channel activation by NO is inhibited by a blockade of cyclic nucleotide-dependent protein kinases, suggesting a key role of these enzymes in NO-induced vasodilation. To further examine this mechanism, we tested the hypothesis that pharmacological stimulation of the cGMP-dependent protein kinase will simulate the activating effect of NO on potassium channels. Indeed, we found that (Sp)-guanosine cyclic 3',5'-phosphorothioate (1 μM), a selective activator of the cGMP-dependent protein kinase, dramatically increased potassium currents measured by the whole-cell patch clamp technique in freshly dispersed pulmonary artery smooth muscle cells. These currents were inhibited by an inhibitor of calcium-gated potassium channels, charybdotoxin. Our results support the hypothesis that the effect of NO on potassium channels is mediated by the cGMP-dependent protein kinase.

Key words

Nitric oxide – Cyclic guanosine 3',5'-monophosphate – Potassium channel – Protein kinase – Patch clamp technique

Introduction

NO is an important endogenous vasodilator in the pulmonary circulation (Archer *et al.* 1994a) and a promising therapeutic agent in pulmonary hypertension (Zapol *et al.* 1994). However, the mechanism of the NO-induced vasodilation is not completely understood. It is known that NO causes vasodilation by activating guanylate cyclase and by increasing cGMP levels in vascular smooth muscle (VSM) cells (Craven and DeRubertis 1978) but how exactly cGMP reduces vascular tone is less clear.

We have proposed a mechanism for NO-induced pulmonary vasodilation, schematically illustrated in Fig. 1. We hypothesize that cGMP synthesized by guanylate cyclase in response to NO or other stimuli activates cGMP-dependent protein kinase, which in turn phosphorylates and thus opens potassium channels in the VSM plasma membrane. Resulting K^+ efflux leads to membrane hyperpolarization, reduced Ca^{2+} influx through the voltage-gated calcium channels, and vasodilation. There is evidence for NO/cGMP-induced VSM hyperpolarization (Furchgott 1984, Tare *et al.* 1990, Garland and McPherson 1992, Krippeit-Drews *et al.* 1992), K channel activation (Fujino *et al.* 1991, Archer *et al.* 1993b, Robertson *et al.* 1993, Archer *et al.* 1994b), and a decrease in intracellular Ca^{2+} concentration (Twort and van Breemen 1988). Recently, we have found that an inhibitor of the cyclic nucleotide-dependent protein kinases, N-[2-(methylamino)ethyl]-5-isoquinlinesulfonamide (H-8), prevents NO/cGMP-induced K channel activation, implying a role for the cyclic nucleotide-dependent protein kinases (Archer *et al.* 1994b). Here we present further evidence for the involvement of protein kinase, specifically the cGMP-dependent type, in the NO/cGMP-induced K channel activation. We stimulated cGMP-dependent protein

kinase by its selective activator, (Sp)-guanosine cyclic 3',5'-phosphorothioate, (Sp)-cGMP[S] (Butt *et al.* 1990), and found that it increased whole-cell K^+ currents (I_K), as described earlier with NO/cGMP.

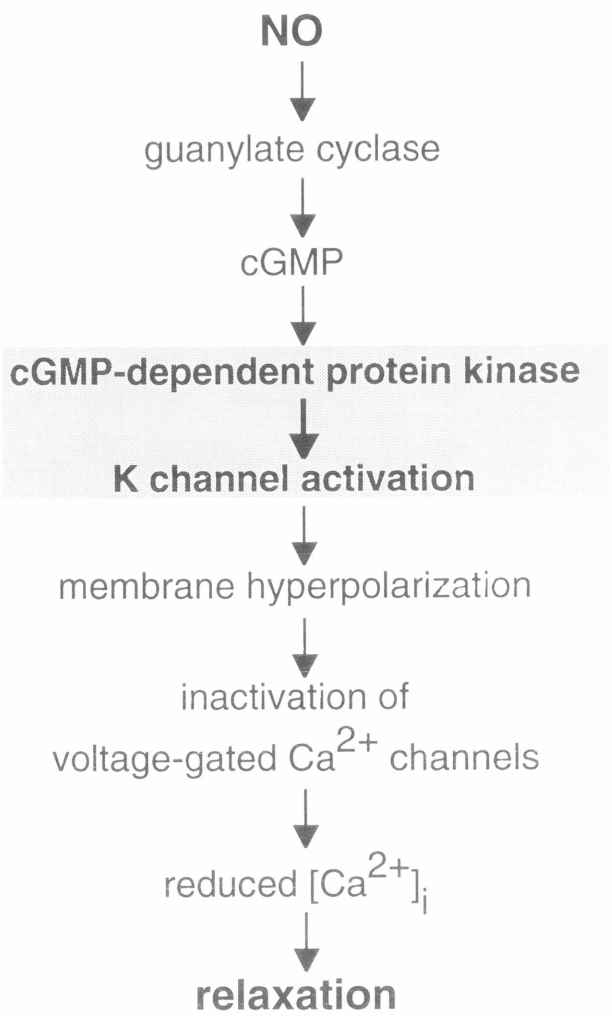

Fig. 1
Proposed mechanism of NO-induced vasodilation. The highlighted area shows the step studied in this report, i.e. the ability of cGMP-dependent protein kinase to activate K channels. See text for detailed discussion.

Methods

Experimental design. I_K was measured by the whole cell patch clamp technique in freshly dispersed rat pulmonary artery VSM cells. The patch pipette contained (Sp)-cGMP[S] (1 μM) in the experimental but not in the control group of cells (n=4 and 5, respectively). The relationship between I_K and membrane potential (I/V) was measured immediately after the membrane rupture with the pipette (before significant drug diffusion throughout the volume of the cell could have occurred) and then every 2 min for 20 min. The type of K channel involved was characterized by quantifying the inhibitory effects of charybdotoxin (CTX; 200 nM), tetraethylammonium (TEA; 10 mM), and 4-aminopyridine (4-AP; 5 mM). CTX is an inhibitor of the calcium-dependent K channel (K_{Ca} channel), whereas 4-AP preferentially inhibits the delayed rectifier K channel. Low dose TEA is somewhat selective for the K_{Ca} channel. The contribution of the ATP-sensitive K channels was excluded by high ATP concentration in the patch pipette (5 mM).

Cell isolation. Adult, male, specific pathogen free, Sprague Dawley rats were anaesthetized with Nembutal (50 mg/kg i.p.) and mechanically ventilated with air. After thoracotomy, the pulmonary artery was cannulated *via* the right ventricular incision. The lungs were then perfused for several minutes at ~10 ml/min with ice-cold Hank's solution (pH=7.3) containing NaCl (145 mM), KCl (5 mM), $MgCl_2$ (1.0 mM), HEPES (10 mM), and glucose (10 mM). The lungs were then removed from the body and placed in the same solution for 10–30 min. Using a dissecting microscope, 2–3 mm long rings of the left and right branches of the main pulmonary artery were isolated. They were placed in oxygenated, ice-cold Hank's solution of the same composition as described above with added $CaCl_2$ (0.05 mM). The connective tissue was removed and small pieces of artery were incubated for 30 min at 37 °C in a nominally Ca^{2+}-free saline solution, followed by a solution containing 50 μM $CaCl_2$, bovine albumin (0.1 %) and protease type XXIV (0.02 %). After 35 min, segments were washed with Ca^{2+}-free solution and cells were separated by trituration. Cells were centrifuged (1000 rpm for 5 min) and resuspended in a saline solution containing 10 mM $CaCl_2$.

Patch clamp measurements. I_K was measured at 22 °C using the standard whole cell patch clamp technique (Hamill *et al.* 1981, Archer *et al.* 1993a). The patch pipette had 1.5 µm outside diameter and resistance ~3 MΩ. The pipette solution (pH=7.2) contained KCl (140 mM), $MgCl_2$ (1.0 mM), HEPES (10 mM), ATP (dipotassium salt, 5 mM), phosphocreatine (disodium salt, 2 mM), and EGTA (1 mM). The cells were studied in a bath solution containing NaCl (145 mM), KCl (5.4 mM), $MgCl_2$ (1.0 mM), $CaCl_2$ (1.5 mM), HEPES (10 mM), and glucose (10 mM). Po_2 was 140 mm Hg, pH was 7.4. This solution was perfused through the chamber housing the cells (on a microscope stage) at 2 ml/min.

The cells were held at a membrane potential of −70 mV. To measure the I/V relationship, the cells were exposed to a series of depolarizing pulses (650 ms each, 10 s intervals) from +70 to −50 mV in 20 mV decrements. Resulting currents were amplified (Axopatch 1D amplifier, Axon Instruments, Foster City, CA, USA), filtered at 2 kHz and recorded at 2.5 kHz on a personal computer using pCLAM 5.1 software and Labmaster TL-1 DMA interface. Series

resistance was compensated (20–60 %) using the compensation controls.

Drugs. (Sp)-cGMP[S] was purchased from Biolog Life Sciences Institute (Bremen, Germany). CTX was from Calbiochem (La Jolla, CA, USA). All other drugs and reagents were from Sigma (St. Louis, MO, USA). All drugs were dissolved in normal saline.

Statistics. Results are reported as the means ± S.E.M. The I/V curves were evaluated and compared between groups with the repeated measures ANOVA using a StatView 4.02 computer program (Abacus Concepts, Berkeley, CA, USA). $P<0.05$ was considered statistically significant.

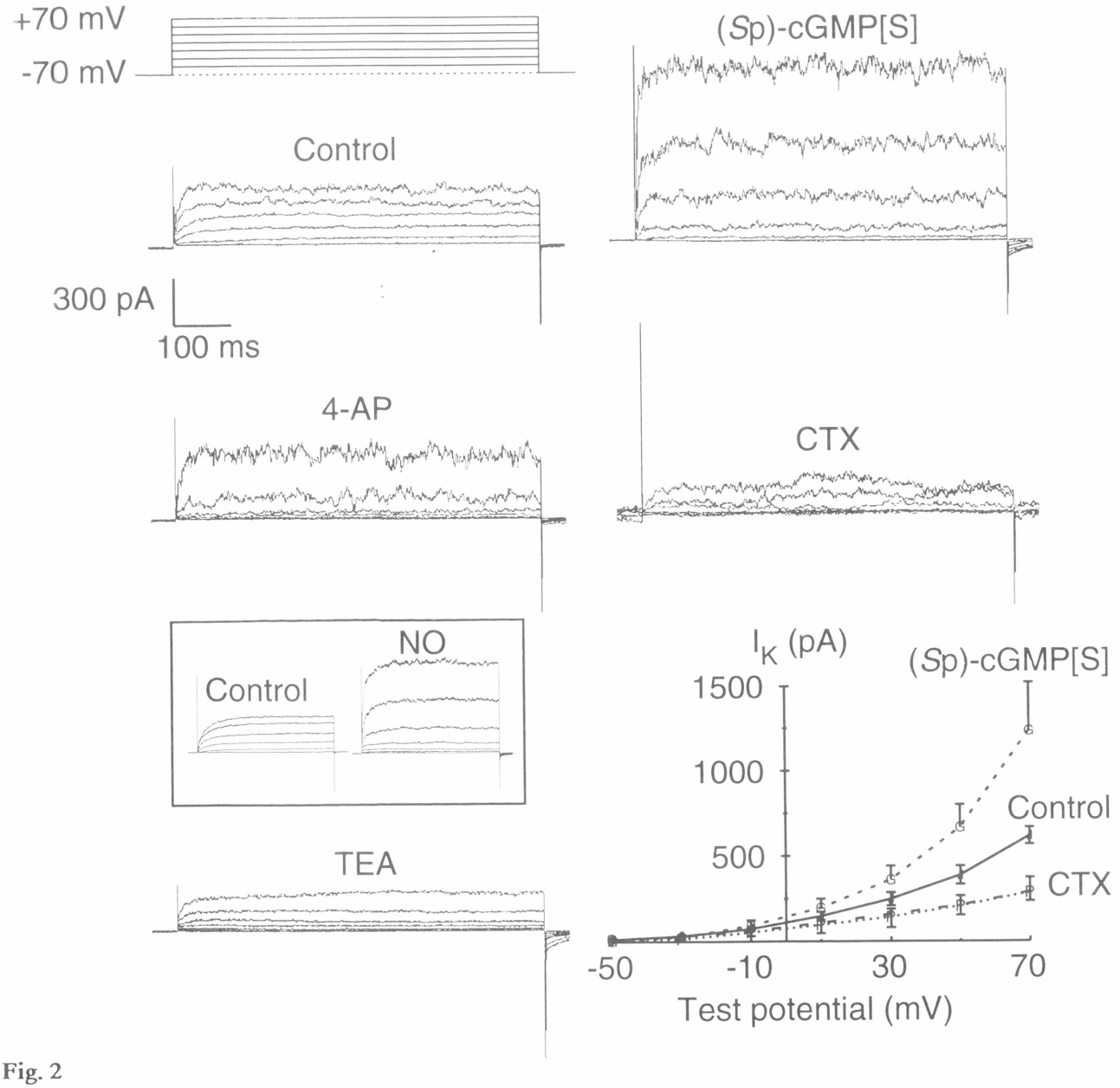

Fig. 2

A selective activator of cGMP-dependent protein kinase, (Sp)-cGMP[S], opens charybdotoxin (CTX)-sensitive K channels. The upper row shows the families of K^+ currents (I_K) elicited in one cell by a series of depolarizations from −70 to +70 mV (schematically illustrated in the upper left corner) immediately after obtaining a stable seal (control) and 18 min later after (Sp)-cGMP[S] diffused from the patch pipette into the cytosol. The middle row shows that I_K in the same cell is more sensitive to CTX than 4-aminopyridine (4-AP). The left lower panel shows the sensitivity of I_K in the same cell to low dose TEA. The right lower panel shows the relationship between membrane potential and I_K (mean ± S.E.M.) in a control group of cells ($n=5$) and in cells exposed to (Sp)-cGMP[S] for 20 min. Note that the curves overlap when the cells are treated with CTX. The control, (Sp)-cGMP[S], and CTX curves differ significantly by repeated measures ANOVA ($P<0.05$). The inset in the frame shows, for comparison, an example of the I_K response to NO.

Results

All of the cells studied displayed I_K dominated by K_{Ca} channel as judged from greater sensitivity to CTX and low dose TEA than to 4-AP (Fig. 2). The activation threshold potential of −40 mV was not altered by (Sp)-cGMP[S]. In the control group, I_K was stable for the entire study period (>20 min).

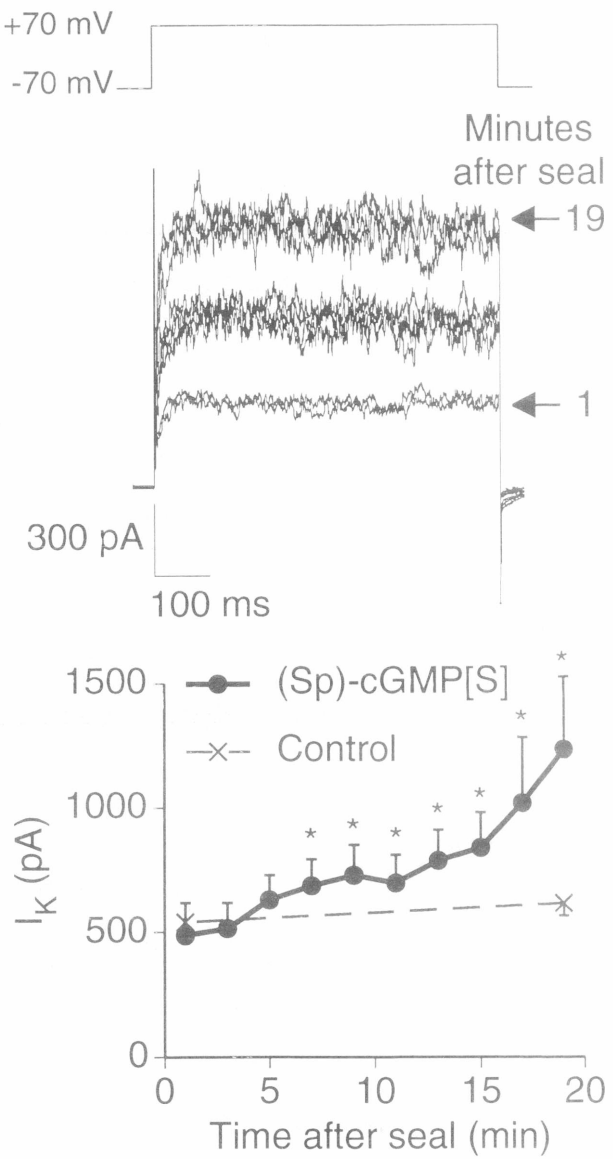

Fig. 3
(Sp)-cGMP[S] increases K^+ currents in a time-dependent manner. The upper panel shows K^+ currents elicited in a single cell by depolarization from −70 to +70 mV (schematically illustrated above the panel) in 2 min intervals starting 1 min after obtaining a seal (the lowermost trace). The lower panel shows the mean ± S.E.M. values for the whole group treated with (Sp)-cGMP[S] (n=4) compared with the control group (n=5). * $P<0.05$ vs. time = 1 min.

In the group of cells where the patch pipette contained (Sp)-cGMP[S], the I_K was not significantly different from the control group immediately after a stable seal has been obtained, confirming that the two groups consisted of comparable cells. However, unlike in the control cells, the I_K rose in the cells treated with (Sp)-cGMP[S] as the drug diffused from the pipette into the cytosol. The rise started right after obtaining a seal and continued throughout the entire study period (Fig. 3). By the 7th min I_K was significantly greater than immediately after obtaining a seal. The I/V curves were compared statistically between control and (Sp)-cGMP[S] groups after 20 min, and the difference was found significant (Fig. 2). This effect of (Sp)-cGMP[S] resembled the previously described effect of NO and cGMP on these cells (Fig. 2 inset).

Discussion

In context of an effort to clarify the mechanism of NO/cGMP-induced vasodilation, this study tested the hypothesis that stimulation of cGMP-dependent protein kinase activates K_{Ca} channels in VSM isolated from the rat pulmonary artery. As expected, the relatively selective activator of cGMP-dependent protein kinase, (Sp)-cGMP[S], greatly increased I_K, and this effect was inhibited by CTX.

We and others have shown previously that agents which increase cGMP levels in VSM, including NO, activate K channels (Fujino *et al.* 1991, Archer *et al.* 1993b, Robertson *et al.* 1993, Archer *et al.* 1994b). In the pulmonary artery VSM cells, the K channels activated by NO/cGMP were inhibited by CTX but not by 4-AP (Archer *et al.* 1994b), indicating that the K channel sensitive to NO/cGMP is the K_{Ca} channel rather than delayed rectifier.

cGMP is a physiologic activator of the cGMP-dependent protein kinase, although it can also stimulate the cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (Schmidt *et al.* 1993). Cyclic nucleotide-dependent protein kinases are abundant in smooth muscles and phosphorylate many intracellular proteins (Thomas *et al.* 1990, Lincoln and Cornwell 1993). Cyclic nucleotide-dependent protein kinase mediation thus appears to be one possible mechanism of cGMP-induced K channel activation. An alternative option would be a direct gating of the K channel by cGMP, as described in the retinal rod photoreceptor cells (Kaupp 1991). Recently, we attempted to distinguish between these two possibilities by employing an inhibitor of cyclic nucleotide-dependent protein kinases, H-8. We found that H-8 prevented the K_{Ca} channel activation by NO/cGMP (Archer *et al.* 1994b), indicating that, in the pulmonary artery VSM cells, a functional cyclic nucleotide-dependent protein kinase is necessary for the effect of cGMP on the K_{Ca} channel. Thus cGMP-gated

K channel does not appear to exist in the pulmonary artery VSM cells. However, although H-8 preferentially inhibits cGMP-protein kinase, it can also block the cAMP-protein kinase. Hence, those experiments could not conclusively distinguish between the role of cGMP- and cAMP-dependent protein kinases.

In the present experiment we used an opposite approach: rather than inhibiting protein kinases, we utilized a selective activator of cGMP-dependent protein kinase to see whether it will mimic the effects of NO/cGMP on K_{Ca} channels. Our positive finding – I_K dramatically increased by (Sp)-cGMP[S] – confirms that cGMP-dependent protein kinase has the ability to mediate the effect of cGMP on K_{Ca} channels, and suggests that an additional contribution by cAMP-dependent protein kinase is not necessary. Furthermore, although cGMP-dependent protein kinase may directly phosphorylate the K channel, the possibility that other proteins could be important intermediary kinase targets cannot be excluded (Minami *et al.* 1993).

The current results are an additional piece in the mosaic of experimental evidence (Archer *et al.* 1994b) supporting the concept of NO-induced pulmonary vasodilation outlined in Fig. 1. The important role of K_{Ca} channels was proved by showing that NO increases CTX-sensitive K currents in pulmonary artery VSM cells and that CTX reduces NO-induced relaxation of pulmonary artery rings. Similar effects of NO and cGMP on K_{Ca} channels, as well as the inhibition of the NO action by guanylate cyclase blocker methylene blue, indicate that the effect of NO is mediated by cGMP rather than by a direct effect of NO on K channels suggested by Bolotina *et al.* (1994). Preventing and mimicking the effect of NO, respectively, by H-8 and (Sp)-cGMP[S] support the obligatory involvement of cyclic nucleotide-dependent protein kinases, especially the cGMP-dependent type.

The NO-cGMP-protein kinase-K_{Ca} channel vasodilatory pathway investigated in this study probably may not be the only mechanism of NO/cGMP-induced vasodilation. It could act synergistically with other mechanisms, such as the protein kinase-dependent phosphorylation of the myosin light chain (Lincoln and Cornwell 1993) or reduction in calcium release from the sarcoplasmic reticulum mediated by cyclic ADP-ribose (Lee 1994).

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Reprint Requests

V. Hampl, Ph.D., Research Service (151), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417, USA