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Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase

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ABSTRACT **Nitric oxide (NO)-induced relaxation is associated with increased levels of cGMP in vascular smooth muscle cells. However, the mechanism by which cGMP causes relaxation is unknown. This study tested the hypothesis that activation of Ca-sensitive K (K_{Ca}) channels, mediated by a cGMP-dependent protein kinase, is responsible for the relaxation occurring in response to cGMP. In rat pulmonary artery rings, cGMP-dependent, but not cGMP-independent, relaxation was inhibited by tetraethylammonium, a classical K-channel blocker, and charybdotoxin, an inhibitor of K_{Ca} channels. Increasing extracellular K concentration also inhibited cGMP-dependent relaxation, without reducing vascular smooth muscle cGMP levels. In whole-cell patch-clamp experiments, NO and cGMP increased whole-cell K current by activating K_{Ca} channels. This effect was mimicked by intracellular administration of (S_P)-guanosine cyclic 3′,5′-phosphorothioate, a preferential cGMP-dependent protein kinase activator. Okadaic acid, a phosphatase inhibitor, enhanced whole-cell K current, consistent with an important role for channel phosphorylation in the activation of NO-responsive K_{Ca} channels. Thus NO and cGMP relax vascular smooth muscle by a cGMP-dependent protein kinase-dependent activation of K channels. This suggests that the final common pathway shared by NO and the nitrovasodilators is cGMP-dependent K-channel activation.**

Nitric oxide (NO) and nitrovasodilators cause vasodilatation by activating guanylate cyclase and increasing cGMP in vascular smooth muscle (VSM) (1). The mechanism by which cGMP reduces vascular tone has been uncertain. Several experiments suggest that cGMP-mediated vasodilation is associated with changes in membrane potential. (*i*) KCl, which depolarizes VSM cells, inhibits endothelium-dependent vasodilatation (2). (*ii*) NO itself hyperpolarizes VSM in many (3–5), but not all (6, 7), studies. Finally, agents that increase cGMP can activate K channels (8–10). K-channel activity is the main determinant of membrane potential, and K efflux resulting from K-channel opening causes hyperpolarization, inhibits voltage-gated Ca channels, and promotes relaxation (Fig. 1).

The current investigation evaluated two hypotheses: (*i*) K-channel activation is essential for cGMP-induced VSM relaxation and (*ii*) increases in cGMP activate K channels by stimulating cGMP-dependent protein kinase (cGK).

To precisely characterize the role of NO/cGMP-activated K channels in vascular relaxation, it is necessary to combine studies of vascular tone [isolated pulmonary artery (PA) rings] and electrophysiology (whole-cell patch-clamp studies of PA VSM). These studies prove that NO and agents that increase cGMP cause relaxation in large part by a cGK-mediated activation of Ca-sensitive K (K_{Ca}) channels.

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MATERIALS AND METHODS

Drugs. Drugs and reagents were from Sigma and were dissolved in normal saline unless otherwise stated. Bath concentrations of solvents were <0.1% and all vehicles were tested to exclude nonspecific effects. Saturated NO solutions (2–3 mM) were prepared and concentrations were determined as described (11).

Vascular Rings. Second-division PA rings were dissected from adult male specific-pathogen-free Sprague–Dawley rats anesthetized with Nembutal (50 mg/kg i.p.) and mounted at a resting tension of 800 mg in Earle's solution (12). Rings were denuded of endothelium by rubbing with a metal probe in all experiments except those involving zaprinast, a cGMP phosphodiesterase inhibitor that caused little relaxation unless the endothelium was intact (12). Rings constricted with norepinephrine (NE, 31 nM) were given acetylcholine (ACh, 10^{-8} to 10^{-5} M), which reduced tension in rings with ($-90.2 \pm 5.2\%$) but not without ($-4.7 \pm 2.7\%$) endothelium. Denuded rings were constricted with KCl (80 mM) or NE (31 nM) and relaxed with 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM), a cyclic nucleotide phosphodiesterase inhibitor, or authentic NO (10^{-8} to 10^{-4} M). The effect of pretreatment with K-channel inhibitors tetraethylammonium (TEA, 30 mM), 4-aminopyridine (4-AP, 10 mM), or charybdotoxin (CTX, 200 nM; Calbiochem) ($n \geq 5$ rings per group) on IBMX- and NO-induced relaxation was compared to control (no inhibitor) in both NE- and KCl-constricted rings. The doses of K-channel blockers used have been shown to inhibit whole-cell K current (I_K) in PA VSM cells (13). Rings were flash frozen in liquid N_2 at the nadir in tension for cGMP analysis (14). To exclude nonspecific impairment of relaxation, the effect of TEA was compared in rings relaxed with the cGMP phosphodiesterase inhibitor zaprinast (10^{-7} to 10^{-4} M), gift from Rhône-Poulenc Rorer, Dagenham, England, dissolved in dimethyl sulfoxide vs. papaverine (10^{-7} to 10^{-5} M), a cGMP-independent vasodilator that inhibits the voltage-gated Ca channel (15, 16).

Electrophysiology. Enzymatically dispersed rat VSM cells were obtained from second-division PAs (13). I_K was measured at 22°C using whole-cell patch-clamp technique (17). The microelectrodes had a 1.5-μm o.d. and a resistance of $\approx$3 MΩ (13). The pipette solution contained 140 mM KCl, 1.0 mM $MgCl_2$, 10 mM Hepes, 5 mM ATP (dipotassium salt), 2 mM phosphocreatine (disodium salt), and 5 mM EGTA (pH 7.2). When it became apparent that NO/cGMP-responsive

Abbreviations: NO, nitric oxide; 8-Br-cGMP, 8-bromo-cGMP; VSM, vascular smooth muscle; K_{Ca} channel; Ca-sensitive K channel; cGK, cGMP-dependent protein kinase; PA, pulmonary artery; CTX, charybdotoxin; TEA, tetraethylammonium; I_K, whole-cell K current; (S_P)-cGMP[S], (S_P)-guanosine cyclic 3′,5′-phosphorothioate; 4-AP, 4-aminopyridine; NE, norepinephrine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinlinesulfonamide; ACh, acetylcholine; IBMX, 3-isobutyl-1-methylxanthine.

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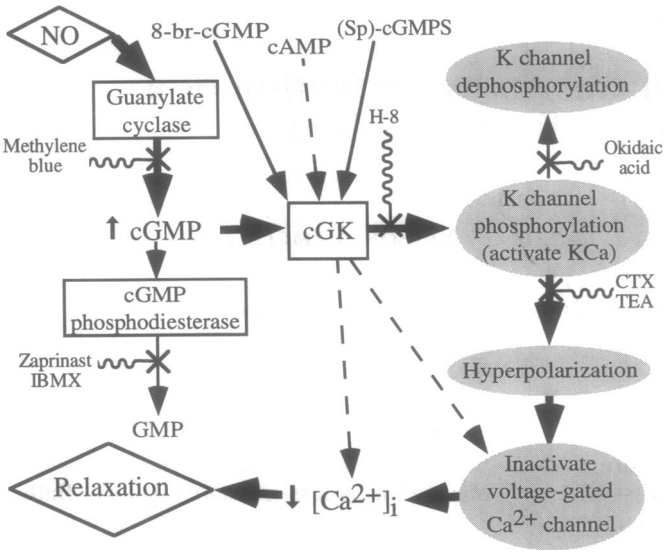


FIG. 1. Proposed mechanism for NO/cGMP-mediated VSM relaxation. cGMP activates cGK, which promotes opening of K_{Ca} channels. This leads to membrane hyperpolarization, inhibition of the voltage-gated Ca^{2+} channel, and relaxation. $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

cells displayed a current dominated by K_{Ca} channels, a lower EGTA concentration (1 mM) was used in further experiments, including those with okadaic acid, (S_P)-guanosine cyclic 3',5'-phosphorothioate {(S_P)-cGMP[S]}, and an additional control group. This did not alter the percent of cells that were sensitive to NO and CTX.

The chamber containing the cells was perfused (2 ml/min; Po_2, 140 mmHg) on a microscope stage with a solution containing 145 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM Hepes, and 10 mM glucose (pH 7.4). Cells were held at −70 mV and then stepwise changes in holding potential up to +70 mV were made in 20-mV increments at 10-s intervals (pulse duration, 650 ms). Currents were amplified (Axopatch 1D amplifier; Axon Instruments, Foster City, CA), filtered at 2 kHz, and recorded at 2.5 kHz on a microcomputer using Axopatch 5.1 software and interface. Series resistance was compensated (20–60%) using the compensation controls. In most experiments, the cell served as its own control (e.g., the I_K after intervention were normalized to the I_K at +70 mV under control conditions). This corrects for variations in the magnitude of I_K among cells of different sizes. For cGMP, methylene blue, and okadaic acid, which were added to the patch pipette, the initial I_K on penetration of the cell was considered the "control" value for normalization. In six control cells (no agents given), I_K was stable for >30 min.

The effects of increasing cGMP by three strategies were studied. (*i*) Authentic NO (2 μM) was given close to the cell surface with perfusion suspended. (*ii*) cGMP was given in the patch pipette (1 mM) or in the bath solution as a membrane-permeable analog, 8-bromo-cGMP (8-Br-cGMP, 2 mM) (18). (*iii*) cGMP phosphodiesterase was inhibited by IBMX (0.1 mM) or zaprinast (0.2 mM).

To determine whether NO activated K channels through its ability to increase cGMP, NO was given to five cells while the patch pipette contained the guanylate cyclase inhibitor methylene blue (0.1 mM). The type of K channel activated by intracellular cGMP was characterized by noting the effects of CTX (200 nM), TEA (10 mM), and 4-AP (5 mM) on the responses to NO and 8-Br-cGMP.

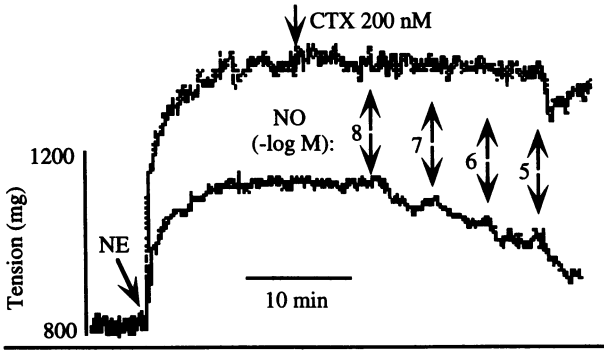
To ascertain whether K-channel activation depended on cGK, NO and 8-Br-cGMP were given to cells (five cells per group) 25 min after a stable seal was obtained with a pipette containing the cyclic nucleotide-dependent protein kinase inhibitor H-8 {*N*-[2-(methylamino)ethyl]-5-isoquinlinesulfonamide in 0.1% ethanol; 0.3 mM; Calbiochem} (19). The effects of (S_P)-cGMP[S], a preferential cGK activator (20) (1 μM in the pipette; BioLog Life Sciences Institute, Bremen, Germany), and okadaic acid, a protein phosphatase inhibitor (21) (2 μM, in the patch pipette), on I_K were assessed.

Statistics. Values are the mean ± SEM. Intergroup differences were assessed by two-tailed paired *t* tests or ANOVA (factorial or repeated measures, as appropriate) with post hoc analysis using a Fisher's protected least significant difference test or means comparison contrast (STATVIEW and SUPERANOVA, Abacus Concepts, Berkeley, CA). A $P \leq 0.05$ was considered statistically significant.

RESULTS

Vascular Rings. Increasing extracellular K concentration virtually eliminated IBMX- and NO-induced relaxation (Fig. 2). Relaxation to IBMX was −7 ± 5% in rings precontracted with KCl, as opposed to −100 ± 18% in those contracted with NE ($P < 0.05$). However, cGMP levels were similar in IBMX-treated denuded rings constricted with NE or KCl (2.1 ± 0.7 and 2.7 ± 1.0 pmol/g of tissue, respectively; $P = 0.59$). Rings receiving both NO and IBMX had similar cGMP content whether constricted with NE or KCl (8.9 ± 4.9 and 5.5 ± 1.4 pmol/g of tissue, respectively; $P = 0.44$).

TEA and CTX decreased the cumulative relaxation to NO (10^{-8} to 10^{-5} M) (Fig. 2) and shifted the threshold response from $<10^{-7}$ M to $\geq 10^{-6}$ M. However, at high doses of NO (10^{-4} M), K-channel inhibition could be partially overcome. The net fall in tension caused by 10^{-4} M NO was −432 ± 51 mg in control rings compared to −268 ± 59 mg with 4-AP, −346 ± 70 mg with TEA, and −259 ± 66 mg with CTX ($P = 0.16$). Even 10^{-4} M NO was ineffective in relaxing rings constricted with KCl (net fall in tension, −88 ± 37 mg). TEA impaired relaxation to IBMX (−16 ± 7% compared to −100 ± 18% in controls; $P < 0.05$) and zaprinast but did not inhibit papaverine-induced relaxation (Fig. 3).



	BL (mg)	NE/KCl (mg)	K block (mg)	ΔNO (mg)	ΔNO (%)
NE/control	801±11	1239±46		-220±29	-52±9
NE/TEA	794±8	1248±112	1279±105	-109±66	-22±6
NE/4-AP	803±10	1177±78	1168±84	-144±41	-41±7†
NE/CTX	787±24	1352±73	1359±98	-21±61*	-4±24*
KCl/control	800±10	1030±39		-33±13*	-17±6*

FIG. 2. CTX reduces NO-induced relaxation. Traces are from representative experiments. Values are the mean ± SEM (n = 6–8 rings per group). BL, baseline tension. NE/KCl and K block give the tension present after giving NE, KCl, or a K-channel blocker. ΔNO (mg) and ΔNO (%) are the absolute and percentage cumulative relaxation in response to NO (10^{-8} to 10^{-5} M). * and †, $P < 0.05$ that value differs from NE/control and NE/CTX, respectively.

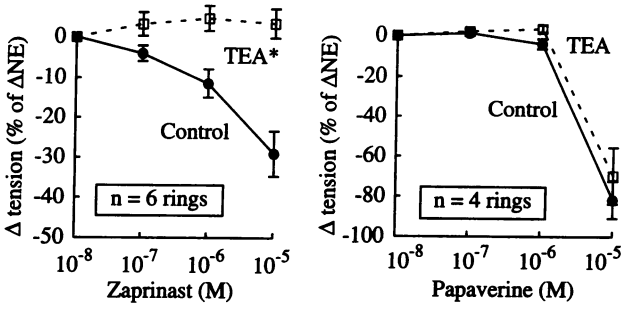


FIG. 3. TEA impairs relaxation to zaprinast but not papaverine. The change in tension is expressed as a percentage of the active tension generated by NE. *, $P < 0.001$ that the curve differs from control.

Whole-Cell Patch Clamp. NO, cGMP, and 8-Br-cGMP all increased I_K (Figs. 4 and 5) without changing the activation threshold potential of −40 mV. PA VSM cells displayed two patterns of I_K (Fig. 5). Approximately two-thirds of the cells had a low-noise slow-inactivating current, more sensitive to 4-AP than CTX or TEA, consistent with a major contribution by a delayed-rectifier-type K channel (Fig. 5*C*). The other cells had current traces displaying spontaneous spiking and considerable inhibition of I_K by CTX or TEA, consistent with a greater contribution of K_{Ca} channels. These CTX-sensitive cells responded to NO with a rapid increase in I_K (Fig. 5*A*) lasting for 15–22 min.

Pretreatment with methylene blue completely inhibited the effects of NO. The I_K elicited by the largest voltage pulse, +70 mV, was 99 ± 7% of control (methylene blue and NO) vs. 184 ± 31% (NO alone, $P < 0.05$). IBMX increased basal I_K and this was enhanced by addition of NO (Fig. 6). The synergistic increase in I_K caused by the addition of NO to IBMX-treated cells mirrored the higher cGMP levels in rings treated with NO and IBMX compared to rings treated with IBMX alone (8.9 ± 4.0 and 2.1 ± 0.7 pmol/g of tissue, respectively; $P = 0.05$). Zaprinast produced identical effects to IBMX in two additional cells (data not shown). NO and 8-Br-cGMP induced increases in I_K were completely inhibited by H-8. The I_K elicited by the largest voltage pulse was 97 ± 5% (H-8 and NO) vs. 184 ± 31% (NO alone, $P < 0.05$). Intracellular administration of (S_P)-cGMP[S] (Fig. 7) or okadaic acid (Fig. 8) enhanced I_K in cells with an I_K dominated by K_{Ca} channels.

DISCUSSION

The major finding of this study is that increasing cGMP levels in VSM cells increase I_K and this cGK-mediated effect is essential to NO- and cGMP-induced relaxation. I_K is increased whether cGMP is elevated by activating guanylate cyclase, by inhibiting cGMP phosphodiesterase, or by direct augmentation of cGMP levels. Fig. 1 illustrates the proposed mechanism of relaxation utilized by NO and cGMP. Although single-channel studies of excised patches showed that exogenous cGK increases I_K (9), this study defines the cGMP–cGK–K-channel cascade in the whole cell and proves the functional significance of this pathway for VSM relaxation.

Endothelial-derived relaxing factor, now thought to be NO (22), increases levels of cGMP in VSM prior to the onset of vasodilatation (1). The link between cGMP-dependent vasodilatation and membrane potential was unclear despite evidence that depolarization inhibited the effects of endothelial-derived relaxing factor (2) [e.g., KCl impairs pulmonary vasodilatation to ACh and bradykinin (23)]. In the current study, KCl nearly eliminates relaxation in response to NO and IBMX (Fig. 2). The failure of NO and IBMX to relax KCl-constricted rings is due to elimination of the chemical gradient for K efflux, not impaired cGMP synthesis (cGMP levels were similar in KCl- and NE-constricted rings).

Pharmacological blockade of K channels also inhibits cGMP-induced relaxation but does not impair the response to a cGMP-independent vasodilator, papaverine (Fig. 3). Presumptive identification of the subtype of K channel involved

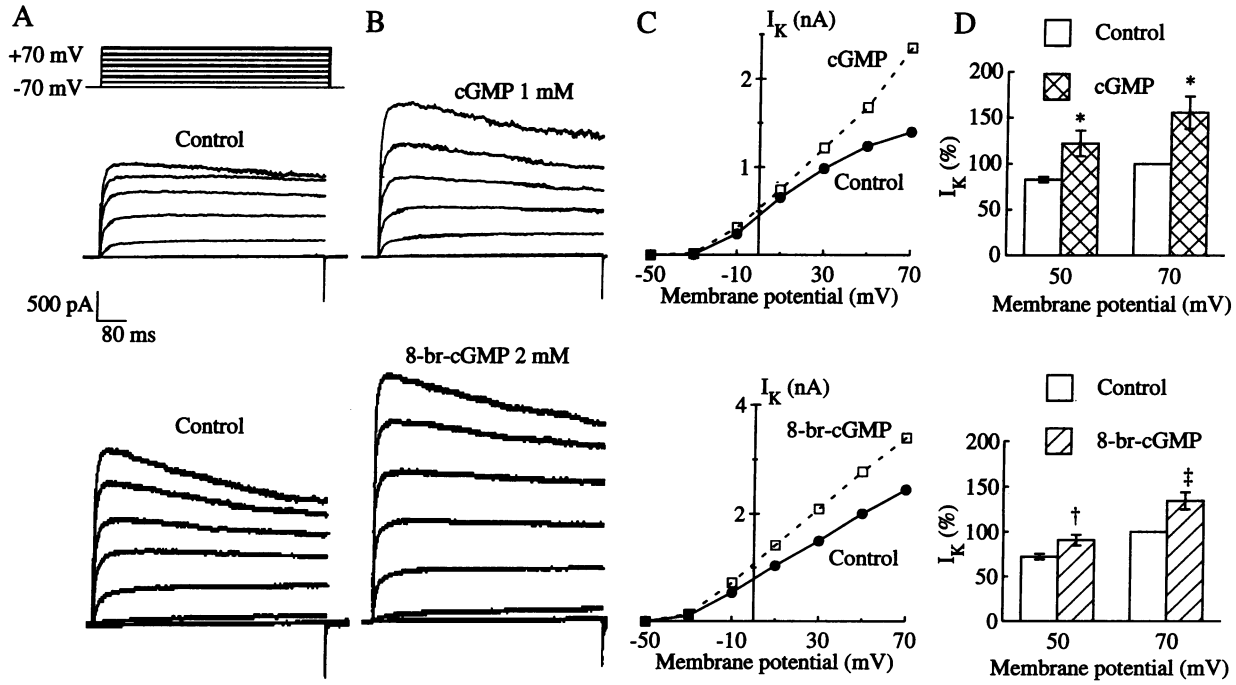


FIG. 4. cGMP and 8-Br-cGMP increase I_K. (*A*) Representative I_K family under control conditions. Traces in *A* and *B* were created by averaging three consecutive runs, which blurred the spontaneous spiking morphology (visible in Fig. 5*A*). (*B*) I_K family after cGMP or 8-Br-cGMP. (*C*) Representative current–voltage plots. (*D*) The I_K (mean ± SEM) elicited by depolarization to +50 and +70 mV before (control) and after cGMP ($n = 5$ cells) and 8-Br-cGMP ($n = 10$ cells) treatment. *, †, and ‡, $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, that the value differs from control.

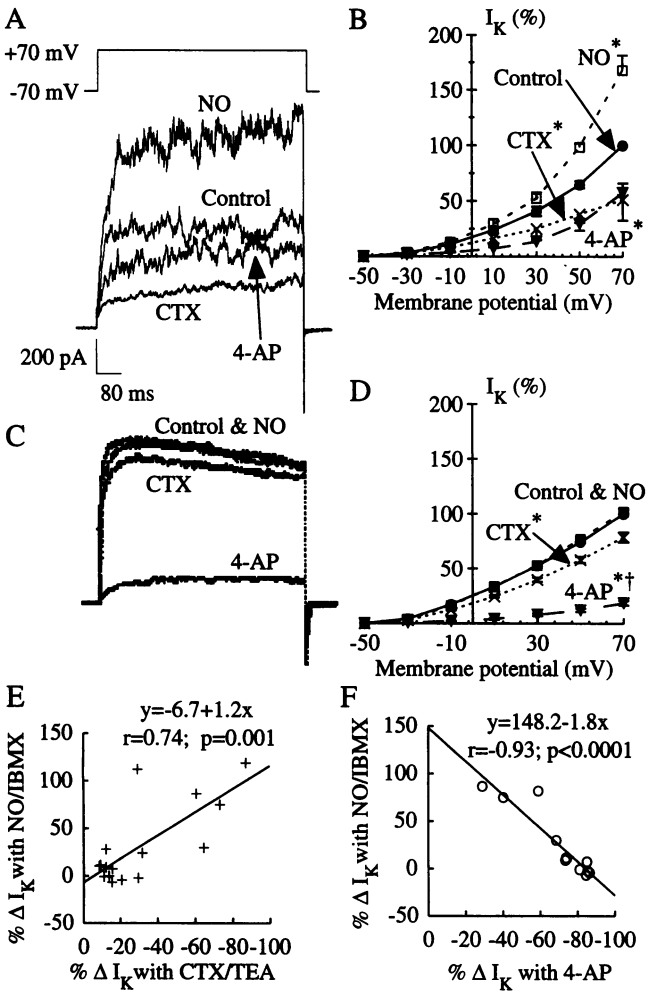


FIG. 5. Relative contribution of K_{Ca} vs. delayed rectifier channels to I_K predicts its responsiveness to NO and cGMP. (*A*) Representative currents in a NO-responsive cell (note spontaneous spiking and CTX-sensitivity, consistent with significant K_{Ca}-channel activity). (*B*) Current–voltage plot (mean ± SEM) of four NO-responsive cells. Although not apparent on this scale, I_K was consistently elevated by NO even at the membrane potential of −30 mV. (*C*) Representative currents in an NO-unresponsive cell (note lack of spontaneous spiking and lesser CTX sensitivity). (*D*) Current–voltage plot of eight NO-unresponsive cells. (*E*) Direct correlation between cGMP-dependent increases in I_K and CTX- or TEA-induced I_K inhibition. (*F*) Inverse correlation between the cGMP- and 4-AP-induced changes in I_K. * and †, $P < 0.005$ that the curve differs from control and CTX, respectively.

is provided by the observation that CTX, a relatively specific inhibitor of K_{Ca} channels (24), reduces NO-induced relaxation (Fig. 2). Thus, these observations suggest that NO/cGMP-induced relaxation is largely mediated by K_{Ca}-channel activation, although the reduction of IBMX-induced relaxation by 4-AP raises the possibility that a delayed rectifier or other K channel may also be involved.

Micropuncture measurements of membrane potential in intact vascular tissues have yielded conflicting results as to whether NO does (3–5) or does not (6, 7) cause membrane hyperpolarization. This technique does not lend itself to dissection of the intracellular pathway for cGMP-mediated hyperpolarization or identification of the subclasses of ion channels involved. Consequently, direct measurement of K-channel activity, using the patch-clamp technique, was chosen to assess the mechanism by which NO and cGMP alter VSM-cell electrophysiology. The current study defines a central role for cGMP, rather than NO itself, in K-channel activation. Focusing on cGMP makes it easier to understand how agents that elevate cGMP, such as ACh, may hyperpolarize and relax VSM without an obligatory role for NO. While NO-induced hyperpolarization is inhibited by the NO inactivator, hemoglobin, ACh-induced hyperpolarization in the same vascular rings is preserved (6, 7). Furthermore, in the adult rat pulmonary circulation, ACh causes vasodilatation that is not inhibited by NO synthase inhibitors (25) and does not elicit NO synthesis (12). cGMP may be the common link among hyperpolarizing vasodilators.

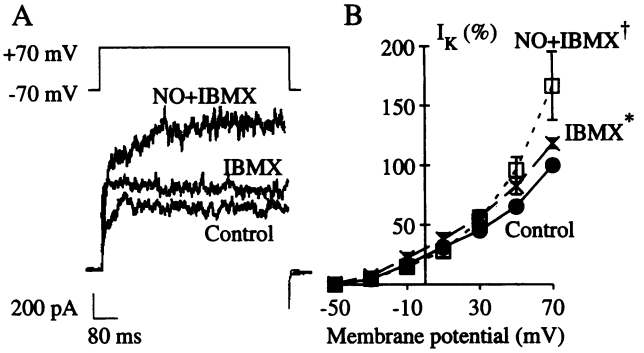


FIG. 6. cGMP phosphodiesterase inhibition increases I_K. (*A*) Representative I_K under control conditions and after IBMX and NO. (*B*) Current–voltage plot (mean ± SEM; $n = 4$–6). * and †, $P < 0.05$ and $P < 0.01$, respectively, that the curve differs from control.

The K_{Ca} channels are logical targets for a hyperpolarizing factor as they are ubiquitous and have large conductance so that activation of only a few channels in VSM cells, which have high input resistance, would promote hyperpolarization. One of the first demonstrations that cGMP can activate K channels was the finding that nitroglycerin activates K_{Ca} channels in coronary artery VSM (8). Subsequently, it has been reported that cGMP activates K_{Ca} channels in cerebral and coronary VSM by a mechanism involving cGK (9, 26). These elegant electrophysiologic studies did not establish that the increase in open probability of K_{Ca} channels was essential for the actions of nitroglycerin or NO on vascular tone. The current study found that activation of K channels contributes significantly to the relaxation response.

Although all rings relaxed in response to IBMX, zaprinast, and NO, only a third of all cells displayed a significant NO-induced increase in I_K. It appears the activation of channels in responsive cells (those with CTX-sensitive I_K) is able to influence the vascular tone of the entire ring. Tare *et al.* (3) observed that NO-induced hyperpolarization was erratic unless vessels were first partially depolarized (with NE). In the current study, NO and cGMP relaxation was, by

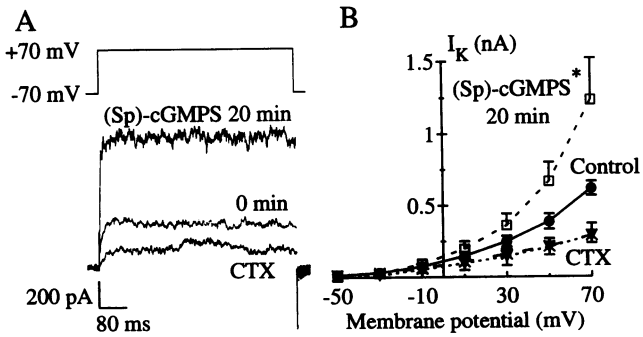


FIG. 7. (S_P)-cGMP[S] increases I_K. (*A*) Representative I_K traces immediately (0 min) and 20 min after membrane rupture with a patch pipette containing (S_P)-cGMP[S]. CTX was given externally after the (S_P)-cGMP[S] measurement. (*B*) Current–voltage plot (mean ± SEM) obtained from four cells 20 min after a membrane rupture with a pipette containing (S_P)-cGMP[S] and from five cells not exposed to (S_P)-cGMP[S] (control). CTX was given to both groups. *, $P < 0.05$ that the curve differs from control and CTX.

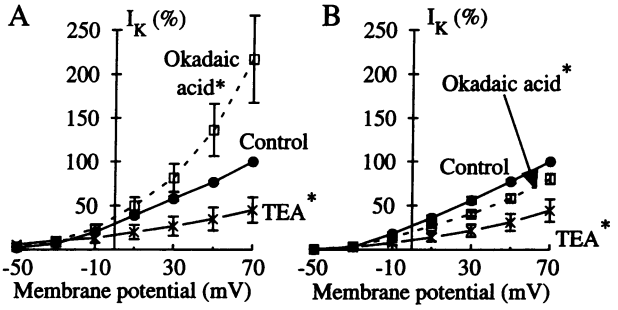


FIG. 8. Okadaic acid increases I_K in K_{Ca}-predominant cells. (*A*) Current–voltage plot from seven cells with I_K dominated by K_{Ca} channels. I_K was recorded immediately (control) and 20 min (okadaic acid) after membrane rupture. Subsequently, TEA was given externally. (*B*) Current–voltage plot from six cells with the I_K dominated by a delayed rectifier type of K channel. *, $P < 0.05$ that the curve differs from control.

necessity, studied in NE-preconstricted rings, whereas NO's effect on I_K was determined in cells that were not exposed to NE. NE may partially depolarize the VSM cells and increase cytosolic Ca concentration; both effects would promote activation of the K_{Ca} channel by NO. Failure to recognize responsive and unresponsive cells could lead to variable assessments of the effects of NO and cGMP on membrane electrophysiology.

The central role of cGMP (as opposed to guanylate cyclase or a direct NO effect) in K_{Ca}-channel activation is proven by the ability of cGMP or 8-Br-cGMP to increase I_K (Fig. 4). Methylene blue's inhibition of NO-induced K-channel activation argues against a direct effect of NO on the K channel.

There is a report that GMP, rather than cGMP, increases the activity of K_{Ca} channels (27). However, phosphodiesterase inhibition minimizes GMP production while augmenting cGMP levels and yet phosphodiesterase inhibitors cause relaxation and increase I_K (Fig. 6). This implies that cGMP is more important than GMP in augmenting I_K.

The increase in I_K caused by NO and cGMP requires cGK activity and is inhibited by H-8. Although cGK may directly phosphorylate the K channel, the possibility that other proteins could be important intermediary kinase targets cannot be excluded. Furthermore, H-8 is not entirely specific and may inhibit both cGK and cAMP-dependent protein kinase. The predominant role of cGK in the NO/cGMP dilator response is supported by the dramatic increase in I_K caused by (S_P)-cGMP[S] (Fig. 7), mimicking the effects of NO and cGMP. (S_P)-cGMP[S] is quite a specific activator of cGK with minimal effects on cAMP-dependent protein kinase (20).

Kinases modulate the activity of many intracellular proteins by enhancing phosphorylation. Homeostasis is maintained by phosphatases that dephosphorylate the target proteins, reversing the effects of the kinases. Okadaic acid slows dephosphorylation and increases I_K in NO-sensitive cells (Fig. 8), indicating that stabilization of protein phosphorylation is important to the basal activity of cGMP-sensitive K channels.

Limitations. Although the cGMP levels measured in rings (pmol/g of tissue) cannot be directly compared to molar cGMP concentration used in patch pipette, it is likely that we administered higher cGMP doses than occur physiologically. Nevertheless, increasing cGMP by low doses of NO or phosphodiesterase inhibition increased I_K, suggesting that K channels are activated by physiologically relevant cGMP levels.

Many vasodilators increase both cGMP and cAMP production. A role for cAMP in NO-induced relaxation (28) cannot be excluded as both IBMX and H-8 have some effect on the cAMP pathway and cGK can be activated by high levels of cAMP (29).

cGMP might also cause relaxation through its well-established ability to reduce cytosolic Ca^{2+} concentration (30), an effect that could have been obscured by the large doses of KCl and TEA used in the current experiment. However, the relaxation caused by papaverine, which inhibits the voltage-gated Ca channel and lowers cytosolic Ca^{2+} concentration (16), was not inhibited by the same dose of TEA. It is probable that effects on cytosolic Ca^{2+} concentration and K_{Ca} channels occur in the VSM.

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