Mathematical modeling of the nitric oxide/cGMP pathway in the vascular smooth muscle cell

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The nitric oxide (NO)/cGMP pathway in the vascular smooth muscle cell (VSMC) is an important cellular signaling system for the regulation of VSMC relaxation. We present a mathematical model to investigate the underlying mechanisms of this pathway. The model describes the flow of NO-driven signal transduction: NO activation of soluble guanylate cyclase (sGC), sGC- and phosphodiesterase-catalyzed cGMP production and degradation, cGMP-mediated regulation of protein targets including the Ca\(^{2+}\)-activated K\(^+\) (KCa) channel, and the myosin contractile system. Model simulations reproduce major NO/cGMP-induced VSMC relaxation effects, including intracellular Ca\(^{2+}\) concentration reduction and Ca\(^{2+}\) desensitization of myosin phosphorylation and force generation. Using the model, we examine several testable principles. 1) Rapid sGC desensitization is caused by end-product cGMP feedback inhibition; a large fraction of the steady-state sGC population is in an inactivated intermediate state, and cGMP production is limited well below maximum. 2) NO activates the KCa channel with both cGMP-dependent and -independent mechanisms; moderate NO concentration affects the KCa via the cGMP-dependent pathway, whereas higher NO concentration is accommodated by a cGMP-independent mechanism. 3) Chronic NO synthase inhibition may cause underexpressions of KCa channels including inward rectifier and KCa channels. 4) Ca\(^{2+}\) desensitization of the contractile system is distinguished from Ca\(^{2+}\) sensitivity of myosin phosphorylation. The model integrates these interactions among the heterogeneous components of the NO signaling system and can serve as a general modeling framework for studying NO-mediated VSMC relaxation under various physiological and pathological conditions. New data can be readily incorporated into this framework for interpretation and possible modification and improvement of the model.

cell signaling; smooth muscle relaxation; calcium desensitization; signal transduction; integrative model

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incorporation of new experimental data on specific mechanisms or pathways.

**METHODS**

**sGC activation by NO.** Figure 1 shows NO-activated transition of sGC from the basal state to the activated state (five-coordinate, 5c). NO stimulates the activity of sGC (300- to 700-fold) by binding to the heme of the enzyme. Recent studies suggest that this binding mechanism involves a two-step process (4, 9, 46). During the first step, NO binds to basal sGC to form an intermediate six-coordinate (6c) ferrous nitrosyl heme complex, which subsequently decays to a fully activated (5c) complex. The first step in NO binding to sGC is expressed as a reversible biochemical reaction process: 

$$E_b + NO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{6c}$$

with rate constant $k_2$.

For the NO-dependent pathway, a second NO binding site at the heme of sGC was proposed by Zhao et al. (46), which facilitates cleavage of the Fe-His105 bond. This process can be described as an enzymatic reaction based on traditional Michaelis-Menten kinetics (i.e., $E + S \rightleftharpoons ES \rightarrow P + E$, where $E$ is enzyme, $S$ is substrate, and $P$ is product). The process can be described as an enzymatic reaction based on traditional Michaelis-Menten kinetics (i.e., $E + S \rightleftharpoons ES \rightarrow P + E$, where $E$ is enzyme, $S$ is substrate, and $P$ is product). The process can be described as an enzymatic reaction based on traditional Michaelis-Menten kinetics (i.e., $E + S \rightleftharpoons ES \rightarrow P + E$, where $E$ is enzyme, $S$ is substrate, and $P$ is product).
NO with the isolated heme of sGC are rapid (the association rate constant was reported as 7.1 ± 2 × 10^8 M⁻¹·s⁻¹ at 4°C in Ref. 46, and no intermediate was observed in the experiment), breakage of the His105 residue becomes the rate-limiting step in the process (the rate constant of this NO-dependent transition step was measured as 2.4 × 10³ M⁻¹·s⁻¹ at 4°C in Ref. 46), where the rate constant is three orders of magnitude smaller than the association constant for NO binding of the heme. Consequently, we assume that the NO-dependent pathway can be represented as a single-step irreversible process with rate constant k₄:

\[
E₅ₖ + NO \rightarrow E₅ₖ \rightarrow E₆ₕ + NO
\]

Another aspect of NO-sGC interaction is the dissociation of NO from the heme domain of sGC, i.e., the deactivation of E₅ₖ and the recovery of the basal sGC condition. We describe this process according to a first-order, irreversible kinetics as:

\[
k₃ \quad E₅ₖ \rightarrow E₆ₕ + NO
\]

where the turnover rate k₃ can potentially be modulated by several factors in vivo, including the sGC proximity to NO scavengers, concentrations of substrates, and [Ca²⁺] (22). Bellamy et al. (5) reported that sGC desensitization was observed in intact cells but not in lysed cells where substrates and products were diluted. In response to NO stimulation, the rate of cGMP production has a biphasic shape, in lysed cells where substrates and products were diluted. In response to NO stimulation, the rate of cGMP production has a biphasic shape, where the rate constant for NO binding to sGC is smaller than the association constant for NO binding of the heme.

Consequently, we assume that the NO-dependent pathway can be represented as a single-step irreversible process with rate constant k₄:

\[
E₅ₖ + NO \rightarrow E₅ₖ \rightarrow E₆ₕ + NO
\]

NO, Together with NO scavenging, the total NO consumption rate including NO binding to sGC may be approximated as a first-order kinetics. The dynamics of this process is expressed as:

\[
\frac{d[NO]}{dt} = J_{no} - k_{int}[NO]
\]

where Jₙₒ is the endogenous or exogenous NO influx representing the physiological NO availability or NO analog introduced experimentally and t is time. Here, kₐₒ denotes the lumped NO consumption rate constant that reflects the activity of NO scavengers. Vaughn et al. (41) have estimated the rate of NO consumption in VSMC as 0.01 s⁻¹ for first-order kinetics.

**cGMP production and degradation.** Because the synthesis of cGMP begins after the catalytically active sGC E₅ₖ is formed (46), we assume that cGMP production from GTP depends primarily on the degree of E₅ₖ activity. The lifetime of cGMP is also regulated by the multiple isoforms of cyclic nucleotide phosphodiesterases (PDEs), which hydrolyze the cyclic nucleotide into GMP. The production and degradation of cGMP can both be modeled with Michaelis-Menten kinetics:

\[
\text{GTP} + E₅ₖ \rightleftharpoons \text{GTP} \cdot E₅ₖ \rightarrow \text{cGMP} + E₅ₖ
\]

\[
\text{cGMP} + \text{PDE} \rightleftharpoons \text{cGMP} \cdot \text{PDE} \rightarrow \text{GMP} + \text{PDE}
\]

Assuming that the presence of GTP is abundant, cGMP production kinetics mainly depends on the sGC concentration. Study has shown that multiple types of PDE with different substrate affinities, subcellular locations, and kinetic properties (especially isoforms of PDE5 in VSMC) catalyze cGMP hydrolysis in VSMC (8). Here, we simply assume that PDE has a constant intracellular concentration and homogeneous catalytic affinity to its substrate cGMP. The balance equation for cGMP is written as:

\[
\frac{d[cGMP]}{dt} = V_{max,sGC} E₅ₖ - \frac{[cGMP]V_{max,pde}}{K_m,pde + [cGMP]}
\]

where Vₘₐₓₕₐₜ,ₕₐₜ,ₕₐₜ represents the maximal rate of cGMP production when E₅ₖ = 1, which implies that all available enzyme molecules participate in the catalysis, and Vₘₐₓₕₐₜ,ₕₐₜ,ₕₜₜ is maximum cGMP hydrolysis rate. Vₘₐₓₙₙ,ₕₐₜ,ₕₜₜ is proportional to the total sGC concentration [E₅ₖ], which is conserved in our model. Hence, as shown in Eq. 2, the cGMP production and degradation rates are vₕₜ = Vₘₐₓₕₐₜ,ₕₜₜ,ₕₚₜₑₜₜ [E₅ₖ] and vₜₛ = [cGMP]Vₘₐₓₕₐₜ,ₕₜₜ,ₕₚₜₑₜₜ / Kₘₕₚₜₑₜₜ + [cGMP]), respectively. The Michaelis-Menten constant Kₘₕₚₜₑₜₜ was measured in experimental studies by Turko et al. (39, 40) (see Table 1). Recent studies have shown that the basal level of PDE activity for cGMP hydrolysis is relatively low. However, this activity is greatly enhanced (up to 9- to 11-fold) after cGMP binding to its regulatory NH²-terminal domain (GAF domain) (23, 33, 34). The PDE activation process by cGMP can be described as cGMP + PDEinactive ↔ PDEactive. Consequently, the concentration of active PDE depends on the level of cGMP, because in a Michaelis-Menten kinetics Vₘₐₓₙₜ,ₚₜₑₜₜ is proportional to the total concentration of active PDE, which we assume, is approximately proportional to cGMP concentration. This is a reasonable approximation if the PDE level is high compared with cGMP concentration. Therefore, we describe the maximum cGMP hydrolysis rate Vₘₐₓₕₜₜ,ₕₜₜ,ₕₚₜₑₜₜ as being proportional to [cGMP]:

\[
V_{max,pde} = K_{pde}[cGMP]
\]

**NO/cGMP-dependent VSMC relaxation.** Smooth muscle force development primarily depends on the cross-bridge recruitment between myosin and actin filaments, which is regulated by MLCK phosphorylation, activated and inhibited by MLCK kinase (MLCK) and MLCP, respectively. It has been proposed that the NO/cGMP pathway leads to VSMC relaxation by having two major effects: reduction of the cytoplasmic [Ca²⁺] and Ca²⁺ desensitization of the contractile system (8). The reduction in [Ca²⁺] is accomplished by decreasing extracel-
Table 1. Model parameters

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<tr>
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See text for parameter descriptions.

where $Q$ represents the basal condition and $Q_s$ represents the maximal change that can be induced by cGMP. $Q$ can represent a variety of physical quantities including an ionic channel conductance, an enzymatic reaction rate constant, or an electrophysiological term (e.g., half-activation voltage) in vivo. In addition, both $Q_b$ and $Q_s$ can fluctuate as physiological conditions change.

Ca$^{2+}$ entry reduction. Intracellular Ca$^{2+}$ is modulated by several NO-mediated ion channels on the VSMC membrane. Here, we only consider the major channel target for cGMP: the large-conductance KCa channel. Activation of the KCa channel by cGMP induces membrane hyperpolarization, which subsequently deactivates the voltage-gated Ca$^{2+}$ channel, shifting the inward Ca$^{2+}$ flux and bringing about relaxation. Studies by Onoue and Katusic (28) on canine middle cerebral arteries indicate that VSMC relaxation to NO via activation of KCa channels may involve both cGMP-dependent and cGMP-independent pathways. A patch-clamp study by Gerzani et al. (14) shows similar results in rat cerebral arteries.

In this study, we assume that NO regulates KCa channels via both the cGMP-dependent and cGMP-independent pathways. Specific activation of the KCa channel may be due to phosphorylation of channel proteins or its regulatory proteins (8). Studies by Zhou et al. (47) suggest that PKG stimulates the activity of two isoforms of the KCa channel proteins, which results in a shift of the voltage dependence of the single-channel open probability ($P_o$) toward more negative potentials. Other studies (31, 36) report similar cGMP-induced decreases in the voltage and Ca$^{2+}$ thresholds of $I_{KCa}$. Therefore, we have chosen to model the regulatory effect of NO/cGMP on voltage dependence of the channel $P_o$ described as:

$$P_o(V_m) = \frac{1}{1 + e^{-\gamma(V_m-V_o)}}$$

where $V_m$ denotes the membrane potential, $V_o$ is the half-activation voltage, and $S_{cNO}$ is the steepness factor. In our model, $V_o$ is the major regulatory target for Ca$^{2+}$, NO, and cGMP. The above $P_o(V_m)$ relationship can be shifted by modulating $V_o$. We hypothesize that NO exerts its effect by shifting the $P_o(V_m)$ relationship via both cGMP-dependent and cGMP-independent mechanisms. This is expressed as:

$$V_o = V_o + \gamma \log([Ca^{2+}]_i) - V_{gmax} R_{cNO}[K_{CaNO}, n_{NO}]$$

$$= V_o + R_{cNO}(K_{NO}, n_{NO}) - V_o$$

where the linear relationship between $V_o$, and the logarithm of [Ca$^{2+}$] (VCa, and $V_o$) is given in our original VSMC model (42). Single-channel studies by Robertson et al. (31) on VSMC in rabbit cerebral arteries and Stockand and Sansom (36) on human mesangial cells found that $V_o$ was shifted by cGMP-activated PKG toward a more negative voltage range, whereas the steepness factor $S_{cNO}$ remained unchanged.

Ca$^{2+}$ desensitization of MLC phosphorylation. Smooth muscle contraction and relaxation directly depend on actin-myosin interaction and are regulated by two competing processes: phosphorylation of the regulatory 20-kDa MLC by MLCK and dephosphorylation by MLCP. The activity of MLCK is a function of the concentration of the Ca$^{2+}$-calmodulin complex (CaCM), whereas MLCP can be activated directly by cGMP/PKG. MLCK activation and subsequent contractile force generation can be regulated by balancing the activities of the two enzymes MLCK and MLCP, i.e., the balance between MLC phosphorylation and dephosphorylation (20). It is well established that by activating MLCP, PKG phosphorylates the M110 regulatory subunit of MLCP and/or an MLCP inhibitor, decreases the MLC phosphorylation level, and desensitizes the responses of contractile system to Ca$^{2+}$, without affecting MLCK activity. This causes the inhibition of MLC phosphorylation without altering [Ca$^{2+}$], (8, 19, 26). Increased MLCP activity shifts the balance between two MLC phos-
phorylation regulatory enzymes, MLCK and MLCP, which leads to a contractile system less responsive to Ca$^{2+}$.

In our previous study of the VSMC (42), we modified a kinetic model of MLCP phosphorylation and cross-bridge formation originally developed by Hai and Murphy (16). The four-state model of contractile system kinetics is illustrated in Fig. 1, in which MLCP is regulated by the CaCM. Therefore, MLCP phosphorylation was only a Ca$^{2+}$-dependent process. In this study, we associate the MLCP dephosphorylation rate constant with cGMP concentration to model the cGMP effect on MLC activity:

$$k_{\text{dep}} = k_{\text{dep}}^o + k_{\text{dep}}^p[C\text{GMP}](K_{\text{dep}}^{\text{pH}})$$

where $k_{\text{dep}}$ is the basal MLCP dephosphorylation rate constant and $k_{\text{dep}}^p$ is a first-order rate constant for cGMP-regulated MLCP dephosphorylation. The rate constant of MLCP phosphorylation is Ca$^{2+}$-dependent and defined as $k_{\text{ph}} = k_{\text{ph}}(\text{Ca}^{2+})$. If we approximate the four-state myosin phosphorylation model (Fig. 1) with a reduced two-state phosphorylation kinetics:

$$M \rightarrow M_p$$

(with the conservation constraint $M + M_p = 1$), we have, at equilibrium,

$$M_p = \frac{k_{\text{ph}}}{k_{\text{dep}} + k_{\text{ph}}} = \frac{\text{[Ca}^{2+}]^\gamma}{\text{[Ca}^{2+}]^\gamma + k_{\text{dep}}/k_{\text{ph}}}$$

Ca$^{2+}$ desensitization of the VSMC contractile system is widely interpreted as a reduction of MLCP phosphorylation at the same or a comparable Ca$^{2+}$ level. The Ca$^{2+}$ sensitivity of MLCP phosphorylation can be formally defined as a nondimensional quantity $\Theta_{\text{ca}}$, the magnitude of the relative change of MLCP phosphorylation with regard to the relative change of [Ca$^{2+}$]:

$$\Theta_{\text{ca}} = \frac{\text{[Ca}^{2+}]^\gamma}{\text{[Ca}^{2+}]^\gamma + K_{\text{dep}}^p/\text{K}_{\text{dep}}^p}$$

where $K_{\text{dep}}$ = $k_{\text{dep}}/k_{\text{dep}}$ is the desensitization factor for the VSMC contractile system, which reflects the competing roles of MLCK and MLCP in regulating the MLCP phosphorylation that is conserved by Ca$^{2+}$ and cGMP. The term $\gamma$ is the order of Ca$^{2+}$ influence on MLCP phosphorylation. According to the above definition, the value of $\Theta_{\text{ca}}$ indicates Ca$^{2+}$ sensitivity of MLCP phosphorylation at the corresponding Ca$^{2+}$ level. In the four-state model, the phosphorylation level (P) and the relative force (f) generated by cross-bridge interaction are calculated as $P = M_p + AM_p$ and $f = AM_p + AM$, respectively, where M is the free form of MLC, $M_p$ is the fraction of phosphorylated myosin, $AM_p$ is the fraction of myosin attached to the actin filament, and $AM$ is the fraction of attached myosin cross-bridges but dephosphorylated.

Model protocols and parameter assignments. Model simulations are undertaken at a temperature of 20°C with extracellular ionic concentrations held constant: extracellular Na$^+$ concentration ([Na$^+$]) = 140 mM, extracellular K$^+$ concentration ([K$^+$]) = 5.0 mM, and extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) = 2.0 mM. Cell volume is constant as 1 pl. Model parameters are listed in Table I. VSMC precontraction is induced by one of two methods: 1) increasing membrane Ca$^{2+}$ permeability by increasing L-type Ca$^{2+}$ channel conductance $g_{\text{CaL}}$, which simulates the effect of substances such as UTP, and 2) increasing the Ca$^{2+}$ sensitivity of contractile apparatus by increasing the MLC phosphorylation rate $k_{\text{ph}}$, which simulates the effects of phenylephrine (PE). Equations for modeling the four-state MLCP phosphorylation and contractile kinetics are given in Eq. 20 in Ref. 42. All other model equations, parameters, and initial conditions of VSMC membrane channels can be found in the APPENDIX of Ref. 42.

The entire NO/cGMP signaling pathway is described by a system of deterministic ordinary differential equations programmed with MATLAB and solved numerically on a personal computer with an Intel-based processor. The numerical values of the majority of model parameters assigned are based on parameters adopted from the published literature, and the rest are identified within physiological ranges with a nonlinear least-square optimization technique by fitting reported experimental data (see Table 1).

RESULTS

sGC desensitization: a local feedback inhibition. NO-induced VSMC relaxation becomes deactivated during the latter phase of a short-term exposure of VSMC to NO (in seconds) (5, 13). Such a nitrate tolerance effect may be due to reduced sGC sensitivity to NO. This suggests that cellular regulation tends to stabilize the NO/cGMP pathway within a relatively wide range of NO availability and that sGC-activated cGMP accumulation and PDE-mediated degradation are two mechanisms that may regulate a variety of patterns of cGMP responses to NO. Changes in sGC sensitivity to NO indicate an immediate local feedback inhibition while the total sGC is maintained constant. As described in METHODS, we assume that sGC desensitization is primarily caused by end-product cGMP feedback inhibition.

Figure 2 shows the sGC desensitization effect with linear feedback ($m = 1$, Eq. 1) under conditions of constant NO stimulation (220 nM, as indicated in Ref. 5). Figure 2A shows that the model simulation reproduces the measured cGMP accumulation data (see Fig. 3b in Ref. 5). The cGMP production rate exhibits a biphasic time response similar to the analysis by Bellamy et al. (5): $v_p$ sharply increases to its peak in <5 s and subsequently drops to a significantly lower level in ~1 min (Fig. 2B), which indicates rapidly reduced sGC activity. As shown in Fig. 2C, the major fraction of the sGC population (>50%) is shifted to the intermediate form $E_{ci}$ after...
a quick transient in $E_{c}$, peaking in <5 s. This accumulation of $E_{c}$ is due to 1) an enhanced $E_{c}$ decay to $E_{i}$ mediated by cGMP and 2) fast NO binding to $E_{b}$ (i.e., high affinity of sGC for NO), which competes with the rate-limiting process converting $E_{dc}$ to $E_{sc}$. In Fig. 2C, a low $E_{sc}$ suggests that the steady-state cGMP level is well below the maximum production capacity.

To study how cGMP feedback inhibition causes sGC desensitization and to what extent, model simulations were conducted with no feedback ($m = 0$) and with second-order feedback ($m = 2$). Figure 3 shows that different $v_{p}, v_{d}$, and sGC response patterns as our model simulations produce fits of a quality similar to the same cGMP accumulation data shown in Fig. 2A (fitting results are not shown). Without cGMP feedback, the model does not generate a transient output of cGMP synthesis rate $v_{p}$ but rather a response that reaches its plateau gradually. Here, $v_{p}$ is balanced by the rising degradation rate $v_{d}$ as cGMP concentration levels off at the steady state (Fig. 3A). In this case, $E_{sc}$ comprises the majority fraction of the sGC population (Fig. 3B). For second-order cGMP feedback, the transient $v_{p}$ response exhibits a greater decline as the result of a more pronounced decrease in $E_{sc}$ from a higher peak levels compared with that of the first-order feedback shown in Fig. 2. These results show that, given cGMP accumulation data alone, one cannot sufficiently determine the exact cGMP regulatory mechanism because varied sGC and PDE activity patterns that modulate cGMP synthesis and degradation may generate an identical NO-induced cGMP accumulation. Model parameter values used for each simulation with different orders of cGMP feedback are listed in Table 2.

Figure 4 shows the steady-state cGMP production and $E_{sc}$ in response to systematically elevated NO with linear ($m = 1$) cGMP feedback inhibition. Figure 4A shows that large increases in NO synthesis only result in moderate increases in cGMP production with a Hill-like sigmoid-shaped response, whose magnitude depends on the level of sGC activity reflected by $E_{sc}$ (Fig. 4B). Blockade of the cGMP feedback ($m = 0$, with all other parameters unchanged) shifts both NO-cGMP and NO-$E_{sc}$ relationships toward the lower NO range (smaller $E_{c0}$, higher NO potency) with slightly steeper slopes. This agrees qualitatively with experimental reports on platelets by Mo et al. (23), in which NO had higher potency for cGMP production in lysed platelets (diluted cGMP concentration may presumably diminish feedback inhibition) than in intact cells.

$K_{Ca}$ channel: cGMP-dependent and -independent effects. It has been suggested that under physiological conditions NO brings about relaxation by activating sGC. However, certain pathological conditions (e.g., cerebral ischemia) cause excessive endogenous NO production. Here, NO may directly activate $K_{Ca}$ channels and thus initiate VSMC relaxation via a cGMP-independent pathway (28, 29). In the previous simulation, we studied rapid sGC desensitization, in which cGMP production inhibited by negative feedback limits the role of NO in VSMC relaxation via the cGMP-dependent pathway. Logically, a high rate of NO biosynthesis carrying pathological signals might be accommodated by an alternative and thus cGMP-independent pathway for the VSMC to have protective responses. This alternative pathway could consist of a direct NO activation of regulatory targets, or other second messengers may be involved. Recent studies suggest that 20-hydroxyeicosatetraenoic acid (20-HETE, a product from cytochrome P-450 4A substrate: arachidonic acid) constitutively maintains VSMC tone in cerebral arteries by inhibiting large-conductance $K_{Ca}$ channels (32). Thus NO can inhibit 20-HETE production by deactivating enzyme cytochrome P-450 (1), which may contribute to the cGMP-independent activation of $K_{Ca}$ channels (2, 37).

The quantities $V_{Ca}$, $V_{GMP}$, and $V_{s}$ in Eq. 4 are determined based on data from the study of Stockand and Sansom (36), in which cGMP-dependent PKG shifted $V_{s}$ from 42 mV at control level to −34 mV at [Ca$^{2+}$]$_{i}$ = 1.0 μM. Figure 5, A and B, shows the model-generated $P_{o}$ vs. $V_{m}$ and $P_{o}$ vs. [Ca$^{2+}$]$_{i}$ relationships, which compare favorably to measured data. This experiment consists of an all-or-none testing of the cGMP effect, in which the cGMP effect is tested at a saturated cGMP level with $R_{NO}$ ($K_{Ca}$, $K_{M,NO}$, $V_{GMP}$, $P_{NO}$, $R_{GMP}$) = 1 without involving the cGMP-independent NO mechanism ($R_{NO}$ in Eq. 4 set to 0).

By incorporating direct activation of the $K_{Ca}$ channel by NO, the model simulation results (Fig. 6A) show NO-MLC phosphorylation steady-state relationships under different sGC activity levels. Elevated NO reduces MLC phosphorylation under

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Table 2. Model parameters used for fitting data

Fig. 3. Different orders of cGMP feedback. A and B: without cGMP feedback ($m = 0$). A: cGMP synthesis and degradation rates $v_{p}$ and $v_{d}$. B: responses of fractional populations of 3 sGC forms. C and D: second-order cGMP feedback ($m = 2$). Exogenous NO source is simulated at a holding 220 nM concentration as indicated in Ref. 5.

By incorporating direct activation of the $K_{Ca}$ channel by NO, the model simulation results (Fig. 6A) show NO-MLC phosphorylation steady-state relationships under different sGC activity levels. Elevated NO reduces MLC phosphorylation under
both control and sGC inhibition conditions. These results qualitatively agree with measured data from Ref. 29, in which elevated NO at high concentration completely relaxed canine middle cerebral arteries to the basal level when sGC activity was partially or entirely blocked by 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (ODQ) and cGMP production was consequently minimal. In our simulations, the VSMC is preconditioned to develop contraction by increasing the L-type Ca\textsuperscript{2+} channel permeability by 10-fold to simulate the UTP effect used in the experimental protocol (29). sGC activity is blocked by inhibiting the cGMP production rate with decreased \( V_{\text{max, sGC}} \) to simulate the effect of the sGC-specific inhibitor ODQ. Figure 6B shows [Ca\textsuperscript{2+}], reduction in response to hyperpolarization (not shown) induced by increasing \( I_{\text{KCa}} \) channel activity. Figure 6, C and D, shows that without the NO direct effect on \( I_{\text{KCa}} \) channel, at any level of sGC activity the cGMP-dependent mechanism alone cannot reduce the MLC phosphorylation back to minimal levels. Rather, it is brought to a residue phosphorylation level near 20% for the high [NO] range (>500 nM), where [Ca\textsuperscript{2+}], remains significantly high. As expected, the ability of cGMP to reduce [Ca\textsuperscript{2+}], and MLC phosphorylation is gradually lost as sGC becomes increasingly more inhibited (Fig. 6, C and D). These model results suggest that a cGMP-independent mechanism that senses higher [NO] may exist in certain cell types where cGMP production is limited by sGC desensitization as discussed in sGC desensitization: a local feedback inhibition.

Effect of basal NO and K\textsuperscript{+} channels. Under basal conditions, endothelium-released NO assists in maintenance of smooth muscle tone. If endogenous production of NO is inhibited (e.g., endothelium damage), hypertension may occur (7, 15). In this case, the impaired capability of NO-induced membrane hyperpolarization brings about a higher [Ca\textsuperscript{2+}]. This may lead to an elevated VSMC tone and potentially contribute to development of a hypertensive condition.

Membrane depolarization can be induced by elevating \([K^+]_o\), as a result of reduced outward K\textsuperscript{+} current because of the reduced K\textsuperscript{+} gradient across the membrane and the equilibrium potential for K\textsuperscript{+} according to the Nernst equation. However, elevation of \([K^+]_o\), affects currents through all types of K\textsuperscript{+} channels including the \( I_{\text{KCa}} \) channel, the inward rectifier (\( I_{\text{Ki}} \)) channel, and the background K\textsuperscript{+} channel. Model simulations shown in Fig. 7 demonstrate this phenomenon, as well as the role played by \( I_{\text{KCa}} \) and \( I_{\text{Ki}} \) channel current (\( I_K \)) (Fig. 7, B and C). The log-linear relationship between \( V_m \) and \([K^+]_o\) shown in Fig. 7A is qualitatively consistent with experimental measurement of the \( V_m-[K^+]_o \) relationship by Bratz et al. (7) in wild-type and NOS inhibitor-treated rats. The underlying currents, however, exhibit complex and nonlinear patterns. A reduction of inward rectifier conductance by 50% causes an increase in basal membrane potential and a decrease in the slope of the \( V_m-[K^+]_o \) relationship (Fig. 7A). Same-magnitude reduction of \( I_{\text{KCa}} \) channel expression induces a much smaller depolarization effect (simulation not shown) because of the low activity of \( I_{\text{KCa}} \) channels at basal NO and Ca\textsuperscript{2+} levels. This result suggests that a reduced expression of the \( I_{\text{KCa}} \) channel caused by long-term NOS inhibition may be a more effective way to cause membrane depolarization than activating the \( I_{\text{KCa}} \) channel. The multiphasic characteristics of \( I_{\text{KCa}} \), \( I_{\text{Cal}} \), and [Ca\textsuperscript{2+}], shown in Fig. 7, B, D, and E, are caused by the voltage-dependent activation and inactivation windows of the L-type Ca\textsuperscript{2+} channel operated in the voltage range between −30 and −20 mV (see Fig. 3 in Ref. 42).

\( Ca^{2+} \) sensitivity and \( Ca^{2+} \) desensitization. In isolated muscle strip studies on rabbit femoral arteries, Lee et al. (19) induced maximum steady-state force in the preparation by administrating high [K\textsuperscript{+}]\( _o \) (154 mM at PE = 100 \( \mu \)M). Accordingly, in our model, the maximum steady-state force \( f_m \) can be produced by elevating the rate constant \( k_{\text{MLCK}} \) to a level that saturates the MLCK activity at the condition of zero cGMP
effect [i.e., \( R_{cGMP}(K_m, mlcp/H, mlcp) = 0 \)] and recording the force \( f_m = AM_p + AM \). The relative force is then calculated as \( R_f = f/f_m \).

Figure 8, A and B, shows comparisons between model-produced results and measured data (19). The contractile system responds to cGMP by shifting the steady-state \( \text{Ca}^{2+} \)-force and \( \text{Ca}^{2+} \)-MLC phosphorylation relationships into a range of higher \( \text{Ca}^{2+} \) levels. The \( \theta_{\text{Ca}}[\text{Ca}^{2+}]_i \) curve as shown in Fig. 8C shifts toward the range of higher \( \text{Ca}^{2+} \) levels after the application of cGMP. The curve shifting in Fig. 8C represents the actual \( \text{Ca}^{2+} \) desensitization effect. The contractile system is least sensitive (minimal \( \theta_{\text{Ca}} \)) to relative change in \( \text{Ca}^{2+} \) at saturated \( \text{Ca}^{2+} \) levels and most sensitive (maximum \( \theta_{\text{Ca}} \)) at

Fig. 6. NO-induced cGMP-independent VSMC relaxation under control and 10% and 1% sGC activity conditions. A and B: with NO-directed activation of \( K_{\text{Ca}} \) channel. A: MLC phosphorylation (\( M_p + AM_p \)); B: \( [\text{Ca}^{2+}]_i \). C and D: without NO-directed effect. Preconstrictions were induced by a 10-fold increase in the L-type \( \text{Ca}^{2+} \) channel conductance \( g_{\text{CaL}} \). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) inhibition effect on sGC activity is simulated by reducing the enzyme-catalyzing factor to 10% and 1% of its control level.
minimal Ca\(^{2+}\) levels. It should be noted that MLC phosphorylation becomes more sensitive to \([\mathrm{Ca}^{2+}]_{i}\) variations when cGMP concentration increases and the contractile system is, however, desensitized (shown as decreases in relative force and MLC phosphorylation in Fig. 8, A and B). Here, we can see the conceptual difference between the \([\mathrm{Ca}^{2+}]_{i}\) sensitivity of MLC phosphorylation (quantitatively represented by \(\Theta_{\text{Ca}}\)) and the

Fig. 7. Basal NO influences on K\(^{+}\) currents and membrane potentials under control conditions (solid lines) and inward rectifier current (\(I_{\mathrm{k}}\)) reduced by 50\% (dashed lines). A: membrane depolarization induced by increased extracellular K\(^{+}\) concentration ([K\(^{+}\)]\(_{o}\)). B: Ca\(^{2+}\)-activated \(I_{\mathrm{KCa}}\). C: \(I_{\mathrm{k}}\). D: \([\mathrm{Ca}^{2+}]_{i}\). E: \(I_{\mathrm{CaL}}\).

Fig. 8. Ca\(^{2+}\) desensitization effects induced by cGMP and Ca\(^{2+}\) sensitivity of MLC phosphorylation. A: relative force \(R_{f}\), B: MLC phosphorylation. C: Ca\(^{2+}\) sensitivity (\(\Theta_{\text{Ca}}\)) under control condition \([R(K_{\text{m,mlcp}},R_{H,mlcp}) = 0, \text{ where } R_{H} \text{ is Hill coefficient}]\) and after application of cGMP analog 8-bromo-guanosine 3',5'-cyclic monophosphate (8-BrcGMP) at 10 \(\mu\)M \([R(K_{\text{m,mlcp}},R_{H,mlcp}) = 1]\) in the model because 8-BrcGMP reached the maximum regulatory effect at 10 \(\mu\)M in the experiments by Lee et al. (19). Model simulations, solid lines; \(\bullet\), data in control condition; \(\blacksquare\), with cGMP. Dashed line, \(R(K_{\text{m,mlcp}},R_{H,mlcp}) = 0.33\); dashed-dotted line, \(R(K_{\text{m,mlcp}},R_{H,mlcp}) = 0.67\).
Ca\textsuperscript{2+} desensitization/sensitization of the contractile system. The term “Ca\textsuperscript{2+} desensitization” used in most of the literature refers to MLC phosphorylation and force reduction at the same Ca\textsuperscript{2+} level, whereas the term “Ca\textsuperscript{2+} sensitization” indicates changes of MLC phosphorylation induced by Ca\textsuperscript{2+} variations.

**DISCUSSION**

The NO-induced VSMC relaxation mechanism is portrayed as an integrated cell signaling system. Our single cell model characterizes the complex interactions that take place among the biochemical NO/cGMP pathway, the electrophysiology of the cell membrane, and protein contractile kinetics.

The signal carried by NO is detected by sGC in terms of a ligand-receptor interaction process with two-step sGC activation kinetics (Fig. 1). The model structure adopts the scheme proposed by Zhao et al. (46). To interpret the measured data of Bellamy et al. (5), we proposed a cGMP feedback-controlled sGC decay from activated form $E_a$ to basal form $E_b$. The model predicts that the intermediate $E_d$ is the dominant steady-state species of sGC under physiological NO stimulation (Fig. 2). This model prediction suggests that the sGC desensitization by cGMP feedback may limit or stabilize cGMP production within a wide range of [NO] (Figs. 3 and 4), which may contribute to the robustness of the VSMC response to small NO perturbations. Recovery of sGC desensitization is a much slower process that was completed in ~10 min, as reported by Bellamy et al. (5). In our model, the recovery rate of desensitized sGC to control level directly depends on the rate of the cGMP degradation (Eq. 1). We showed that the $k_{sp}$ parameter varies under different orders of cGMP feedback inhibition of sGC to produce the same NO-induced cGMP accumulation responses (see Table 2), which determine different cGMP degradation rates. Therefore, cGMP feedback order $m$ in our model may be resolved by comparing the time constant of cGMP degradation to that of sGC recovery from desensitization.

cGMP regulation is a complex mechanism. Increasing amounts of experimental evidence suggest that basal PDE activity for cGMP hydrolysis is at a relatively low level. It can be activated (up to 9- to 11-fold) after cGMP binding to its regulatory NH\textsubscript{2}-terminal domain (so-called GAF domain) and further enhanced by PKG phosphorylation (23, 33, 34). On the other hand, it has been suggested that sGC can be deactivated by PKG-dependent phosphorylation, which provides another negative-feedback inhibition to tune down cGMP production (27). Studies by Mullershausen et al. (24, 25) showed that cGMP can have a transient response to sustained NO and drop further to a lower level in a later phase. Such a biphasic cGMP response indicates that the rate of cGMP synthesis is outcompeted by the degradation rate because of acceleration in PDE-mediated hydrolysis, a persistent inhibition of sGC activity, or the presence of both mechanisms. It can be also caused by the depletion of the sGC substrate GTP. This evidence would tend to indicate that the intracellular regulation and control of the NO-induced cGMP response in VSMC is the result of coordinated actions of all the competing factors. The current model could be greatly improved if these factors could be incorporated to analyze the molecular interactions and their biochemical roles, which can help to further elucidate the underlying mechanisms governing the cGMP regulation.

There is still much controversy regarding the existence of cGMP-independent NO mechanisms. Studies on rat basilar arteries suggest that NO causes vasodilation by a cGMP-dependent pathway without affecting the activity of the KCa channel (2, 35). In rat middle cerebral arteries, it appears that NO activates KCa channels via a cGMP-independent pathway (6, 45). The specific mechanism involved in the cGMP-independent pathway for NO-induced KCa channel activation is still unclear and is under investigation. In the present study, the model parameters for the KCa channel (Eq. 4) were identified by fitting the channel $P_{o}$ data ($P_{o}$-Ca\textsuperscript{2+} and $P_{o}$-V\textsubscript{m} relationships, Fig. 5) from Stockand and Sansom (36). Our model shows that sGC desensitization limits the capability of sGC to catalyze cGMP production and higher NO must be handled via an alternative mechanism to the cGMP pathway. The model hypothesizes the coexistence of both cGMP-dependent and cGMP-independent mechanisms. Our model simulations show that a direct NO effect via the KCa channel reduces MLC phosphorylation under the conditions of sGC inhibition (Fig. 6), which agrees qualitatively with the experimental study by Onoue and Katusic (29).

The model also suggests that long-term regulation of the expression of K\textsuperscript{+} channels (including KCa and K\textsubscript{v} via inhibition of NO) plays an important role in hypertension caused by VSMC membrane depolarization due to chronic endogenous NO deficiency (Fig. 7). Because membrane depolarization induced by elevating [K\textsuperscript{+}]o, can be attributed to all types of K\textsuperscript{+} channels expressed on the VSMC membrane, other K\textsuperscript{+} channels [e.g., ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channel] that were not represented in our model could also be potentially affected by long-term NO inhibition.

In addition, we investigated the Ca\textsuperscript{2+} desensitization effect of the VSMC contractile system by cGMP. The model simulations closely agree with data by Lee et al. (19) for cGMP effects on MLC Ca\textsuperscript{2+}-phosphorylation and contractile Ca\textsuperscript{2+}

Other membrane targets for cGMP. cGMP can modulate behaviors of other membrane channels and lead to Ca\textsuperscript{2+} reduction. These include deactivation of the L-type Ca\textsuperscript{2+} channel (38), activation of membrane Ca\textsuperscript{2+}-ATPase (44), and inhibition of the Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channel.

NO/cGMP effects on Ca\textsuperscript{2+} sparks. Ca\textsuperscript{2+} sparks released from the sarcoplasmic reticulum may contribute to global Ca\textsuperscript{2+} concentrations and/or cause membrane hyperpolarization by
activating K$_{Ca}$ channels (18). A recent study shows that NO inhibits the activity of norepinephrine on these sites partly via a cGMP-independent pathway but does not increase spark activity or recruit new so-called frequent discharge sites (30). Other studies, however, suggest that cGMP-mediated VSMC relaxation may occur partially through an increase in the frequency of Ca$^{2+}$ sparks and a subsequent increase in the activity of K$_{Ca}$ channels (18). Our modeling study would require the knowledge of the mechanisms of Ca$^{2+}$ spark modulations (e.g., frequency modulation and amplitude modulation) by NO/cGMP-related factors.

Despite the above limitations, our model represents an extensible framework for the study of the important NO/cGMP signaling mechanisms. It also can potentially be incorporated into a multiscaled model to study blood vessel behavior under various physiological and pathological conditions (43). Considering the large variety of VSMC types across different tissues and species, NO/cGMP mechanisms are bound to vary and have designs that are specific to cell types. Certain regulatory targets present in one cell type may not be necessarily present in another, or may respond differently to NO signals. Moreover, in most cases, reported experimental results in vitro or on purified molecules were detected under conditions different from those in vivo. Thus the model can serve as a general modeling framework for the study of NO/cGMP effects on the VSMC as a complex cell signaling system. This framework can be tailored to address particular situations and can also be expanded to incorporate newly observed mechanisms.

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