Complex interactions of NO/cGMP/PKG systems on Ca\(^{2+}\) signaling in afferent arteriolar vascular smooth muscle

Susan K. Fellner and William J. Arendshorst

Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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Fellner SK, Arendshorst WJ. Complex interactions of NO/cGMP/PKG systems on Ca\(^{2+}\) signaling in afferent arteriolar vascular smooth muscle. Am J Physiol Heart Circ Physiol 298: H144–H151, 2010. First published October 30, 2009; doi:10.1152/ajpheart.00485.2009.—Little is known about the effects of nitric oxide (NO) and the cyclic GMP (cGMP)/protein kinase G (PKG) system on Ca\(^{2+}\) signaling in vascular smooth muscle cells (VSMC) of resistance vessels in general and afferent arterioles in particular. We tested the hypotheses that cGMP- and Ca\(^{2+}\)-dependent big potassium channels (BKCa\(_{\text{Ca}^{2+}}\)) buffer the Ca\(^{2+}\) response to depolarization by high extracellular KCl and that NO inhibits adenosine diphosphoribose (ADPR) cyclase, thereby reducing the Ca\(^{2+}\)-induced Ca\(^{2+}\) release. We isolated rat afferent arterioles, utilizing the magnetized microsphere method, and measured cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) with fura-2, a preparation in which endothelial cells do not participate in [Ca\(^{2+}\)]\(_i\) responses, KCIs (50 mM)-induced depolarization causes an immediate increase in [Ca\(^{2+}\)]\(_i\), of 151 nM. The blockers N\(^{\text{N}}\)-nitro-L-arginine methyl ester (of nitric oxide synthase), 1,2,4-oxadiazolo-[4,3-\(a\)]quinolin-1-one (ODQ, of guanylyl cyclase), KT-5823 (of PKG activation), and iberiotoxin (IBX, of BKCa\(_{\text{Ca}^{2+}}\) activity) do not alter the [Ca\(^{2+}\)]\(_i\) response to KCIs, suggesting no discernible endothelial NO production under basal conditions. The NO donor sodium nitroprusside (SNP) reduces the [Ca\(^{2+}\)]\(_i\), response to 77 nM; IBX restores the response to control values. These data show that activation of BKCa\(_{\text{Ca}^{2+}}\) in the presence of NO/cGMP provides a brake on KCIs-induced [Ca\(^{2+}\)]\(_i\) responses. Experiments with the inhibitor of cyclic ADPR 8-bromo-cyclic ADPR (8-Br-cADPR) and SNP + downstream inhibitors of PKG and BKCa\(_{\text{Ca}^{2+}}\) suggest that NO inhibits ADPR cyclase in intact arterioles. When we pretreat afferent arterioles with 8-bromoguanosine 3',5'-cyclic mono-phosphate (8-Br-cGMP; 10 

FOR NEARLY 20 YEARS, investigators have explored mechanisms by which nitric oxide (NO) causes vasodilatation and blunts vasoconstriction. Nearly all studies of the renal microcirculation have examined renal blood flow (RBF) in intact animals or arteriolar diameter changes in isolated vessels or other in vitro preparations. To our knowledge, no laboratory has studied the Ca\(^{2+}\) signaling events that occur as a consequence of exposure to NO or to the subsequent formation of cyclic GMP (cGMP) and activation of protein kinase G (PKG) in a renal resistance vessel.

The vasodilatory properties of the NO/cGMP/PKG system have been ascribed to stimulation or inhibition of numerous Ca\(^{2+}\) signaling pathways in vascular smooth muscle cells (VSMC). Inhibition of inositol trisphosphate receptor (IP\(_{3}\)R), the Na\(^+/Ca\(^{2+}\) exchanger in both forward and reverse modes, voltage-gated L-type channels, adenosine diphosphoribose (ADPR) cyclase, phosphodiesterase 3 (responsible for cAMP degradation), canonical transient receptor potential (TRPC)6, and myosin light chain (MLC) phosphatase have been reported (1, 13, 14, 19, 34, 48). NO/cGMP/PKG activates maxi or big Ca\(^{2+}\)-activated K channels (BKCa\(_{\text{Ca}^{2+}}\)) in nonrenal vessels (34, 39, 41). Changes in activity of Na\(^+/Ca\(^{2+}\) exchange, BKCa\(_{\text{Ca}^{2+}}\), TRPC6, and L-type channels affect Ca\(^{2+}\) entry, whereas inhibition of IP\(_{3}\)R and ADPR cyclase diminish Ca\(^{2+}\) mobilization. Alterations in Ca\(^{2+}\) sensitization occur in response to NO/cGMP/PKG but are not involved in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) levels per se.

A key candidate for NO-induced vasodilatation in many vascular beds has been activation of BKCa\(_{\text{Ca}^{2+}}\) and subsequent hyperpolarization (34, 39, 41). Evidence for the presence of BKCa\(_{\text{Ca}^{2+}}\) has been demonstrated by immunohistochemistry in isolated afferent arterioles (26) and physiologically in the blood-perfused juxtaglomerular nephron preparation (19, 49). In single VSMC isolated from rabbit arcuate artery, the current amplitude of BKCa\(_{\text{Ca}^{2+}}\) at positive voltages is inhibited by iberiotoxin (IBX) (37). The vast majority of studies of nonrenal vessels suggest that cGMP and PKG facilitate activation of BKCa\(_{\text{Ca}^{2+}}\) (30, 41). However, some have shown that 1,2,4-oxadiazolo-[4,3-\(a\)]quinolin-1-one (ODQ) inhibition of guanylyl cyclase fails to alter activation of BKCa\(_{\text{Ca}^{2+}}\) channels by the NO donor sodium nitroprusside (SNP), suggesting a direct effect of NO on mesenteric artery VSMC (29). Studies of afferent arteriolar diameter utilizing the blood-perfused juxtaglomerular vascular model report that stimulation of BKCa\(_{\text{Ca}^{2+}}\) with the agonist NS-1619 causes an increase in baseline diameter and inhibits the vasoconstrictor effect of angiotensin II (ANG II). On the other hand, inhibition of BKCa\(_{\text{Ca}^{2+}}\) activity with tetraethylammonium reduces lumen diameter (49) but does not alter the response to ANG II (7). Experiments measuring changes in RBF with ANG II fail to show an effect of IBX, a specific inhibitor of BKCa\(_{\text{Ca}^{2+}}\), but when the channel is stimulated with the agonist NS-1619 the vasoconstrictor response to ANG II is diminished (26). Thus controversy exists in defining the physiological functional role of BKCa\(_{\text{Ca}^{2+}}\) in the contractile responses in the renal microcirculation.

We designed our experiments to test the hypothesis that activation of BKCa\(_{\text{Ca}^{2+}}\) provides an important brake on depolarization-related increases in [Ca\(^{2+}\)]\(_i\), in afferent arterioles when components of the NO/cGMP/PKG system are active. An increase in [Ca\(^{2+}\)]\(_i\), is considered a major driving force for contraction of VSMC. We chose high extracellular KCl as the agent to increase [Ca\(^{2+}\)]\(_i\), in afferent arteriolar VSMC to simplify the model. Because G protein-coupled receptors are not stimulated, there should be no activation of IP\(_{3}\)R, no receptor-operated Ca\(^{2+}\) entry or activation of TRPC channels, and no

Address for reprint requests and other correspondence: S. K. Fellner, Dept. of Cell and Molecular Physiology, Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545 (e-mail: sfellner@med.unc.edu).

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products of arachidonic acid metabolism. KCl-induced increases in [Ca\textsuperscript{2+}]i alter VSMC Ca\textsuperscript{2+} sensitivity primarily at the level of MLC phosphorylation by modulation of MLC phosphatase activity and possibly by modulation of MLC kinase activity as well (38). However, because we are studying changes in [Ca\textsuperscript{2+}]i, and not arteriolar contraction, the effect of NO on the MLC sensitivity to Ca\textsuperscript{2+} is not an issue. KCl does, of course, cause plasma membrane depolarization. We chose SNP as our source of NO because SNP is degraded inside cells to yield NO, thereby avoiding problems with spontaneous degradation in stock solutions or issues of diffusion time into the cell (1, 17). Because certain processes may contribute to or oppose the effects of the NO/cGMP/PKG system on Ca\textsuperscript{2+} signaling in VSMC, we employed a variety of pharmacological inhibitors to study these pathways.

METHODS

All studies were approved by and performed in compliance with the guidelines and practices of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Preparation of fresh afferent arterioles. We used the magnetized polystyrene microsphere-sieving technique as previously described in our laboratory (9) to isolate afferent arterioles (<20 μm in diameter) from 4.5- to 5-wk-old (80–110 g) male Sprague-Dawley rats maintained in the Chapel Hill colony. Phosphate-buffered saline (PBS) containing (in mM) 137 NaCl, 4.1 KCl, 0.66 KH\textsubscript{2}PO\textsubscript{4}, 3.4 Na\textsubscript{2}HPO\textsubscript{4}, 2.5 NaHCO\textsubscript{3}, 1.0 MgCl\textsubscript{2}, and 5 glucose was adjusted daily to pH 7.4 at 4°C and 23°C. The vessel segments in PBS containing 0.1% bovine serum albumin (BSA) were treated with collagenase type IV (Worthington, Lakewood NJ; 374 U/mg, 4–5 μg/ml) for 18 min at 34°C. Arterioles were loaded with fura-2 AM (3 μM) and 0.1% BSA for 55 min at 23°C in the dark. After arterioles were washed with PBS, the suspension was kept in Ca\textsuperscript{2+} (1.1 mM)-containing buffer on ice.

Measurement of cytosolic free calcium concentration. We measured [Ca\textsuperscript{2+}]i, as previously described (9). To enhance immobilization of vessels to the coverslips, we coated the glass with CellTak (BD Bioscience, Bedford, MA). Afferent arterioles were identified by their morphology and measured external diameter of 15–20 μm. As well, we required visualization of microspheres in the lumen of the afferent arteriole or in the proximal branch of an interlobular artery from which it arose, to exclude the possibility that the vessel was an efferent arteriole. A segment of an afferent arteriole was centered in a small window of the optical field that was free of glomeruli or tubular fragments. There was heterogeneity of sampling sites along the vessel length.

The VSMC were excited alternately with light of 340- and 380-nm wavelength from a dual-excitation wavelength Delta-Scan equipped with dual monochrometers and a chopper [Photon Technology International (PTI), Birmingham, NJ]. After signals passed through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. Signal intensity was acquired, stored, and processed by an IBM-compatible Pentium computer and Felix software (PTI). Background subtraction was performed in all studies. There was no interruption in the recording during the addition of reagents to the chamber. A video camera projected images of afferent arterioles onto a video monitor, permitting visualization of contraction of vessel segments.

We previously demonstrated that application of fura-2 and pharmacological agents on the abluminal side of the afferent arteriole results in no detectable contribution to the [Ca\textsuperscript{2+}]i signal from endothelial cells (EC). Bradykinin (10\textsuperscript{-7}–10\textsuperscript{-5} M) increases [Ca\textsuperscript{2+}]i, by 10–25 nM, which is not significantly different from baseline. N\textsuperscript{ω}-nitro-L-arginine methyl ester (l-NAME) does not change the [Ca\textsuperscript{2+}]i response to the endothelin-B (ET\textsubscript{B}) receptor agonist sarafotoxin in our preparation (8), to high KCl in isolated microperfused afferent arterioles (51), or to ANG II in afferent arterioles isolated with the agarose gel infusion technique (16). We do not know whether the microspheres (4.5-μm diameter) in our preparation, during their passage through arterioles and ending in glomeruli, damage EC.

Reagents. Reagents were added to the bath as concentrated solutions to achieve a final desired concentration in the buffer. When vessels were pretreated with an agent, at least 2 min elapsed before addition of KCl. We purchased 8-bromo-cyclic ADPR (8-Br-cADPR), IBX, SNP and l-NAME from Sigma Aldrich (St. Louis, MO), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), KT-5823, and ODQ from CalBiochem (San Diego, CA), fura 2-AM from Molecular Probes (Eugene, OR), and magnetized microspheres (4.5 μm) from Spherotech (Libertyville, IL).

Rationale for pharmacological agents. We used SNP to supply a fixed exogenous amount of NO. SNP is commonly used at a concentration of 100 μM to generate NO in vitro (52, 55). Measured levels of NO in EC are in the high nanomolar range (22). The extent to which SNP liberates NO in VSMC in our preparation is unknown but likely is in the micromolar range.

IBX (100 nM) is a specific inhibitor of the BKCa\textsubscript{2+}-channel (45). To evaluate actions of NO and the cGMP/PKG system independent of activation of BKCa\textsubscript{2+}, we pretreated arterioles with the inhibitor.

When we wished to examine the effects of cGMP independent of those due to NO itself, we employed 8-Br-cGMP (10 μM), which resists hydrolysis and is able to enter the cell (27).

To block the activation of the cGMP/PKG system, we used two agents. KT-5823 (1 μM) blocks the activation of PKG; ODQ (10 μM) antagonizes soluble guanylyl cyclase. Such intervention isolates the actions of NO independent of the cGMP/PKG system.

To eliminate the possibility of a contribution of endogenous NO, we used l-NAME (10 μM) to inhibit NO synthase and the formation of NO (5, 24, 36, 51).

We previously showed that the response of afferent arterioles to KCl depends in part on endogenous cADPR and the ryanodine receptor (RyR) to amplify Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) (11). 8-Br-cADPR antagonizes the action of cADPR on the RyR (55). Because NO has been reported to inhibit ADPR cyclase in coronary VSMC, we wanted to compare the effects of inhibiting the enzyme versus blocking its product (55).

KCl was used at a concentration of 50 mM; higher concentrations produce only modest increments in the [Ca\textsuperscript{2+}]i, response. Furthermore, we are concerned that concentrations of 100 mM lower the extracellular Na\textsuperscript{+} concentration sufficiently to activate reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange.

In no case did any of the aforementioned reagents, alone or in combination, cause a statistical difference in baseline values of [Ca\textsuperscript{2+}], in afferent arterioles.

Statistics. Individual arterioles were studied only once and then discarded; thus each recording originates from a separate vessel. At least six arterioles from a minimum of two rats per experiment on two different days were examined for each data set. Data are presented as means ± SE (Table 1). Group comparisons were evaluated by oneway ANOVA, with P < 0.05 considered statistically significant (GraphPad Prism Software, La Jolla, CA).

RESULTS

Measured baseline [Ca\textsuperscript{2+}]i for the entire data set was 110 ± 5 nM. In no experimental subgroup was baseline [Ca\textsuperscript{2+}]i different from control baseline (109 ± 2 nM) or from the grand mean.

[Ca\textsuperscript{2+}]i, response to KCl. Afferent arterioles respond to KCl (50 mM) with a prompt peak increase of 151 ± 3 nM followed by a sustained plateau 50–75 s later of Δ101 ± 2 nM above baseline (n = 28, Fig. 1, Table 1).
Table 1. Summary of increases in cytosolic calcium concentration of afferent arterioles in response to high extracellular KCl and pharmacological agents

<table>
<thead>
<tr>
<th>Group</th>
<th>Peak [Ca(^{2+})]</th>
<th>Plateau [Ca(^{2+})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>151 ± 3 (28)</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>KCl + l-NAME</td>
<td>126 ± 21 (6)</td>
<td>97 ± 20</td>
</tr>
<tr>
<td>KCl + ODQ</td>
<td>124 ± 15 (4)</td>
<td>90 ± 16</td>
</tr>
<tr>
<td>KCl + KT-5823</td>
<td>105 ± 10 (5)</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>KCl + IBX</td>
<td>134 ± 2 (5)</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>KCl + SNP</td>
<td>77 ± 8 (12)</td>
<td>60 ± 8 (&lt;0.001)</td>
</tr>
<tr>
<td>KCl + SNP + IBX</td>
<td>126 ± 18 (10)</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>KCl + SNP + KT-5823</td>
<td>54 ± 1</td>
<td>45 ± 1 (&lt;0.001)</td>
</tr>
<tr>
<td>KCl + SNP + IBX + KT-5823</td>
<td>75 ± 3 (9)</td>
<td>60 ± 2 (&lt;0.001)</td>
</tr>
<tr>
<td>KCl + SNP + ODQ</td>
<td>66 ± 6 (5)</td>
<td>47 ± 5 (&lt;0.001)</td>
</tr>
<tr>
<td>KCl + SNP + 8-Br-cADPR</td>
<td>81 ± 3 (11)</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>KCl + 8-Br-cGMP</td>
<td>143 ± 8 (9)</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>KCl + 8-Br-cGMP + IBX</td>
<td>340 ± 27 (9)</td>
<td>137 ± 7 (&lt;0.001)</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of observations. [Ca\(^{2+}\)] = cytosolic calcium concentration; l-NAME = nitro-l-arginine methyl ester; ODQ, 1,2,4-oxadiazole-3,4-di-N-[3-quinolinyl]-l-one; IBX, iberiotoxin; SNP, sodium nitroprusside; 8-Br-cADPR, 8-bromo-cyclic adenosine diphosphoribose; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate. *P vs. KCl: ANOVA for “peak” P < 0.001, ANOVA for “plateau” P < 0.001; †P vs. KCl + SNP: ANOVA for “peak” P < 0.001, ANOVA for “plateau” P < 0.001; NS, not significant (P > 0.05).

Effect of interruption of NO/cGMP system on [Ca\(^{2+}\)] responses to KCl. We showed previously (8) that bradykinin and l-NAME do not alter the [Ca\(^{2+}\)] response to sarafotoxin (ET\(_B\) receptor agonist) or endothelin-1 in afferent arteriolar VSMC. In the presence of l-NAME, the peak response to KCl is Δ126 ± 21 nM and the plateau is 97 ± 20 nM [P not significant (NS) for both vs. control]. To further explore a possible role of endogenous NO in our afferent arterioles, we used two inhibitors of the cGMP/PKG system, ODQ and KT-5823. In the presence of KT-5823, the peak [Ca\(^{2+}\)] response to KCl is Δ105 ± 10 nM and the plateau is 77 ± 10 (P = NS vs. control). When arterioles are pretreated with ODQ, the [Ca\(^{2+}\)] response is Δ124 ± 15 nM (n = 6, P > 0.5 vs. control). These results further substantiate our assumption that EC are not physiologically active (producing endogenous NO to affect Ca\(^{2+}\) signaling) in our preparation of afferent arterioles (Fig. 1B).

Inhibition of BK\(_{Ca}\) and KCl responses. Because BK\(_{Ca}\) are present in afferent arterioles and because activation of the channels with NO/cGMP or an increase in [Ca\(^{2+}\)], should lead to hyperpolarization and a subsequent reduction in [Ca\(^{2+}\)], entry, we tested the response to KCl in the presence of the specific inhibitor IBX (100 nM). IBX has no net effect on the peak or plateau [Ca\(^{2+}\)], response to KCl Δ134 ± 2 and Δ108 ± 4 nM respectively; both P = NS; Figs. 1B and 2B, first 2 pairs of bars). Thus, under control conditions and in the absence of exogenous NO, there is no discernible activation of the BK\(_{Ca}\) to alter [Ca\(^{2+}\)], responses during KCl-induced depolarization.

[Ca\(^{2+}\)], responses to KCl in afferent arterioles pretreated with SNP to generate NO. As noted above, SNP has no effect on baseline [Ca\(^{2+}\)] (109 ± 1 control vs. 114 ± 3 nM with SNP). In the presence of SNP, however, the peak [Ca\(^{2+}\)], response to KCl is reduced by ~50% to Δ77 ± 8 nM and the plateau by ~40% to Δ60 ± 2 nM (both P < 0.001; Fig. 2, Table 1). Thus exogenous NO exerts a pronounced inhibitory effect on the KCl-induced increase in [Ca\(^{2+}\)].

Effect of iberiotoxin in presence of NO. The peak increase in the [Ca\(^{2+}\)], response to KCl in the presence of IBX and SNP is Δ126 ± 18 nM, and the plateau is Δ86 ± 10 nM [P < 0.05 vs. SNP without IBX for peak and P = NS for plateau, but neither is different from control responses to KCl, P = NS; Fig. 2B]. These data show that exogenously administered NO can activate BK\(_{Ca}\): to limit Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (VGCC) activated by KCl-induced depolarization and reduce the overall [Ca\(^{2+}\)] response. The specific BK\(_{Ca}\) inhibitor IBX negates this group of related processes.

Inhibition of activation of PKG. The peak [Ca\(^{2+}\)], response to KCl in the presence of SNP and KT-5823 is Δ54 ± 1 nM (P < 0.01 vs. control, P = NS vs. KCl + SNP; Fig. 3, Table 1). Addition of IBX to SNP and KT-5823 results in a peak [Ca\(^{2+}\)], response of KCl of 75 ± 3 nM (P < 0.01 vs. control, P = NS vs. KCl + SNP). These results suggest the presence of a PKG-dependent system that, when inhibited, is associated with a fall rather than the anticipated increase in the [Ca\(^{2+}\)], response to KCl. Moreover, this intriguing process is masked when BK\(_{Ca}\) is inhibited with IBX, that is, the [Ca\(^{2+}\)], responses rise back to the values with SNP alone (P = 0.02). That plateau [Ca\(^{2+}\)], values fall suggests that Ca\(^{2+}\) entry is inhibited.

Experiments were conducted with ODQ to block the formation of cGMP, and the results were very similar to those with KT-5823 (Table 1). To further delineate the nature of the cGMP/PKG-dependent process, we performed experiments with exogenous 8-Br-cGMP.

[Ca\(^{2+}\)], response to KCl in presence of 8-Br-cGMP. Exposure of afferent arteriolar VSMC to cGMP could result in multiple effects, such as stimulation of BK\(_{Ca}\) or inhibition of voltage-gated L-type channels. In both cases, one would expect cGMP to decrease the [Ca\(^{2+}\)], response to KCl. This does not occur, however, when KCl stimulates VSMC pretreated with 8-Br-cGMP (10 μM). Neither the peak [Ca\(^{2+}\)], response to KCl of Δ143 ± 8 nM nor the plateau response of 109 ± 8 nM in the presence of 8-Br-cGMP is different from control KCl responses (Fig. 4A, Table 1). The data do not exclude, however, the possibility that equal and opposite mechanisms are operative, that is, a combination of pathways inhibited by and stimulated by cGMP.
When arterioles are treated with both 8-Br-cGMP and IBX, the peak \([\text{Ca}^{2+}]_i\) response to KCl is more than doubled, increasing to \(340 \pm 27 (P = 0.001\text{ vs. } 8\text{-Br-cGMP without IBX; Fig. 4).}\) The plateau \([\text{Ca}^{2+}]_i\) response is increased by 25%. These data strongly support the idea that there is a cGMP-dependent pathway that enhances the \([\text{Ca}^{2+}]_i\) response to KCl in afferent arterioles. We are unaware of any currently identified process in which NO or cGMP increases \([\text{Ca}^{2+}]_i\) in VSMC. These unusual results raise the question of whether \(\text{Ca}^{2+}\) entry via activation of a \(\text{Ca}^{2+}\)-, cGMP-dependent Cl\(^-\) channel (Cl\(_{\text{Ca}^{2+},cGMP}\)) is a possible candidate for this process (vide infra).

ADPR cyclase and NO. We designed this protocol to test the action of NO, independent of activation of BK\(_{\text{Ca}^{2+}}\) and other downstream consequences of cGMP/PKG signaling. By pre-treating afferent arterioles with both KT-5823 and IBX, we avoid these other \(\text{Ca}^{2+}\)-related mechanisms (Fig. 5). Under these conditions, the peak \([\text{Ca}^{2+}]_i\) response to KCl is \(\Delta 75 \pm 3\) nM (\(P = \text{NS}\) vs. SNP alone, \(P < 0.001\) vs. control). Although NO has been implicated in the activation of BK\(_{\text{Ca}^{2+}}\)-independent of cGMP/PKG, the presence of IBX eliminates this possibility (29). When 8-Br-cADPR is added to SNP, the peak \([\text{Ca}^{2+}]_i\) response to KCl is \(\Delta 81 \pm 3 (P = \text{NS}\) vs. both SNP and SNP + KT-5823 + IBX), demonstrating that the specific cADPR inhibitor causes no further inhibition in the presence of NO. Thus NO alone, in the absence of other downstream effects, inhibits the \([\text{Ca}^{2+}]_i\) response to KCl by ~45%, a value very similar to that of the effect of 8-Br-cADPR on KCl-induced \([\text{Ca}^{2+}]_i\) responses in our previous studies (11). Because inhibition of ADPR cyclase (and formation of cADPR) diminishes mobilization of \(\text{Ca}^{2+}\) via the RyR from the sarcoplasmic reticulum, one would expect a greater effect on initial peak rather than plateau values of \([\text{Ca}^{2+}]_i\). It is generally accepted that mobilization accounts for at least half of peak...

Fig. 1. Changes in cytosolic \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) of isolated afferent arterioles to KCl (50 mM). A: representative recording of \([\text{Ca}^{2+}]_i\) response to KCl. B: summary data of increases in peak (left bar in each pair) and plateau (right bar in each pair) \([\text{Ca}^{2+}]_i\), in afferent arterioles in response to KCl (50 mM) in afferent arteriolar vascular smooth muscle cells (VSMC) in the absence or presence of the inhibitors \(\text{N}^\circ\)-nitro-L-arginine methyl ester (L-NAME; nitric oxide synthase), 1,2,4-oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ; soluble guanylyl cyclase), KT-5823 (protein kinase G), or iberiotoxin (IBX; \(\text{Ca}^{2+}\)-dependent big potassium channel (BK\(_{\text{Ca}^{2+}}\)), \(P = \text{not significant (NS) vs. control for each agent, both peak and plateau. See Table 1 for } P \text{ and } n \text{ values.}

8-Br-cGMP plus IBX. When arterioles are treated with both 8-Br-cGMP and IBX, the peak \([\text{Ca}^{2+}]_i\) response to KCl is more than doubled, increasing to \(\Delta 340 \pm 27 (P < 0.001\text{ vs. } 8\text{-Br-cGMP without IBX; Fig. 4).}\) The plateau \([\text{Ca}^{2+}]_i\) response is increased by 25%. These data strongly support the idea that there is a cGMP-dependent pathway that enhances the \([\text{Ca}^{2+}]_i\) response to KCl in afferent arterioles. We are unaware of any currently identified process in which NO or cGMP increases \([\text{Ca}^{2+}]_i\) in VSMC. These unusual results raise the question of whether \(\text{Ca}^{2+}\) entry via activation of a \(\text{Ca}^{2+}\)-, cGMP-dependent Cl\(^-\) channel (Cl\(_{\text{Ca}^{2+},cGMP}\)) is a possible candidate for this process (vide infra).

ADPR cyclase and NO. We designed this protocol to test the action of NO, independent of activation of BK\(_{\text{Ca}^{2+}}\) and other downstream consequences of cGMP/PKG signaling. By pre-
channel should counteract an increase in \([\text{Ca}^{2+}]\), and depolarization that follows agonist stimulation and therefore could be important in the control of renal vascular tone. Although the channel is typically viewed as \(\text{Ca}^{2+}\) dependent, it is also voltage sensitive and activated by NO/cGMP/PKG. The weight of evidence supports activation of \(\text{BK}_{\text{Ca}^{2+}}\) by PKG rather than NO per se (30, 34, 39, 44). Patch clamp studies in renal artery EC show strong voltage and cGMP dependence of \(\text{BK}_{\text{Ca}^{2+}}\). In the absence of exogenous cGMP or at neutral voltage, high micromolar concentrations of \(\text{Ca}^{2+}\) are required to open the channel (4). Depolarization enhances the open probability. The resting membrane potential in rat afferent arterioles is between −40 and −65 mV, depending on the experimental preparation (25, 53). High extracellular KCl will, of course, depolarize the membrane. Thus assessment of the role of \(\text{BK}_{\text{Ca}^{2+}}\) in any experimental model must take into account four variables: [\(\text{Ca}^{2+}\)], responses, whereas entry is largely responsible for the plateau phase. This is indeed the case for this group of experiments (Table 1).

Table 1 summarizes the peak and plateau values for increase in \([\text{Ca}^{2+}]\) from baseline values and \(P\) values based on ANOVA for each data set.

**DISCUSSION**

We designed the present study to test the hypothesis that activation of \(\text{BK}_{\text{Ca}^{2+}}\) provides an important brake on increases in \([\text{Ca}^{2+}]\) in VSMC of afferent arterioles that are depolarized by high extracellular KCl. Our new data demonstrate the importance of \(\text{BK}_{\text{Ca}^{2+}}\) in the global \([\text{Ca}^{2+}]\) response to KCl and uncover some of the complexities of the NO/cGMP/PKG system in \(\text{Ca}^{2+}\) signaling in this important renal resistance arteriole.

The traditional view of the role of EC-produced NO is that NO diffuses out of the EC plasma membrane through the VSMC cell membrane and into the VSMC cytosol. More recently, it has been shown that aquaporin-1 transports NO across EC and VSMC membranes, facilitating the rate of transfer compared with simple diffusion (18). Once transported, NO may interact directly or via the cGMP/PKG signaling system with a variety of channels or enzymes, the net result of which leads to some reversal of vasoconstriction, a major fraction of which is due to a reduction in \([\text{Ca}^{2+}]\). Key candidates for counteracting a vasoconstrictor-induced rise in \([\text{Ca}^{2+}]\) are reduced \(\text{Ca}^{2+}\) entry secondary to activation of \(\text{BK}_{\text{Ca}^{2+}}\) channels and/or inhibition of voltage-gated L-type channels and diminished \(\text{Ca}^{2+}\) mobilization caused by inhibition of ADPR cyclase. cADPR is required for optimal operation of the RyR to produce CICR. To explore the integrated effects of NO/cGMP on afferent arteriolar VSMC, we elected to use 50 mM KCl to cause membrane depolarization and to initiate \(\text{Ca}^{2+}\) signaling cascades. By so doing, we avoid consideration of participation of IP3R activation, receptor-operated \(\text{Ca}^{2+}\) mechanisms, and arachidonic acid metabolites.

In VSMC of afferent arterioles and interlobular arteries, although \(\text{BK}_{\text{Ca}^{2+}}\) is highly expressed, its physiological function is poorly understood (26). Conceptually, operation of this
membrane potential, \([Ca^{2+}]\), cGMP concentration, and PKG activity. In our preparation, the afferent arterioles are not pressurized; the global \([Ca^{2+}]\) does not rise above \(\approx 400\) nM, and the supply of NO/cGMP is exogenous. We acknowledge that \([Ca^{2+}]\) may be higher in certain intracellular microenvironments.

The extent of depolarization caused by KCl (50 mM) in an afferent arteriolar VSMC is unknown. The results of the control experiments with KCl and IBX in the absence of SNP or 8-Br-cGMP are not different from those with KCl alone. Thus it appears that the increase in \([Ca^{2+}]\) and the level of depolarization following addition of KCl to afferent arteriolar VSMC is insufficient to activate BK\(_{Ca}\). Our data clearly show, however, that in the presence of putative physiological concentrations of cGMP, BK\(_{Ca}\) has a prominent braking effect on the increase in \([Ca^{2+}]\), initiated by KCl in afferent arterioles.

To explore the contribution of the cGMP/PKG system to KCl-induced \([Ca^{2+}]\), signaling in afferent arterioles independent of BK\(_{Ca}\), we treated the vessels with 8-Br-cGMP (10 \(\mu\)M) in the presence of IBX. 8-Br-cGMP does not alter the control \([Ca^{2+}]\), response to KCl. When arterioles are pretreated with 8-Br-cGMP plus IBX, however, the \([Ca^{2+}]\) response to KCl nearly doubled, suggesting that the cGMP/PKG system of VSMC has both stimulatory and inhibitory components to \([Ca^{2+}]\) signaling. These are surprising and novel findings.

To our knowledge, there is no previously described \(Ca^{2+}\) signaling pathway that responds to stimulation with cGMP to increase \([Ca^{2+}]\) in VSMC. Although the NO/cGMP/PKG system has been shown to activate skeletal muscle RyR1 via \(S\)-nitrosylation and cardiac RyR2 through \(S\)-nitrosogluthathione, it has no effect on caffeine stimulation of the RyR in pulmonary and aortic VSMC (21, 32, 47). Our results with 8-Br-cGMP in the presence of IBX are in the opposite direction of the traditional view that the cGMP/PKG system causes a decrease in \([Ca^{2+}]\).

It is well accepted that L-type VGCC provide a major pathway for \(Ca^{2+}\) entry in arteriolar arterioles. cGMP has been reported to inhibit L-type \(Ca^{2+}\) channel activity in portal vein and cultured aortic VSMC (2, 40). To date, there are no studies that examine the specific effects of cGMP/PKG on VGCC in arteriolar VSMC. If cGMP/PKG were a major pathway for reducing \([Ca^{2+}]\), one would anticipate reversal of SNP inhibition of \([Ca^{2+}]\), responses to KCl in the presence of KT-5823 or ODQ. Instead, we observe the opposite effect—that is, blockade of cGMP/PKG with KT-5823 or ODQ inhibits the \([Ca^{2+}]\) response to KCl in afferent arterioles, supporting the concept that there is a cGMP/PKG-dependent process that increases \([Ca^{2+}]\).

Although some investigators believe that \(Ca^{2+}\) stimulation of \(Ca^{2+}\)-activated Cl\(^-\) channels (Cl\(_{Ca}\)) causes sufficient depolarization to activate VGCC, we (12) and others (43) note that inhibitors of this classic Cl\(_{Ca}\) fail to alter the response to ANG II in afferent arterioles. Many of the drugs utilized to inhibit Cl\(_{Ca}\) are nonspecific and, depending on concentration, may activate BK\(_{Ca}\) (15, 35). The classic Cl\(_{Ca}\) is voltage dependent and requires \(\approx 650\) nM \([Ca^{2+}]\), at a voltage of \(-80\) mV and \(300\) nM at \(+100\) mV for activation (reviewed in Ref. 31). It is an open question whether there is sufficient depolarization and high enough \([Ca^{2+}]\) to immediately activate the Cl\(_{Ca}\) after agonist stimulation of afferent arteriolar VSMC.

A cGMP-dependent \(Ca^{2+}\)-activated Cl\(^-\) channel (Cl\(_{Ca}\)) has been identified according to its effects on vasomotion and electrophysiological characteristics in small mesenteric arterial VSMC (3, 27, 33, 36). In contrast to the classic Cl\(_{Ca}\), Cl\(_{Ca}\) has an EC\(_{50}\) for \([Ca^{2+}]\), of \(\approx 74\) nM, is activated by 3–6 \(\mu\)M cGMP, is voltage- and time independent, is not inhibited by niflumic acid or by DIDS (<200 \(\mu\)M), and is inhibited by 2–6 \(\mu\)M Zn\(^{2+}\) (28, 36). Subsequently, electrophysiological evidence for a Cl\(_{Ca}\), cGMP has been found in a variety of vessels such as renal, cerebral, and superior mesenteric arteries and femoral and portal veins (28). The low IC\(_{50}\) for \(Ca^{2+}\) suggests that the channel may be partially activated at rest and contribute to maintenance of membrane potential.

Our study raises the question of a possible role of Cl\(_{Ca}\), cGMP in \(Ca^{2+}\) signaling in afferent arteriolar VSMC. Zn\(^{2+}\) (3 \(\mu\)M) consistently inhibits the responses to KCl in the present study (data not shown), but because Zn\(^{2+}\) also inhibits voltage-gated L-type channels, we cannot conclude that inhibition of a Cl\(_{Ca}\), cGMP is occurring (46). Future studies designed to explore a functional role for Cl\(_{Ca}\), cGMP in \(Ca^{2+}\) signaling in VSMC are required.

The importance of the role of cADPR in \(Ca^{2+}\) signaling VSMC and in endothelial cells has been summarized in a recent review (56). A growing body of literature demonstrates a critical role for cADPR in the \(Ca^{2+}\) response to angiotensin, endothelin, and superoxide generation (8, 10, 56). The ADPR cyclase of VSMC differs from the cyclase of sea urchin eggs, Aplysia, and HL-60 cells by being inhibited rather than stimulated by NO and Zn\(^{2+}\) (6, 55). There is convincing evidence for a direct inhibitory effect of NO on ADPR cyclase activity in membrane fragments of coronary VSMC and airway smooth muscle cells (54, 55). Because the active site of ADPR cyclase is ectocellular, NO need only exit the EC to initiate \(S\)-nitrosylation of the enzyme (23). Independence from entry into the VSMC plasma membrane, and from a requirement for formation of cGMP, might predict early responses to inhibition by NO in vivo, but the concentration of NO needed for \(S\)-nitrosylation is not yet known. A study with gaseous NO in the isolated, perfused hydronephrotic kidney shows an ODQ-insensitive component to ANG II constriction at concentrations of \(>1\) \(\mu\)M NO (50). Whether this vasorelaxant effect of NO occurs because of inhibition of ADPR cyclase is not yet known.

Fig. 5. Percent inhibition of peak \([Ca^{2+}]\), responses to KCl in afferent arteriolar VSMC. 8-Bromo-cyclic ADPR (8-Br-cADPR) inhibits the response by 44% (see Ref. 11). KT-5823 and IBX block downstream effects of the NO donor SNP, demonstrating the inhibitory effect of NO alone. No further inhibition occurs with addition of 8-Br-cADPR to KCl + SNP.
We find that the [Ca\(^{2+}\)]\(_i\) response of afferent arterioles to KCl in combination with SNP is not different from that achieved when both KT-5823 and IBX are blocking downstream effects of cGMP and of BK\(_{Ca}^{\text{2+}}\), or different from the response to KCl in the presence of the cADPR inhibitor 8-Br-cADPR. Under both of these experimental conditions, it is the peak rather than the plateau response that is largely affected, suggesting an inhibition of mobilization of [Ca\(^{2+}\)]\(_i\) from the SR. Thus our data are compatible with a direct effect of NO on ADPR cyclase activity in afferent arterioles. We previously showed (11) that the [Ca\(^{2+}\)]\(_i\) response of afferent arterioles to KCl involves CICR and that blockade of cADPR with 8-Br-cADPR diminishes the response by \(\sim 45\%\). Further studies are needed to explore the effects of NO on the ADPR cyclase pathway in VSMC of resistance vessels. Because the VSMC cyclase differs from that of many other cell types and because at least two ADPR cyclases have been identified in mammals (CD38 and CD157), differentiation of the distribution of enzymatic subtypes in EC and VSMC presents a particular challenge.

In summary, our study of Ca\(^{2+}\) signaling in afferent arteriolar VSMC provides new information showing that KCl-mediated increases in [Ca\(^{2+}\)]\(_i\), in the presence of exogenous NO or cGMP, strongly activate BK\(_{Ca}^{\text{2+}}\), suggesting an important role for this channel when intact renal resistance vessels are stimulated with depolarizing agonists. Our data are also suggestive of an inhibitory role of NO on ADPR cyclase to block the formation of cADPR and its effect on the RyR. A surprising finding is that a yet to be defined PKG-dependent pathway appears to be responsible for an increase in [Ca\(^{2+}\)]\(_i\). Future studies need to be designed to utilize physiological depolarizing and vasoconstrictor agonists such as ANG II to further investigate the role of the NO/cGMP/PKG system in arteriolar VSMC.

**REFERENCES**

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**GRANTS**

**NO/cGMP/PKG AND Ca\(^{2+}\) SIGNALLING IN AFFERENT ARTERIOLE**


