Mechanism of glucose-6-phosphate dehydrogenase-mediated regulation of coronary artery contractility

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Ata H, Rawat DK, Lincoln T, Gupte SA. Mechanism of glucose-6-phosphate dehydrogenase-mediated regulation of coronary artery contractility. Am J Physiol Heart Circ Physiol 300: H2054–H2063, 2011. First published March 11, 2011; doi:10.1152/ajpheart.01155.2010.—We previously identified glucose-6-phosphate dehydrogenase (G6PD) as a regulator of vascular smooth muscle contraction. In this study, we tested our hypothesis that G6PD activated by KCl via a phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-protein kinase C (PKC) pathway increases vascular smooth muscle contraction and that inhibition of G6PD relaxes smooth muscle by decreasing intracellular Ca2+ ([Ca2+]i) and Ca2+ sensitivity to the myofilament. Here we show that G6PD is activated by membrane depolarization via PKC and PTEN pathway and that G6PD inhibition decreases intracellular free calcium ([Ca2+]i) in vascular smooth muscle cells and thus arterial contractility. In bovine coronary artery (CA), KCl (30 mmol/l) increased PKC activity and doubled G6PD Vmax without affecting Km. KCl-induced PKC and G6PD activation was inhibited by bisperoxo(pyridine-2-carboxyl)oxovanadate (Bpvi; 10 μmol/l), a PTEN inhibitor, which also inhibited (P < 0.05) KCl-induced CA contraction. The G6PD blockers 6-aminonicotinamide (6AN; 1 mmol/l) and epiprostosterone (EPI; 100 μmol/l) inhibited KCl-induced increases in G6PD activity, [Ca2+]i, Ca2+-dependent myosin light chain (MLC) phosphorylation, and contraction. Relaxation of precontracted CA by 6AN and EPI was not blocked by calnoinx (10 μmol/l), a plasma membrane Ca2+ ATPase inhibitor or by lowering extracellular Na+, which inhibits the Na+/Ca2+ exchanger (NCX), but cyclopiazonic acid (200 μmol/l), a sarcoplasmic reticulum Ca2+-ATPase inhibitor, reduced (P < 0.05) 6AN- and EPI-induced relaxation. 6AN also attenuated phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Ser855, a site phosphorylated by Rho kinase, inhibition of which reduced (P < 0.05) KCl-induced CA contraction and 6AN-induced relaxation. By contrast, 6AN increased (P < 0.05) vasodilator-stimulated phosphoprotein (VASP) phosphorylation at Ser239, indicating that inhibition of G6PD increases PKA or PKG activity. Inhibition of PKG by RT-8-Br-PET-cGMPs (100 mmol/l) diminished 6AN-evoked VASP phosphorylation (P < 0.05), but RT-8-Br-PET-cGMPs increased 6AN-induced relaxation. These findings suggest G6PD inhibition relaxes CA by decreasing Ca2+ influx, increasing Ca2+ sequestration, and inhibiting Rho kinase but not by increasing Ca2+ extrusion or activating PKG.

pentose phosphate pathway; hexosemonophosphate shunt; NADPH; vascular smooth muscle; calcium; Rho kinase; protein kinase G; myosin phosphatase target subunit 1; vasodilator-stimulated phosphoprotein; phosphorylation

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD)-derived NADPH catalyzes reduction of oxidized (GSSG) to reduced (GSH) glutathione in all cell types, promotes nitric oxide synthesis in endothelial cells, and facilitates fatty acid synthesis in the liver (18, 40). It has been proposed that the pentose phosphate pathway is also involved in glucose metabolism in vascular smooth muscle (VSM) (1). Moreover, we (8) recently showed that G6PD is a novel regulator of VSM contractility. In that study, we demonstrated that G6PD is activated by membrane depolarization or by stimulation of receptor-mediated signaling pathways via protein kinase C (PKC). The activated G6PD elicits increases in intracellular free Ca2+ ([Ca2+]i), thereby enhancing contraction of VSM. Conversely, inhibition of G6PD activity reduces [Ca2+]i levels, thereby relaxing precontracted VSM (9). The mechanism by which membrane depolarization activates PKC and G6PD and how inhibition of G6PD reduces [Ca2+]i remain unclear, however.

A variety of PKC isoforms are expressed in the coronary artery (CA), and we have shown that PKCζ interacts with and activates G6PD by phosphorylating Ser210, near the catalytic domain, and that their interaction in CA is promoted by stimulation with KCl (8). PKCζ is activated by increases in diacylglycerol (DAG), but not by increases in [Ca2+]i. We therefore suggest (Fig. 1) that PKCζ and G6PD are activated by membrane depolarization in Ca2+-independent manner via the tumor suppressor enzyme phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a major enzyme that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) (29) and DAG pathway, which is increased by depolarization (17), resulting in the stimulation of PKC activity. In the present study, therefore, we tested our hypothesis that G6PD activated by KCl via a PTEN-PKC pathway increases VSM contraction and that inhibition of G6PD relaxes smooth muscle by decreasing [Ca2+]i and Ca2+ sensitivity to the myofilament. It is clear that inhibition of G6PD reduces [Ca2+]i, in precontracted arteries (9), but the mechanism is not well understood. Increases in VSM [Ca2+]i, are triggered by influxes of Ca2+ through several plasma membrane Ca2+ channels and by release of Ca2+ from stores (6, 7, 35). Conversely, [Ca2+]i is reduced by Ca2+ extrusion via plasma membrane Ca2+ ATPase (PMCA) and NCX and through sequestration of Ca2+ in the sarcoplasmic reticulum (SR) via sarcoplasmic/endoplasmic Ca2+ ATPase (SERCA) (14). Although less information is available about the effects of membrane depolarization on Ca2+ sequestration (SERCA) and extrusion (PMCA or NCX), it is known that inhibition of NCX increases the membrane potential and elevates [Ca2+]i (22). Therefore, as illustrated in a schematic (Fig. 1), we speculated that inhibition of G6PD reduces Ca2+ influx and facilitates the clearance of Ca2+ through all three aforementioned mechanisms and Ca2+ sensitivity to the myofilament. Our results show that G6PD inhibition relaxes CA mainly by reducing Ca2+ influx and enhanc-
antibodies mentioned in RESULTS (see Figs. 2–9) was performed as lysis buffer after which Western blot analysis using specific Chemical (Ann Arbor, MI).

Depolarization of VSM membrane results in activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol trisphosphate (IP3). Of the cleaved products, DAG is important in activation of protein kinase C (PKC), which phosphorylates and activates G6PD. Activated G6PD then stimulates rise in intracellular calcium concentration ([Ca2+]i) to induce contraction of VSM. As phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3) to yield PIP2, we hypothesized that membrane depolarization activates PTEN to result in G6PD activation and contraction of VSM. Furthermore, pharmacological inhibition of G6PD by 6-amino nicotinamide (6AN) or epiandrosterone (EPI) is known to relax precontracted VSM. We, thus, questioned whether this is mediated by [Ca2+]i sequestration and extrusion, along with a concomitant decrease in Ca2+ influx; and 2) inhibition of the Rho kinase pathway and activation of the protein kinase G (PKG) pathway, well-established mediators of vascular contraction and relaxation, respectively. PI3K, phosphotidylinositol 3-kinase.

on excitation at a single wavelength and expressed as the ratio of the time-resolved fluorescence variation from the basal fluorescence (at 480 nm; F480/520). [Ca2+]i, was also determined from the fura 3PE 340 nm/380 nm fluorescence ratio as previously described (8).

Contraction of CA. Isolated, endothelium-denuded left anterior descending CA rings were prepared from bovine hearts and studied for changes in isometric force as described previously (8). Low extracellular Na+ ([Na+]o) experiments were performed using previously published methods by replacing NaCl (120 mmol/l) with N-methyl-d-glucamine (120 mmol/l) in Kreb’s solution (22).

Phosphorylation of myosin phosphatase target subunit 1 and myosin light chain. Phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Ser1605, which is catalyzed by Rho kinase (37), in CA was assessed by Western blot analysis using anti-phospho-MYPT1 antibodies (Cell Signaling). In addition, phosphorylation of myosin light chain (MLC) at Ser20 in CA was assessed by Western blot analysis using anti-phospho-MYPT1 antibodies (Cell Signaling). The levels of NADP(H) in CA were determined for changes in isometric force as described previously (8). Low extracellular Na+ ([Na+]o) experiments were performed using previously published methods by replacing NaCl (120 mmol/l) with N-methyl-d-glucamine (120 mmol/l) in Kreb’s solution (22).

Vasodilator phosphoprotein phosphorylation. CAs were pretreated appropriately for each condition and homogenized. The tissue homogenate was subjected to Western blot analysis using an anti-phospho-vasodilator phosphoprotein (VAP) antibodies (Cell Signaling) to assess phosphorylation of VASP at Ser239. NADPH levels. The levels of NADPH in CA were determined by HPLC (Elite LaChrom Chromatography System; Hitachi, Tokyo, Japan) using previously published methods (10).

PKC activity. Activation of PKC in CA smooth muscle was assessed using a non-radioactive assay kit purchased from Stressgen (Ann Arbor, MI). ELISA was performed as per the manufacturer’s protocol, and activity was reported as antibodies per milligrams of protein.

Results

Depolarization activates G6PD. Consistent with our recent findings (8), we observed that G6PD is activated by membrane depolarization in CAs. G6PD assays (Fig. 2A) and enzyme kinetic analysis (Fig. 2B) showed that KCl (30 mmol/l)-
induced membrane depolarization led to a doubling of \( V_{\text{max}} \), although \( k_m \) was unaffected.

Depolarization stimulates PTEN signaling and activates G6PD via PKC. We recently observed that PKC\( \theta \) interacts with and activates G6PD (8). Here we examined possible upstream regulators of PKC involved in depolarization-induced activation of G6PD. Because PKC\( \theta \) requires DAG for its activation, our focus was on identifying the roles played by PTEN and phosphotyrosine 3-kinase (PI3K), two key regulators of PI\( \beta \)2 levels (29). We found that KCl activates PKC and that PKC was further activated by inhibition of PI3K using 2-morpholin-4-yl-8-phenylchromen-4-one (LY294002; 10 \( \mu \)mol/l; Sigma), a tyrosine phosphatase inhibitor (16), reduced PKC activity, compared with time-matched controls (Fig. 2A). The response was increased by the PI3K inhibitor LY294002 (10 \( \mu \)mol/l; \( n = 8 \)) and reduced by the PTEN inhibitor bisproxo(pyridine-2-carboxyl)oxovanadate (Bpv; 10 \( \mu \)mol/l; \( n = 8 \)) but not by the serine-threonine phosphatase inhibitor cocktail 1 (PIC1; 10 \( \mu \)mol/l; \( n = 8 \)). Aortic relaxation induced by inhibition of G6PD is attenuated in G6PD-deficient mice. To determine whether the apparent regulation of CA contractility by G6PD is unique to bovine species, we used genetically mutated G6PD-deficient mice to examine the effects of G6PD inhibitors on precontracted aortas. We first established the utility of this model for our application by characterizing G6PD activity levels in homogenates of heart from the mice (Fig. 4A). In heart, both G6PD\( ^{+/+} \) (mutation in one allele; 0.088 ± 0.075 nmol·min\(^{-1} \)·mg protein\(^{-1} \)) and G6PD\( ^{+/−} \) (mutation in both the alleles; 0.047 ± 0.071 nmol·min\(^{-1} \)·mg protein\(^{-1} \)) mice showed less G6PD activity than G6PD\( ^{−/−} \) mice (no mutation; 0.59 ± 0.22 nmol·min\(^{-1} \)·mg protein\(^{-1} \)). When aortas from G6PD\( ^{+/+} \) and G6PD\( ^{+/−} \) mice were then used to assess the effect of G6PD inhibitors on vessels precontracted with KCl, we found that...
both 6AN and EPI dose dependently relaxed precontracted aortas from G6PD−/− mice (Fig. 4B), as they did with bovine CA (Fig. 3C). By contrast, 6AN (1 mmol/l)-induced relaxation was reduced by 74.6% in precontracted aortas from G6PD−/− mice, compared with G6PD+/+ mice (Fig. 4C), while EPI (100 μmol/l)-induced relaxation was inhibited to a similar degree (Fig. 4D).

Inhibition of G6PD attenuates depolarization-induced phosphorylation of MLC. We next investigated the effect of 6AN and EPI on depolarization-induced increases in [Ca2+]i and MLC phosphorylation. Our data show that in isolated CASMCs depolarization increased [Ca2+]i, from 80 to 150 nmol/l, and that this effect was significantly attenuated by EPI (100 μmol/l; Fig. 5A). Consistently, 6AN (1 mmol/l; Fig. 5B) also decreased KCl-induced elevation of [Ca2+]i in freshly isolated CA rings. As Ca2+-dependent phosphorylation of MLC is a key step in excitation-contraction coupling in VSM, we used Western analysis to test whether G6PD inhibition alters levels of p-MLC (Fig. 5C). We found that depolarization increased p-MLC levels, compared with untreated controls, and that the effect was completely abolished under Ca2+-free conditions. The depolarization-induced increase in p-MLC was also re-
duced by 6AN, which brought p-MLC levels down to those seen in the untreated control.

NCX and SERCA are activated during depolarization-induced CA contraction but PMCA is not. [Ca^{2+}]_i is regulated by three major mechanisms: Ca^{2+} influx, extrusion, and sequestration. We selectively inhibited different Ca^{2+} pumps to investigate which of these mechanisms are activated during depolarization-induced CA contraction. KCl-induced CA contractions were unaffected by inhibition of PMCA with caloxin (AnaSpec; 10 \mu mol/l; Fig. 6A), but reducing \([Na^{+}]_o\) or inhibiting SERCA with cyclopiazonic acid (CPA; 200 \mu mol/l) elicited a doubling of contractile force (Fig. 6A). To confirm the effectiveness of the Ca^{2+} pump inhibitors, we tested the effects of caloxin and CPA on U46619-induced CA contractions. Figure 6B shows that U46619 (100 nmol/l)-induced CA contractions were augmented by PMCA or SERCA inhibition, confirming the efficacy of these inhibitors at the respective doses used in our experiments.

6GPD inhibitors relax depolarization-induced CA contractions by increasing Ca^{2+} sequestration and reducing influx. To assess their involvement in 6AN-induced relaxation of precontracted CA, we tested the effect of selectively inhibiting different Ca^{2+} pumps on this relaxation (Fig. 6C). We found that inhibiting SERCA with CPA reduced 6AN-induced relaxation, while reducing \([Na^{+}]_o\) unexpectedly accelerated CA relaxation. Inhibiting PMCA with caloxin had no effect on 6AN-induced relaxation.

Reverse mode Na^{+}/Ca^{2+} exchange activates SERCA to regulate CA contraction and relaxation. To study the role of Ca^{2+} extrusion during 6AN-induced relaxation of precontracted CA, we first assessed the effect of low \([Na^{+}]_o\) on 6GPD

![Fig. 6](http://ajpheart.physiology.org/)

**Fig. 6.** Roles Na^{+}/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+} ATPase (PMCA), and sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) in regulating contraction and 6-AN-induced relaxation of CA. Contribution of Ca^{2+} extrusion to 6AN-induced relaxation was determined by inhibiting PMCA, NCX, and SERCA by caloxin (10 \mu mol/l), lowering extracellular Na^{+} (\([Na^{+}]_o\)) from 143 to 23 mmol/l, and cyclopiazonic acid (CPA; 200 \mu mol/l), respectively. These inhibitors increased CA contraction evoked by KCl (30 mmol/l; A) or U46619 (100 nmol/l; B). Interestingly, 6AN-evoked relaxation of CAs was reduced by CPA and increased by lowering \([Na^{+}]_o\); inhibition of PMCA had no effect (C); \(n = 10–15\) in each group. *\(P < 0.05\) vs. control.
activity and then its effects on CA contraction and relaxation. Reducing [Na\(^+\)]\(_o\), is known to inhibit forward mode Na\(^+\)/Ca\(^2+\) exchange, activate reverse mode Na\(^+\)/Ca\(^2+\) exchange, depolarize VSM cells (als) and increase [Ca\(^2+\)]\(_i\) (14). Consistent with those observations, we found that incubating CAs in low [Na\(^+\)]\(_o\), Kreb’s solution evoked contractions (2.5–3.0 g), though they were weaker than contractions induced by KCl (Fig. 7A). When CAs were treated with KCl (30 mmol/l) in low [Na\(^+\)]\(_o\) solution, the evoked contractions were about twice as strong as those evoked by KCl alone (Fig. 7A). Inhibition of SERCA in low [Na\(^+\)]\(_o\), solution induced contractions similar to those induced by KCl (Fig. 7A).

We recently demonstrated that activation of G6PD by membrane depolarization increases [Ca\(^2+\)]\(_i\), and induces CA contraction (8). We therefore tested whether low [Na\(^+\)]\(_o\),-induced membrane depolarization would increase G6PD activity and whether G6PD inhibition would relax low [Na\(^+\)]\(_o\),-induced CA contractions. We found that low [Na\(^+\)]\(_o\), did indeed increase G6PD activity in CA and that the activity was increased further by KCl or CPA (Fig. 7B). When we then assessed the roles of Na\(^+\)/Ca\(^2+\) exchange and Ca\(^2+\) sequestration in regulating CA function, we found that inhibiting SERCA in the presence of low [Na\(^+\)]\(_o\), increased the sensitivity of CA to 6AN-induced relaxation (Fig. 7, C and D).

Rho kinase but not PKG mediates 6-AN-induced relaxation CA precontracted with KCl. To investigate the signaling cascade involved in G6PD inhibitor-induced relaxation of CAs precontracted with KCl, we examined the possible involvement of PKG and Rho kinase, two well-established mediators of vasorelaxation and vasoconstriction, respectively. As shown in Fig. 8A, KCl-induced CA contractions were significantly inhibited by the Rho kinase inhibitor 4-[(1R)-1-aminoethyl]-N-4-pyridinyl-trans-cyclohexanecarboxamide, dihydrochloride (Y27632; 10 \(\mu\)mol/l). Inhibition of Rho kinase also reduced 6AN-induced relaxation of precontracted CAs (Fig. 8B). KCl treatment increased phosphorylation of MYPT1 at Ser855, which was blocked by 6AN (Fig. 8, C and D). On the other hand, there was no significant difference between contractions evoked by 30 mmol/l KCl under control conditions and in the presence of the specific PKG inhibitor 8-bromo-\(\beta\)-phenyl-1,3,5-triethenoguanosine-3,5-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-PET-cGMPS; BioLog, Bremen, Germany; 100 nmol/l; Fig. 9A). As expected, inhibition of PKG reduced CA relaxation induced by sodium nitroprusside, a nitric oxide donor (Fig. 9B), but surprisingly PKG inhibition enhanced CA relaxation evoked by 6AN (Fig. 9C), although 6AN increased phosphorylation of VASP at Ser239, compared with untreated and KCl-treated CA. This effect was blocked by Rp-8-Br-PET-cGMPS or staurosporine (Fig. 9, D and E).

DISCUSSION

We previously showed that membrane depolarization activates PKC\(\beta\), which mediates KCl-induced activation of G6PD in isolated CA (8). Our present findings show that pretreating CAs with Bpv, a PTEN inhibitor, reduces KCl-stimulated increases in PKC and G6PD activity and that it also inhibits KCl-induced CA contraction. In addition, we provide evidence that inhibiting G6PD reduces KCl-induced CA contraction by reducing Rho kinase-mediated phosphorylation of MYPT1 and PLC and that activation of PKG does not contribute to the VSM relaxation evoked by 6AN. Our data thus suggest a novel role for PTEN and G6PD in regulating VSM tone.

PI3K and PTEN are expressed in all mammalian cells, including VSMCs, and are involved in regulating VSM contraction evoked by KCl or electrical stimulation (31, 33). PTEN is a dual phosphatase that dephosphorylates PIP3 to PIP2, which is in turn cleaved to IP3 and DAG by phospholipase C. Both of these products are second messengers, and DAG is well known to activate various PKC isoforms in VSM. We found that Bpv inhibited KCl-evoked increases in PKC and G6PD activity in CA. By contrast, KCl-evoked PKC activity in CA was enhanced by inhibition of PI3K, which suggests KCl-evoked increases inPIP2 are responsible for stimulating...
PKC activity. The precise mechanism via which depolarization induces activation of PTEN was not investigated in the present study; however, PTEN is known to be activated by phosphorylation/dephosphorylation of its COOH-terminal region, which serves as an electrostatic switch in controlling the membrane translocation of the protein (5) or by phosphorylation in a putative S/TX(X)T motif by Rho kinase during cell chemotaxis (19, 36). That said, Rho kinase-dependent phosphorylation is unlikely to activate PTEN or PKC-dependent G6PD activity, as the Rho kinase inhibitor Y27632 does not affect KCl-induced PKC and G6PD activation in CA (8). Alternatively, PTEN activation by KCl or membrane depolarization could result from PIP2 acting as a feedback activator (4). In addition, PTEN is predominantly cytosolic and lacks the four-transmembrane voltage-sensor domain characteristic of voltage-gated ion channels (24), which makes it unlikely that

Fig. 8. 6AN relaxes CA in part by suppressing Rho kinase-catalyzed myosin phosphatase target subunit 1 (MYPT1) phosphorylation. Y-27632 (10 μmol/l; n = 10) suppressed KCl (30 mmol/l; 30K; n = 10)-induced contractions (A) and 6AN-induced relaxation (B). C and D: Western blot analysis with anti-phospho-MYPT1 and anti-MYPT1 showing that 6AN (3 mmol/l) reduces KCl-induced phosphorylation of MYPT1 at S855, a Rho kinase-specific phosphorylation site. Blot shown is representative blot of 5 experiments. Note: gel was loaded in a blinded fashion and so 2 lanes containing irrelevant information/sample to the study were removed from the final blot figure (indicated by a gap). Also, original capture was flipped to get final left to right orientation as shown. *P < 0.05 vs. control; @P < 0.05 vs. 30K.

Fig. 9. PKG does not participate in 6AN-induced CA relaxation. Rp-8-Br-PET-cGMPS (100 nmol/l), a PKG inhibitor, increased (NS; n = 10) KCl (30 mmol/l)-induced contraction of CAs (A) and reduced (P < 0.05; n = 10) relaxation induced by a nitric oxide donor, sodium nitroprusside (SNP; B). C: Rp-8-Br-PET-cGMPS (100 nmol/l) paradoxically increased 6AN-induced relaxation of CAs precontracted with KCl (n = 10–15). D and E: Western blot analysis using anti-phospho-vasodilator-stimulated phosphoprotein (VASP) and anti-VASP showing that 6AN (3 mmol/l) reduces KCl-induced phosphorylation of VASP at Ser239, a PKG-specific phosphorylation site. Blot shown is representative blot of 5 experiments. Note: gel was loaded in a blinded fashion and so 2 lanes containing irrelevant information/sample to the study were removed from the final blot figure (indicated by a gap). Also, original capture was flipped to get final left to right orientation as shown. *P < 0.05 vs. control; @P < 0.05 vs. 30K.
PTEN functions as a membrane voltage-sensing protein and implies the existence of other voltage-sensing proteins that initiate the signaling cascade to stimulate PTEN. For instance, the striking sequence homology between the catalytic domains of PTEN and VSP, a novel voltage-sensing non-ion channel protein identified in Ciona intestinalis, is noteworthy (24). Moreover, a Zebrafish ortholog of voltage sensing protein (VSP) was recently shown to have both voltage-sensing and tyrosine phosphatase capabilities (13). Given our findings, we cannot exclude the possibility that an as yet unidentified mammalian VSP was activated by depolarization. Consistent with this notion, our results indicate that Bpv and the tyrosine phosphatase inhibitor PIC2 each inhibited KCl-induced CA contraction.

Depolarization of VSMCs causes rapid Ca\(^{2+}\) influx through L-type voltage-gated channels with subsequent activation of MLC kinase and a Rho-Rho kinase pathway that mediates an increase in the Ca\(^{2+}\) sensitivity of the contractile proteins. The Ca\(^{2+}\) sensitization primarily involves phosphorylation of MLC by Ca\(^{2+}\)-calmodulin-activated MLC kinase and is negatively regulated by the cyclic nucleotide-dependent kinases PKA and PKG. It is known that MLC is also phosphorylated in a Ca\(^{2+}\)-independent manner (12). In the present study, incubation of CAs under extracellular [Ca\(^{2+}\)]-free conditions completely inhibited KCl-evoked MLC phosphorylation, which suggests MLC phosphorylation is dependent on either the entry of extracellular Ca\(^{2+}\) and/or release of stored Ca\(^{2+}\) from the SR. In addition, we recently observed that depolarization induced Ca\(^{2+}\)-independent activation of G6PD mediated by PKC\(\delta\) increases CA contractility (8). Consistent with that report, we observed in the present study that the increase in [Ca\(^{2+}\)] in CA and CASMCs following their depolarization can be significantly attenuated by 6AN or EPI and that 6AN nearly completely blocks KCl-induced phosphorylation of MLC in CA. These data support the idea that depolarization-induced, Ca\(^{2+}\)-independent activation of G6PD is at least partly responsible for the increase in [Ca\(^{2+}\)], and the Ca\(^{2+}\)-dependent steps involved in VSMC contraction, including the increased Ca\(^{2+}\) sensitivity of the myofilaments.

The mechanisms by which Ca\(^{2+}\) is handled in VSM are not fully understood but are known to involve a variety of Ca\(^{2+}\) channels and transporters. One Ca\(^{2+}\) transporter involved is the NCX, which transports Na\(^{+}\) and Ca\(^{2+}\) into or out of cells, depending on the extracellular and intracellular concentrations of the two cations. In reverse mode, it pumps Na\(^{+}\) out of and Ca\(^{2+}\) into the cells (14). It has been speculated that the NCX operating in reversed mode contributes to the generation of myogenic tone and the maintenance of blood pressure (22). Our results show that decreasing extracellular Na\(^{+}\), which forces the NCX to operate in the reverse mode, is sufficient to substantially increase G6PD activity and CA contraction. Reverse-mode Na\(^{+}/Ca\(^{2+}\) exchange also augments depolarization-induced CA contractions, supporting the idea that it is important for generation of smooth muscle tone (28, 39).

Our results are consistent with earlier observations indicating forward mode of Na\(^{+}/Ca\(^{2+}\) exchange is involved in clearing Ca\(^{2+}\) from the cytoplasm (22, 28). We also found that inducing reverse-mode Na\(^{+}/Ca\(^{2+}\) exchange favors VSM membrane depolarization and Ca\(^{2+}\) entry and that inhibition of SERCA using CPA further increases both depolarization- and NCX-induced CA contraction. This finding is consistent with the earlier observations that CPA augments KCl-induced peak and sustained tension, as well as increases in [Ca\(^{2+}\)]\(_i\) induced by KCl or lowering [Na\(^{+}\)]\(_o\) in urinary bladder (21) and aorta (22). We also found that CPA augments low [Na\(^{+}\)]\(_o\)-induced increases in G6PD activity in CA. Based on previous studies showing that CPA and another SERCA inhibitor, thapsigargin, are able to induce depolarization of VSMCs (32) and endothelial cells (38), respectively, and that membrane depolarization leads to G6PD activation (8), we suggest that CPA may increase G6PD activity in a [Ca\(^{2+}\)]\(_i\)-independent manner by enhancing low [Na\(^{+}\)]\(_o\)-induced depolarization. As expected, CPA also reduced the rate of 6AN-induced relaxation in precontracted CA, suggesting sequestration of Ca\(^{2+}\) by the SR via SERCA is activated by inhibition of G6PD. Furthermore, the enhancement of 6AN-induced relaxation of CA precontracted with KCl and CPA under low [Na\(^{+}\)]\(_o\) condition is attributable to a reduction of Ca\(^{2+}\) influx via the NCX or voltage-gated Ca\(^{2+}\) channels and an increase in sequestration of Ca\(^{2+}\) into the SR.

We found that PMCA inhibition had no effect on either depolarization-induced contractile force or 6AN-induced relaxation of CAs precontracted with KCl. This suggests that PMCA is not involved in the development of contractile force or modulating relaxation in precontracted CA under the conditions tested in this study. Consistent with that idea, PMCA4 gene ablation had no significant effect on KCl-induced peak contractions in urinary bladder (21). On the other hand, the rate of KCl-induced contraction was significantly diminished in PMCA4 gene ablated bladder, and the time constant for its relaxation in Ca\(^{2+}\)-free solution showed a marginal but significant increase. Therefore, a role for PMCA in modulating the rate of depolarization-induced contraction cannot be ruled out.

![Fig. 10. Schematic illustration of G6PD-mediated VSM contraction and relaxation pathway. Depolarization of membrane potential increases G6PD activity via a PTEN-PKC pathway. Stimulated G6PD increases [Ca\(^{2+}\)]\(_i\) and p-MLC resulting in elevation of VSM contraction. Inhibition of G6PD by 6AN or EPI decrease [Ca\(^{2+}\)]\(_i\) and p-MLC by partially enhancing Ca\(^{2+}\) sequestration or via PKG.](https://ai.journals.org/doi/10.1152/ajpheart.00143.2011)
completely, although our data indicate that \( \text{Cr}^{2+} \) extrusion via \( \text{Na}^+/	ext{Ca}^{2+} \) exchange and sequestration by SERCA are likely the important mechanisms for maintaining appropriate \( [\text{Ca}^{2+}] \), levels during depolarization-induced contraction of CA.

Our investigation into downstream effectors of G6PD revealed that phosphorylation of MYPT1 at Ser855, a target of Rho kinase (37), was increased and phosphorylation of VASP at Ser239 (3), a target of PKG, was slightly decreased by KCl-induced depolarization. Upon inhibition of G6PD in CA precontracted with KCl, MYPT1 phosphorylation decreased and VASP phosphorylation increased, which suggests both of these kinases are affected by inhibition by G6PD. Consistent with that idea, the activities of both Rho kinase and PKG are increased by oxidation of NADPH to NADP⁺ or by increased NADPH-dependent \( \text{H}_2\text{O}_2 \) generation (2, 11, 26, 27).

It is well known that activation of Rho kinase increases VSM contraction (12) and PKG promotes its relaxation (20). Consistent with that finding, inhibition of Rho kinase by Y27632 reduced KCl-induced CA contraction, as well as 6AN-induced relaxation of CA precontracted with KCl. To our surprise, however, inhibition of PKG by Rp-8-Br-PET-cGMPs reduced 6AN-induced VASP phosphorylation, while 6AN-evoked relaxation was enhanced and the dose-response curve was shifted to the left. It has been proposed that PKG phosphorylates VASP and that this is important for the regulation of actin cytoskeletal dynamics (15). Among proteins inactivated by PKG-dependent phosphorylation is Rho A (25), which activates Rho kinase and increases VSM contraction (34). In this way, the actions of PKG oppose those of Rho kinase in VSM. It remains unclear why inhibition of Rho kinase and PKG should, respectively, decrease and increase 6AN-induced CA relaxation. However, our findings suggest that changes in cellular redox associated with G6PD inhibition reduce Rho kinase-dependent phosphorylation of MYPT1 and, in turn, contraction of CA and that increases in PKG activation due to G6PD-inhibition may not play a major role in regulating vasomotor function.

In summary, our present study looking into the molecular mechanisms involved in depolarization-induced activation of G6PD and subsequent contraction of CA found that 1) PTEN mediates depolarization-induced activation of PKC and G6PD; 2) G6PD-inhibition-induced relaxation of CA is mediated by inhibition of \( \text{Ca}^{2+} \) influx and enhanced \( \text{Ca}^{2+} \) sequestration into the SR but not by altered \( \text{Ca}^{2+} \) extrusion through either the PMCA or NCX; 3) G6PD inhibition reduces the \( \text{Ca}^{2+} \) sensitivity of myofilaments, perhaps through reduction in Rho kinase activity and MYPT1 phosphorylation; and 4) Rho kinase and PKG are regulated by G6PD-inhibition. Therefore, as proposed in a schematic illustration (Fig. 10), increase in G6PD activity, which modulates intracellular redox, by depolarization of the membrane potential via a PTEN-PKC pathway plays a physiological role in increasing \([\text{Ca}^{2+}]_i\), \( \text{Ca}^{2+} \) sensitivity to the myofilament, and contractility. Conversely, G6PD inhibition relaxes CA by decreasing \( \text{Ca}^{2+} \) influx, increasing \( \text{Ca}^{2+} \) sequestration, and inhibiting Rho kinase but not by increasing \( \text{Ca}^{2+} \) extrusion or activating PKG.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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