

Research Article

Inhibition of large conductance calcium-dependent potassium channel by Rho-kinase contributes to agonist-induced vasoconstriction

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Keywords:

Rho, smooth muscle, hyperpolarization, vascular reactivity, mesenteric artery, patch clamp

ABSTRACT

We tested the hypothesis that Rho-kinase inhibits the large-conductance, calcium and voltage-dependent potassium (BKCa) channels thereby promoting vasoconstriction. Our results show that the Rho-kinase inhibitor, Y-27632, induced concentration-dependent relaxation in rat mesenteric artery. The selective BKCa channel inhibitors, iberiotoxin (0.1 mM) and tetraethylammonium (10 mM) increased the EC₅₀ of Y-27632 more than 2-fold and decreased Y-27632-induced maximum relaxation (P<0.05). In the inside-out patch clamp configuration, constitutively active Rho-kinase (1 mg/ml) attenuated BKCa channel activity induced by protein kinase G (PKG) (P<0.05). Y-27632 (10 mM) reversed the inhibitory effect of active Rho-kinase (P<0.01). Furthermore, in the presence of Y-27632, addition of active Rho-kinase had no effect on PKG-stimulated BKCa channel activity. Taken together, our data suggest that Rho-kinase negatively regulates BKCa channels, thus providing a novel mechanism through which Rho-kinase increases smooth muscle contraction.

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INTRODUCTION

Abundant evidence has suggested that RhoA/Rho-kinase pathway mediated calcium-sensitization is involved in vasoconstriction (Chrissobolis and Sobey, 2006; Hilgers and Webb, 2005; Jin et al., 2006; Lee et al., 2004). Myosin light chain (MLC) phosphatase is identified as a downstream target of Rho-kinase. By phosphorylation of MLC phosphatase, Rho-kinase inhibits MLC phosphatase activity and increases the phosphorylation levels of MLC, leading to vascular smooth muscle contraction. Recent data also suggest that RhoA/Rho-kinase influences ion channel activity including potassium channels (Cachero et al., 1998;

Luykenaar et al., 2004; Rossignol and Jones, 2006). The large-conductance, calcium and voltage-dependent potassium (BKCa) channel is the predominant potassium channel in vascular smooth muscle cells and plays a pivotal role in regulating of smooth muscle contractility of resistance arteries (Mistry and Garland, 1998). It is activated by membrane depolarization and intracellular calcium concentration elevation, which leads to membrane hyperpolarization and subsequent smooth muscle relaxation. Decreased BKCa channel activity increases vasoconstriction and is associated with various cardiovascular diseases such as hypertension (Bratz et al., 2005; Callera et al., 2004).

Here, we hypothesized that Rho-kinase inhibits the BKCa channel activity thereby promoting constriction of mesenteric artery smooth muscle. The experiments were carried out in isolated mesenteric artery by measuring isometric force changes in the presence of inhibitors for Rho-kinase or BKCa channels as well as in freshly isolated mesenteric artery smooth muscle cells using patch clamp technique in the inside-out membrane configuration at room temperature.

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MATERIALS AND METHODS

Isometric force measurement

Male Sprague-Dawley rats (275-299 g, Harlan Laboratories) were anesthetized with pentobarbital sodium (50 mg/kg ip) and mesenteric arteries were rapidly removed and cleaned of periadventitial adhering fat in a cold physiological saline solution of the following composition (in mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.18, CaCl₂·2H₂O 1.6, MgSO₄·7H₂O 1.6, NaHCO₃ 25, dextrose 5.5, EDTA 0.03.

Third order segments of mesenteric arteries were mounted in muscle baths and allowed to equilibrate for 60 min under a passive tension of 5 mN in the physiological saline solution gassed with 95% O₂/5% CO₂ at 37 °C. The rings were pre-contracted by phenylephrine (1 mM) and the relaxation response to acetylcholine (10 mM) was obtained on each ring for assessment of endothelium function. After washing and re-equilibration, the artery segments were pre-incubated with vehicle (0.1% DMSO) or BKCa channel inhibitors including iberiotoxin (0.1 mM) and tetraethylammonium (10 mM) for 15 min. A dose-response curve to Rho-kinase inhibitor Y-27632 (0.1 mM to 10 mM) was constructed after phenylephrine-induced smooth muscle contractions reached peak plateau phase. Data are expressed as percentage relaxation from phenylephrine-induced maximum relaxation.

Cell isolation

Smooth muscle cells were isolated as described previously (White et al., 1995). Briefly, cells were incubated at 37°C in a buffer containing papain (6 mg/ml), dithiothreitol (4 mg/ml), collagenase (2 mg/ml), and 0.02% bovine serum albumin. After 30 min of gentle shaking, the muscle strips were lightly triturated. The enzyme solution was centrifuged at 500 rpm for 15 min and the pellet was re-suspended in fresh medium (in mM): NaCl 110, KCl 5, CaCl₂ 2, MgCl₂ 2, HEPES 10, NaHCO₃ 10, KH₂PO₄ 0.5, glucose 10, EDTA 0.49, and taurine.

Patch clamp

For inside-out patch studies, the bathing solution exposed to the cytoplasmic surface of the membrane consisted of the following (in mM): K₂SO₄ 60, KCl 30, MgCl₂ 2, CaCl₂ 0.16, 1,2-bis(o-Aminophenoxy)ethane-N,N,N,N-tetraacetic Acid Tetra(acetoxymethyl) Ester (pCa 7) 1, HEPES 10, ATP 5, and glucose 10 (pH 7.4; 22-25 °C). The pipette

solution was Ringer's solution (in mM): NaCl 110, KCl 5, MgCl₂ 1, CaCl₂ 2 and HEPES 10. BKCa channel activity was stimulated by 400 U/ml of purified protein kinase G (PKG) in the presence of cGMP (100 mM) and ATP (100 mM). Recombinant active Rho-kinase (1 mg/ml) or Y-27632 (10 mM) was subsequently applied according to specific experiment requirements. Single-channel data were recorded and analyzed at a potential of +40 mV where BKCa channel openings are easily distinguished from other channel species, thus permitting more accurate statistical analysis. Average channel activity (NPo) in patches with multiple BKCa channels was determined as described previously (Dimitropoulou et al., 2002).

Data analysis

Data are expressed as mean ± standard error of the mean (S.E.M). The Student's *t*-test and one-way analysis of variance with Bonferroni comparison post test were performed. When *P* < 0.05, differences were considered to be significant.

RESULTS

Effect of BKCa channel inhibitors on Y-27632-induced relaxation curve

To determine whether blockade of BKCa channels reduces the efficacy of the Rho-kinase inhibitor Y-27632, mesenteric rings were pre-incubated with vehicle (0.1% DMSO) or a selective BKCa channel inhibitor, iberiotoxin (0.1 mM) for 15 min. Cumulative concentrations of Y-27632 (0.1 to 10 mM) were added when the contraction of the rings reached maximum response to phenylephrine. The results show that Y-27632 induced concentration-dependent relaxation of vehicle treated rings at an EC₅₀ value of 1.8 mM (Figure 1A). Iberiotoxin shifted the curve to the right (*P*<0.01) and increased the EC₅₀ of Y-27632 more than 2-fold (EC₅₀=4.0 mM). Iberiotoxin also significantly decreased Y-27632-induced maximum relaxation from -87 ± 2% to -76 ± 3% (*P*<0.05). These results were confirmed by another BKCa channel inhibitor, tetraethylammonium (10 mM), which increased the EC₅₀ of Y-27632 from 2.0 to 4.0 mM and reduced the maximum relaxation response to Y-27632 (Figure 1B).

Effect of constitutive active Rho-kinase on BKCa channel activity

Previously, we have identified BKCa channel in rat mesenteric smooth muscle cells that this channel has a

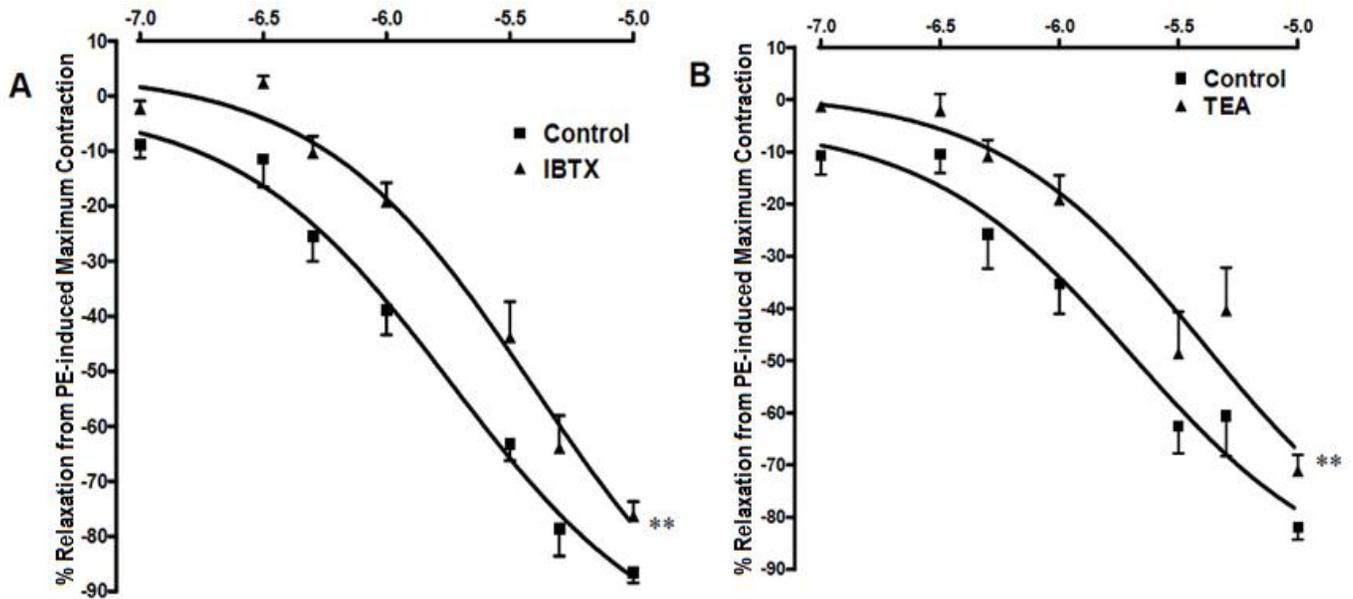


Fig. 1.

Effects of BKCa channel inhibitors on Y-27632-induced relaxation curve. A) Iberitoxin increased EC50 of Y-27632 and decreased the maximum relaxation response to Y-27632; B) Tetraethylammonium increased EC50 of Y-27632 and decreased the maximum relaxation response to Y-27632. n=6-11, ** P<0.01 vs. Control.

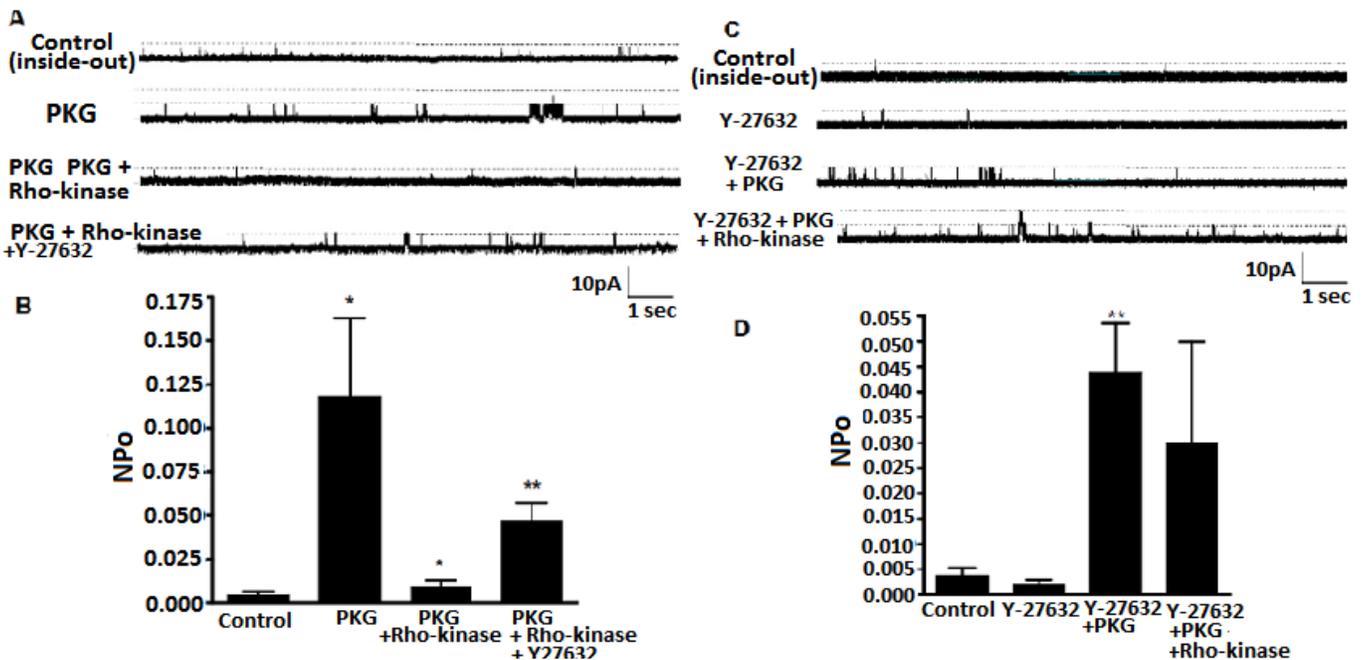


Fig. 2.

Effects of active Rho-kinase and Y-27632 on BKCa channel activity. A) Representative tracings for BKCa channel activity in control, PKG-treated, PKG+Rho-kinase-treated, or addition of Y-27632 to PKG plus Rho-kinase-treated conditions. B) A summary of BKCa channel opening probability (NPo). n=10, *P<0.05: control vs. PKG, PKG vs. PKG+Rho-kinase; **P<0.01: PKG+Rho-kinase vs. PKG+Rho-kinase+Y-27632. C) Representative tracings for BKCa channel activity in control, Y-27632-treated, Y-27632+PKG-treated, or addition of Rho-kinase to Y-27632+Rho-kinase-treated conditions. D) A summary of BKCa channel NPo. n=10, **P<0.01: Y-27632 vs. PKG+Y-27632.

high conductance (nearly 200pS) and is stimulated by increasing calcium concentration at the cytoplasmic surface of the membrane (Dimitropoulou et al., 2001). The activity of BKCa channel was minimal when the patch was excised into the inside-out configuration with the normal inside-out bathing solution. At basal level (Figure 2A&B), the channel open probability (N_{po}) was 0.0043 ± 0.0021 (+40 mV). Addition of 400 U/ml of purified PKG to the bath solution in the presence of cGMP (100 mM) and ATP (100 mM) significantly increased BKCa channel activity to 0.118 ± 0.045 (Figure 2A&B; $P < 0.05$ vs. basal N_{po}). PKG-stimulated channel activity was attenuated by constitutively active Rho-kinase (1 mg/ml) in the presence of ATP (100 mM), of which N_{po} was decreased to 0.0088 ± 0.0037 ($P < 0.05$ vs. PKG-increased N_{po}). The Rho-kinase inhibitor, Y-27632 (10 mM) reversed the inhibitory effect of active Rho-kinase on BKCa channels ($P < 0.01$).

In the next set of experiments, mesenteric smooth muscle cells were incubated with Y-27632 before the cells were stimulated with PKG. Y-27632 did not have a significant effect on basal activity of BKCa channels (N_{po} = 0.0038 ± 0.0015 vs 0.0019 ± 0.0011 at basal level). In the presence of Y-27632, addition of active Rho-kinase slightly decreased PKG-stimulated BKCa channel activity; but there was no statistical difference (N_{po} = 0.0510 ± 0.0390 vs. 0.0298 ± 0.0201 PKG-stimulated, Figure 2C&D)

DISCUSSION

Potassium channels are the main determinant of membrane potential, contributing to the regulation of smooth muscle tone. The mechanisms of RhoA/Rho-kinase induced vasoconstriction through inhibition of MLC phosphatase have been extensively studied, yet little information is available on the effects of RhoA/Rho-kinase on potassium channels. It has been reported that the voltage-dependent delayed rectifying channel (KDR) activity is inhibited by RhoA/Rho-kinase. Studies by Cachero et al (Cachero et al., 1998) suggest that RhoA mediated the inhibitory effects of m1 muscarinic receptor on KDR. Furthermore, co-immunoprecipitation of RhoA and Kv1.2 provides the direct evidence that RhoA physically binds to KDR. Electrophysiology experiments suggested that both wild type and constitutively active RhoA reduced activity of Kv1.2 but not the dominant negative RhoA. In addition, Luykenaar et al. (Luykenaar et al., 2004)

observed that UTP caused depolarization and vasoconstriction in isolated cerebral arteries. RhoA or Rho-kinase inhibitors not only attenuated UTP-induced vasoconstriction but also abolished the inhibitory effect of UTP on KDR activity.

Some evidence suggests that RhoA/Rho-kinase may also affect the inward rectifying potassium channels. Transfection with constitutively active RhoA decreased basal Kir2.1 activity in human embryonic kidney 293T cells; whereas dominant negative RhoA abolished the inhibitory effect of carbachol, a m1 muscarinic receptor agonist, on Kir2.1 (Jones, 2003). However, this effect is not mediated by Rho-kinase since the Rho-kinase inhibitor did not block the inhibitory effect of carbachol on Kir2.1. Further studies by the same group suggest that all three types of the Kir2 channel are negatively regulated by RhoA (Rossignol and Jones, 2006).

BKCa channels have been shown to mediate the actions of important vasodilators such as nitric oxide and endothelium-derived hyperpolarizing factors. The BKCa channel consists of two types of subunits: α - and β -subunit. Four α -subunits containing calcium-sensitive regions form the channel pore, and association with β -subunit enhances the channel sensitivity to calcium and voltage. It is well characterized that BKCa channels are activated by increased intracellular calcium and membrane depolarization. The results from our study suggest that BKCa channels may be also regulated by protein kinases such as Rho-kinase. In isometric tension experiments, we found that inhibition of BKCa channels by iberiotoxin or tetraethylammonium increased both the EC₅₀ of Y-27632 and reduced the maximum relaxation caused by Y-27632 in the mesentery artery. Furthermore, we demonstrated that constitutively active Rho-kinase reduced the N_{po} of BKCa channels, and Y-27632 reversed this inhibitory effect of Rho-kinase on BKCa channels.

It has been reported that protein kinases are able to bind BKCa channel subunits and regulate the channel activity through phosphorylation. Studies show that both cyclic AMP-dependent protein kinase (PKA) and PKG induce BKCa channels activation by phosphorylation of α - and/or β -subunits whereas protein kinase C inhibits BKCa channels (Toro et al., 1998). Whether the protein kinases interact directly or indirectly with BKCa channels remains controversial. Some studies suggest that BKCa channel subunits have

direct phosphorylation sites for the protein kinases (Alioua et al., 1998); others have proposed that the protein kinases act on a protein phosphatase because phosphatase inhibitors diminished the effect of PKG or PKA on BKCa channels (Imig et al., 2008; White et al., 1991; Zhou et al., 1996). Whether Rho-kinase directly acts on channels or through regulation of the phosphatase activity requires further investigation.

In conclusion, these data suggest that Rho-kinase negatively regulates BKCa channels, thus providing a novel mechanism through which Rho-kinase increases smooth muscle contraction. Increased RhoA/Rho-kinase activity and impaired BKCa channel function have been implicated in many diseases such as hypertension, urinary incontinence and erectile dysfunction. Our results revealed the interaction between these two important players in the regulation of vascular tone, offering new insight to understand smooth muscle contraction and possible new therapeutic treatment.

ACKNOWLEDGEMENTS

This study was supported by grants from the American Heart Association Scientist Development grant (0530007N) and the National Institutes of Health (DK-73531 and HL-74167).

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