Inhibition of TRPC1/TRPC3 by PKG contributes to NO-mediated vasorelaxation

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Chen J, Crossland RF, Noorani MM, Marrelli SP. Inhibition of TRPC1/TRPC3 by PKG contributes to NO-mediated vasorelaxation. Am J Physiol Heart Circ Physiol 297: H417–H424, 2009. First published June 5, 2009; doi:10.1152/ajpheart.01130.2008.—Nitric oxide (NO) inhibits transient receptor potential channel 3 (TRPC3) channels via a PKG-dependent mechanism. We sought to determine 1) whether NO inhibition of TRPC3 occurs in freshly isolated smooth muscle cells (SMC); and 2) whether NO inhibition of TRPC3 channels contributes to NO-mediated vasorelaxation. We tested these hypotheses in freshly isolated rat carotid artery (CA) SMC using patch clamp and in intact CA by vessel myograph. We demonstrated TRPC3 expression in whole CA (mRNA and protein) that was localized to the smooth muscle layers. TRPC1 protein was also expressed and coimmunoprecipitated with TRPC3. Whole cell patch clamp demonstrated nonselcetive cation channel currents that were activated by UTP (60 μM) and completely inhibited by a TRPC3 channel inhibitor, La3+ (100 μM). The UTP-stimulated current (\(I_{\text{UTP}}\)) was also inhibited by intracellular application of anti-TRPC3 or anti-TRPC1 antibody, but not by anti-TRPC6 or anti-TRPC4 control antibodies. We next evaluated the NO signaling pathway on \(I_{\text{UTP}}\). Exogenous NO ([((-(N-methyl-N-(N-methylammoniohexyl)-aminol)diaz-1-ium-1,2-diolate (MAHMA NONOate)) or a cell-permeable cGMP analog (8-bromo-cGMP) significantly inhibited \(I_{\text{UTP}}\). Preapplication of a PKG inhibitor (KT5823) reversed the inhibition of MAHMA NONOate or 8-bromo-cGMP, demonstrating the critical role of PKG in NO inhibition of TRPC1/TRPC3. Intact CA segments were contracted with UTP (100 μM) in the presence or absence of La3+ (100 μM) and then evaluated for relaxation to an NO donor, sodium nitroprusside (1 μM). Relaxation to sodium nitroprusside was significantly reduced in the La3+ treatment group. We conclude that freshly isolated SMC express TRPC1/TRPC3 channels and that these channels are inhibited by NO/cGMP/PKG. Furthermore, NO contributes to vasorelaxation by inhibition of La3+-sensitive channels consistent with TRPC1/TRPC3.

transient receptor potential channel; protein kinase G; transient receptor potential 3; transient receptor potential 1

THE CANONICAL TRANSIENT RECEPTOR potential channels (TRPCs) are nonselective cation channels that have been demonstrated to play a variety of roles in regulation of vascular tone (23). TRPC3 is one of the less well-characterized members of the TRPC family (TRPC1–7) within the vasculature. Nevertheless, TRPC3 has recently been shown to be involved in receptor-mediated constriction within the smooth muscle cells (SMCs) of cerebral arteries (18). Activation of the TRPC3 channel permits Ca2+ and Na+ entry into the cell; however, the major mechanism of Ca2+ entry in SMC is thought to be secondary to Na+ entry and smooth muscle depolarization (18). Depolarization from Na+ entry leads to activation of L-type Ca2+ channels with subsequent Ca2+ entry and smooth muscle contraction.

Nitric oxide (NO) is well known as a vasodilator. However, the mechanism by which NO promotes smooth muscle relaxation is still incompletely resolved. At this time, it is believed that NO leads to smooth muscle relaxation by multiple pathways, including activation of potassium channels (6), activation of the sarco(endo)plasmic reticulum Ca2+-ATPase (8), inhibition of Rho kinase (7, 20), and stimulation of myosin light chain phosphatase (16). Recent TRPC3 studies indicate that these channels are regulated by NO as well (13), raising the question of whether inhibition of TRPCs may be yet another pathway by which NO regulates smooth muscle relaxation.

Initial studies by Kwan et al. (13) demonstrated that TRPC3 overexpressed in human embryonic kidney (HEK)-293 cells can be inhibited by NO through PKG-dependent phosphorylation of the protein at positions T11 and S263. Since structurally related TRPC6 and TRPC7 share several of these PKG phosphorylation consensus sites, these channels have been proposed to share the same regulatory potential of the NO signaling pathway (13). Indeed, a more recent examination of TRPC6 has shown that PKG-dependent phosphorylation does occur in overexpressed TRPC6 channels, as well as in a rat aorta SMC line, A7r5 cells (22).

While the evidence for negative regulation of TRPCs by the NO signaling pathway in vascular smooth muscle is mounting, it is still incomplete. Even with the recent reporting of NO/cGMP/PKG negative regulation of TRPC6 in A7r5 cells (22), data regarding similar regulation of TRPC3 or TRPC7 in SMC are lacking. Furthermore, the existing data regarding this potentially novel aspect of NO-mediated vasorelaxation is entirely derived from studies performed with a SMC line (22). This is an especially relevant point, since it is known that expression of proteins involved in Ca2+ handling (including TRPC3 and TRPC6) changes significantly in SMC culture (3). Thus studies are needed in freshly isolated vascular SMCs (VSMCs) to demonstrate that this mechanism is not unique to SMC culture or cell lines. Additionally, the proposed model of NO inhibition of TRPC3 needs to be examined in intact arteries to determine whether this mechanism comprises a meaningful component of NO-mediated vasorelaxation.

The present study was originally designed to test the following hypotheses: 1) NO/cGMP/PKG-mediated inhibition of TRPC3 channels occurs in native SMCs; and 2) NO-mediated vasorelaxation is partially dependent on inhibition of TRPCs in intact carotid artery (CA). However, in the course of these studies, we obtained novel data suggesting that TRPC3 might act within a TRPC1/TRPC3 heteromer. We subsequently modi-
ified hypothesis 1 to incorporate this new data as follows: NO/cGMP/PKG-mediated inhibition of TRPC1/TRPC3 channels occurs in native SMCs. These hypotheses were tested in the rat CA at the cellular level (whole cell patch clamp) and at the whole artery level (isolated vessel myograph). These studies show for the first time that VSMC exhibit TRPC1/TRPC3 feedback system that is functionally involved in NO-mediated vasorelaxation of intact arteries.

MATERIALS AND METHODS

Experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Protocol Review Committee at Baylor College of Medicine. Male Long-Evans rats (230–330g) from Charles River Laboratories were anesthetized with isoflurane. RT-PCR analysis. Total RNA from CA was extracted using the RNeasy MicroKit (Qiagen) per manufacturer’s instructions. The mRNA was converted to cDNA using SuperScript III (Invitrogen), according to manufacturer instructions. RNase H was added and incubated for 20 min at 37°C to degrade any remaining RNA. Control according to manufacturer instructions. RNase H was added and mRNA was converted to cDNA using SuperScript III (Invitrogen), RNeasy MicroKit (Qiagen) per manufacturer’s instructions. The

PCR amplification was performed using Platinum Taq DNA polymerase (Invitrogen) according to the recommended protocol. Amplification parameters were as follows: 97°C activation (2 min) followed by 94°C dissociation (15 s), 56°C anneal (45 s), and 72°C elongation (45 s) for 33 cycles. Primer pairs were as follows: TRPC3 forward: 5'-ctggaacagagtgttcgttt-3', TRPC3 reverse: 5'-caggatacagctgccacaa-3', TRPC6 forward: 5'-ggccttcgttcacttcatc-3', TRPC6 reverse: 5'-ctgccgtgtctgctgctgcc-3', TRPC7 set 1 forward: 5'-ctaggggtttgtcctca-3', TRPC7 set 1 reverse: 5'-ctgctgctgctgtcctgat-3', TRPC7 set 2 forward: 5'-tgctaatcagctgatagcgc-3', TRPC7 set 2 reverse: 5'-aaaggagacacgaggttc-3'. Negative controls were performed with RT− samples to confirm the absence of genomic DNA amplification.

Immunohistochemistry. The CA was removed and immediately frozen in 2-methylbutane. Frozen sections were mounted in a cryostat (−20°C) and cut into 10-μm sections. The sections were fixed with 4% paraformaldehyde for 10 min and then washed with phosphate-buffered saline (PBS) (3 x 5 min). Sections were then permeabilized and blocked in PBS containing goat serum (10%), BSA (0.5%), and Tween 20 (0.1%) for 30 min at RT. Incubation with rabbit anti-TRPC3 antibody (Sigma) was performed overnight at 4°C. The next day, washed with PBS (3 x 5 min) and incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary (Jackson Immuno-Research, West Grove, PA). The secondary detection antibody was incubated overnight at 4°C, covered at room temperature (60 min) at 1:1,000 dilution in the blocking solution above. Sections were next washed with PBS (3 x 5 min) and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary (Jackson Immuno-Research, West Grove, PA). The secondary detection antibody was incubated covered at room temperature (60 min) at 1:1,000 dilution. The sections were washed with PBS (5 x 10 min) and stained with 4,6-diamidino-2-phenylindole (1:10,000 dilution) before coverslips were applied. Imaging was performed using an Olympus IX81 motorized fluorescence microscope with Retiga 2000RV camera and Slidebook 4.2 software. Sections were acquired at 0.3-μm intervals using a ×60 oil objective (numerical aperture 1.40).

Patch-clamp electrophysiology. The whole-cell patch-clamp configuration was used for measurements of whole-cell currents in freshly isolated SMC using a MultiClamp 700B amplifier (Axon Instruments) and pCLAMP 10 software (Axon Instruments, Union City, CA), as reported previously (4). Patch electrodes were pulled from borosilicate glass (1.65 outer diameter, 1.28 inner diameter; Warner Instruments) and polished to a pipette resistance of ~2 MΩ. In some instances, currents were normalized to the cell membrane capacitance and presented as current densities (pA/pF). The pipette buffer contained (in mM) 156 NaCl, 1.8 CaCl2, 8.8 MgCl2, 2 EGTA, 7.85 d-glucose, and 10 HEPES; pH was adjusted to 7.2 with CsOH. The calculated free Ca2+ concentration was 131 nM. The bath buffer contained (mM) 156 NaCl, 1.8 CaCl2, 10 glucose, and 10 HEPES; pH was adjusted to 7.4 with NaOH. The isolated cells were placed in a chamber on the stage of an inverted microscope and continually superfused with bath buffer. Whole-cell currents were recorded in the voltage-clamp mode over the voltage range of –110 to +80 mV (sweep rate of 0.131 mV/ms, –50 mV holding potential). Whole cell ionic currents were measured in the absence or presence of 60 μM UTP. Lanthanum
chloride (100 μM) was added 5 min after UTP stimulation. In some cases, 10 μM gadolinium chloride was added instead of lanthanum chloride. Data were digitized and filtered at 1 kHz. All recordings were performed at room temperature (20–22°C).

Anti-TRPC3 antibody targeted to an intracellular region of the TRPC3 protein near the COOH-terminus, anti-TRPC1 antibody targeted to an intracellular epitope, as well as anti-TRPC4 and anti-TRPC6 antibodies. These antibodies were thus added to the pipette solution to allow access to the antigenic region of the channels. Since the anti-TRPC antibodies contain sodium azide (NaN₃) as preservative, we also investigated the effect of 0.00025% NaN₃ (concentration as 10⁻⁹ M NaN₃) when anti-TRPC antibodies are diluted at 1:200 in pipette solution. This concentration of NaN₃ did not alter the evoked UTP currents. UTP stimulation was started 10 min after obtaining whole cell access. 1-Oleoyl-2-acetyl-sn-glycerol (OAG) and KT 5823 (BIOMOL, Plymouth Meeting, PA) were dissolved in DMSO. (Z)-1-[(N-methyl-N-[6(N-methylammoniohexyl)amino)]diazen-1-ium-1,2-diolate (MAHMA NONOate) (Cayman, Ann Arbor, MI) was dissolved in distilled water. (8-BrcGMP) (Tocris, Elliville, MO) and other compounds were dissolved in DMSO. This concentration of NaN₃ did not alter the evoked UTP responses when anti-TRPC antibodies are diluted at 1:200 in pipette solution. The presence of 0.01 mM NaOH and added in both bath and pipette solution. The presence of 0.01 mM NaOH (concentration as 10 μM MAHMA NONOate) in pipette solution does not alter the basal current or UTP-stimulated current (IᵢUTP) (data not shown). 8-Bromo-cGMP (8-BrcGMP) (Tocris, Elliville, MO) and other compounds were dissolved in distilled water.

Isometric tension recordings. Isolated CAs from Long Evans rats were placed in ice-cold physiological solution containing the following (in mM): 137 NaCl, 5.6 KCl, 1 MgCl₂, 10 glucose, 2.5 CaCl₂, and 10 HEPES (pH 7.4). Each artery was subsequently cut in four ring segments that were 2.5 mm in length. The rings were then mounted in an eight-channel artery myograph (ChuelTech, Houston, TX) containing the same physiological solution at 37°C. This bicarbonate-free buffer was used in these experiments due to precipitation of LaCl₃ in these cases, 10 μM MAHMA NONOate in the presence or absence of LaCl₃ (100 μM). This concentration of UTP was used because the level of tension did not differ between groups. Following preconstriction with UTP, concentration responses were generated with sodium nitroprusside (SNP, 1 × 10⁻⁹ to 1 × 10⁻⁶ M) in log steps. In the summary plot, relaxations were normalized according to the formula ($T_{SNP} - T_{UTP}$)/(100, where $T_{base}$ is the resting tension, $T_{UTP}$ is the tension in response to 100 μM UTP, and $T_{SNP}$ is the tension in response to each concentration of SNP. Data was digitized and analyzed with Powerlab/SP with Chart version 4.24.

Statistical analysis. Data are presented as means ± SE. Comparisons between treatment groups containing single points were made by two-way repeated measures ANOVA; comparison between individual points was evaluated by the Holm-Sidak method. Comparisons between treatment groups containing single points were made by one-way ANOVA (SigmaPlot 11).

RESULTS

Demonstration of TRPC3/6/7 mRNA and protein in CA. We first examined CA for expression of TRPC3/6/7 mRNA by RT-PCR. Single products of expected size were obtained for TRPC3 and TRPC6; however, no product was obtained for TRPC7 with either of two sets of PCR primers (Fig. 1A). The TRPC7 primers were validated using cDNA obtained from whole brain.

We next determined whether TRPC3 protein was expressed in CA by Western analysis. A doublet corresponding to the glycosylated and unglycosylated forms of the protein was obtained at the expected mass of TRPC3 (Fig. 1B) (10). Heart protein was used as a positive control and yielded a lower mass band of ~84 kDa (not shown), consistent with a known splice

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variant expressed in heart (17). TRPC6 protein was not detected in CA, although it was detected in cerebral arteries and in brain (Supplemental Fig. 1). (The online version of this article contains supplemental data.) TRPC7 was not examined for protein due to the lack of mRNA.

We performed immunofluorescence with CA frozen sections to determine which cells within the CA expressed TRPC3 (Fig. 1C). An autofluorescence recording in the green channel demonstrates the multiple elastic layers within the CA. The innermost elastic layer (luminal side, Lu) reflects the boundary between the endothelial and smooth muscle layers. The 4,6-diamidino-2-phenylindole-stained nuclei are visible in the blue channel. TRPC3 immunofluorescence (red channel) was present throughout the smooth muscle layers, but was not detected in the endothelium.

**Demonstration of TRPC1/TRPC3 protein interaction.** During the course of these studies, data emerged suggesting a role for TRPC1 or a TRPC1-containing heteromer. As TRPC1 and TRPC3 have demonstrated the capacity to form a heteromeric channel in expression systems and native embryonic brain tissue (15, 21), we investigated this possibility in CA. TRPC1 protein was detected by Western as a single band at ~88 kDa (Fig. 2A). Next, coimmunoprecipitation was performed using anti-TRPC3 as the precipitating antibody, and the Western blot was probed with anti-TRPC1. A prominent single band was detected corresponding to the expected mass of TRPC1 (Fig. 2B). A total of three separate co-IP experiments were performed with protein extracts from freshly isolated CA. We similarly found a protein interaction of TRPC1/TRPC3 in cultured CA SMCs by coimmunoprecipitation (Supplemental Fig. 2).

**Demonstration of TRPC1/TRPC3 currents in isolated VSMCs.** We utilized whole cell patch clamp to determine whether TRPC1/TRPC3 channel currents were present in freshly isolated CA SMCs. Initial studies were performed using OAG (100 μM) to stimulate TRPC currents. OAG, a cell-permeable analog of diacylglycerol, is a known direct activator of the TRPC family. OAG produced increased inward and outward currents, as would be expected (Supplemental Fig. 3). We next evaluated the potential of UTP to stimulate TRPC currents. This agonist has recently been demonstrated to stimulate TRPC3 channel currents in rat cerebral artery SMCs (18). Figure 3A shows raw current-voltage recordings at rest, after UTP (60 μM), and following addition of a nonselective TRPC1 and TRPC3 channel blocker, 100 μM La3+. The I_{UTP} was primarily inward and reversed near 0 mV in these conditions. A summary of the subtracted currents (UTP − basal and UTP + La3+ − basal) normalized for capacitance are shown in Fig. 3B. The I_{UTP} was abolished by the application of La3+.

To confirm that UTP was indeed stimulating TRPC1/TRPC3 currents in CA SMCs, we applied TRPC1 or TRPC3 channel antibody to the inside of the cell via the patch pipette solution (Fig. 4A). These antibodies recognize an intracellular epitope and thus were expected to interfere with normal channel function, as previously demonstrated (1, 2, 11, 12, 19). Application of TRPC1 channel antibody (3 μg/ml) or TRPC3 channel antibody (3 μg/ml) produced a significant decrease in I_{UTP}. In contrast, application of other TRPC antibodies (TRPC4 and TRPC6) did not decrease I_{UTP}, demonstrating the functional role for TRPC1 and TRPC3, but not TRPC6. TRPC4 antibody was used as a negative control, as there was no expectation that TRPC4 channels were involved in the smooth muscle response to UTP. As an additional control, application of sodium azide (a preservative present in antibody solutions) also did not decrease I_{UTP}. The summary I_{UTP} are presented as subtracted currents normalized to cell capacitance, as in Fig. 3B. The I_{UTP} at −110 mV is summarized for the different antibodies in Fig. 4B. The peak inward I_{UTP} with either TRPC1 or TRPC3 antibody is significantly reduced compared with control or to TRPC4 and TRPC6 antibodies. When taken with the coimmunoprecipitation results, these data suggest that I_{UTP} is carried by a TRPC1/TRPC3 heteromer in rat CA SMC.

**Demonstration of TRPC1/TRPC3 current inhibition by NO/PKG.** From the above studies, we demonstrated that the I_{UTP} in CA SMCs was largely dependent on TRPC1/TRPC3 channels. We next sought to determine whether the TRPC1/TRPC3 channel current was inhibited by NO or products of the NO-signaling pathway (Fig. 5A). Application of a NO donor, MAHMA NONOate (10 μM), results in a significant inhibition of I_{UTP}. This concentration of MAHMA NONOate produces a near, but not complete, dilation of cerebral arteries and arterioles, suggesting that this concentration of NO donor is sufficient but not excessive to promote dilation of an artery (28). Similar reduction of I_{UTP} was obtained with the cell-permeable cGMP analog, 8-BrcGMP (100 μM). To evaluate the role of PKG in the mechanism, we used a PKG inhibitor (KT5823; 1 μM) to reverse the effect of MAHMA NONOate or 8-BrcGMP. Thus, if NO and cGMP act through PKG activation, inhibition of PKG should reverse the inhibitory effect of MAHMA NONOate and 8-BrcGMP. Figure 5A further demonstrates that KT5823 does indeed inhibit the effect of MAHMA NONOate and 8-BrcGMP to block I_{UTP}. Notice that 8-BrcGMP and MAHMA NONOate significantly reduce I_{UTP}, whereas I_{UTP} is restored to near control levels when the PKG inhibitor is present. Thus the TRPC1/TRPC3 current is inhib-
edited by NO and cGMP. Furthermore, these regulatory effects are mediated by PKG. The peak inward current at $-110\, \text{mV}$ are summarized in Fig. 5B. Peak inward currents are significantly reduced compared with control, as well as the KT5823/MAHMA NONOate or KT5823/8-BrcGMP groups.

Role of TRPC-like currents in NO-mediated vasorelaxation. We used CA segments in an isometric tension ring bath to evaluate the role of TRPC1/TRPC3 channels in NO-mediated vasorelaxation. $N^\text{G}$-nitro-L-arginine methyl ester (50 $\mu\text{M}$) was included to inhibit endogenous production of NO. UTP (100 $\mu\text{M}$) was used to preconstrict arteries in the absence or presence of La$^{3+}$ (100 $\mu\text{M}$). This was the highest concentration of UTP that still produced comparable tension in all groups. Relaxations were then elicited by the delivery of SNP (10 to $10^{-6}\text{M}$). Figure 6A shows a representative experiment, including a control (no La$^{3+}$), 100 $\mu\text{M}$ La$^{3+}$, and a time control (no SNP added) to evaluate the stability of the UTP constriction. The summarized data (Fig. 6B) demonstrate a significant inhibition of the NO-mediated relaxation by 100 $\mu\text{M}$ La$^{3+}$.

DISCUSSION

Our goal in this study was to test the following two hypotheses regarding the regulation of vascular tone by NO: 1) NO/cGMP/PKG-mediated inhibition of TRPC1/TRPC3 channels occurs in native SMC; and 2) NO-mediated vasorelaxation is partially dependent on inhibition of TRPCs in intact CA. As a result of these studies, we conclude that CA smooth muscle expresses a TRPC1/TRPC3 heteromer, which can be activated in response to UTP stimulation. The TRPC1/TRPC3 heteromer is significantly inhibited by addition of exogenous NO or a cell-permeable analog of cGMP. Furthermore, this inhibition requires the specific activation of PKG. Last, we present evidence that this mechanism of TRPC1/TRPC3 channel inhibition contributes to the mechanism of NO-mediated vasorelaxation in the intact artery. These findings are summarized in Fig. 7 and are discussed further below.
however, we considered all of the TRPC3/6/7 channels as potentially NO sensitive.

In the present study, we evaluated native CA smooth muscle for the expression of TRPC3/6/7. We demonstrated the presence of TRPC3 and TRPC6 mRNA in whole CA. However, expression of TRPC7 was not detected with either of two validated sets of PCR primers. TRPC3 protein was readily detected by Western analysis and localized to the SMC by immunofluorescence. Interestingly, we did not detect TRPC3 expression in the endothelial cells in this artery, even though it has been shown to be expressed in the endothelium of some other vascular beds (22a, 26) and in cultured endothelial cells (17a, 20a). TRPC6 protein was not detectable in CA by Western analysis, although it was detectable at low levels in whole cerebral artery and brain protein extracts (Supplemental Fig. 1). While these findings demonstrated a significant presence of TRPC3 in carotid smooth muscle, we could not exclude some low level of expression of TRPC6 as well. Therefore, while studies continued...

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Fig. 5. A: summary I-V plots showing $I_{UTP}$ normalized to cell capacitance (current density). Recordings were performed with UTP alone (circles) or following pretreatment with 8-bromo-cGMP (8-BrcGMP; diamonds), (Z)-1-[(N-methyl-N-[6-(N-methylammoniohexyl)amino)]diazen-1-ium-1,2-diolate (MAHMA NONOate; squares), KT5823 + 8-BrcGMP (inverted triangles), or KT5823 + MAHMA NONOate (stars). The $I_{UTP}$ was inhibited by the nitric oxide (NO) donor, as well as the cGMP analog. Note that the inhibition of the NO donor or cGMP analog was reversed in the presence of a PKG inhibitor. B: summary of UTP-stimulated current at $-110$ mV evaluating regulation by the NO signaling pathway. *$P < 0.05$ vs. control.

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Fig. 6. A: representative artery myograph traces from isolated CA segments. CA segments were preconstricted with 100 μM UTP before addition of sodium nitroprusside (SNP) in the absence (control) or presence of 100 μM La$^{3+}$. The arrows indicate the points at which SNP was delivered. A time control was performed, in which SNP was not administered, to demonstrate the stability of the UTP response over the time period of the SNP study. B: summary of the SNP-mediated relaxation responses for control (circles) and for 100 μM La$^{3+}$ (triangles). A time control was performed in which no SNP was administered (squares). The La$^{3+}$ treatment group was significantly different from the controls. *$P < 0.05$ vs. control and time control groups.
predominantly with TRPC3, we continued to consider a possible role for TRPC6 in our experimental design (see below).

The functional expression of TRPC3 channels in carotid SMCs was evaluated by patch clamp. We used UTP to elicit TRPC currents, given that this agonist is reported to produce mainly TRPC3 channel activation in cerebral cortex SMC (18). UTP (60 μM) produced reproducible currents that reversed at ~0 mV and shared similar rectification compared with other reported TRPC3 recordings (10, 18). Since there is a lack of selective pharmacological tools with which to study TRPC3 and TRPC6 function and RNA silencing proved ineffective in the CA smooth muscle, we evaluated the role of these channels in I_{UTP} by inhibition with selective TRPC antibodies delivered via the patch pipette solution. This type of strategy has been used successfully by others to inhibit TRPC1, TRPC3, and TRPC6 channels (1, 2, 11, 12, 19, 25). Surprisingly, we found significant inhibition of I_{UTP} by intracellular incubation with anti-TRPC1 or anti-TRPC3 antibodies. Given the unexpected finding suggesting a role for TRPC1 in the I_{UTP}, we performed coimmunoprecipitation studies to evaluate the possible existence of TRPC1/TRPC3 heteromeric channel. These studies suggested a prominent protein interaction between TRPC1 and TRPC3, consistent with the existence of TRPC1/TRPC3 heteromers in native SMCs. Although a TRPC1/TRPC3 heteromer has not previously been demonstrated in SMC, a TRPC1/TRPC3 heteromer has been reported in embryonic rat brain (21), as well as by coexpression studies (15). Lintschinger et al. (15) further demonstrated that the TRPC1/TRPC3 heteromer channel takes on characteristics that are unique from the respective homomeric channels. In particular, the TRPC1/TRPC3 channel adopts a more inwardly rectifying current compared with the homomeric TRPC3 channel, and the basal activity is increased by the removal of extracellular calcium (15). Our studies clearly demonstrate an inwardly rectifying current (Fig. 2B), as well as potentiation of basal current following the removal of extracellular bath calcium (not shown). Although Reading et al. (18) concluded that UTP stimulated a TRPC3 channel in cerebral SMC, their data could still be explained by a TRPC1/TRPC3 heteromer. Reading et al. (18) knocked down TRPC3 by RNA silencing and demonstrated loss of I_{UTP} by patch clamp. However, it seems highly plausible that knockdown of one of the components of a TRPC1/TRPC3 heteromer might also have profound effects on the functionality of the channel and produce the same functional knockdown. Thus, when evaluated as a whole, our data are consistent with TRPC3 acting within a TRPC1/TRPC3 heteromeric channel in native SMC.

Inhibition of TRPC1/TRPC3 by the NO signaling pathway in native SMCs. Inhibition of TRPCs by the NO signaling pathway was first shown with TRPC3 heterologously expressed in HEK-293 cells (13), and more recently with TRPC6 in A7r5 cells (22). The present study extends these findings to TRPC1/TRPC3 channels in freshly isolated SMCs, thus demonstrating that this NO/TRPC feedback system is not unique to cell culture or overexpression systems. We have demonstrated that endogenous TRPC1/TRPC3 channels responsible for I_{UTP} could be significantly inhibited by exogenous NO or a cell-permeable analog of cGMP in native SMCs. Furthermore, we demonstrated the obligatory role of PKG in the signaling pathway by reversing the inhibitory effect of NO and cGMP by PKG inhibition. While NO has also been reported to modulate TRPCs through cysteine S-nitrosylation (27), the nearly complete reversal of NO inhibition by inhibiting PKG suggests that the action of NO in this system is predominantly through activation of cGMP/PKG.

PKG phosphorylation of TRPC3 is clearly supported by studies evaluating homomeric TRPC3 channels. In a TRPC1/TRPC3 heteromeric channel, PKG regulation could be imparted via the regulatory sites of TRPC3 alone or in combination through yet-to-be-described sites on TRPC1. In support of the latter possibility, an evaluation of predicted PKG phosphorylation sites with rat TRPC1 returns two possible sites (KinasePhos). Future studies will be needed to determine whether either of these predicted phosphorylation sites contributes to TRPC1 channel regulation.

Inhibition of NO-mediated vasorelaxation by TRPCs. As stated earlier, a functional role of NO-dependent inhibition of TRPCs in vasorelaxation has not previously been reported. We used vessel myography to evaluate the role of TRPCs in NO-mediated vasorelaxation in intact CA. The arteries were precontracted with UTP in the presence or absence of La^{3+}, a broadly acting TRPC blocker. Based on our patch clamp data, we expected activation of TRPC1/TRPC3 channels by UTP. When an exogenous NO donor was delivered, significantly less...
relaxation occurred in the La\(^{3+}\)-treated arteries compared with controls. These data are consistent with the involvement of a La\(^{3+}\)-sensitive channel that is stimulated by UTP and inhibited by NO. As La\(^{3+}\) can act to inhibit multiple pathways of Ca\(^{2+}\) entry, further studies will be required to definitively prove the specific role of TRPC1/TRPC3. Nevertheless, the isolated smooth muscle data and the intact artery myograph data are consistent with NO inhibition of a Ca\(^{2+}\) entry pathway leading to UTP-mediated constriction. The data thus far are consistent with TRPC1/TRPC3 as the molecular target for NO-mediated inhibition of Ca\(^{2+}\) entry.

**Physiological significance.** We have presented evidence for a feedback system involving NO and TRPC1/TRPC3 channels in the control of vascular tone (see Fig. 7). This system involves receptor-mediated activation of TRPC1/TRPC3 channels, which, in turn, contributes to SMC depolarization and increased vascular tone. NO contributes to vasorelaxation through multiple previously described mechanisms, and we now add inhibition of TRPC1/TRPC3 channels to that list.

**GRANTS**

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