cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells

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We provide here the first direct evidence that cGMP-PK can activate K\textsubscript{Ca} channels in arterial smooth muscle cells. We demonstrate that NO and a membrane-permeable analogue of cGMP can activate K\textsubscript{Ca} channels in on-cell patches approximately twofold. Furthermore, cGMP-PK, in the presence of ATP and cGMP added directly to the intracellular surface of inside-out patches, increases channel activity by approximately eightfold. These results suggest that cGMP-PK-mediated activation of K\textsubscript{Ca} channels may contribute to the actions of NO and other nitrovasodilators.

We present the first direct evidence that cGMP-PK can activate K\textsubscript{Ca} channels in inside-out patches of smooth muscle from rabbit cerebral arteries. cGMP-PK was effective only in the presence of cGMP and ATP, suggesting that cGMP-PK acts through phosphorylation of the K\textsubscript{Ca} channel or an associated protein. Furthermore, we show that NO and a membrane-permeable analogue of cGMP can also activate K\textsubscript{Ca} channels in on-cell patches. These results suggest that activation of K\textsubscript{Ca} channels may contribute to vasodilating actions of nitrovasodilators including NO.

METHODS

New Zealand White rabbits were overdosed with pentobarbital sodium, and the entire lengths of the basilar arteries were removed. The arteries were cleaned in an isolation medium containing (in mM) 80 L-glutamic acid (monosodium salt), 55 NaCl, 6 KCl, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2 MgCl\textsubscript{2}, and 10 glucose, as well as 70 \mu M CaCl\textsubscript{2}, pH 7.3. The arteries were then cannulated and perfused at a constant flow with the above isolation medium also containing 0.4 mg/ml elastase (Sigma) and 2 mg/ml collagenase (type 1A, Sigma). After an incubation time of ~45 min, the tissue was removed and stored in the above solution with 200 \mu M CaCl\textsubscript{2} at 4°C. Single cells were released from the artery by trituration. The bathing solution contained (in mM) 140 KCl, 2 MgCl\textsubscript{2}, 3 ethylene glycol-bis(\textalpha-aminooxyethyl) ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 HEPES, and 2.29 CaCl\textsubscript{2}, pH 7.4. Free calcium was calculated as 300 nM using a computer program (15) and absolute reaction constants (12). These constants were corrected for temperature (22°C) and ionic strength. The following apparent reaction constants were calculated at pH 7.4: pK\textsubscript{Ca\textsubscript{EGTA}} = 7.17, pK\textsubscript{Ca\textsubscript{EGTA}} = 1.53. The pipette solution was (in mM) 120 NaCl, 20 KCl, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, and 10 HEPES, pH 7.4.
All experiments were performed at room temperature (22°C). Pipettes were pulled from borosilicate glass (Sutter Instruments) with a resistance of 5–20 MΩ. Single-channel currents in both the cell-attached and inside-out configurations (6) were measured using an Axopatch-1C amplifier and recorded simultaneously on a Sony DAT recorder (DTC-700). The data were then filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices) and digitized at 5 kHz. Data were acquired using an Axotape program (Axon Instruments) and analyzed using ASCD software (G. Droogmans Lab. Fysiologie, KU Leuven, Belgium) on a Gateway 386 PC. Gaussian distributions fitted to current amplitude histograms were used to calculate single-channel currents. Average channel activity (NP,) in patches was determined from recordings of several minutes by

\[ NP, = \left( \sum_{j=1}^{N} t_j \right) / T \]

where \( P_0 \) is the open state probability, \( T \) is the duration of the recording, \( t_j \) is the time spent with \( j = 1, 2, \ldots, N \) channels open, and \( N \) is the maximal number of channels observed in conditions of high levels of \( P_0 \). Steady-state \( NP_0 \), estimates were made from 5- to 15-min recordings under each condition.

Materials. HEPES and TEA+ were obtained from Sigma Chemical. cGMP-PK was obtained from Promega and 8-(4-chlorophenylthio)-guanosine-3,5'-cyclic monophosphate (CPT-cGMP) from Biolog Life Science Institute, as well as a gift from Prof. U. Walter, Würzburg, Germany. The molsidomine derivative 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) was a gift from Dr. M. Murphy (University of Vermont). The active metabolite of molsidomine, SIN-1A, arises from a hydroxyl-induced nonenzymatic ring opening of SIN-1. SIN-1A is then oxidized, releasing NO (4). Stock solutions of SIN-1 were heated to 37°C for 10 min before use to activate SIN-1 to SIN-1A. All results are expressed as means ± SE. All potentials mentioned in the text and figures are membrane potentials.

RESULTS

NO (SIN-1) activates Kc₃ channels in patches on intact single smooth muscle cells. Kc₃ channels were identified by their conductance over the voltage range of -30 to +30 mV (132.4 ± 5.0 pS; \( n = 10 \)), unitary current (10.9 ± 0.25 pA; \( n = 5 \)), voltage sensitivity (e-fold increase in \( P_0 \), every 12.0 mV; \( n = 2 \)), and sensitivity to 200 μM externally applied TEA+ (40% reduction in unitary current at +50 mV, from 10.9 ± 0.25 to 6.5 ± 0.89 pA; \( n = 3 \); see also Ref. 2).

To test the hypothesis that Kc₃ channels are involved in the actions of cGMP-PK, the effects of NO on Kc₃ channels in patches on intact single smooth muscle cells from cerebral arteries were investigated. Nitrovasodilators have been reported to lower intracellular calcium (11), which would lead to a decrease in the activity of Kc₃ channels. We attempted to minimize changes in calcium flux across the membrane by bathing the cells in low extracellular calcium (300 nM) and by examining Kc₃ channels at membrane potential (+50 mV) near the calcium equilibrium potential. NO was increased in the bath by adding SIN-1 to the extracellular solution. At 22°C, 50 μM SIN-1 releases NO at a rate of 0.12 μM/min. SIN-1 at 5 μM caused complete relaxation of pressurized myogenic cerebral arteries (unpublished observations). In the experiment shown in Fig. 1, SIN-1 (50 μM) increased activity (measured as \( NP_o \)) of Kc₃ channels from 0.00046 to 0.00112 or by 2.43-fold at +50 mV. The mean increase in \( NP_o \) under these conditions was 2.21 ± 0.28-fold (\( n = 5 \)) at +50 mV. SIN-1 had no effect on unitary currents. Mean single channel currents before and after SIN-1 were 11.4 ± 0.3 and 11.4 ± 0.4 pA, respectively, at +50 mV (\( n = 5 \)).

Membrane-permeable cGMP analogue activates Kc₃ channels in patches on intact single smooth muscle cells. The activation of Kc₃ channels by NO and the observations of others that NO activates guanylyl cyclase suggest that elevation of intracellular cGMP can open Kc₃ channels in smooth muscle. These results would predict that elevation of intracellular cGMP by other means should also activate Kc₃ channels. We tested this hypothesis by examining the effects of a membrane-permeable analogue of cGMP (CPT-cGMP), a specific and potent activator of cGMP-PK (9), on Kc₃ channels in patches on intact smooth muscle cells from cerebral arteries. CPT-cGMP has the following advantages over other membrane-permeable analogues of cGMP such as 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP): 1) it has higher lipophilicity so that it should permeate the cell membrane at higher rates; 2) it has a high degree of specificity of the cGMP-PK; it has little effect on cGMP-regulated phosphodiesterases; and 3) it is resistant to degradation by phosphodiesterases (3). In the experiment shown in Fig. 2, CPT-cGMP (400 μM), when added to the bath solution, increased \( NP_o \) from 0.0016 to 0.0030 or 1.9-fold at +30 mV. The mean increase in \( NP_o \) to CPT-cGMP (400 μM) was 2.91 ± 0.80-fold (\( n = 9 \); Fig. 2). CPT-cGMP had no effect on unitary currents (7.4 ± 0.1 pA before CPT-cGMP and 7.4 ± 0.2 after; \( n = 4 \); +30 mV). The activation of Kc₃ channels by CPT-cGMP appears to be an indirect effect, since cGMP applied to the intracellular side of Kc₃ channels in inside-out excised patches had no effect on \( NP_o \) (see Fig. 3).

CPT-cGMP PK activates Kc₃ channels in excised inside out patches. SIN-1 (NO) and CPT-cGMP activation of Kc₃ channels in patches on intact single cells lends support to the idea that cGMP-PK can activate Kc₃ channels. We tested this possibility directly by applying CPT-cGMP-PK to the intracellular side of Kc₃ channels in excised inside-out patches (Fig. 3). In the experiment shown in Fig. 3A,
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Fig. 2. Activation of KCa channel by soluble cGMP analogue. Cell-attached configuration, +30 mV membrane potential. 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (CPT-cGMP; 400 μM) added to external surface increased Np, from 0.0016 to 0.0030, a 1.88-fold increase at +30 mV.

Fig. 3. cGMP-dependent protein kinase (cGMP-PK) activates KCa channel. A: inside-out configuration, +10 mV membrane potential. Ki- 

nase (5 U/ml) increased Np, from 0.114 to 0.737 at +10 mV, a 6.46-fold increase. B: combined results from 7 experiments. ATP was added first, and effect compared with control condition (normalized to 1). Subsequently, cGMP was added to ATP, and channel activity compared with ATP alone. Finally, kinase was added, and effect compared with 2 nucleotides. Reverse order was also performed, where kinase was added first, followed by ATP and cGMP together. It can be seen that ATP, cGMP, and kinase were all required together to produce an effect; 8.85 ± 2.90-fold increase in channel activity.

cGMP-PK (5 U/μl) with ATP (100 μM) and cGMP (100 μM) increased Np, from 0.114 to 0.737 at +10 mV, a 6.46-fold increase. The mean increase in Np, to cGMP-PK with ATP and cGMP was 8.85 ± 2.90-fold at +10 mV (n = 7; Fig. 3B). ATP alone (100 μM; n = 4), cGMP alone (100 μM; n = 4), cGMP and ATP together (100 μM each; n = 4), and cGMP-PK alone (5 U/ml; n = 30) did not increase channel activity (Fig. 3B). Thus the activation of these channels required both cGMP and ATP to be present, suggesting that the cGMP is stimulating the kinase, which in turn is activating the channel by phosphorylation. The activated cGMP-PK had no effect on unitary currents (7.8 ± 0.2 pA before and 7.6 ± 0.1 pA after; +10 mV; n = 4).

cGMP-PK could theoretically elevate Np, by increasing the number of functional channels (N), Pp, or both. To determine whether cGMP-PK increased N, the activation of KCa channels by cGMP-PK was investigated at low and high levels of Pp, by varying the holding potential at a fixed level of calcium. In these experiments (n = 6) the cGMP-PK was preincubated with the cGMP and ATP for 10 min before applying to the bath solution. Figure 4A shows the effect of cGMP-PK on channel activity at +10 and +50 mV, i.e., when Pp is low and high. At +10 mV, cGMP-PK with ATP and cGMP increased Np, from 0.14- to 1.04- or 7.42-fold. However, at +50

Fig. 4. Effect of cGMP-PK at different membrane potentials. A: at +10 mV, kinase increased Pp, from 0.070 to 0.501 (7.16-fold). At +50 mV, where channel activity was high in control condition (Pp, = 0.564), increase was only to 0.849, a 1.50-fold increase. B: single-channel activation curve. Effect of cGMP-PK is far greater when Pp, value is low in control condition. These results suggest that increase in channel activity is due to increase in Pp, rather than recruitment of channels. Data were fitted with Boltzman relationship: Pp, = Pmax (1 + exp[(Vm - V0.5)/k]), where Pp, is peak Pp, V0.5 is membrane potential at which peak Pp, is one-half Pmax, and k is steepness factor, which indicates voltage sensitivity of Pp,. Midpoint of activation shifted leftward by 37 mV. Pmax for control condition was 0.88 and was 0.92 for cGMP-PK. Steepness factor for both conditions was 12 mV.
mV, when $P_o$ was high (0.564), cGMP-PK increased $N_p$ only 1.50-fold. This small increase in $N_p$, due to an elevation of $P_o$ from 0.564 to 0.849 without a change in $N$, was 2. When $P_o$ was >0.75, the increase in channel activity was only 1.01 ± 0.01-fold (n = 12). Figure 4B shows the effect of cGMP-PK on $P_o$ over a wider range of membrane potentials. The effect of cGMP-PK on $K_c$ channels in this patch could be explained by the leftward shift in the activation curve, with a change in the midpoint of activation from $+58$ to $+21$ or by $-37$ mV. Thus cGMP PK appears to act through a shift in the activation curve of $K_c$ channels and not by increasing the number of functional channels.

**DISCUSSION**

We provide the first direct evidence that $K_c$ channels in arterial smooth muscle can be opened through activation of cGMP-dependent protein kinase. cGMP-PK activation of $K_c$ channels required both cGMP and ATP, suggesting that cGMP-PK acts through phosphorylation. Furthermore, we demonstrate that NO and the membrane-permeable cGMP analogue (CPT-cGMP) can also open $K_c$ channels. We therefore propose the following scheme of regulation of $K_c$ channels by nitrovasodilators: 1) activation of guanylyl cyclase, 2) elevation of cGMP, 3) stimulation of cGMP-PK, 4) phosphorylation of $K_c$ channels or an associated regulatory protein, and 5) activation of $K_c$ channels caused by an ~30 mV leftward shift in the activation curve. These results suggest that activation of $K_c$ channels may contribute to the actions of NO and other vasodilators.

Fujino et al. (5) demonstrated that nitroglycerin as well as 8-BrcGMP could activate $K_c$ channels in coronary artery smooth muscle cells grown in explants. Furthermore, these investigators also found that cGMP, alone, could increase channel activity in inside-out patches. We found no effect of cGMP on $K_c$ channels in inside-out patches (see Fig. 3D). It is possible that in their preparation the kinase was present in the membrane, whereas in ours it was not. $K_c$ channels in aortic smooth muscle can be activated by ANF and nitroprusside (18), which increases cGMP. In this case, the membrane-permeable cGMP analogue, dibutyryl cGMP (1 mM) also activated $K_c$ channels. However, these investigators found that cGMP (1000 μM) could also activate $K_c$ channels in inside-out patches. Neither Fujino et al. (5) nor Williams et al. (18) directly tested the effects of cGMP-PK kinase on $K_c$ channels. Here we used a membrane-permeant analogue of cGMP (CPT-cGMP) that, unlike dibutyryl cGMP or other cGMP analogues, is very specific for cGMP-PK and is resistant to degradation by cGMP phosphodiesterases. We also directly tested the effects of cGMP-PK.

$K_c$ channels in smooth muscle can also be activated by cAMP-dependent protein kinase (10, 14) and G proteins (9). Furthermore, guanosine 5′-O-(3-thiotriphosphate) has been shown to shift the activation curve of $K_c$ channels from myometrium smooth muscle by about ~30 mV (17). The mean increase in $P_o$ caused by cGMP-PK (with ATP and cGMP) of 8.85-fold could be explained by a shift in the activation curve of $K_c$ channels of ~26 mV (see also Fig. 4B). This shift could be the result of either an increase in calcium affinity of the channel or a shift in the voltage dependence.

In conclusion, these are the first results showing cGMP-PK to have a direct effect on $K_c$ channels, providing supporting evidence for the modulation of this channel by NO.

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