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Intracellular signalling mechanism responsible for modulation of sarcolemmal ATP-sensitive potassium channels by nitric oxide in ventricular cardiomyocytes

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Key points

- Both the ATP-sensitive potassium (K_{ATP}) channel and the gaseous messenger nitric oxide (NO) play fundamental roles in protecting the heart from injuries related to ischaemia.
- NO has previously been suggested to modulate cardiac K_{ATP} channels; however, the underlying mechanism remains largely unknown.
- In this study, by performing electrophysiological and biochemical assays, we demonstrate that NO potentiation of K_{ATP} channel activity in ventricular cardiomyocytes is prevented by pharmacological inhibition of soluble guanylyl cyclase (sGC), cGMP-dependent protein kinase (PKG), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and extracellular signal-regulated protein kinase 1/2 (ERK1/2), by removal of reactive oxygen species and by genetic disruption of CaMKIIδ.
- These results suggest that NO modulates cardiac K_{ATP} channels via a novel cGMP–sGC–cGMP–PKG–ROS–ERK1/2–calmodulin–CaMKII (δ isoform in particular) signalling cascade.
- This novel intracellular signalling pathway may regulate the excitability of heart cells and provide protection against ischaemic or hypoxic injury, by opening the cardioprotective K_{ATP} channels.

Abstract The ATP-sensitive potassium (K_{ATP}) channels are crucial for stress adaptation in the heart. It has previously been suggested that the function of K_{ATP} channels is modulated by nitric oxide (NO), a gaseous messenger known to be cytoprotective; however, the underlying mechanism remains poorly understood. Here we sought to delineate the intracellular signalling mechanism responsible for NO modulation of sarcolemmal K_{ATP} (sarcK_{ATP}) channels in ventricular cardiomyocytes. Cell-attached patch recordings were performed in transfected human embryonic kidney (HEK) 293 cells and ventricular cardiomyocytes freshly isolated from adult rabbits or genetically modified mice, in combination with pharmacological and biochemical approaches. Bath application of the NO donor NOC-18 increased the single-channel activity of Kir6.2/SUR2A (i.e. the principal ventricular-type K_{ATP}) channels in HEK293 cells, whereas the increase was abated by KT5823 [a selective cGMP-dependent protein kinase (PKG) inhibitor], mercaptopropionyl glycine [MPG; a reactive oxygen species (ROS) scavenger], catalase (an H_2O_2-degrading enzyme), myristoylated autocamtide-2 related inhibitory peptide (mAIP) selective for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and U0126 [an extracellular signal-regulated protein kinase 1/2 (ERK1/2) inhibitor], respectively. The NO donors NOC-18 and *N*-(2-deoxy-α,β-D-glucopyranose-2-)-N^2-acetyl-*S*-nitroso-D,L-penicillaminamide

D.-M. Zhang and Y. Chai contributed equally to this study.

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(glycol-SNAP-2) were also capable of stimulating native sarcK_{ATP} channels preactivated by the channel opener pinacidil in rabbit ventricular myocytes, through reducing the occurrence and the dwelling time of the long closed states whilst increasing the frequency of channel opening; in contrast, all these changes were reversed in the presence of inhibitors selective for soluble guanylyl cyclase (sGC), PKG, calmodulin, CaMKII or ERK1/2. Mimicking the action of NO donors, exogenous H_2O_2 potentiated pinacidil-preactivated sarcK_{ATP} channel activity in intact cardiomyocytes, but the H_2O_2-induced K_{ATP} channel stimulation was obliterated when ERK1/2 or CaMKII activity was suppressed, implying that H_2O_2 is positioned upstream of ERK1/2 and CaMKII for K_{ATP} channel modulation. Furthermore, genetic ablation (i.e. knockout) of CaMKIIδ, the predominant cardiac CaMKII isoform, diminished ventricular sarcK_{ATP} channel stimulation elicited by activation of PKG, unveiling CaMKIIδ as a crucial player. Additionally, evidence from kinase activity and Western blot analyses revealed that activation of NO–PKG signalling augmented CaMKII activity in rabbit ventricular myocytes and, importantly, CaMKII activation by PKG occurred in an ERK1/2-dependent manner, placing ERK1/2 upstream of CaMKII. Taken together, these findings suggest that NO modulates ventricular sarcK_{ATP} channels via a novel sGC–cGMP–PKG–ROS(H_2O_2)–ERK1/2–calmodulin–CaMKII (δ isoform in particular) signalling cascade, which heightens K_{ATP} channel activity by destabilizing the long closed states while facilitating closed-to-open state transitions. This pathway may contribute to regulation of cardiac excitability and cytoprotection against ischaemia–reperfusion injury, in part, by opening myocardial sarcK_{ATP} channels.

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Abbreviations APD_{90}, action potential duration at 90% repolarization; CaMKII, calcium/calmodulin-dependent protein kinase II; E_K, equilibrium potential for potassium; ERK, extracellular signal-regulated kinase; 5-HD, 5-hydroxydecanoate; HEK293, human embryonic kidney 293 (cell line); H_2O_2, hydrogen peroxide; IRK, inwardly rectifying Kir2.x (channel); K_{ATP}, ATP-sensitive potassium (channel); KCO, potassium channel opener; Kir, inwardly rectifying potassium (channel); mAIP, myristoylated autocamtide-2 related inhibitory peptide selective for CaMKII; MAPK, mitogen-activated protein kinase or MAP kinase; MEK, mitogen-activated protein kinase kinase or MAPK kinase; mitoK_{ATP}, mitochondrial K_{ATP} (channel); MPG, *N*-(2-mercaptopropionyl)glycine; NO, nitric oxide; NOC-18, DETA NONOate; NP_o, open probability; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; p-CaMKII, autophosphorylated CaMKII; PIP_2, phosphatidylinositol-4,5-bisphophate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; sarcK_{ATP}, sarcolemmal K_{ATP}; sGC, soluble guanylyl cyclase; glycol-SNAP-2, *N*-(2-deoxy-α,β-D-glucopyranose-2-)-N^2-acetyl-*S*-nitroso-D,L-penicillaminamide; SNAP, *S*-nitroso-*N*-acetyl penicillamine; SUR, sulfonylurea receptor; V_m, membrane potential.

Introduction

Vital in the adaptive response to (patho)physiological stress, the ATP-sensitive potassium (K_{ATP}) channel functions as a high-fidelity metabolic sensor, which couples intracellular metabolic state to membrane excitability (Ashcroft, 1988; Miki & Seino, 2005; Nichols, 2006) and serves a homeostatic role ranging from blood glucose regulation to cardioprotection (Olson & Terzic, 2010). The K_{ATP} channel is a hetero-octameric protein composed of four inwardly rectifying potassium channel subunits (Kir6.x) and four sulphonylurea receptors (SURx; Shyng & Nichols, 1997; Babenko *et al.* 1998), whose molecular (subunit) composition exhibits tissue specificity. For example, in cardiac (ventricular) and skeletal muscles the K_{ATP} channels are composed of Kir6.2 and SUR2A subunits (Inagaki *et al.* 1996; Okuyama *et al.* 1998), whereas in central neurons and pancreatic β-cells they consist of Kir6.2 and SUR1 subunits (Aguilar-Bryan *et al.* 1998). Whilst it is appreciated that K_{ATP} channels are directly regulated by intracellular ATP, MgADP (Nichols, 2006) and phosphatidylinositol-4,5-bisphophate (PIP_2; Fan & Makielski, 1997; Baukrowitz *et al.* 1998; Shyng & Nichols, 1998), how these important channels are modulated by more complicated intracellular signalling processes is far less understood.

The gaseous messenger nitric oxide has a fundamental biological role in protecting the heart against ischaemia–reperfusion injury (Bolli, 2001). It has been suggested that NO shortens action potential duration

$(APD)_{90}$ and increases maximal diastolic potential in the heart, by activating sarcolemmal K_{ATP} (sarcK_{ATP}) channels via a cGMP-dependent mechanism (Baker *et al.* 2001). Nitric oxide also potentiates the action of potassium channel openers (KCOs) on the K_{ATP} channel in single ventricular cells, yet with conflicting findings on whether cGMP is involved (Shinbo & Iijima, 1997; Han *et al.* 2002). The intracellular mechanism by which NO modulates cardiac K_{ATP} channels has remained largely unknown.

In the present study, we combined single-channel patch-clamp recordings with pharmacological and biochemical approaches to delineate the intracellular signalling mechanism responsible for NO modulation of cardiac sarcK_{ATP} channels. Human embryonic kidney (HEK) 293 cells expressing recombinant cardiac-type K_{ATP} (i.e. Kir6.2/SUR2A) channels and ventricular cardiomyocytes freshly isolated from adult rabbits as well as from CaMKIIδ gene-null and wild-type mouse models expressing endogenous K_{ATP} channels were used. Specifically, we investigated the involvement in NO signal transduction of soluble guanylyl cyclase (sGC), cGMP-dependent protein kinase (PKG), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), calmodulin, calcium/calmodulin-dependent protein kinase II (CaMKII) and extracellular signal-regulated protein kinase (ERK)1/2 of the mitogen-activated protein kinase (MAPK) family. Here we show that functional modulation of ventricular sarcK_{ATP} channels by NO induction is mediated by intracellular signalling via a novel sGC–cGMP–PKG–ROS(H_2O_2)–ERK1/2–calmodulin–CaMKII (CaMKIIδ isoform in particular) signalling pathway that alters the open and closed properties of the channel, enhancing channel activity.

Methods

Ethical approval

All protocols involving animals were approved by the institutional Animal Care and Use Committee at the University of California, Davis, and experiments were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals* 8th edition (2011) of the National Research Council, USA and conformed to the principles of UK regulations as described by Drummond (2009).

Construction of cDNAs

To reconstitute cardiac ventricular-type K_{ATP} channels, cDNAs encoding the pore-forming subunit Kir6.2 (mouse; gift from Dr. Susumu Seino at Kobe University, Chuo-ku, Japan) and the regulatory subunit SUR2A (rat; gift from Dr. Joseph Bryan at Baylor College of Medicine, Houston, TX, USA) were subcloned into mammalian expression vectors pIRES-EGFP (Clontech, Mountain View, CA, USA) and pcDNA3 (Invitrogen, Carlsbad, CA, USA), respectively. The plasmids to be used for transient transfection were prepared with Qiagen maxipreps and verified by DNA sequencing (Qiagen, Valencia, CA, USA).

Mammalian cell culture and transient transfection

The HEK293 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium DMEM/F12 (Mediatech, Herndon, VA, USA; supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin) at 37°C in humidified air supplemented with 5% CO_2. Cells were transiently transfected with expression plasmids containing cDNAs of interest using a modified calcium phosphate–DNA coprecipitation method (Chen & Okayama, 1987; Jordan *et al.* 1996). Positive transfection was marked by cistronic EGFP expression provided by the vector pIRES-EGFP. The cells were replated the following day at a density of 5000–20,000 cells per dish onto 12 mm glass coverslips precoated with fibronectin (~0.5 μg per coverslip, or 0.5 $\mu g\ cm^{-2}$; Sigma-Aldrich, St Louis, MO, USA) to be recorded 48–72 h after transfection as previously described (Lin *et al.* 2000).

Isolation of ventricular cardiomyocytes

Rabbits. Left ventricular myocytes were enzymatically isolated from adult New Zealand White rabbits as described before (Chai *et al.* 2011). Rabbits were deeply anaesthetized by intravenous injection of pentobarbital sodium (80–100 mg kg^{-1}). Hearts were excised and quickly placed on a Langendorff apparatus and perfused retrogradely for 5–7 min with nominally Ca^{2+}-free Dulbecco's minimal essential medium solution. Perfusion was then switched to the same solution containing 1 mg ml^{-1} collagenase with up to 0.1 mg ml^{-1} neutral protease. Once the heart became flaccid (~15–30 min), the ventricles were dispersed and filtered. The cell suspension was washed several times with medium containing ~150 μM Ca^{2+}.

Mice. CaMKIIδ-null mice (generated as reported previously; Ling *et al.* 2009) and their littermate/wild-type controls were anaesthetized with isoflurane at 3–5% in 100% oxygen via a Bickford veterinary vapourizer with a flow rate of 1–2 l min^{-1}, followed by decapitation. Hearts were excised, and myocytes were dissociated from ventricles by enzymatic treatment. Isolated ventricular myocytes were subsequently plated on 12 mm glass coverslips freshly coated with laminin (~1 μg per coverslip, or 1 $\mu g\ cm^{-2}$; Invitrogen, Carlsbad, CA, USA) to enhance cell adhesion. Rod-shaped cells with clear margin and striation were used for immediate recordings.

Electrodes, recording solutions and single-channel recordings

The recording electrodes were pulled from thin-walled borosilicate glass with an internal filament (MTW150F-3; World Precision Instruments, Sarasota, FL, USA) using a P-97 Flaming Brown puller (Sutter Instrument Co., Novato, CA, USA) and were firepolished to a resistance of 5–10 MΩ. Cell-attached single-channel recordings (Hamill *et al.* 1981) were performed using a recording chamber (RC26; Warner Instruments, Hamden, CT, USA) filled with the intracellular (bath) solution, and the recording pipette was filled with the extracellular solution. For HEK293 cells, the intracellular (bath) solution consisted of (mM): KCl, 110; $MgCl_2$, 1.44; KOH, 30; EGTA, 10; HEPES, 10; and sucrose, 30; pH adjusted to 7.2 with KOH. The extracellular (intrapipette) solution consisted of (mM): KCl, 140; $MgCl_2$, 1.2; $CaCl_2$, 2.6; and HEPES, 10; pH adjusted to 7.4 (with KOH). For cardiomyocytes, the intracellular (bath) solution consisted of (mM): KCl, 127; $MgCl_2$, 1; KOH, 13; EGTA, 5; HEPES, 10; and glucose, 10; pH adjusted to 7.2 (with KOH). The extracellular (intrapipette) solution consisted of (mM): KCl, 140; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 10; and glucose, 10; pH adjusted to 7.4 (with KOH). The use of symmetrical recording solutions (140 mM K^+) resulted in an equilibrium potential for potassium (E_K) and a resting membrane potential (V_m) around 0 mV, as determined from the *I–V* relationship of the K_{ATP} channel. All recordings were carried out at room temperature, and all patches were voltage clamped at −60 mV (i.e. with +60 mV intrapipette potentials) unless specified otherwise. Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Molecular Devices: Axon Instruments, Sunnyvale, CA, USA), low-pass filtered (3 dB, 2 kHz) and digitized at 20 kHz online using Clampex 9 software (Axon Instruments) via a 16 bit A/D converter (Digidata acquisition board 1322A; Axon Instruments).

Preparations of drugs

Working solutions of *N*-(2-deoxy-α,β-D-glucopyranose-2-)-N^2-acetyl-*S*-nitroso-D,L-penicillaminamide (glycol-SNAP-2), DETA NONOate (NOC-18), 1,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one (zaprinast), pinacidil, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), KT5823, *N*-(2-mercaptopro pionyl)glycine (MPG), 5-hydroxydecanoate (5-HD), fluphenazine-N-2-chloroethane (SKF-7171A), myristoylated autocamtide-2 related inhibitory peptide for CaMKII (mAIP), 1,4-diamino-2,3-dicyano-1,4-*bis* (2-aminophenylthio)butadiene (U0126) and 2′-amino-3′-methoxyflavone (PD98059) were diluted from aliquots with bath recording solutions prior to use. Stock solutions were prepared as follows: zaprinast, pinacidil, KT5823, ODQ, SKF-7171A, U0126 and PD98059 in DMSO; and glycol-SNAP-2, NOC-18, MPG, 5-HD and mAIP in H_2O; all were stored at −80°C in aliquots. Working solutions of catalase (human erythrocyte) and H_2O_2 were prepared directly from original stocks immediately before use. All working drug solutions were put on ice and kept away from light. Drugs were applied through a pressure-driven perfusion system (BPS-8; ALA Scientific Instruments, Westbury, NY, USA) to the recording chamber via a micromanifold positioned closely to the patches. Reagents and chemicals were purchased from EMD Millipore (Calbiochem, Billerica, MA, USA) or Sigma-Aldrich (St Louis, MO, USA). For pharmacological blockade, individual groups of cells were pretreated with respective inhibitors (except catalase) at room temperature for at least 15 min before being subjected to functional assays.

Electrophysiological data analysis

Data were analysed as described before (Lin *et al.* 2000, 2004; Mao *et al.* 2007; Chai & Lin, 2008, 2010; Lin & Chai, 2008; Chai *et al.* 2011), using individual data files of 120 s durations.

Single-channel currents. Individual, digitized single-channel records of 120 s duration (gap-free) were detected with Fetchan 6.05 (events list) of pCLAMP (Axon Instruments) using the 50% threshold crossing criterion and analysed with Intrv5 (gift from Dr. Barry S. Pallotta, formerly at University of North Carolina, Chapel Hill, NC, USA, and Dr. Janet Fisher at University of South Carolina, Columbia, SC, USA). Analysis was performed at the main conductance level (approximately 70–80 pS) for K_{ATP} channels. Only patches with infrequent multiple-channel activity were used for single-channel analysis. Duration histograms were constructed as described by Sigworth & Sine (1987), and estimates of exponential areas and time constants were obtained using the method of maximal likelihood estimation. The number of exponential functions required to fit the duration distribution was determined by fitting increasing numbers of functions until additional components could not significantly improve the fit (Horn, 1987; McManus & Magleby, 1988). Events with duration less than 1.5 times the system dead time were not included in the fit. Mean durations were corrected for missed events by taking the sum of the relative area (a) of each exponential component in the duration frequency histogram multiplied by the time constant (τ) of the corresponding component. Each of the single-channel properties was then normalized to the corresponding controls obtained in individual patches (taken as one).

Multiple-channel currents. In patches where multiple-channel activities of K_{ATP} channels were observed for more than 10% of the recording time, the digitized current records were analysed using Fetchan 6.05 (browse) of pCLAMP to integrate currents in 120 s segments. The current amplitude (I) values (current amplitude = integrated current/acquisition time) were then normalized to the corresponding controls obtained from the same patches to yield normalized open probability (NP_o; control value taken as one), because the normalized current amplitude is equivalent to the normalized NP_o obtained from single-channel analysis when the single-channel conductance remains the same (Mao *et al.* 2007). The normalized NP_o values obtained from both single-channel and multiple-channel patches were then pooled. In Fig. 1 and all other figures illustrating raw single-channel current records, representative traces (taken from individual 120 s files used for data analysis) with segments marked with a horizontal bar on top are displayed at increasing temporal resolution in successive traces (arranged from top to bottom).

CaMKII activity assay

Isolated rabbit ventricular myocytes were treated with NOC-18 (300 μM; chemical NO donor) or zaprinast (50 μM; selective inhibitor of cGMP-specific phosphodiesterases V and IX, capable of activating PKG) in the absence and presence of KT5823 (1 μM; selective PKG inhibitor) or U0126 [10 μM; selective mitogen-activated protein kinase kinase or MAPK kinase (MEK) inhibitor] for up to 30 min at room temperature. Immediately after the treatment, myocytes were homogenized using sonication in an ice-cold lysis buffer containing 50 mM HEPES, pH 7.5, 2 mg ml^{-1} bovine serum albumin, 5 mM EDTA and phosphatase inhibitor cocktail. CaMKII activity assays were then performed on fresh lysates as previously described (Wu *et al.* 2002; Erickson *et al.* 2008). Briefly, CaMKII activity was measured as a function of ^{32}P-ATP incorporation into a synthetic substrate, syntide-2, by scintillation counter. Assays were performed at 30°C. Background measurements lacking syntide-2 were subtracted from experimental values. Kinase activity is expressed relative to baseline radiation from samples containing no cellular lysate. Each experiment was done in triplicate and repeated three times, unless otherwise noted.

Western blot analysis

Rabbit ventricular myocytes were treated and lysed as described under CaMKII activity assay (above). Immunoblotting for total and T287 phosphorylated CaMKII was performed via standard protocols. Equal amounts of protein were loaded and electrophoresed on 10% SDS–polyacrylamide gel before being transferred to a polyvinylidene difluoride membrane. Total CaMKII antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:1000 dilution. Phospho-specific CaMKII antibody (Affinity Bioreagents, Golden, CO, USA) was used at 1:1500 dilution. After incubation with the horseradish peroxidase-labelled secondary antibody, blots were developed using enhanced chemiluminescence (Pierce SuperSignal; Thermo Scientific, Rockford, IL, USA).

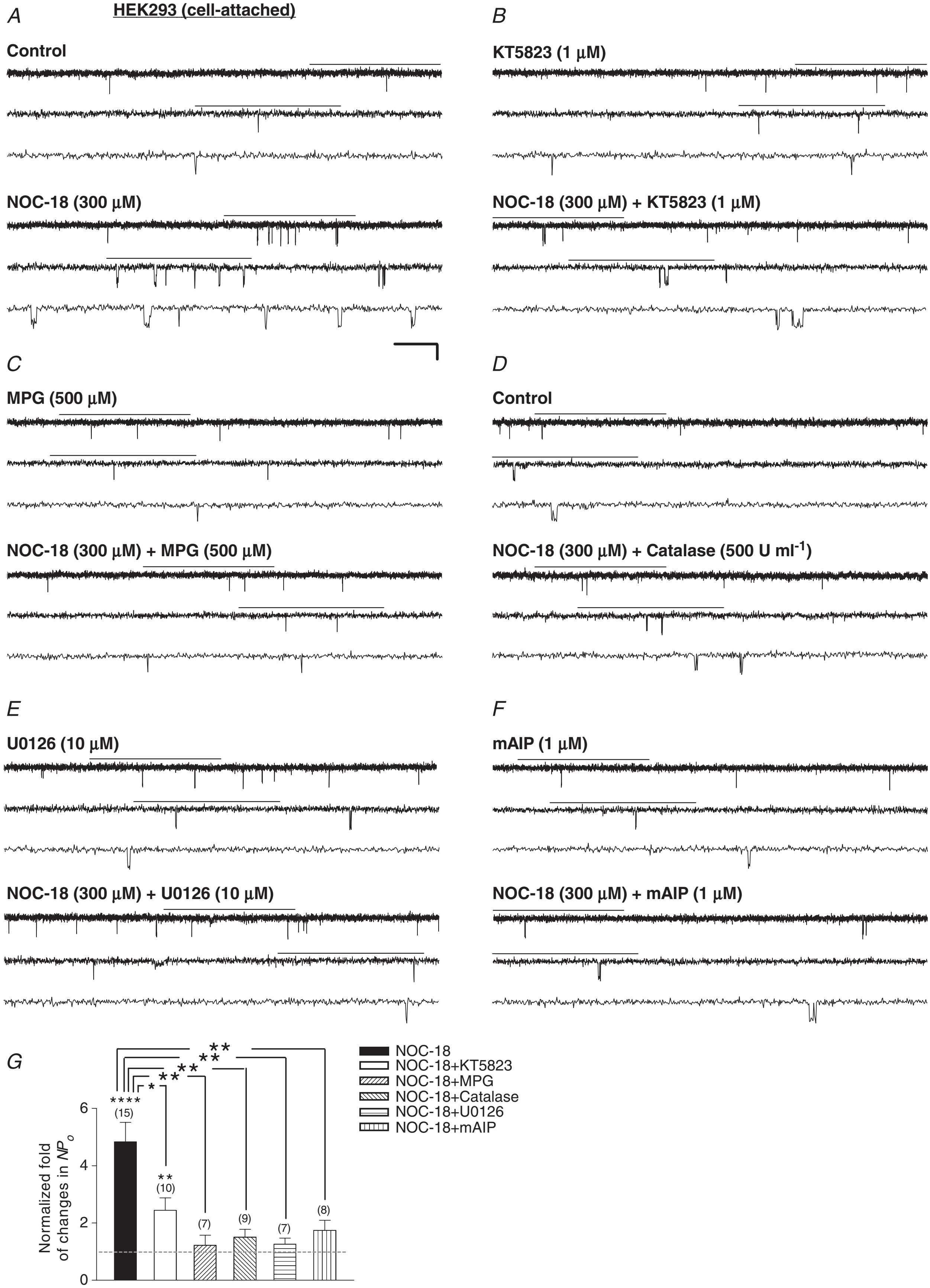
Statistics

Data are presented as means ± SEM. Statistical comparisons were made using Student's two-tailed one-sample, paired or unpaired t tests, or one-way ANOVA followed by Dunnett's multiple comparison tests to test the significance of difference in the following: normalized data (in response to treatment) in individual groups (Student's one-sample t tests); raw data pairs obtained before and during treatment in the same group (Student's paired t tests); normalized data between two separate groups (Student's unpaired t tests); or normalized data among multiple groups (one-way ANOVA followed by Dunnett's multiple comparison tests). Significance was assumed when $P < 0.05$. Statistical comparisons were performed using Prism (GraphPad Software, San Diego, CA, USA).

Results

Stimulation of Kir6.2/SUR2A channels by NO induction in intact HEK293 cells depends on PKG activation

To elucidate the underlying mechanism responsible for functional modulation of cardiac K_{ATP} channels by NO, we first examined how Kir6.2/SUR2A (i.e. ventricular-type K_{ATP}) channels transiently expressed in HEK293 cells respond to NO induction. Single-channel recordings were performed in the cell-attached patch configuration to preserve integrity of the intracellular milieu for potential signalling. Bath perfusion of NOC-18 (300 μM), an NO donor which spontaneously releases NO in aqueous solution, markedly enhanced the single-channel activity of Kir6.2/SUR2A channels (Fig. 1*A* shows a representative patch); the apparent opening frequency and the open duration were both increased, whereas the single-channel conductance remained the same. The averaged normalized NP_o (i.e. relative channel activity) was increased to 4.84 ± 0.68 (control taken as one; Fig. 1*G*, filled bar; $P < 0.0001$, Student's two-tailed, one-sample t test; $n = 15$). In contrast, although pretreatment with the selective PKG inhibitor KT5823 did not alter the basal activity of these channels (Fig. 1*A* and *B*), K_{ATP} channel stimulation evoked by NOC-18 was reduced by more than 50% in the presence of 1 μM KT5823 (following 15 min pretreatment; Fig. 1*B* and *G*, open bar; $P < 0.01$; $n = 10$), revealing significant attenuation of the NOC-18 effect by KT5823 (Fig. 1*G*, filled *vs.* open bars; $P < 0.05$, Dunnett's multiple comparison test following one-way



A
HEK293 (cell-attached)
Control
NOC-18 (300 μM)
B
KT5823 (1 μM)
NOC-18 (300 μM) + KT5823 (1 μM)
C
MPG (500 μM)
NOC-18 (300 μM) + MPG (500 μM)
D
Control
NOC-18 (300 μM) + Catalase (500 U ml-1)
E
U0126 (10 μM)
NOC-18 (300 μM) + U0126 (10 μM)
F
mAIP (1 μM)
NOC-18 (300 μM) + mAIP (1 μM)
G
Normalized fold of changes in NPo
NOC-18
NOC-18+KT5823
NOC-18+MPG
NOC-18+Catalase
NOC-18+U0126
NOC-18+mAIP
(15)
(10)
(7)
(9)
(7)
(8)

ANOVA). The specificity of KT5823 at 1 μM to selectively inhibit activation of PKG but not that of cAMP-dependent protein kinase (PKA) has been verified in our recent study (Chai & Lin, 2010). These data thus indicate that NOC-18 stimulated Kir6.2/SUR2A channels in intact HEK293 cells primarily via activation of PKG.

Effects of ROS scavengers and catalase on Kir6.2/SUR2A channel stimulation by NO induction

ROS are identified as important mediators in intracellular signalling (Dröge, 2002; Finkel, 2011). The NO donor *S*-nitroso-*N*-acetyl penicillamine (SNAP) has been shown to induce ROS generation in isolated rat cardiomyocytes (Xu *et al.* 2004). Are ROS involved in cardiac K_{ATP} channel stimulation by NO? We evaluated this possibility by examining whether ROS removal affects the action of NO donors on Kir6.2/SUR2A channels. Following pretreatment for at least 15 min, MPG (500 μM; an ROS scavenger) was applied together with NOC-18 (300 μM) to cell-attached patches obtained from transfected HEK293 cells. Coapplication of NOC-18 and MPG did not alter the single-channel currents of Kir6.2/SUR2A channels (Fig. 1*C* and *G*, third bar from left), in sharp contrast to the increase rendered by NOC-18 when applied alone (Fig. 1*G*, filled *vs.* third bars; $P < 0.01$). We also examined the effect of the H_2O_2-decomposing enzyme catalase on NO donor-induced channel stimulation. H_2O_2 is a relatively stable form of ROS, an attractive candidate for cell signalling (Scherz-Shouval & Elazar, 2007). In the presence of catalase (500 U ml^{-1}), which provides a sink for endogenously generated H_2O_2, NOC-18 (300 μM) failed to elevate Kir6.2/SUR2A channel activity (Fig. 1*D* and *G*, fourth bar from left), showing nearly complete blockade of the NOC-18 effect (Fig. 1*G*, filled *vs.* fourth bars; $P < 0.01$). These data indicate that ROS, and especially H_2O_2, were indispensible signals for NO stimulation of cardiac-type K_{ATP} channels in intact HEK293 cells.

Inhibition of ERK1/2 abrogates Kir6.2/SUR2A channel stimulation by NO induction

ERK1/2, a member of the MAPK family, is ubiquitously expressed and has many diverse cellular and physiological functions (Rose *et al.* 2010). ERK1/2 may be activated by H_2O_2 (Nishida *et al.* 2000). We showed above that NO stimulation of Kir6.2/SUR2A channels required ROS/H_2O_2; however, little is known about whether ERK plays a signalling role in acute NO modulation of ion channel function. To address this question, following pretreatment with U0126, which blocks activation of ERK1/2 through selectively inhibiting MEK1 and MEK2, cell-attached recordings were conducted in the continuous presence of U0126. Intriguingly, we found that NOC-18 (300 μM) was incapable of facilitating Kir6.2/SUR2A channel opening when U0126 (10 μM) was coadministered (Fig. 1*E* and *G*, fifth bar from left); that is, the increase in the normalized NP_o by NOC-18 was abrogated by blocking ERK1/2 activation (Fig. 1*G*, filled *vs.* fifth bars; $P < 0.01$). These data indicate that ERK1/2, presumably activated downstream of ROS, was required for NO stimulation of cardiac-type K_{ATP} channels.

Effect of CaMKII inhibitory peptides on NO stimulation of Kir6.2/SUR2A channels

Calcium/calmodulin-dependent kinases (CaMKs) influence processes as diverse as gene transcription, cell survival, apoptosis, cytoskeletal reorganization and learning and memory. CaMKII is the CaMK isoform predominantly found in the heart (Maier, 2009). Nevertheless, the potential involvement of CaMKII in NO signalling for cardiac K_{ATP} channel modulation has never been investigated. In this set of experiments, we tested whether blocking CaMKII activation with mAIP (1 μM), a myristoylated autocamtide-2 related inhibitory peptide for CaMKII, interferes with Kir6.2/SUR2A channel

Figure 1. Stimulation of Kir6.2/SUR2A channels by NO induction in transfected HEK293 cells requires activities of cGMP-dependent protein kinase (PKG), reactive oxygen species (ROS), H_2O_2, extracellular signal-regulated kinase (ERK1/2) and calcium/calmodulin-dependent protein kinase II (CaMKII)
A–*F*, representative single-channel current traces of Kir6.2/SUR2A obtained from cell-attached patches before (upper panel of traces) and during (lower panel of traces) application of DETA NONOate (NOC-18, 300 μM; *A*) or NOC-18 plus one of the following: the KT5823 (1 μM; *B*); *N*-(2-mercaptopropionyl)glycine (MPG, 500 μM; *C*); catalase (500 U ml^{-1}; *D*); U0126 (10 μM; *E*); or myristoylated autocamtide-2 related inhibitory peptide selective for CaMKII (mAIP, 1 μM; *F*), showing that the NO donor NOC-18 increases recombinant Kir6.2/SUR2A channel activity in intact HEK293 cells, whereas the increase induced by NOC-18 is abated when PKG, ROS, H_2O_2, ERK1/2 or CaMKII is selectively inhibited. Patches were voltage clamped at −60 mV. Downward deflections represent openings from closed states. Segments of current traces (taken from individual 120 s data files) marked with a horizontal line atop are displayed in successive traces at increasing temporal resolution. Horizontal scale bars represent 1 s, 300 ms and 100 ms (top to bottom in each three-trace group), and vertical scale bars represent 4 pA. *G*, averaged normalized open probability (NP_o) of Kir6.2/SUR2A channels obtained from individual groups (control taken as one, indicated by dashed line; mean ± SEM of 7–15 patches), demonstrating that the stimulatory effect of NOC-18 on the normalized NP_o (i.e. relative channel activity) of Kir6.2/SUR2A channels is dependent on PKG, ROS, H_2O_2, ERK1/2 and CaMKII. *$P < 0.05$, **$P < 0.01$ and ****$P < 0.0001$ (Student's two-tailed, one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups).

stimulation induced by NOC-18 (300 μM). Subsequent to 15 min pretreatment with mAIP, coapplication of NOC-18 and mATP resulted in no significant change in the activity of Kir6.2/SUR2A channels acquired in cell-attached patches (Fig. 1*F* and *G*, sixth bar from left), uncovering that mAIP nullified the stimulatory action of NOC-18 (Fig. 1*G*, filled *vs.* sixth bars; $P < 0.01$). These results thus indicate that NO modulation of Kir6.2/SUR2A channels in intact HEK293 cells relied on activation of CaMKII.

Effect of NO induction on sarcK$_{ATP}$ channels in intact rabbit ventricular myocytes: the dependence on sGC and PKG

To evaluate the physiological relevance of NO signalling in cardiac K$_{ATP}$ channel modulation, cell-attached recordings as performed on HEK293 cells were conducted on ventricular cardiomyocytes freshly isolated from adult rabbits. In these native cells, pinacidil (100–200 μM), a KCO, was applied first to induce baseline sarcK$_{ATP}$ channel activity comparable to that seen in transfected HEK293 cells. The NO donors glyco-SNAP-2 (300 μM; Fig. 2*A*) and NOC-18 (300 μM; Fig. 2*B*) were then added, and both evoked marked increases in the opening and bursting frequencies and the bursting duration of ventricular sarcK$_{ATP}$ channels; the normalized NP_o was raised to 8.29 $\pm$ 2.71 (control value in pinacidil taken as one; Fig. 2*E*, grey bar; $P < 0.05$) and 5.79 $\pm$ 1.51 (Fig. 2*E*, filled bar in black; $P < 0.01$), respectively, whereas the single-channel conductance remained unchanged. Moreover, to ensure that the stimulatory effect of NO induction on the normalized single-channel activity of rabbit ventricular sarcK$_{ATP}$ channels is not biased toward increases due to the low basal activity in the cell-attached patch configuration, the absolute NP_o (i.e. NP_o without normalization) values obtained in control and NOC-18-treated conditions were directly compared (Supplemental Fig. S1; a scatter plot). The averaged absolute NP_o values were significantly increased, manifesting a positive effect of NOC-18 (nine data pairs; $P < 0.05$); the shift in the median points (Supplemental Fig. S1, golden bars) was also consistent with an upward change caused by NOC-18. These results thus indicate that NO induction stimulated pinacidil-preactivated sarcK$_{ATP}$ channels in native ventricular cardiomyocytes, reinforcing our findings made on recombinant cardiac K$_{ATP}$ channels. By contrast, NOC-18 did not increase sarcK$_{ATP}$ channel activity in excised, inside-out patches (data not shown), excluding the possibility that the stimulation results from direct chemical modification of the channel by NO.

To identify signalling partners involved in NO modulation of the channel in native cardiomyocytes, we next examined whether NO modulation of ventricular sarcK$_{ATP}$ channels requires activation of sGC and PKG, by applying NOC-18 (300 μM) together with the selective sGC inhibitor ODQ (50 μM) or the PKG inhibitor KT5823 (1 μM), following pretreatment with respective inhibitors. The NOC-18 did not potentiate the single-channel activity of sarcK$_{ATP}$ channels preactivated by pinacidil in the presence of ODQ (Fig. 2*C* and *E*, open bar) or KT5823 (Fig. 2*D* and *E*, hatched bar), revealing annihilation of the stimulatory effect of NO donors (Fig. 2*E*, $P < 0.05$ *vs.* filled bar in black). These results indicate that NO induction was capable of enhancing the function of sarcK$_{ATP}$ channels in native ventricular cardiomyocytes and that the enhancement was sGC- and PKG-dependent.

Suppression of ERK1/2 activity obliterates sarcK$_{ATP}$ channel stimulation elicited by NO donors in intact ventricular cardiomyocytes

Our findings obtained from the cloned K$_{ATP}$ channel Kir6.2/SUR2A expressed in HEK293 cells (see Fig. 1) revealed, for the first time, that ERK1/2 was required for NO modulation of cardiac K$_{ATP}$ channels. To substantiate these findings in a native cell setting, cell-attached patch-clamp recordings were conducted on rabbit ventricular myocytes pretreated with the ERK1/2 inhibitor U0126. Application of NOC-18 (300 μM) in the continuous presence of U0126 (10 μM) failed to elevate pinacidil-preactivated sarcK$_{ATP}$ single-channel activity (Fig. 3*A* and *E*, open bar); the increase in the normalized NP_o induced by NOC-18 was completely abolished (Fig. 3*E*, filled *vs.* open bars; $P < 0.05$). Likewise, in ventricular myocytes pretreated with PD98059, another ERK1/2 inhibitor, NOC-18 was unable to stimulate sarcK$_{ATP}$ channels when PD98059 (20 μM) was coapplied (Fig. 3*B* and *E*, third bar from left; $P < 0.05$ *vs.* filled bar). These data consistently supported our hypothesis that activation of ERK1/2 mediates NO stimulation of sarcK$_{ATP}$ channels in ventricular myocytes.

Effects of antagonizing calmodulin and CaMKII on ventricular sarcK$_{ATP}$ channel stimulation caused by NO donors

To define the roles played by calmodulin (a ubiquitous calcium-binding protein) and CaMKII (activation of which depends on Ca^{2+}/calmodulin binding) for sarcK$_{ATP}$ channel stimulation elicited by NO in ventricular cardiomyocytes, SKF-7171A, a selective calmodulin antagonist, and mAIP, the membrane-permeable inhibitory peptide for CaMKII, were respectively coapplied with NOC-18

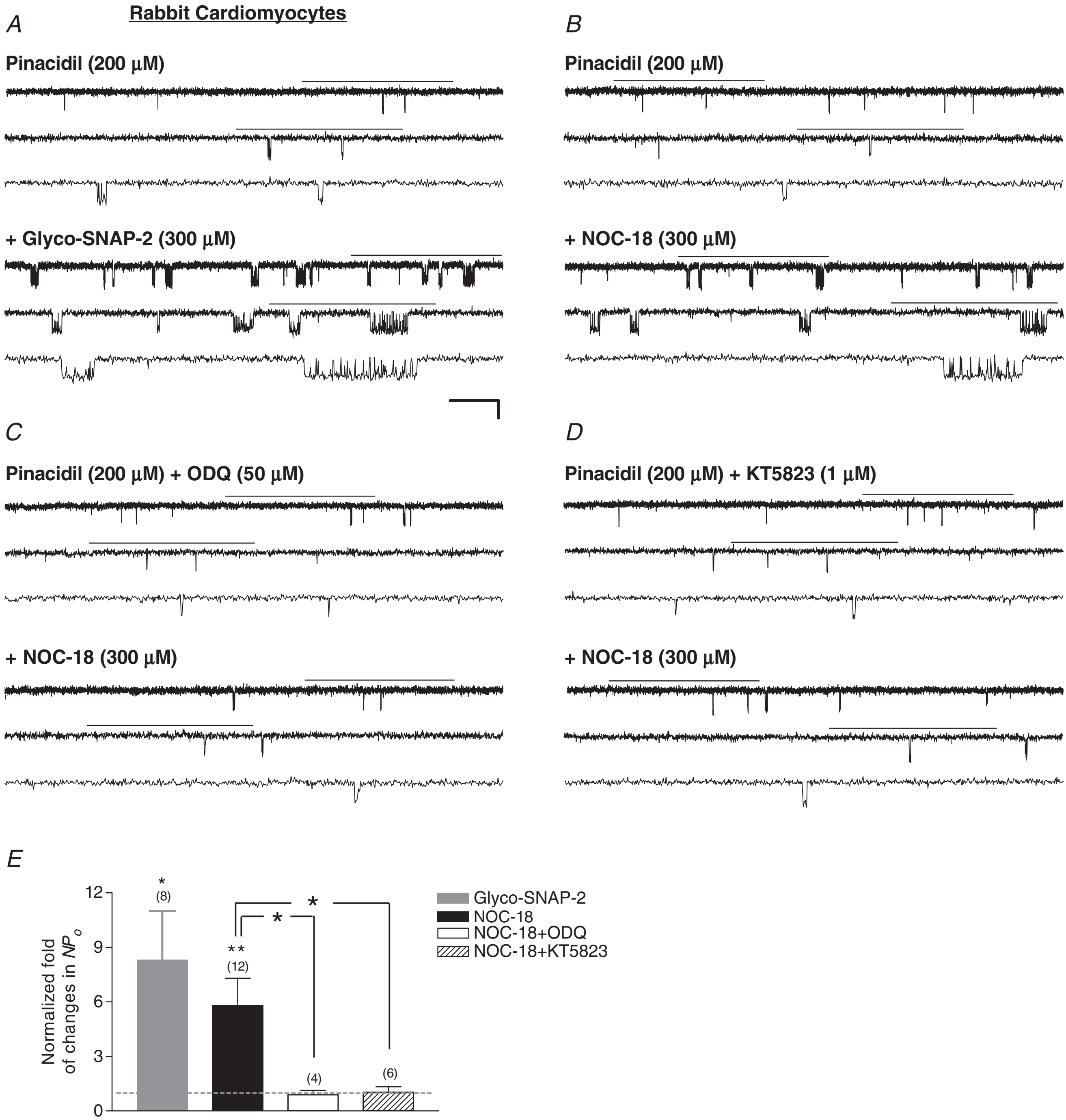


Figure 2. NO induction potentiates sarcolemmal K_{ATP} ($sarcK_{ATP}$) channel activity in intact adult rabbit ventricular cardiomyocytes in a soluble guanylate cyclase (sGC)- and PKG-dependent manner
A–D, representative single-channel current traces of ventricular $sarcK_{ATP}$ channels induced by pinacidil (200 μM) in cell-attached patches obtained from rabbit cardiomyocytes before and during addition of glycol-SNAP-2 (300 μM; *A*), NOC-18 (300 μM; *B*), or NOC-18 plus 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 50 μM; *C*) or KT5823 (1 μM; *D*), illustrating that NO donors enhance ventricular $sarcK_{ATP}$ channel activity but the enhancement is reversed in the presence of inhibitors selective for sGC or PKG. Recording settings and scale bars are the same as described in the legend to Fig. 1. *E*, averaged, normalized NP_o in individual groups of cell-attached patches ($n = 4$–12), showing that the significant increase of $sarcK_{ATP}$ single-channel activity in intact ventricular cardiomyocytes induced by NO donors is abolished by inhibition of sGC or PKG. $^*P < 0.05$; $^{**}P < 0.01$ (Student's one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups).

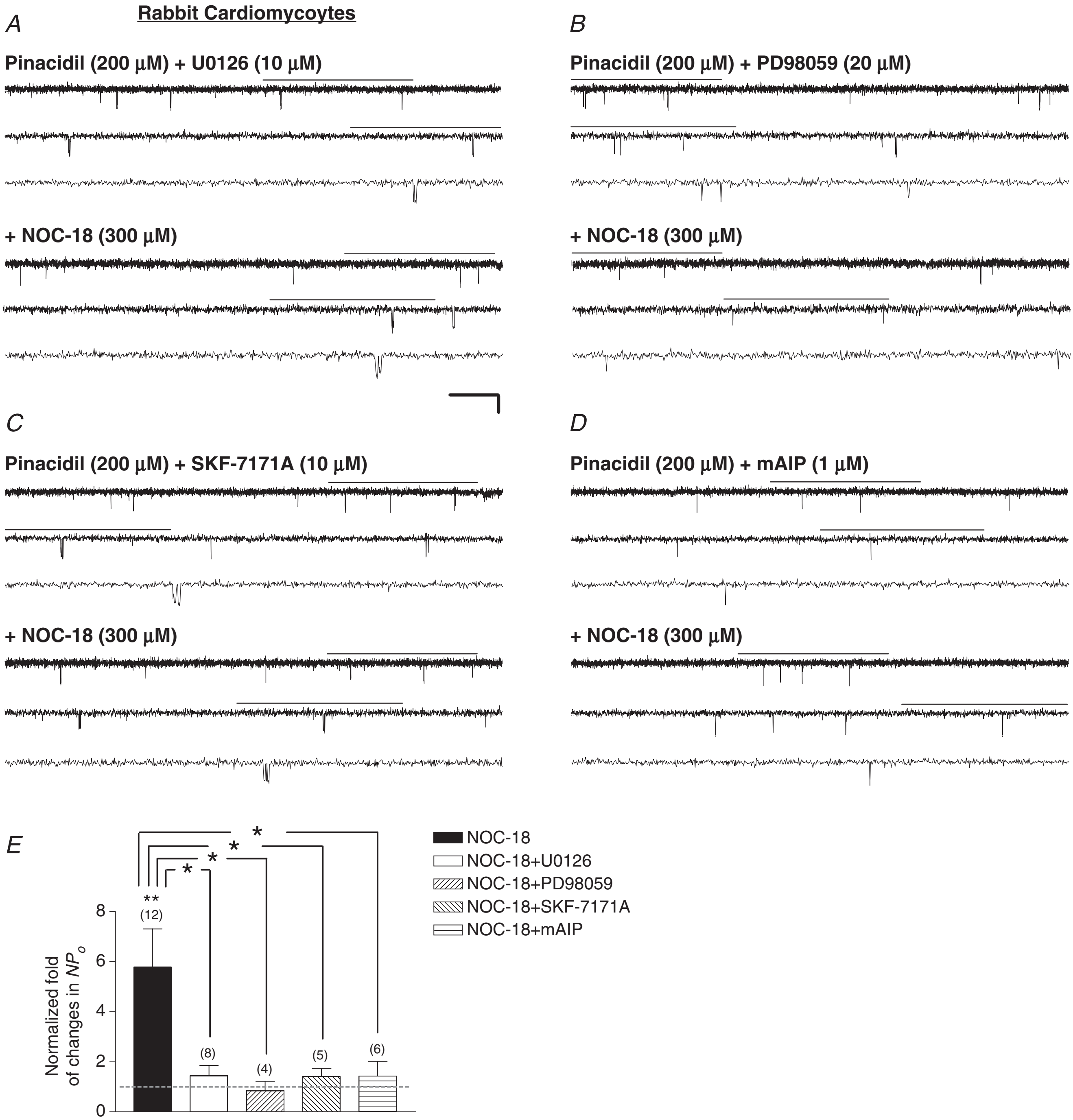


Figure 3. Activation of ERK1/2, calmodulin and CaMKII mediates NO stimulation of sarcK$_{ATP}$ channels in rabbit ventricular cardiomyocytes

A–D, representative single-channel current traces of pinacidil-preactivated sarcK$_{ATP}$ channels in cell-attached patches before and during addition of NOC-18 (300 μM) together with one of the following inhibitors: U0126 (10 μM; *A*); PD98059 (20 μM; *B*); SKF-7171A (10 μM; *C*); or mAIP (1 μM; *D*), illustrating that the stimulatory effect of NOC-18 on native ventricular sarcK$_{ATP}$ channels is nullified when ERK1/2, calmodulin or CaMKII activity is suppressed. See Fig. 1 for definition of scale bars. *E*, summary data of the averaged normalized NP_o obtained in individual groups of cell-attached patches ($n = 4$–12), demonstrating that stimulation of sarcK$_{ATP}$ channels by NO induction in intact ventricular cardiomyocytes requires activities of ERK1/2, calmodulin and CaMKII. The NOC-18 group data, the same as those shown in Fig. 2, are included here for comparison purposes. $^*P < 0.05$; $^{**}P < 0.01$ (Student's one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups).

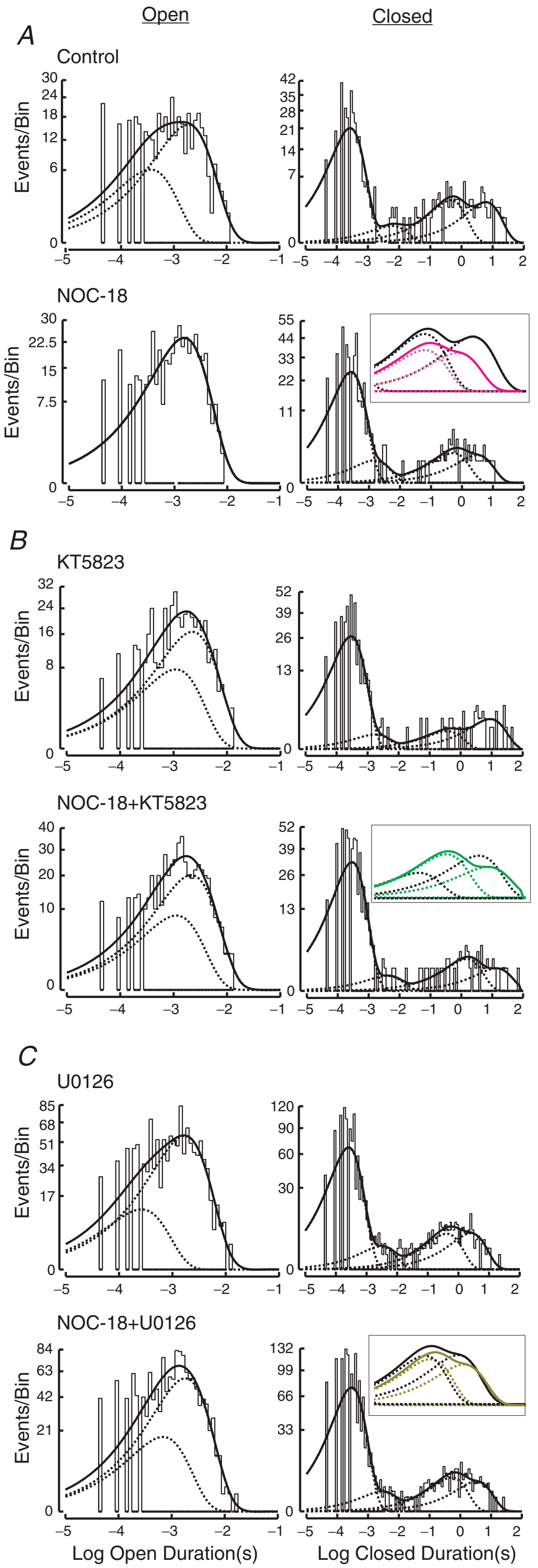
during cell-attached patch-clamp recordings (following pretreatment). When coapplied with SKF-7171A (10 μM; Fig. 3*C*) or mAIP (1 μM; Fig. 3*D*), NOC-18 (300 μM) did not enhance ventricular sarcK_{ATP} channel currents pre-activated by pinacidil (Fig. 3*E*, fourth and fifth bars from left), yielding significant abrogation of the stimulatory effect of NOC-18 (Fig. 3*E*; $P < 0.05$ *vs.* filled bar for both groups). In agreement with the findings made in HEK293 cells (see Fig. 1), these results indicate that the stimulatory action of NO induction on ventricular sarcK_{ATP} channels required activation of calmodulin and CaMKII.

Inhibition of ERK and CaMKII abolishes potentiation of sarcK_{ATP} channel activity rendered by exogenous H_2O_2 in ventricular cardiomyocytes

We showed in the preceding subsections that inhibition of ROS/H_2O_2, ERK and CaMKII could blunt functional stimulation of ventricular K_{ATP} channels induced by NO donors in intact cells, revealing the involvement of these molecules as intracellular signalling partners mediating K_{ATP} channel stimulation downstream of NO (induction). It is important to determine how ERK1/2 and CaMKII are positioned relative to ROS in the NO signalling pathway that enhances K_{ATP} channel function. To address this, we examined whether the ability of exogenous H_2O_2 to stimulate ventricular K_{ATP} channels in intact cells is affected by inhibition of ERK1/2 and CaMKII (Supplemental Fig. S2). The rationale is as follows. If H_2O_2 is generated endogenously after, and hence positioned downstream of, activation of ERK1/2 and CaMKII, the effectiveness of exogenous H_2O_2 to stimulate sarcK_{ATP} channels should not be compromised by suppression of either kinase. The same outcome is expected in the event that H_2O_2 modulates sarcK_{ATP} channels independently of these kinases. Conversely, if H_2O_2 stimulates sarcK_{ATP} channels via activation of ERK and/or CaMKII, the K_{ATP} channel-potentiating capability of exogenous H_2O_2 ought to be hampered by functional suppression of respective kinases. Interestingly, while application of H_2O_2 (1 mM) reliably enhanced sarcK_{ATP} single-channel activity pre-activated by pinacidil in cell-attached patches obtained from rabbit ventricular cardiomyocytes, H_2O_2 failed to elicit changes in K_{ATP} channel activity when the MEK1/2 inhibitor U0126 (10 μM) or the CaMKII inhibitory peptide mAIP (1 μM) was coapplied (Supplemental Fig. S2), revealing total abolition of the stimulatory action of H_2O_2 by inhibition of ERK1/2 and CaMKII ($P < 0.05$ *vs.* H_2O_2 applied without kinase inhibitors). These results indicate that both ERK1/2 and CaMKII were crucial for exogenous H_2O_2 to potentiate ventricular K_{ATP} channel activity successfully, hence placing ERK1/2 and CaMKII downstream of H_2O_2 for stimulation of K_{ATP} channels in intact ventricular cardiomyocyes.

Effects exerted by NO signalling on ventricular sarcK_{ATP} single-channel open and closed properties

Our foregoing data indicate that NO donors enhanced the activity of ventricular K_{ATP} channels via intracellular signalling. To delineate whether NO signalling affects the gating (i.e. opening and closing) of ventricular sarcK_{ATP} channels, we analysed K_{ATP} single-channel activity to determine whether the NO donor NOC-18 causes more frequent entry into the open state (i.e. increases the opening frequency), prolongs stay in the open state (i.e. increases the open time constant of certain open state), decreases dwelling time in the closed states (i.e. decreases the closed time constant of certain closed state), stabilizes or destabilizes the occurrence of a particular state (i.e. shifts the relative distribution among states) or induces any combination of the above. The fitting results revealed that in the control condition, the open- and closed-duration distributions of rabbit ventricular sarcK_{ATP} channels in the cell-attached patch configuration could be described best by a sum of two open components and a sum of four closed components, respectively (Fig. 4*A*, control; a representative patch), implying that there are at least two open states and four closed states. Moreover, NOC-18 treatment altered the closed duration distribution (Fig. 4*A*, closed; top *vs.* bottom panels); the relative areas and/or the time constants under the longer and longest closed states were reduced [Fig. 4*A*, inset; magenta colour (depicting NOC-18-treated condition) *vs.* black (depicting control)], while the shorter closed states were stabilized, resulting in shortening of the mean closed duration to 231.1 from 734.3 ms in this representative patch. Indeed, pooled data showed that NOC-18 decreased the normalized mean closed duration (control taken as one; 0.31 ± 0.07; $P < 0.0001$; $n = 7$) and increased the normalized opening frequency (5.10 ± 1.60; $P < 0.05$), thereby elevating the normalized NP_o (i.e. relative channel activity; see Figs. 1*G* and 2*E*). Meanwhile, the longer open state became increasingly prominent in the presence of NOC-18 (Fig. 4*A*, open; top *vs.* bottom panels), although neither the corrected mean open duration (1.65 ms in control condition *vs.* 1.75 ms in NOC-18) obtained from this patch nor the normalized mean open duration averaged from the whole group (control taken as one; 1.16 ± 0.15; $n = 7$) was significantly increased. By contrast, NOC-18 failed to evoke similar changes in the opening frequency (data not shown) and the open and closed duration distributions of ventricular sarcK_{ATP} channels when the PKG inhibitor KT5823 (Fig. 4*B*), the ERK1/2 inhibitor U0126 (Fig. 4*C*) or the CaMKII inhibitory peptide mAIP (data not shown) was coadministered, explicating the



absence of NOC-18-induced increases in NP_o in these conditions (see Figs. 1*G*, 2*E* and 3*E*). Our findings thus indicate that NO induction potentiated ventricular K_{ATP} channel activity by shortening and destabilizing long closures, whilst increasing the opening transitions of the channel, in a PKG-, ERK1/2- and CaMKII-dependent manner.

Genetic ablation of CaMKIIδ prevents PKG stimulation of ventricular sarcK_{ATP} channels

The predominant CaMKII isoform in the heart is CaMKIIδ (Tobimatsu & Fujisawa, 1989). To evaluate the role of CaMKIIδ in mediating cGMP/PKG stimulation of cardiac K_{ATP} channels, a CaMKIIδ-null mouse model (plus littermate controls) was employed. Application of the PKG activator zaprinast (50 μM) to cell-attached patches obtained from wild-type mouse ventricular myocytes significantly enhanced the activity of sarcK_{ATP} channels preactivated by pinacidil (Fig. 5*A* and *C*, filled bar; normalized $NP_o = 4.57 \pm 1.29$; $P < 0.05$); however, this stimulatory effect was absent in CaMKIIδ-null cardiomyocytes (Fig. 5*B* and *C*, open bar); that is, genetic ablation of CaMKIIδ prevented ventricular sarcK_{ATP} channel stimulation caused by activation of PKG (Fig. 5*C*, filled *vs.* open bars; $P < 0.01$). These results indicate that CaMKIIδ was required for functional enhancement of ventricular sarcK_{ATP} channels elicited by PKG activation in intact cells, unveiling a previously unrecognized role played by CaMKIIδ. As activation of PKG represented a key step linking NO induction to functional enhancement of cardiac K_{ATP} channels (see Figs. 1 and 2), the findings obtained from CaMKIIδ-null ventricular cardiomyocytes thus lend additional support to our hypothesis that CaMKII, especially CaMKIIδ, is indispensable in the NO–PKG signalling cascade for functional modulation of myocardial K_{ATP} channels.

Figure 4. NO signalling alters the open- and closed-duration distributions of ventricular sarcK_{ATP} channels
A–C, frequency histograms of open-duration and closed-duration distributions fitted from events detected before (upper panels) and during (lower panels) application of NOC-18 (300 μM; *A*), NOC-18 plus KT5823 (1 μM; *B*) or NOC-18 plus U0126 (10 μM; *C*) in representative cell-attached patches obtained from rabbit ventricular myocytes. Insets show superimposed curve fittings of duration distributions of the two longer closed components in control (black) *versus* treatment conditions (colours) to highlight NOC-18 effects. The NOC-18 left-shifts the longest closed component and reduces the relative areas under longer/longest closed components, effectuating destabilization of the longer/longest closed components. By contrast, inhibition of PKG (with KT5823) or ERK1/2 (with U0126) prevents these changes induced by NOC-18 from occurring, which demonstrates that the NO donor effects on duration distributions are mediated by intracellular signalling through activation of PKG and ERK1/2.

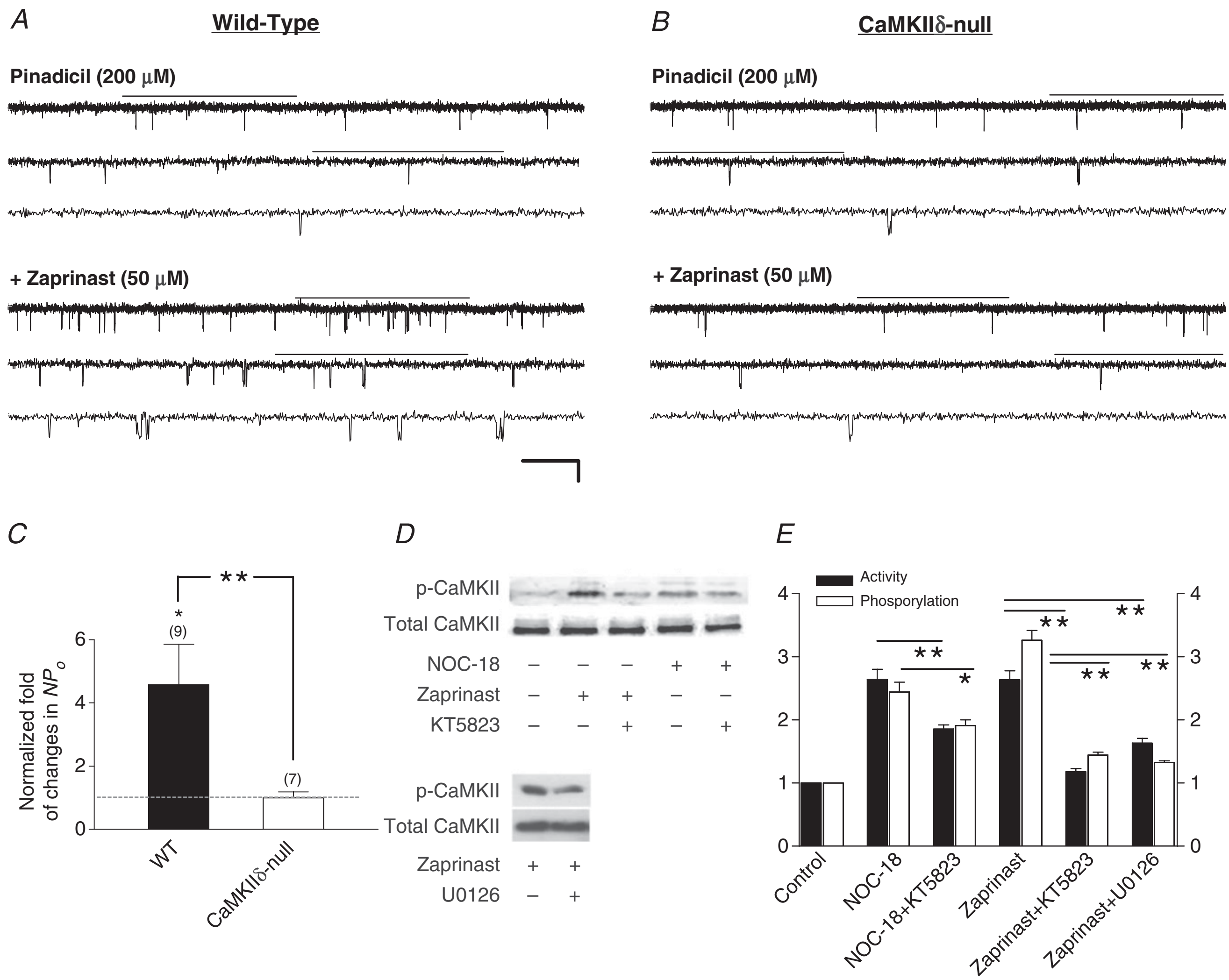


Figure 5. Role of CaMKII in NO/PKG signalling: genetic ablation of CaMKIIδ abolishes PKG stimulation of ventricular sarcK_{ATP} channels, whilst CaMKII activity is increased by NO–PKG activation in an ERK1/2-dependent manner

A–C, electrophysiological analysis of sarcK_{ATP} channel activity in response to PKG activation in intact ventricular myocytes isolated from CaMKIIδ-null *versus* littermate/wild-type (WT) mice, showing that genetic ablation of CaMKIIδ obliterates PKG stimulation of ventricular sarcK_{ATP} channels. Representative single-channel current traces of pinacidil-preactivated sarcK_{ATP} channels in response to addition of zaprinast (50 μM; PKG activator) in cell-attached patches obtained from the wild-type (*A*) and CaMKIIδ-null mouse ventricular myocytes (*B*) illustrate that potentiation of pinacidil-preactivated ventricular sarcK_{ATP} single-channel activity by zaprinast is obliterated in CaMKIIδ-null mouse cardiomyocytes. Recording settings and scale bars are the same as described in Fig. 1. Summary data (*C*) obtained from individual groups demonstrate that, compared with wild-type counterparts, the increase in the averaged normalized NP_o (control taken as one; dashed line) by PKG activation is diminished in CaMKIIδ-null ventricular myocytes ($n = 7$–9). *$P < 0.05$; **$P < 0.01$ (Student's one-sample *t* test within groups, and unpaired *t* test between groups). *D* and *E*, biochemical analysis of CaMKII activity, showing that the activity of CaMKII in intact rabbit ventricular myocytes is increased by NO–PKG activation in an ERK1/2-dependent manner. Cardiomyocytes were treated with NOC-18 (300 μM) or zaprinast (50 μM) in the absence and presence of KT5823 (1 μM) or U0126 (10 μM) for 30 min, followed by preparation of cell lysates. The CaMKII activity was then assayed by Western blotting of phospho-CaMKII (p-CaMKII) relative to total CaMKII and by estimating ^{32}P incorporation of a synthetic CaMKII substrate. Representative Western blots (*D*) and the mean densitometric values of relative CaMKII activity (*E*) estimated by ^{32}P incorporation (filled bars) and by Western blots (p-CaMKII relative to total CaMKII values; open bars; $n = 3$) reveal that CaMKII activity in cardiomyocytes is elevated by NO induction and PKG activation, but the increase is attenuated when PKG or ERK1/2 activity is inhibited. Values are means ± SEM of three experiments of independent cell preparations. The kinase activity assay was conducted in triplicate each time. *$P < 0.05$; **$P < 0.01$ (Student's one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups).

Effects of NO induction and PKG activation on CaMKII activity in ventricular myocytes: involvement of ERK1/2

To seek direct evidence for CaMKII activation by NO and PKG in intact cells, two independent biochemical assays, Western blotting that measures autophosphorylation of CaMKII at T287 (p-CaMKII) and a kinase activity assay that detects ^{32}P-ATP incorporation into syntide-2, a synthetic substrate for CaMKII, were conducted. Isolated adult rabbit ventricular myocytes were treated with the NO donor NOC-18 (300 μM) and the PKG activator zaprinast (50 μM), respectively, for 30 min in the absence and presence of KT5823 (1 μM; PKG inhibitor) or U0126 (10 μM; ERK1/2 inhibitor), followed by preparation of cell lysates for subsequent assays to estimate CaMKII activity. Western blotting assays revealed that both zaprinast and NOC-18 elevated the p-CaMKII level (relative to total CaMKII; Fig. 5*D*, upper panel, lanes 2 and 4 from left; Fig. 5*E*, open bars; $P < 0.01$, Student's two-tailed, one sample *t* test; control taken as one); however, these increases were attenuated by KT5823 (Fig. 5*D*, upper panel, lanes 3 and 5 from left; Fig. 5*E*, open bars; $P < 0.01$ for NOC-18 *vs.* NOC-18 + KT5823 and $P < 0.05$ for zaprinast *vs.* zaprinast + KT5823, Dunnett's multiple comparison test following one-way ANOVA) and by U0126 (Fig. 5*D*, lower panel; Fig. 5*E*, $P < 0.01$ for zaprinast *vs.* zaprinast + U0126). In accordance with Western blot data, CaMKII activity measured by ^{32}P-ATP incorporation was also increased by NOC-18 and by zaprinast (Fig. 5*E*, filled bars; three independent runs of triplicates each time; $P < 0.01$ for both treatment groups), and the changes were significantly abated when KT5823 or U0126 was coadministered (Fig. 5*E*, filled bars; $P < 0.01$ *vs.* NOC-18 or zaprinast administered alone). These results indicate that CaMKII was activated by NO–PKG signal transduction in ventricular cardiomyocytes; additionally, the ERK1/2 dependence of CaMKII activation implies that ERK1/2 is likely to be positioned upstream of CaMKII in the signalling cascade triggered by NO–PKG.

Discussion

sGC and PKG are required for NO stimulation of cardiac K_{ATP} channels

NO represents one of the most important defenses against myocardial ischaemia–reperfusion injury (Jones & Bolli, 2006); meanwhile, the K_{ATP} channel has been regarded as mandatory in acute and chronic cardiac adaptation to imposed haemodynamic load, protecting against congestive heart failure and death (Yamada *et al.* 2006). NO may potentiate the action of KCOs on K_{ATP} channels in ventricular cardiomyocytes (Shinbo & Iijima, 1997; Han *et al.* 2002) and activate sarcK_{ATP} channels in normoxic and chronically hypoxic hearts (Baker *et al.* 2001). However, little is known about the intracellular mechanism responsible for NO modulation of cardiac K_{ATP} channels. In the present study, we showed that induction of NO by chemical donors resulted in increases in Kir6.2/SUR2A (i.e. recombinant cardiac-type K_{ATP}) and KCO-induced native sarcK_{ATP} single-channel activities in cell-attached patches obtained from intact HEK293 cells and ventricular cardiomyocytes, respectively. Moreover, the stimulatory action of NO donors was attenuated or abolished by selective inhibition of sGC and PKG, suggesting that NO induction enhances the function of cardiac K_{ATP} channels in intact cells via activation of sGC and PKG. In contrast to a K_{ATP}-potentiating effect observed in intact cells, NO donors did not increase ventricular sarcK_{ATP} channel activity in excised, inside-out patches (data not shown), which is consistent with a working model that NO modulates K_{ATP} channel function via intracellular signalling rather than direct chemical modification of the channel.

ROS, in particular H_2O_2, act as intermediate signals in NO-induced stimulation of cardiac K_{ATP} channels

ROS are generated by all aerobic cells, and most endogenously produced ROS are derived from mitochondrial respiration (Liu *et al.* 2002). They have been shown to contribute to cardioprotection afforded by ischaemic preconditioning (Baines *et al.* 1997). Among all ROS, H_2O_2 is an attractive candidate for cell signalling, because it is relatively stable and long lived and its neutral ionic state allows it to exit the mitochondria easily (Scherz-Shouval & Elazar, 2007). In the present study, increases in Kir6.2/SUR2A channel activity rendered by NO donors in intact HEK293 cells were aborted not only by the ROS scavenger MPG but also by the H_2O_2-decomposing enzyme catalase. These results suggest that ROS, and in particular H_2O_2, presumably produced downstream of PKG activation, mediate NO-induced stimulation of cardiac K_{ATP} channels in intact cells. In line with our findings that support an NO–PKG–ROS signalling model, the NO donor SNAP has been demonstrated to increase ROS generation in isolated cardiomyocytes, which, importantly, requires activation of PKG (Xu *et al.* 2004). It has also been shown that late and early preconditioning induced by NO donors is blocked by the ROS scavenger MPG, implying that ROS are involved in cardioprotection induced by (exogenous) NO (Takano *et al.* 1998; Nakano *et al.* 2000); in light of the present findings, protection by NO in the heart may involve ROS-dependent activation of myocardial sarcK_{ATP} channels.

In addition to ROS, an involvement of the putative mitochondrial K_{ATP} (mitoK_{ATP}) channel in mediating NO stimulation of cell-surface cardiac K_{ATP} channels was also investigated. Opening of mitoK_{ATP} channels has been suggested as a downstream event of PKG activation (Xu *et al.* 2004). Our findings indicate that 5-hydroxydecanoate (5-HD), the specific antagonist for the putative mitoK_{ATP} channel, significantly attenuated the increase in Kir6.2/SUR2A channel activity rendered by NOC-18 in intact HEK293 cells (Supplemental Fig. S3). The results thus suggest that the mitoK_{ATP} channel (or 'the 5-HD-sensitive factor'; see Chai & Lin, 2010), like ROS, is an intermediate signal crucial for mediating functional enhancement of cardiac K_{ATP} channels caused by NO. Activation of the mitoK_{ATP} channel and ROS generation may be sequential or parallel events induced by NO. However, because ROS scavengers in intact cells completely abolish the stimulatory effect on cardiac K_{ATP} channels rendered by NO induction (Fig. 1) and by activation of PKG (Chai *et al.* 2011), whereas the stimulatory effect of exogenous H_2O_2 on cell-surface K_{ATP} channels is unaffected by 5-HD treatment (Chai & Lin, 2010), it is conceivable that the mitoK_{ATP} channel or the 5-HD-sensitive factor is positioned upstream of, not in parallel to, ROS/H_2O_2 (generation) for K_{ATP} channel modulation in the NO–PKG signalling pathway. Collectively, these results support our working model (Fig. 6), where the putative mitoK_{ATP} channel mediates ROS generation induced by NO induction to stimulate cell-surface K_{ATP} channel activity. MitoK_{ATP} channels and ROS are implicated in the cardioprotective effect of ischaemic preconditioning (Vanden Hoek *et al.* 1998; Pain *et al.* 2000) and the anti-infarct effect of NO in intact, isolated heart (Xu *et al.* 2004). It is possible that NO exerts its cardiac protection by activating sarcK_{ATP} channels via a PKG–mitoK_{ATP}–ROS signalling mechanism.

ERK1/2 mediates NO- and H_2O_2-induced stimulation of cardiac K_{ATP} channels

ERKs play pivotal roles in many aspects of cell functions and are activated by oxidative stress in some types of cells (Aikawa *et al.* 1997; Nishida *et al.* 2000). Our present investigation revealed that increases in cardiac K_{ATP} single-channel activity induced by NO donors in both ventricular cardiomyocytes and transfected HEK293 cells were abolished by inhibition of MEK1 and MEK2 (both upstream kinases of ERK1/2) with U0126 or PD98059. These results thus suggest that, like ROS, ERK1/2 is a key

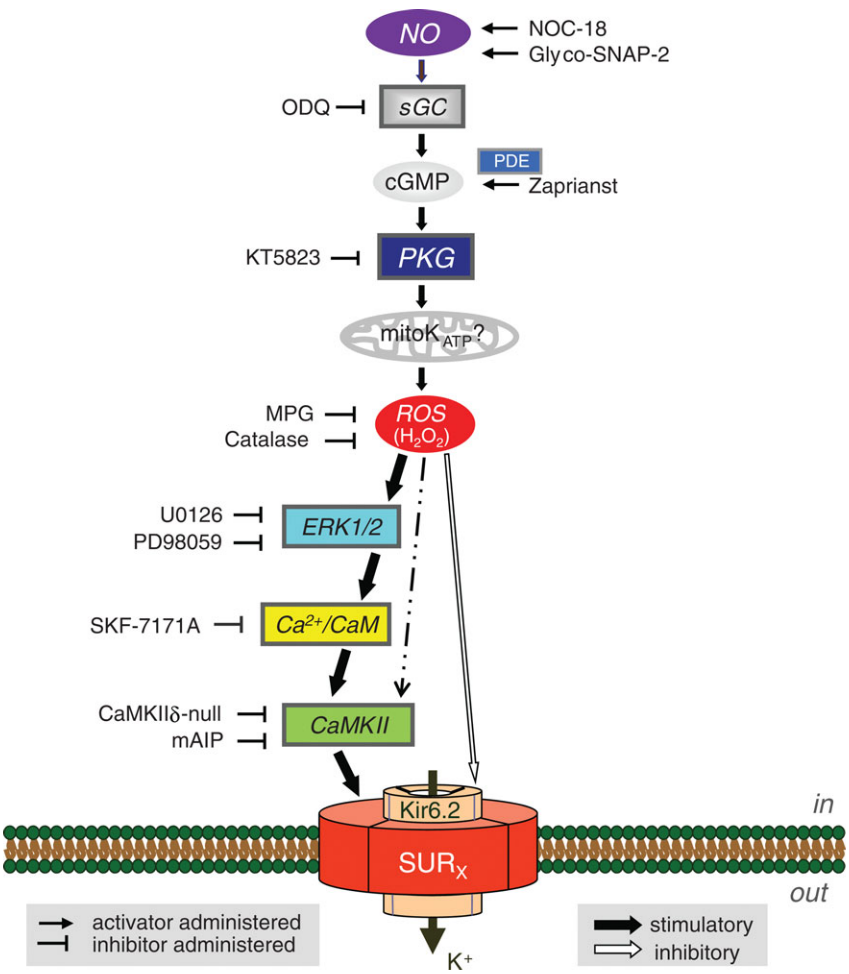


Figure 6. Working model of the NO signalling pathway for functional modulation of ventricular sarcK_{ATP} channels
Based on evidence obtained from the present study, we suggest that induction of NO leads to sGC activation and cGMP generation, which in turn activates PKG and triggers downstream signalling that consists of (in sequence) ROS, ERK1/2, calmodulin and CaMKII, resulting in sarcK_{ATP} channel stimulation. Signalling components involved are shown in rectangular or oval shapes (shaded); pharmacological reagents or genetic ablation employed in the present study targeting individual signalling components are also depicted, with inhibitory approaches positioned on the left and activators on the right.

relay signal evoked by NO to mediate cardiac K_{ATP} channel stimulation. But what is the relationship between ROS and ERK in the NO–K_{ATP} channel signalling pathway?

Most aspects of oxidant signalling have been linked to the more stable derivative, H_2O_2 (Finkel, 2003). It has been reported that in cardiac myocytes, ERKs are activated by H_2O_2 transiently and in a concentration-dependent manner (Aikawa *et al.* 1997). H_2O_2 may regulate K_{ATP} channel activity in ventricular cardiomyocytes (Goldhaber *et al.* 1989; Ichinari *et al.* 1996; Tokube *et al.* 1996). Befittingly, exogenous H_2O_2 enhances the single-channel activity of pinacidil-preactivated sarcK_{ATP} channels in a concentration-dependent manner in intact rabbit ventricular myocytes (Chai *et al.* 2011). In the present study, we found that the stimulatory action of exogenous H_2O_2 on sarcK_{ATP} channels in intact cardiomyocytes was abrogated when the ERK1/2 inhibitor U0126 was coapplied (Supplemental Fig. S2). These results suggest that ERK1/2 is positioned downstream of H_2O_2 to mediate H_2O_2-induced sarcK_{ATP} channel stimulation in ventricular cardiomyocytes. Complementing evidence presented in the foregoing subsections that ROS/H_2O_2 and ERK1/2 were required for NO stimulation of cardiac K_{ATP} channels, it is therefore conceivable that activation of ERK1/2 takes place following ROS generation in the NO–K_{ATP} channel signalling cascade. Indeed, this hypothesis is compatible with biochemical evidence demonstrated by Xu *et al.* (2004) using isolated cardiomyocytes that the NO donor SNAP enhances phosphorylation of ERK in a ROS scavenger-sensitive manner, which suggests phosphorylation/activation of ERK as the downstream signalling event of NO-induced ROS generation. Collectively, our data suggest that ROS/H_2O_2 activates ERK1/2 in the intracellular signalling cascade initiated by NO induction, leading to ventricular sarcK_{ATP} channel stimulation.

Calmodulin and CaMKII are indispensible for stimulation of cardiac K_{ATP} channels induced by NO and H_2O_2

CaMKII is one of the major regulators of Ca^{2+} homeostasis in the heart, phosphorylating cardiac contractile regulatory proteins and modulating the function of cardiac ion channels (Zhang *et al.* 2004; Wagner *et al.* 2009). Binding of Ca^{2+}/calmodulin activates CaMKII, by disinhibiting the autoregulatory domain of the kinase (Hudmon & Schulman, 2002). We showed in the present study that potentiation of pinacidil-preactivated sarcK_{ATP} channels by NO donors in ventricular cardiomyocytes was diminished by both mAIP, a cell-permeable, inhibitory peptide selective for CaMKII, and SKF-7171A, a potent and irreversible calmodulin antagonist; likewise, mAIP treatment abolished NO donor-induced stimulation of recombinant Kir6.2/SUR2A channels expressed in HEK293 cells. These results coherently suggest that NO induction enhances cardiac K_{ATP} channel function via activation of calmodulin and CaMKII. By contrast, application of CaMKII to excised, inside-out patches did not reproduce the positive action of NO donors on ventricular sarcK_{ATP} channel activity (data not shown); it thus seemed unlikely that direct CaMKII phosphorylation of the channel protein is responsible for NO potentiation of K_{ATP} channel function in intact cells. Additionally, we demonstrated that the increase in ventricular sarcK_{ATP} channel activity rendered by exogenous H_2O_2 was reversed by mAIP in intact cardiomyocytes (Supplemental Fig. S2), implying that activation of CaMKII mediates the stimulatory effect of exogenous H_2O_2. Taken together, these results suggest that CaMKII is positioned downstream of ROS/H_2O_2 in the NO signalling pathway to mediate functional enhancement of cardiac K_{ATP} channels.

On the other hand, activation of CaMKII has recently been reported to promote internalization (endocytosis) of cardiac K_{ATP} channels, reducing surface expression (Sierra *et al.* 2013). It is possible that, through different downstream mechanisms, activity and surface expression of cardiac K_{ATP} channels are differentially regulated by activation of CaMKII, as previously reported for cardiac inwardly rectifying potassium channels, IRK (i.e. cardiac Kir2.x channels that give rise to I_{K1} currents; Wagner *et al.* 2009). Notably, for IRK channels the increase in function predominates over the reduction in expression when CaMKII is activated (Wagner *et al.* 2009), resulting in an overall effect of channel stimulation. Our findings evidently support a working model where calmodulin and CaMKII serve as indispensible elements in the NO signalling pathway mediating functional enhancement, not suppression, of cardiac K_{ATP} channels.

Involvement of CaMKIIδ

The CaMKII family consists of four closely related yet distinct isoforms (α, β, γ and δ). The major isoform of CaMKII in the heart is CaMKIIδ (Tobimatsu & Fujisawa, 1989). Importantly, the present study revealed that genetic ablation of CaMKIIδ (i.e. CaMKIIδ knockout) diminished PKG stimulation of ventricular sarcK_{ATP} channels, suggesting a crucial role of CaMKIIδ in mediating enhancement of ventricular sarcK_{ATP} channel activity elicited by PKG activation. As PKG activation was required for NO stimulation of cardiac K_{ATP} channels, these results thus suggest that CaMKIIδ is primarily responsible for functional effects rendered by NO elevation on sarcK_{ATP} channels in intact ventricular myocytes. Increased short-term CaMKII activity may serve as beneficial negative feedback for calcium on repolarization of cardiomyocyte membranes (Wagner *et al.* 2009). Further study is required to identify the direct target(s) of CaMKII(δ) for K_{ATP} channel stimulation.

Activation of NO signalling modifies the open and closed properties of ventricular sarcK_{ATP} channels to potentiate channel activity

Based on the open- and closed-duration distributions of sarcK_{ATP} channels in intact rabbit ventricular cardiomyocytes, we suggest that the cardiac K_{ATP} channel exhibits at least two open states and four closed states. The enhanced K_{ATP} channel activity (as evidenced by higher NP_o values) observed in the presence of NO donors could be accounted for by an increase in the opening frequency and by shifts in the closed-duration distributions, the latter of which included reductions in the occurrence (i.e. the relative area of individual exponential components shown in the frequency histogram) of the two longer closed states relative to that of the two shorter ones, and a shortened dwelling duration (i.e. the time constant) of the longest closed state. These results suggest that NO potentiates ventricular sarcK_{ATP} channel activity by destabilizing the long closed conformations and by facilitating the closed-to-open transitions. Importantly, the aforementioned changes caused by NO donors in the channel open and closed properties were prevented by the PKG inhibitor KT5823, by the MEK1/2 inhibitor U0126 and by the CaMKII inhibitory peptide mAIP, suggesting the involvement of PKG, ERK1/2 and CaMKII as molecular transducers in mediating the effect of NO on cardiac K_{ATP} channel gating.

NO–PKG signalling augments cardiac CaMKII activity in an ERK1/2-dependent manner

Calcium/calmodulin binding activates CaMKII by disinhibiting the autoregulatory domain, which initiates intraholoenzyme autophosphorylation. Autophosphorylation of CaMKII at T287 produces Ca^{2+}-autonomous activity by preventing reassociation of the kinase domain by the autoinhibitory region (Hudmon & Schulman, 2002). Our biochemical evidence revealed that both the PKG activator zaprinast and the NO donor NOC-18 activated CaMKII in intact rabbit ventricular cardiomyocytes, as manifested by increases in autophosphorylation of CaMKII and incorporation of ^{32}P into CaMKII substrates. Importantly, activation of CaMKII induced by NOC-18 and by zaprinast was significantly attenuated by the PKG inhibitor KT5823, suggesting that CaMKII is activated by NO–PKG signal transduction in ventricular cardiomyocytes. Moreover, enhancement of CaMKII activity by zaprinast was reduced in the presence of the MEK1/2 inhibitor U0126, which further suggests that ERK1/2 mediates PKG-elicited activation of CaMKII, hereupon placing CaMKII downstream of ERK1/2 in the signalling cascade initiated by NO–PKG. In addition, we also examined the effect of coapplication of NOC-18 and zaprinast on CaMKII phosphorylation. Data obtained from this group revealed that coapplication of NOC-18 and zaprinast increased CaMKII phosphorylation (Supplemental Fig. S4; $n = 3$), but the magnitude of increase did not exceed that rendered by zaprinast administered alone (see Fig. 5*D* and *E*). These results thus suggest that PKG and NO act through the same signalling mechanism to enhance CaMKII activity in cardiomyocytes, providing additional evidence supportive of our hypothesis that PKG mediates stimulation of CaMKII activity caused by NO. While H_2O_2 can directly drive autonomous CaMKII activation in a Ca^{2+}/calmodulin-dependent manner (Erickson *et al.* 2008), our electrophysiological data showing that cardiac K_{ATP} channel stimulation by exogenous H_2O_2 and by NO donors was both abrogated by inhibition of ERK1/2, complemented by biochemical evidence discussed above, suggest that ERK is likely to be positioned downstream of ROS/H_2O_2 but upstream of CaMKII in the NO signalling pathway, at least for cardiac K_{ATP} channel modulation. In other words, these results collectively support a working model (see Fig. 6), in which Ca^{2+}/calmodulin-dependent activation of CaMKII takes place after sequential activation of NO (induction), sGC, PKG, ROS/H_2O_2 (generation) and ERK1/2 to mediate cardiac K_{ATP} channel stimulation. In this NO–K_{ATP} channel signalling pathway, the ability of ROS to activate CaMKII directly (Erickson *et al.* 2008) appears to be non-essential.

The residual effect caused by NO donors on K_{ATP} channel potentiation in the presence of KT5823 observed in HEK293 cells (Fig. 1*B* and *G*) seemed to imply that in HEK293 cells, but not in ventricular cardiomyocytes, some yet-to-be-identified signal(s) besides PKG is also activated by NO induction to mediate K_{ATP} channel stimulation. Even if NO induces PKG-independent signalling in addition to activation of PKG, the 'divergent' signals probably converge to one common pathway at or above the level of ROS in HEK293 cells, as evidenced by total abrogation of the NO donor effect by scavenging of ROS, or respective suppression of the more downstream signalling partners ERK1/2 and CaMKII (Fig. 1*C*–*F*). It is worth mentioning that many of the intermediate signals required for mediating K_{ATP} channel potentiation in the signalling mechanism proposed in this study (Fig. 6) may intersect with other signalling pathways in different intracellular conditions, and therefore, our findings do not exclude a possibility that the signalling molecules involved in K_{ATP} channel modulation downstream of NO may also affect K_{ATP} channel activity through some parallel signalling pathways. Further studies will be required to elucidate this possibility.

In conclusion, here we report, for the first time, that the function of ventricular sarcK_{ATP} channels is modulated by NO induction via an intracellular signalling pathway consisting of sGC, PKG, ROS/H_2O_2, ERK1/2, calmodulin and CaMKII (CaMKIIδ in particular) that facilitates opening transitions while destabilizing long closures of the channel. Specifically, our study suggests that ERK1/2 mediates NO/PKG activation of CaMKII, thereby relaying the signal from elevation of NO (and ROS) to the sarcK_{ATP} channel in cardiomyocytes, rendering heightened channel activity. The present study highlights the relevance of intracellular signalling mechanisms as effective functional regulators for K_{ATP} channels. The signalling mechanism described herein may offer the framework to permit fine-tuning of K_{ATP} channel activity in different intracellular conditions. Mechanistic understanding of K_{ATP} channel regulation may provide insights into the development of strategies for the management of cardiovascular injury. It is noteworthy that K_{ATP} channels, NO, PKG, ROS and ERK1/2 have also been implicated in cardiac protection/tolerance against ischaemic injury. Cardiac protection by NO from exogenous sources or endogenously released during the short episode of sublethal ischaemia may be mediated partly by K_{ATP} channel stimulation. Hence, this NO–sGC–PKG–ROS–ERK1/2–calmodulin–CaMKII (CaMKIIδ in particular)–sarcK_{ATP} signalling pathway may regulate cardiomyocyte excitability and contribute to endogenous cytoprotection in the heart.

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Additional Information

Competing interests

None declared.

Author contributions

The experiments in this study were conducted in the University of California Davis at the Department of Physiology and Membrane Biology as well as at the Department of Pharmacology. Y.-F.L. directed the study, contributing to the conception and design of the experiments, analysis and interpretation of the data and drafting of the manuscript. Y.C., D.-M.Z. and J.R.E. contributed to the collection and analysis of the data. D.-M.Z., D.M.B. and J.H.B. critically reviewed the manuscript and contributed to important intellectual content. All authors approved the final version of the manuscript.

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