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Functional Modulation of Sarcolemmal K_{ATP} Channels by Atrial Natriuretic Peptide-Elicited Intracellular Signaling in Adult Rabbit Ventricular Cardiomyocytes

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ABSTRACT

ATP-sensitive potassium (K_{ATP}) channels couple cell metabolic status to membrane excitability and are crucial for stress adaptation and cytoprotection in the heart. Atrial natriuretic peptide (ANP), a cardiac peptide important for cardiovascular homeostasis, also exhibits cytoprotective features including protection against myocardial ischemia-reperfusion injuries. However, how ANP modulates cardiac K_{ATP} channels is largely unknown. In the present study we sought to address this issue by investigating the role of ANP signaling in functional modulation of sarcolemmal K_{ATP} (sarcK_{ATP}) channels in ventricular myocytes freshly isolated from adult rabbit hearts. Single-channel recordings were performed in combination with pharmacological approaches in the cell-attached patch configuration. Bath application of ANP markedly potentiated sarcK_{ATP} channel activities induced by metabolic inhibition with sodium azide, whereas the K_{ATP}-stimulating effect of ANP was abrogated by selective inhibition of the natriuretic peptide receptor type A (NPR-A), cGMP-dependent protein kinase (PKG), reactive oxygen species (ROS), extracellular signal-regulated protein kinase (ERK)1/2, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), or the ryanodine receptor (RyR). Blockade of RyRs also nullified hydrogen peroxide (H_2O_2)-induced stimulation of sarcK_{ATP} channels in intact cells. Furthermore, single-channel kinetic analyses revealed that ANP enhanced the function of ventricular sarcK_{ATP} channels through destabilizing the long closures and facilitating the opening transitions, without affecting the single-channel conductance. In conclusion, here we report that ANP positively modulates the activity of ventricular sarcK_{ATP} channels via an intracellular signaling mechanism consisting of NPR-A, PKG, ROS, ERK1/2, CaMKII and RyR2. This novel mechanism may regulate cardiac excitability and contribute to cytoprotection, in part, by opening myocardial K_{ATP} channels.

Key Words: ANP signaling, Reactive Oxygen Species, Ryanodine receptor, ERK, CaMKII

ABBREVIATIONS

ANP	atrial natriuretic peptide
APD	action potential duration
BNP	B-type natriuretic peptide
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNP	C-type natriuretic peptide
ERK	extracellular signal-regulated protein kinase
H_2O_2	hydrogen peroxide
I_K	delayed rectifier potassium current
I_{Ks}	slow delayed rectifier potassium current
I_{to}	transient outward potassium current
K_{ATP}	ATP-sensitive potassium (channel)
Kir	inwardly rectifying potassium (channel)
mAIP	myristoylated autocamtide-2 related inhibitory peptide for CaMKII
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MPG	*N*-(2-mercaptopropionyl)glycine
NO	nitric oxide
NP	natriuretic peptide
NPo	open probability
NPR-A	natriuretic peptide receptor type A
PDE	phosphodiesterase
pGC	particulate guanylyl cyclase

PIP_2	phosphatidylinositol 4,5-bisphosphate
PKG	cGMP-dependent protein kinase
ROS	reactive oxygen species
RyR	ryanodine receptor
sarcK_{ATP}	sarcolemmal K_{ATP}
sGC	soluble guanylyl cyclase
SUR	sulphonylurea receptor

INTRODUCTION

Atrial natriuretic peptide (ANP) (10) belongs to a family of structurally related peptide hormones that play essential roles in regulating cardiovascular function in normal physiology and in disease states (52). Besides exerting its blood pressure- and blood volume-regulating actions as endocrines (35, 52), ANP also functions locally as autacoids to regulate myocyte growth during chronic hemodynamic overload and to effect anti-ischemic cytoprotection (24). ANP is a natural ligand for the particulate guanylyl cyclase (pGC)-linked natriuretic peptide (NP) receptor type A (NPR-A), which is widely distributed throughout the cardiovascular system, with marked expression in the cardiac atria and ventricles (9); upon activation, the NPR-A signals by catalyzing the synthesis of intracellular signaling molecule cGMP (39).

Vital in the adaptive response to (patho)physiological stress, the K_{ATP} channel operates as a high-fidelity metabolic sensor capable of coupling the intracellular metabolic state to membrane excitability (4, 43, 45), and serves a homeostatic role ranging from blood glucose regulation to cardioprotection (49, 63). The K_{ATP} channel is a hetero-octameric protein composed of four inwardly rectifying potassium channel subunits (Kir6.x) and four sulphonylurea receptors (SURx) (5, 58), whose molecular composition exhibits tissue specificity. For example, in cardiac (ventricular) and skeletal muscles K_{ATP} channels are composed of Kir6.2 and SUR2A subunits (31, 48), whereas in central neurons and pancreatic β-cells they consist of Kir6.2 and SUR1 subunits (1). Whilst direct gating/regulation of K_{ATP} channels by intracellular ATP (inhibitory), MgADP (stimulatory) (49) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (8, 59) has been appreciated, how the function of these important channels is modulated by complex cell signaling mechanisms remains relatively less understood.

Studies conducted in our laboratories and others have demonstrated that various K_{ATP} channel subtypes, such as recombinant K_{ATP} channels of the neuronal/pancreatic-type (14, 15) and cardiac-type (16) isoforms as well as native K_{ATP} channels present in ventricular cardiomyocytes (16), vascular smooth muscle cells (34) and pancreatic β-cells (53), are subject to functional modulation by an increase in intracellular cGMP levels. Additionally, evidence from our prior work suggests that cGMP-dependent protein kinase (PKG), reactive oxygen species (ROS), extracellular signal-regulated protein kinase (ERK)1/2 and calcium/calmodulin-dependent protein kinase II (CaMKII) serve as crucial intracellular signaling partners downstream of nitric oxide (NO), a gaseous messenger that induces intracellular cGMP accumulation via soluble guanylyl cyclase (sGC) activation, to mediate functional stimulation of cardiac K_{ATP} channels (72). ANP, on the other hand, delivers its diverse cellular functions by activating the membrane-associated, pGC-linked NP receptors to increase intracellular cGMP levels (39). ANP has been reported to modulate several ionic currents (44). However, how ANP modulates ventricular myocardial K_{ATP} channels has yet to be determined.

To gain insight into the role of ANP in functional modulation of myocardial K_{ATP} channels and to delineate the intracellular signaling mechanism initiated by ANP, in the present study single-channel recordings of sarcK_{ATP} channels were carried out in combination with pharmacological approaches, using ventricular cardiomyocytes freshly isolated from adult rabbit hearts. Specifically, we investigated the potential involvement of NPR-A, PKG, ROS, CaMKII, ERK1/2 of mitogen-activated protein kinase (MAPK) and the ryanodine receptor (RyR) in ANP-K_{ATP} signal transduction. Effects exerted by ANP on ventricular K_{ATP} channel gating were also analyzed to define the kinetic basis of functional modulation.

MATERIALS AND METHODS

Isolation of ventricular cardiomyocytes. All protocols involving animals were approved by the institutional Animal Care and Use Committee and performed in strict accordance with the Guide for the Care and Use of Laboratory Animal of NIH. Left ventricular myocytes were enzymatically isolated from adult New Zealand White rabbits as described before (16, 72). Rabbits were deeply anesthetized by intravenous injection of pentobarbital sodium (80-100 mg/kg). Hearts were excised and quickly placed on a Langendorff apparatus and perfused retrogradely for 5-7 min with nominally Ca^{2+}-free Dulbecco's minimum essential medium solution. Perfusion was then switched to the same solution containing 1 mg/ml collagenase with up to 0.1 mg/ml neutral protease. Once the heart became flaccid (~15-30 min), the ventricles were dispersed and filtered. The cell suspension was washed several times with a Ca^{2+} (around 150 μM)-containing medium. Isolated ventricular myocytes were subsequently plated on 12-mm glass coverslips freshly coated with laminin (~1 μg per coverslip, or 1 μg/cm^2) (Thermo Fisher Scientific: Invitrogen, Carlsbad, CA) to enhance cell adhesion. Rod-shaped cells with clear margin and striation were used for immediate recordings.

Single-channel recordings. Cell-attached single-channel recordings were performed as described before (72), using a recording chamber (RC26; Warner Instruments, Hamden, CT) filled with the intracellular (bath) solution and the recording pipette was filled with the extracellular solution. The recording electrodes were pulled from thin-walled borosilicate glass with an internal filament (MTW150F-3; World Precision Instruments, Sarasota, FL) using a P-97 Flaming Brown puller (Sutter Instrument, Novato, CA), which were subsequently fire-polished to a resistance of 5-10 MΩ. The intracellular (bath) solution consisted of (in mM): KCl 127, $MgCl_2$ 1, KOH 13, EGTA 5, HEPES 10, glucose 10, pH to 7.2. The extracellular (intrapipette)

solution consisted of (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, pH 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 (Vm) 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), low-pass filtered (2 kHz), and digitized at 20 kHz on-line using Clampex 10 software (Axon) via a 16-bit A/D converter (Digidata acquisition board 1322A; Axon).

Preparations of drugs. Reagents and chemicals were obtained from Sigma-Aldrich (Millipore-Sigma; St. Louis, MO) unless otherwise specified. Working solutions of sodium azide, ANP 1-28 (human), anantin (US Biological; Salem, MA), KT5823, *N*-(2-mercaptopropionyl)glycine (MPG), myristoylated autocamtide-2 related inhibitory peptide for CaMKII (mAIP), U0126 and ryanodine were diluted from aliquots prior to use with bath recording solutions. Stock solutions were prepared as follows: ANP and anantin in acetic acid (5% and 0.95%, respectively), pinacidil, KT5823 and U0126 in DMSO, sodium azide, MPG and mAIP in H_2O, and ryanodine in MeOH; all were stored at -80°C in aliquots, except sodium azide, whose stock solution was stored at 4°C. H_2O_2 was freshly prepared from original stocks. 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) to the recording chamber via a micromanifold positioned closely to the patches. For pharmacological blockade, individual groups of cells were pretreated with respective inhibitors at room temperature for at least 15 min before being subjected to functional assays. These

pharmacological agents were administered at working concentrations well established in the literature, with several of them demonstrated in our prior studies to be effective and selective.

Electrophysiological Data Analysis. Single-channel data were analyzed as described before (14-16, 36, 37, 41, 72), using individual (gap-free) data records of 120-s durations. *Single-channel currents:* Individual, digitized single-channel records of 120-s durations (gap-free) were detected with Fetchan 6.05 (events list) of pCLAMP (Axon Instruments) using 50% threshold crossing criterion and analyzed with Intrv5 (gift from Dr. Barry S. Pallotta formerly at University of North Carolina, Chapel Hill, NC and Dr. Janet Fisher at University of South Carolina, Columbia, SC, USA). Analysis was performed at the main conductance level (approximately 75-80 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 (60) 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. 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. *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 analyzed using Fetchan 6.05 (browse) of pCLAMP to integrate currents in 120-sec 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 current amplitude (control taken as 1), as the normalized current amplitude is equivalent to the relative channel activity (i.e., normalized open probability *NPo*) obtained from single-channel analysis when the single-channel conductance remains the same (41). The relative channel activity values obtained from both single-channel and multiple-channel patches were then pooled. In *Fig. 1* and all the rest of data 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).

Statistics. Data are presented as mean ± 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: normalized data (in response to treatment) in individual groups (*one-sample t tests*), normalized data between two separate groups (*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).

RESULTS

ANP stimulates sarcK_{ATP} channels in adult rabbit ventricular myocytes and the effect requires NPR-A activation. To investigate whether ANP modulates the function of cardiac K_{ATP} channels, we began with experiments examining how native K_{ATP} channels in ventricular cardiomyocytes freshly isolated from adult rabbit hearts respond to the ANP treatment. Single-channel recordings were performed in the cell-attached patch configuration to preserve integrity of the intracellular milieu for potential signaling. Sodium azide (3 mM) (29), a metabolic inhibitor, was applied by bath perfusion to induce baseline K_{ATP} channel activity (Fig. 1A, upper trace). Subsequent addition of human ANP 1-28 (100 nM) (57) in the continuous presence of sodium azide markedly enhanced the single-channel activity of ventricular sarcK_{ATP} channels (Fig. 1A, lower trace). The apparent opening and bursting frequencies were increased by ANP whereas the single-channel conductance remained the same (Fig. 1A), resulting in an averaged relative channel activity of 14.93 ± 3.82 that was significantly enhanced from the control level (control in sodium azide acquired before the addition of ANP taken as 1) (Fig. 1C, ANP; 16 patches; $P < 0.005$, two-tailed one-sample *t* test). In line with a positive effect exerted by ANP on the baseline channel elicited by sodium azide (see Fig. 1A and C), ANP also potentiated the baseline channel activity elicited by pinacidil (200 μM), a K_{ATP} channel opener, in a separate set of cell-attached patches (Fig. 2A; a representative patch); the averaged relative channel activity was significantly increased to 3.99 ± 0.95 when ANP was added (same-patch controls in pinacidil taken as 1) (Fig. 2B, ANP; 4 patches; $P < 0.05$, one-sample *t* test). These findings thus unveiled a stimulatory role of ANP in modulating ventricular sarcK_{ATP} channel function in intact cardiomyocytes, which was substantiated by the time course profiles of K_{ATP} channel activity obtained in the absence and presence of ANP (see Supplemental Fig. 1 for absolute channel

activity presented as a function of time from three representative patches, and Supplementary Fig. 2 for a time course plot of averaged relative channel activity). The NPR-A is a pGC-linked NP receptor for ANP (9). To test whether ANP stimulates ventricular sarcK_{ATP} channels via activation of the NPR-A, anantin, a cyclic peptide from *Streptomyces coerulescens* which acts as a competitive antagonist at the NPR-A (24, 68), was used. In a separate group of cell-attached patches where baseline K_{ATP} channel openings had been elicited with bath perfusion of sodium azide (3 mM), the selective NPR-A antagonist anantin (1 μM) was administered in the continuous presence of sodium azide (3 mM) for at least additional 15 min. Subsequent application of ANP (100 nM) together with anantin and sodium azide produced no potentiation of the single-channel activity of ventricular sarcK_{ATP} channels in comparison with the control level obtained from the same patches (Fig. 1B, upper vs. lower traces; a representative patch); the averaged relative channel activity was 0.82 ± 0.21 (control taken as 1) (Fig. 1C, ANP+Anantin; 5 patches; no significant change), revealing significant suppression of the stimulatory effect of ANP on ventricular sarcK_{ATP} channels by anantin (Fig. 1C, ANP vs. ANP+Anantin; $P < 0.05$, Dunnett's multiple comparison test following one-way ANOVA). The single-channel conductance of the channels examined in the present study ranged between 75-80 pS (with membrane potential clamped at -60 mV in symmetrical 140 mM potassium recording solutions), typical of ventricular myocardial K_{ATP} channels composed of Kir6.2 and SUR2A subunits (6, 72); these channels were responsive to the K_{ATP} channel opener pinacidil (Figs. 2A, 6A and 6B, upper trace) and exhibited ATP sensitivity characteristic of K_{ATP} channels, which was manifested by a positive response to metabolic inhibition in the cell-attached patch configuration as well as by a robust increase in the channel activity upon patch excision into the ATP-free bath (a verification procedure we routinely perform at the end of each cell-attached

patch recording experiment). These data thus indicate that ANP positively modulated sarcK_{ATP} channels in intact adult rabbit ventricular myocytes via activation of NPR-A.

Inhibition of PKG abolishes ANP stimulation of ventricular sarcK_{ATP} channels. ANP has been shown to dose-dependently increase intracellular cGMP levels by activating pGC-linked receptors in a variety of cell types including ventricular cardiomyocytes from adult rabbits (20). Which protein(s) in ventricular myocytes might serve as a direct target for cGMP produced by ANP/NPR-A activation to render the stimulatory effect of ANP on myocardial sarcK_{ATP} channels? It is recognized that the cGMP-dependent kinase PKG represents the principal intracellular mediator of cGMP signals elevated by NPs (9). We have previously demonstrated that NO, a gaseous messenger capable of elevating intracellular cGMP levels by activating sGC, positively modulates the function of recombinant and native K_{ATP} channels via an intracellular signaling mechanism that requires the activity of PKG (15, 72). Considering the evidence presented above that the K_{ATP}-potentiating effect of ANP in intact ventricular cardiomyocytes exhibited a dependence on the activation of the pGC-linked ANP receptor NPR-A (see Fig. 1), we thus proceeded to examine whether the cGMP/PKG system is involved in mediating the modulatory effect of ANP on ventricular sarcK_{ATP} channels. Cell-attached patch-clamp recordings were performed in ventricular cardiomyocytes pretreated with the selective PKG inhibitor KT5823 (1 μM) for at least 15 min at room temperature; in the continuous presence of KT5823, sodium azide (3 mM) was applied to induce baseline K_{ATP} channel openings, followed by addition of ANP (100 nM) (Fig. 3A). ANP failed to elicit significant changes in the single-channel activity of sarcK_{ATP} channels preactivated by sodium azide in the presence of KT5823 (1 μM) (Fig. 3A and C, ANP+KT5823; the ANP data from Fig. 1C was displayed in Fig. 3C for comparison purpose); the averaged relative channel activity was 1.53 ± 0.49 (control taken as 1)

(5 patches; no significant change from the same-patch controls), uncovering a significant blockade of the K_{ATP}-stimulating effect of ANP by KT5823 (Fig. 3C; ANP+KT5823 vs. ANP; $P < 0.05$). The specificity of KT5823 at 1 μM to selectively inhibit activation of PKG but not that of PKA has been verified in our recent study (15). These data thus indicate that ANP enhanced sarcK_{ATP} channel activity in intact ventricular cardiomyocytes via PKG, presumably activated consequent to NPR-A-catalyzed cGMP generation.

Effects of ROS scavenging on ventricular sarcK_{ATP} channel stimulation induced by ANP. It has been demonstrated that PKG activation induces ROS generation in cardiac tissues (69). Findings from our prior studies also suggest that ROS mediate the stimulatory action of PKG on neuronal-type K_{ATP} channels (15) and cardiac-type K_{ATP} channels (16) expressed in mammalian cell lines. We showed above that the ANP treatment enhanced the single-channel activity of ventricular sarcK_{ATP} channels in a PKG-dependent manner (see Fig. 3A and C). Would ROS serve as an intermediate signal inducible upon activation of PKG in the ANP/NPR-A signaling pathway to mediate an acute stimulatory effect of ANP on these channels? To investigate this potential link, we examined the effect of *N*-(2-mercaptopropionyl)glycine (MPG), an ROS scavenger, on ANP-elicited stimulation of ventricular sarcK_{ATP} channels in cell-attached patches. Following pretreatment with MPG (500 μM), sodium azide (3 mM) was applied to induce baseline K_{ATP} channel activity. In the continuous presence of MPG (500 μM) and sodium azide (3 mM), subsequent addition of ANP (100 nM) did not increase the single-channel activity of sarcK_{ATP} channels (Fig. 3B; a representative patch); the averaged relative channel activity was 0.52 ± 0.19 (control taken as 1) (Fig. 3C, ANP+MPG; 5 patches; no significant change), which was significantly different from the marked increase observed in patches treated with ANP in the absence of MPG (Fig. 3C; ANP+MPG vs. ANP; $P < 0.05$).

These data thus indicate that ROS, presumably generated downstream of PKG activation, were crucial signals mediating ANP-elicited potentiation of sarcK_{ATP} channel activities in intact ventricular myocytes.

Suppression of ERK1/2 activity abrogates ventricular sarcK_{ATP} channel stimulation induced by ANP. ERK is a member of the MAPK family that is activated by H_2O_2 (46). As presented above (see Fig. 3B and C), the stimulatory effect of ANP on ventricular sarcK_{ATP} channels in intact cells were ROS-dependent. It is therefore intriguing to find out whether ERK might be involved in mediating the acute stimulatory action of ANP on these channels. To address this question, the effects of ANP on the single-channel activity of sarcK_{ATP} channels acquired in intact myocytes using cell-attached recordings were examined in the absence and presence of U0126, a blocker that suppresses activation of ERK1/2 through selective inhibition of MAPK kinase (MEK) 1 and 2. Cells were pretreated with U0126 (10 μM), and baseline sarcK_{ATP} channel activity was then induced by bath perfusion of sodium azide (3 mM) in the continuous presence of U0126 (10 μM). This was followed by bath perfusion of ANP (100 nM) along with sodium azide and U0126. ANP was incapable of facilitating ventricular K_{ATP} channel opening preactivated by sodium azide in the presence of U0126 (Fig. 4A; a representative patch); the averaged relative channel activity was 0.55 ± 0.10 (control taken as 1) (Fig. 4C, ANP+U0126; 6 patches; no significant change), yielding a total abrogation of ANP's stimulatory effect on the channel (Fig. 4C; ANP+U0126 vs. ANP; $P < 0.05$). Interestingly, with functional analysis of K_{ATP} channels our prior work has revealed a stimulatory role of H_2O_2 for functional modulation of K_{ATP} channels in intact adult rabbit ventricular myocytes that is ERK1/2-dependent (72), implying that ERK1/2 is likely positioned downstream of ROS to mediate K_{ATP} channel modulation. These data thus indicate that ERK1/2, presumably activated after ROS

(generation), was required for the acute stimulatory action exerted by ANP on ventricular sarcK_{ATP} channels in intact cardiomyocytes.

Inhibition of CaMKII reverses ANP stimulation of ventricular sarcK_{ATP} channels. Ca^{2+}/calmodulin-dependent kinases (CaMKs) influence processes as diverse as gene transcription, cell survival, apoptosis, cytoskeletal re-organization and learning and memory. CaMKII is the CaMK isoform predominantly found in the heart (40). Our recent work suggests that CaMKII (especially the δ isoform) mediates the stimulation of cardiac K_{ATP} channels induced by NO and that CaMKII is likely positioned upstream of ERK1/2 in this NO-K_{ATP} signaling pathway (72); a potential involvement of CaMKII in mediating ANP modulation of cardiac K_{ATP} channels, however, has not been investigated. To elucidate this matter, ventricular cardiomyocytes were pretreated with mAIP (1 μM), a myristoylated autocamtide-2 related inhibitory peptide for CaMKII, followed by application of sodium azide (3 mM) to induce K_{ATP} channel opening for detection in the cell-attached patch configuration. Subsequently, ANP (100 nM) was administrated in the continuous presence of mAIP (1 μM) and sodium azide (3 mM). Treatment with ANP under this condition elicited no significant change in the activity of ventricular sarcK_{ATP} channels (Fig. 4B; a representative patch); the averaged relative channel activity was 1.34 ± 0.38 (control taken as 1) (Fig. 4C, ANP+mAIP; 6 patches), reflecting significant abolishment of ANP's stimulatory action by mAIP (Fig. 4C; ANP+mAIP vs. ANP; $P < 0.05$). These results thus indicate that ANP modulated sarcK_{ATP} channels in intact ventricular cardiomyocytes in a CaMKII-dependent manner.

Effects of blocking ryanodine receptors on ANP-elicited stimulation of ventricular sarcK_{ATP} channels in intact cells. RyRs regulate release of Ca^{2+} from intracellular stores, and hence blockade of these receptors interferes with Ca^{2+} mobilization (25). Cardiac RyRs play an

important role in cardiac excitation-contraction coupling (17). We previously reported that caffeine, a RyR agonist (42), enhances the function of recombinant K_{ATP} channels in intact mammalian cells and the K_{ATP}-modulating effect of caffeine is, in part, mediated by intracellular Ca^{2+} (41), potentially through recruiting some Ca^{2+}-dependent mechanism(s) following RyR activation. Findings from our prior work performed on ventricular cardiomyocytes have also implied that the potentiation of native K_{ATP} channel activities by the NO/PKG signaling mechanism is calcium-dependent, considering the crucial roles of calmodulin and CaMKII in the signaling process (16, 72). Along similar lines, in the present study the stimulatory effect of ANP on ventricular sarcK_{ATP} channels in intact cardiomyocytes exhibited a dependency on CaMKII activation (see Fig. 4B and C). Would RyRs be involved in activation of CaMKII (and/or other Ca^{2+}-dependent mediators) in the ANP-K_{ATP} signaling pathway, and thereby contribute to the modulation of cardiac K_{ATP} channel function? To test a potential role of RyRs in mediating ANP-K_{ATP} signaling, the effect of ANP was examined in cell-attached patches obtained from ventricular myocytes which had been pretreated, for 20 min at room temperature, with the plant alkaloid ryanodine (100 μM) to block RyRs. Following the pretreatment with ryanodine, bath perfusion of ANP (100 nM) failed to potentiate sarcK_{ATP} single-channel activities induced by sodium azide (3 mM) (Fig. 5A; a representative patch); the averaged relative channel activity was 1.42 ± 0.42 (6 patches; control taken as 1) (Fig. 5B, ANP+Ryanodine), revealing significant nullification of ANP's stimulatory action on the channel (Fig. 5B; ANP+Ryanodine vs. ANP; $P < 0.05$). These data thus indicate that the activity of cardiac RyR (i.e., RyR2) (42) was required for acute stimulation of ventricular sarcK_{ATP} channels elicited by ANP, implying that Ca^{2+} released from the SR may be crucial for ANP signaling to effect positive modulation of K_{ATP} channel function in ventricular myocardium.

Additionally, we performed a set of cell-attached recording experiments to determine whether RyR activation occurs upstream or downstream of ROS generation in mediating functional modulation of ventricular sarcK_{ATP} channels. Consistent with our earlier reports (16, 72), treatment with exogenous H_2O_2 (1 mM), a relatively stable form of ROS, resulted in an increase in the single-channel activity of ventricular sarcK_{ATP} channels preactivated by K_{ATP} channel opener pinacidil (200 μM) (Fig. 6A and C, H_2O_2), yielding an averaged relative channel activity of 10.88 ± 2.37 (5 patches; $P < 0.05$; control taken as 1). By contrast, the stimulatory effect of H_2O_2 was prevented by pretreatment with ryanodine (100 μM) (Fig. 6B and C, H_2O_2+Ryanodine; averaged relative channel activity = 1.40 ± 0.41; 3 patches), revealing abrogation of H_2O_2's stimulatory action by inhibition of RyR (Fig. 6C; H_2O_2 vs. H_2O_2+Ryanodine; $P < 0.05$). These results indicate that RyR2 was required for the stimulatory effect of H_2O_2 on ventricular sarcK_{ATP} channels observed in intact cells. In other words, RyR2 might be positioned downstream of ROS/H_2O_2 (generation) in the ANP signaling pathway.

Kinetic effects exerted by ANP signaling on ventricular sarcK_{ATP} single-channel open and closed properties. To delineate how ANP influences the gating (i.e., opening and closing) of ventricular sarcK_{ATP} channels, single-channel analysis of K_{ATP} channel activities acquired with cell-attached recordings before and during ANP treatment was conducted. The fitting results revealed that in the control condition the open- and closed-duration distributions of rabbit ventricular sarcK_{ATP} channels at the cell-attached patch configuration could be best described by a single open time component and the sum of three closed time components, respectively (Fig. 7A; a representative patch), implying that there are at least one open state and three closed states. ANP treatment altered the closed duration distribution (i.e., shifted the relative distribution among different closed states), including reductions in the relative area (i.e., the relative

frequency of occurrence) of the longest closed state and the time constant (i.e., the dwelling time) of this closed state, while the shorter closed states were stable (i.e., the relative frequency of occurrence of these states not reduced) (Fig. 7 A and B, Closed), resulting in shortening of the mean closed duration (reduced to 20.73 from 99.64 ms). The apparent opening frequency was increased by ANP (from 8.24 to 38.15 events/s) whereas the open duration distribution and the mean open duration (1.92 ms in control compared with 2.00 ms in when ANP was applied) remained relatively unchanged (Fig. 7A and B, Open). The reduction in the mean closed duration of the longest closed state accompanying an increase in the closed-to-open state transitions thus led to enhanced channel activity (as evidenced by a higher NPo) in the presence of ANP (Fig. 1 and Supplemental Fig. 3, a scatter plot). These findings indicate that ANP stimulated ventricular sarcK_{ATP} channels by shortening long closures, destabilizing the longest closed state and facilitating the closed-to-open transitions of the channel.

DISCUSSION

K_{ATP} channels serve a homeostatic role ranging from glucose regulation to cardioprotection and are vital in the adaptive response to (patho)physiological stress (49). Indeed, genetic disruption of the pore-forming subunit that comprises cardiac K_{ATP} channels renders the knockout mice less tolerant to different types of stress, resulting in abnormal cytosolic calcium handling, susceptibility to developing acute cardiac failure, and sudden cardiac death (32, 64, 73). ANP, a primarily myocardium-driven, 28-amino acid polypeptide which mediates the induction of natriuresis, diuresis and vasorelaxation (18), is also an autacoid cardioprotective mediator, exerting antihypertrophic and antifibrotic functions (47) and conferring protection against ischemia-reperfusion injury in the heart (12). In the present study, we demonstrated for the first time that ANP via activation of NPR-A positively modulated the function of sarcK_{ATP} channels in adult rabbit ventricular cardiomyocytes, an effect mediated by an intracellular signaling mechanism consisting of PKG, ROS (H_2O_2 in particular), ERK1/2, CaMKII, and RyR2. We also defined the kinetic basis on which ANP enhanced the single-channel activity of ventricular sarcK_{ATP} channels and provided novel evidence suggesting that RyR2 (activation) is likely positioned downstream of ROS/H_2O_2 in mediating sarcK_{ATP} channel stimulation in intact cardiomyocytes.

ANP potentiates ventricular sarcK_{ATP} channel activity via activation of NPR-A. K_{ATP} channels and ANP are both involved in cardioprotection against ischemic-reperfusion injuries (12, 62, 63). Whilst ANP and other NPs are best known for their ability to regulate blood volume and fluid homeostasis, it has become apparent that NPs are also important regulators of cardiac electrophysiology (51), effectuated in part by modulating ion channel function (44). Several cardiac ion channels have emerged as targets of ANP, including L-type Ca^{2+} channels,

Na^+ channels and hyperpolarization-activated cyclic nucleotide-gated channels; ANP also increases transient outward K^+ current (I_{to}) in human atrial cells, delayed rectifier outward K^+ current (I_K) in embryonic chick heart cells and the slow component of delayed rectifier K^+ current (I_{Ks}) in guinea pig sino-atrial node cells (For a review, see ref. 44). More recently, with a population approach (i.e., the control and the treatment data being collected from separate groups of samples), B-type NP (BNP; also a ligand for NPR-A) (52) and C-type NP (CNP) have been suggested to suppress an intermediate-conductance ATP-sensitive K^+ current in rat ventricular myocytes (11), whose single-channel conductance (~55 pS in 140 mM K^+ solutions) was much lower than that of classical ventricular myocardial K_{ATP} channels (~75-80 pS; for a review, see ref. 28); a rat cardiac K_{ATP} channel with reduced unitary conductance, as speculated by the authors (11), may possess a molecular composition distinct from that of the classical, regular-conductance ventricular K_{ATP} channel (i.e., Kir6.2/SUR2A) widely reported in the literature. However, to date there have been no reports on how ANP modulates ventricular sarcK_{ATP} channels.

ANP binds to the transmembrane GC receptor, NPR-A, to exert its diverse cellular functions (39). In the present study, we showed that bath application of ANP enhanced sarcK_{ATP} single-channel activities from the corresponding control level in intact rabbit ventricular cardiomyocytes (see Figs. 1A, 1C and 2, and Supplemental Figs. 1-3) in an NPR-A-dependent manner (see Fig. 1B and C). These results suggest that activation of the pGC-linked NPR-A by ANP in the ventricular cardiomyocytes elicits functional enhancement of sarcK_{ATP} channels, presumably via an increase of intracellular cGMP and subsequent activation of cGMP-dependent signaling proteins. In line with our findings obtained from ventricular myocytes, K_{ATP} channels in other tissues/cell types are also regulated by ANP. For example, application of ANP has been

demonstrated to increase the single-channel activity of vascular K_{ATP} channels in myocytes cultured from thoracic aorta (34) and to potentiate, via activation of NPR-A, a glibenclamide-sensitive K^+ currents in follicle-enclosed Xenopus oocytes (54), albeit neither of these studies has explored the intracellular signaling mechanism responsible for the effect of ANP. The enhancement of ventricular sarcK_{ATP} channel function by the ANP/NPR-A system demonstrated in the present study may contribute to the acute cardioprotective action of ANP in reducing ischemia-reperfusion injury.

PKG mediates the stimulation of ventricular sarcK_{ATP} channels evoked by ANP. The observation made in the present study that the stimulatory action of ANP on ventricular sarcK_{ATP} channels was abolished by selective inhibition of PKG (see Fig. 3A and C) led us to suggest that ANP, via interaction with the pGC-linked NPR-A that increases the intracellular cGMP level, activates the cGMP-dependent enzyme PKG to augment cardiac K_{ATP} channel activities in intact cardiomyocytes; in other words, ANP stimulates cardiac sarcK_{ATP} channels via a cGMP/PKG mechanism and hence the ANP's modulatory effect on the channel is PKG-dependent. The cGMP/PKG signaling mechanism is involved in the regulation of smooth muscle relaxation, learning and memory, cell division, and cardioprotection (50, 67). We and others have previously demonstarted that intracellular cGMP elevation, induced by application of membrane-permeable cGMP analogs, cell-permeable cGMP-selective phosphodiesterase (PDE) inhibitors, or NO donors, effectively increases K_{ATP} channel activities in various cell models (14-16, 34, 72); more specifically, the stimulatory effect of cGMP elevation on ventricular sarcK_{ATP} channels is principally mediated by activation of PKG (16, 72). Signaling cascades initiated by NO and NPs play an important role in the maintenance of cardiovascular homeostasis (27). The PKG dependence of ANP's stimulatory action on ventricular sarcK_{ATP} channels reported in the

present study (see Fig. 3A and C) was reminiscent of the K_{ATP}-potentiating effect elicited by the NO/cGMP/PKG signaling mechanism in ventricular myocytes (72), although NO and NPs increase intracellular cGMP via activating sGC and pGC, respectively. PKG also mediates ANP's infarct-sparing effect in rabbit hearts when administered prior to reperfusion (70), which potentially may involve PKG-dependent activation of sarcK_{ATP} channels in the myocardium.

ROS are required for mediating ANP-induced stimulation of ventricular sarcK_{ATP} channels. ROS are generated by all aerobic cells, and most endogenously produced ROS are derived from mitochondrial respiration (38). While oxidative stress caused by high levels of ROS damages cellular components and has been linked to a myriad of pathologies, ROS at a lower concentration serve as important cellular signaling molecules in the maintenance of physiological functions (55) and in cardioprotection afforded by ischemic preconditioning (7). In the present study, the enhancement of sarcK_{ATP} channel activities rendered by ANP in intact ventricular cardiomyocytes was prevented by the ROS scavenger MPG (see Fig. 3B and C). These results suggest that ROS, presumably produced downstream of PKG activation, mediate ANP-elicited stimulation of sarcK_{ATP} channels in intact cardiomyocytes. Indeed, ANP may trigger free radical generation by cardiac cells and the effect is blocked by inhibition of PKG (unpublished observation described in ref. 70), which corroborates our hypothesis that ANP induces production of ROS via activation of PKG for K_{ATP} channel modulation in cardiomyocytes. Bradykinin and acetylcholine, both involved in protection afforded by ischemic preconditioning, induce ROS generation in adult rabbit ventricular myocytes in a PKG-dependent manner (33). Along similar lines, NO donors also incite ROS generation in rat cardiomyocytes in a PKG-dependent manner for its anti-infarct effect (69). We have recently demonstrated that ROS/H_2O_2 generation is required for the stimulation of ventricular K_{ATP}

channels induced by NO in adult rabbit cardiomyocytes (72). Evidence from our prior report further uncovered that ROS (H_2O_2 in particular) are positioned downstream of PKG to mediate PKG stimulation of ventricular K_{ATP} channels in intact cells (16). Collectively, these findings suggest that an ANP/NPR-A/cGMP/PKG/ROS signaling cascade likely accounts for functional modulation of cardiac K_{ATP} channels.

Role of ERK1/2 in ANP-induced stimulation of ventricular sarcK_{ATP} channels. ERKs play pivotal roles in many aspects of cell functions and are activated by oxidative stress in some types of cells (2, 46). ERK1/2 mediates short- and long-term responses elicited by cardioactive substances in the heart (19); it is also involved in the infarct-limiting effect achieved through postconditioning in rabbit hearts (22). Our present investigation revealed that the increase in cardiac K_{ATP} single-channel activity elicited by ANP in ventricular cardiomyocytes was abolished by inhibition of MEK1 and MEK2 (both upstream kinases of ERK1/2) with U0126 (see Fig. 4A and C). These results thus suggest that, like PKG and ROS described above, ERK1/2 is a crucial relay signal evoked by ANP to mediate ventricular K_{ATP} channel stimulation. In line with this, ANP has been shown to induce ERK1/2 activation in neonatal rat ventricular myocytes that is required for its anti-hypertrophic effect (61). ANP's infarct-sparing effect in rabbit harts is also sensitive to inhibition of ERK (70). Interestingly, the anorexic hypothalamic peptide nesfatin-1, acting as a novel cardiac peptide, directly modulates performance of rat hearts, the mechanism of which may involve activation of the pGC-linked NPR-A, the cGMP/PKG pathway, and ERK1/2 (3). These findings consistently support a notion that NPR-A activation in the myocardium is associated with activation of ERK1/2 to yield modulation of cardiac function. Moreover, with functional analysis of K_{ATP} channels our recent study has provided evidence suggesting that ERK1/2 is positioned downstream ROS to mediate

potentiation of K_{ATP} channel function in intact ventricular cardiomyocytes isolated from adult rabbit hearts (72); together with our current findings (Fig. 4A and C), it is conceivable that activation of ERK1/2 may occur after generation of ROS in the signaling process elicited by ANP that potentiates K_{ATP} channel function. We thus suggest that that ERK1/2 is positioned downstream of ROS in the ANP-K_{ATP} signaling cascade to mediate cardiac K_{ATP} channel modulation.

CaMKII mediates stimulation of ventricular K_{ATP} channels induced by ANP.

Ca^{2+}/calmodulin-dependent kinases (CaMKs) influence processes as diverse as gene transcription, cell survival, apoptosis, cytoskeletal re-organization and learning and memory. CaMKII, the CaMK isoform predominantly found in the heart (40), 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 (66). Activation of CaMKII is achieved through direct Ca^{2+}/calmodulin binding which disinhibits the autoregulatory domain of the kinase (30) or by redox modification of the protein (26). In the present study we showed that ANP-induced enhancement of sarcK_{ATP} channel activities preactivated by sodium azide in intact cells was nullified in the presence of mAIP, a membrane-permeable, myristoylated autocamtide-2 related inhibitory peptide selective for CaMKII (see Fig. 4B and C), which suggests that ANP enhances ventricular sarcK_{ATP} channel function in intact cells via activation of CaMKII. Complementary to our current findings, we have previously demonstrated that the stimulatory effect of NO, PKG, or exogenous H_2O_2 on ventricular sarcK_{ATP} single-channel activities is reversed by inhibition of calmodulin or CaMKII (16, 72) and that PKG activation increases CaMKII activity/phosphorylation in an ERK1/2-dependenet manner (72), suggesting that CaMKII is activated downstream of PKG, ROS/H_2O_2 and ERK1/2; these findings lent support to

our current hypothesis that CaMKII is positioned downstream of ROS/H_2O_2 and ERK1/2 to mediate ANP-induced stimulation of ventricular K_{ATP} channels. Increased short-term CaMKII activity may serve as beneficial negative feedback for calcium on repolarization of cardiomyocyte membranes (66). Further research is required to elucidate the physiological or pathophysiological role of CaMKII-dependent modulation of cardiac K_{ATP} channel function elicited by ANP.

RyR activation contributes to ANP-induced stimulation of ventricular K_{ATP} channels. The cardiac RyR (RyR2) plays a critical role in excitation–contraction coupling by providing a pathway for the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol (17). In the present study we showed that blockade of RyRs with ryanodine (100 μM) abolished ANP-elicited potentiation of ventricular sarcK_{ATP} channel activities preactivated by sodium azide (see Fig. 5), revealing that activation of RyR2 is required for ANP's modulatory effect on ventricular K_{ATP} channels. Additionally, we demonstrated that the increase in ventricular sarcK_{ATP} channel activity rendered by exogenous H_2O_2 in intact cardiomyocytes was reversed by blockade of RyRs (see Fig. 6), which implies that RyR2 is positioned downstream of ROS/H_2O_2 to mediate the stimulatory effect of exogenous H_2O_2. Taken together, these findings led us to suggest that RyR2, presumably acting through mobilizing intracellular calcium required for activation of some calcium-dependent process(es), is a crucial mediator activated downstream of ANP/NPR-A/PKG/ROS signaling for functional modulation of K_{ATP} channels in ventricular cardiomyocytes.

Intriguingly, activation of the BNP/NPR-A/cGMP/PKG signaling pathway has been suggested to modulate $GABA_A$ receptors (a chloride channel) via induction of calcium release from ryanodine-sensitive intracellular stores in rat retinal ON-type bipolar cells (71). Evidence

implying a role of some intracellular calcium-dependent process in functional modulation of K_{ATP} channels has been reported before. For example, in our earlier work we have demonstrated that caffeine induces functional stimulation of the Kir6.2/SUR1 channel (a neuronal/pancreatic K_{ATP} channel isoform) expressed in HEK293 cells, in part via activation of some Ca^{2+}-dependent mechanism, as evidenced by attenuation of the effect by buffering of intracellular calcium with BAPTA/AM (41). Caffeine at millimolar concentrations activates the RyR (42), and therefore calcium released from intracellular stores (owing to RyR sensitization/activation) may partly account for the K_{ATP}-stimulating effect of caffeine (41). Intracellular calcium mobilization may also be involved in modulation of K_{ATP} channels in central neurons (56). Moreover, as we have previously shown, the stimulation of recombinant Kir6.2/SUR1 K_{ATP} channels by activation of PKG in intact cells is BAPTA/AM-sensitive, indicating that activation of PKG increases the function of K_{ATP} channels in an intracellular calcium-dependent manner (15). Our current finding that RyR2 activity was required for ANP-elicited stimulation of ventricular sarcK_{ATP} channels (see Fig. 5) is in accordance with cumulating evidence suggesting an involvement of intracellular calcium mobilization and subsequent recruitment of some intracellular calcium-dependent signaling partner(s) in functional modulation of K_{ATP} channels.

RyR2 channels are natural and avid substrates of CaMKII (13) and CaMKII may contribute to basal phosphorylation and activity of RyR2 (21). On the other hand, RyRs are also subject to redox modifications, through which RyR2 activity in cardiac cells is increased under physiologically relevant conditions (such as exercise) (23). Data from our current investigation unveiled a crucial role played by RyR2 in mediating H_2O_2 stimulation of ventricular K_{ATP} channels, suggesting that RyR2 is positioned downstream of ROS/H_2O_2 to mediate the stimulation of ventricular K_{ATP} channels (see Fig. 6). As mentioned earlier, our recent research

has suggested that activation of CaMKII or ERK2 is required for the stimulatory action of exogenous H_2O_2 on cardiac K_{ATP} channels and that activation/autophosphorylation of CaMKII evoked by PKG (activation) is likely mediated by ERK1/2 (72). Accordingly, we speculate that the ANP-K_{ATP} signaling pathway may enlist redox modification of the RyR (following ROS generation), which subsequently induces activation of CaMKII to modulate K_{ATP} channels; or alternatively, the ANP-K_{ATP} signaling pathway may encompass sequential activation of ROS, ERK, CaMKII and RyR2 (phosphorylated by CaMKII), allowing subsequent activation of some yet-to-identified calcium-dependent modulators for the channel (Fig. 8, a working model). The interaction among these multi-functional molecules could be more complex. Further research is required to help define the signaling sequence and interaction among these key mediators in the ANP-K_{ATP} signaling pathway.

ANP modifies the gating properties of ventricular sarcK_{ATP} channels to augment channel activity. Based on the open- and closed-duration distributions of sarcK_{ATP} channels in intact rabbit ventricular cardiomyocytes, we suggest that the ventricular myocardial K_{ATP} channel preactivated by sodium azide exhibits at least one open state and three closed states. Changes caused by ANP on the open- and closed-duration distributions (see Fig. 7) revealed that ANP treatment shifted the closed-duration distribution, by reducing the relative occurrence (i.e., the relative area) as well as the dwelling time (i.e., the time constant) of the longest closed state, and increased the closed-to-open transitions (i.e., the opening frequency), thereby elevating the *NPo* of ventricular sarcK_{ATP} channels. In contrast, neither the single-channel conductance nor the open duration was affected. These changes suggest that ANP exerts its modulatory effect on ventricular sarcK_{ATP} channels by altering the kinetic properties of channel gating, enhancing

channel function through destabilization the longest-duration closed conformation while facilitating the transitions from the closed to the open states.

In conclusion, here we report for the first time that the function of ventricular sarcK_{ATP} channels is positively modulated by ANP via an intracellular signaling pathway consisting of NPR-A, PKG, ROS/H_2O_2, RyR2, ERK1/2 and CaMKII, which process enhances the opening frequency whereas destabilizes the long closures of the channel, thereby heightening channel activity. The present study highlights the relevance of cGMP-dependent intracellular signaling mechanisms as effective regulators of myocardial K_{ATP} channel function. The partially overlapping intracellular signaling mechanisms induced by NO (72) and ANP for ventricular K_{ATP} channel modulation may reflect, to some degree, reinforcement between the ANP- and the NO-signaling systems in regulating cardiac function. Mechanistic understanding of K_{ATP} channel regulation may provide insights into the development or improvement of therapeutic strategies for the management of cardiovascular injury. ANP, K_{ATP} channels, ROS and ERK1/2 have all been implicated in cardiac tolerance against ischemic injury. Functional modulation of sarcK_{ATP} channels by ANP signaling may regulate cardiomyocyte excitability and contribute to endogenous cytoprotective mechanisms in the heart. The pleiotropic ANP also modulates K_{ATP} channels in several cell types beyond cardiomyocytes, such as in pancreatic β-cells (53, 65) and in vascular smooth muscle cells (34), evincing the importance of ANP, in part through functional modulation of K_{ATP} channels, in the homeostatic control under physiological and pathophysiological conditions.

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DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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. D.-M.Z. contributed to the collection and analysis of the data and preparation of the figures. D.-M.Z. and Y.-F.L. approved the final version of the manuscript.

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FIGURE LEGENDS

Fig. 1. Stimulation of sarcK_{ATP} channels by ANP in intact adult rabbit ventricular cardiomyocytes via NPR-A activation. *A-B*: Single-channel current traces of ventricular sarcK_{ATP} channels induced by the metabolic inhibitor sodium azide (3 mM) in cell-attached patches before (*upper panel*) and during (*lower panel*) application of ANP (100 nM) (*A*), or ANP plus the selective NPR-A antagonist anantin (1 μM) (*B*). 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 panel); the vertical scale bar represents 4 pA. *C*: Averaged relative channel activity (i.e., normalized open probability *NPo*) of ventricular sarcK_{ATP} channels (control taken as 1; *dashed line*). Number (n) in parenthesis represents the number of patches in individual groups (n = 5-16). $*P < 0.05$; $**P < 0.01$ (Student's two-tailed one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups). Ventricular sarcK_{ATP} single-channel activity was significantly enhanced by ANP and the enhancement was reversed by selective inhibition of NPR-A.

Fig. 2. ANP stimulates sarcK_{ATP} channels preactivated by K_{ATP} channel openers in intact cardiomyocytes. Cell-attached patches were obtained from adult rabbit ventricular myocytes. *A*: Single-channel current traces of ventricular sarcK_{ATP} channels induced by the K_{ATP} channel opener pinacidil (200 μM) before (*upper panel*) and during (*lower panel*) application of ANP (100 nM) in a representative patch. Recording settings and scale bars are the same as described in *Fig. 1*. *B*: Averaged relative activity of ventricular sarcK_{ATP} channels in response to ANP (n =

4). *$P < 0.05$. Application of ANP resulted in an increase in sarcK_{ATP} single-channel activity elicited by pinacidil in intact ventricular myocytes. These findings were in line with those obtained from K_{ATP} channels preactivated by the metabolic inhibitor sodium azide (see Fig. 1A and C), which collectively support our hypothesis that ANP positively modulates the function of ventricular sarcK_{ATP} channels in intact cells.

Fig 3. ANP stimulates sarcK_{ATP} channels in intact adult rabbit ventricular cardiomyocytes in a PKG- and ROS-dependent manner. *A-B*: Single-channel current traces of ventricular sarcK_{ATP} channels induced by sodium azide (3 mM) in cell-attached patches before and during addition of ANP (100 nM) plus the PKG inhibitor KT5823 (*A*), or ANP plus the ROS scavenger MPG (500 μM) (*B*). Recording settings and scale bars are the same as described in *Fig. 1*. *C*: Averaged relative channel activity in individual groups of patches (n = 5-16). *$P < 0.05$; **$P < 0.01$ (one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups). ANP-induced increases in ventricular sarcK_{ATP} single-channel activity were significantly abrogated by inhibition of PKG or scavenging of ROS.

Fig. 4. Stimulation of rabbit ventricular sarcK_{ATP} channels by ANP is mediated by ERK1/2 and CaMKII. *A-B*: Single-channel current traces of sarcK_{ATP} channels elicited by sodium azide (3 mM) in cell-attached patches before and during addition of ANP (100 nM) together with U0126 (10 μM) (*A*) or mAIP (1 μM) (*B*). Scale bars are the same as in *Fig. 1*. *C*: Averaged relative channel activity (n = 6-16). *$P < 0.05$; **$P < 0.01$ (one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups). ANP

stimulation of ventricular sarcK_{ATP} single-channel activity was prevented in the presence of inhibitors selective for ERK1/2 and CaMKII.

Fig. 5. ANP stimulation of sarcK_{ATP} channels in intact ventricular myocytes requires RyR activation. *A*: Single-channel current traces of sodium azide-induced sarcK_{ATP} channels in cell-attached patches before and during addition of ANP (100 nM) acquired following pretreatment with ryanodine (100 μM). Scale bars are the same as in *Fig. 1*. *B*: Averaged relative channel activity (n = 6-16). *$P < 0.05$; **$P < 0.01$ (one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups). The stimulatory effect of ANP on ventricular sarcK_{ATP} single-channel activity was suppressed by blockade of the RyR.

Fig. 6. Stimulation of ventricular sarcK_{ATP} channels by exogenous H_2O_2 in intact cardiomyocytes requires activities of RyR2. Cell-attached patches were obtained from adult rabbit ventricular cardiomyocytes. *A-B,* Single-channel current traces of ventricular sarcK_{ATP} channels induced by pinacidil (200 μM) before and during addition of H_2O_2 (1 mM) (*A*), or H_2O_2 plus ryanodine (100 μM) (*B*). Recording settings and scale bars are the same as described in *Fig. 1* of the main text. *C,* Averaged relative channel activity in individual groups (n = 3-5). *$P < 0.05$ (one-sample *t* test within groups, and one-way ANOVA followed by Dunnett's multiple comparison tests among groups). Treatment with exogenous H_2O_2 resulted in an increase in the single-channel activity of ventricular sarcK_{ATP} channels preactivated by K_{ATP} channel opener pinacidil, whereas the stimulatory effect of H_2O_2 was prevented by pretreatment with ryanodine, implying that RyR2 activation likely occurs downstream of ROS/H_2O_2 production in the ANP-

K_{ATP} signaling pathway in ventricular cardiomyocytes which ultimately leads to K_{ATP} channel activation.

Fig. 7. Effects of ANP on the open- and closed-duration distributions of sarcK_{ATP} channels in intact rabbit ventricular myocytes. *A-B*: Frequency histograms of open-duration (*left column*) and closed-duration (*right column*) distributions fitted from events before (*A*) and during (*B*) application of ANP (100 nM) in a representative cell-attached patch. Duration histograms were constructed as described in MATERIALS AND METHODS. ANP shortened the time constant of the longest closed component and reduced the relative area under this component; the open duration distribution was not affected.

Figure 8. Working model for the ANP signaling pathway that stimulates sarcK_{ATP} channels in intact ventricular cardiomyocytes. Signaling components involved are shown in rectangular or oval shapes (shaded); pharmacological reagents employed in the present study targeting individual signaling components are also depicted (on the left). Broken lines depict potential, alternative pathways. We suggest that ANP via activation of pGC-linked NPR-A leads to cGMP generation, which in turn activates PKG and triggers downstream signaling that consists of ROS, ERK1/2, CaMKII and RyR2, resulting in functional enhancement of sarcK_{ATP} channels.

Figure 1

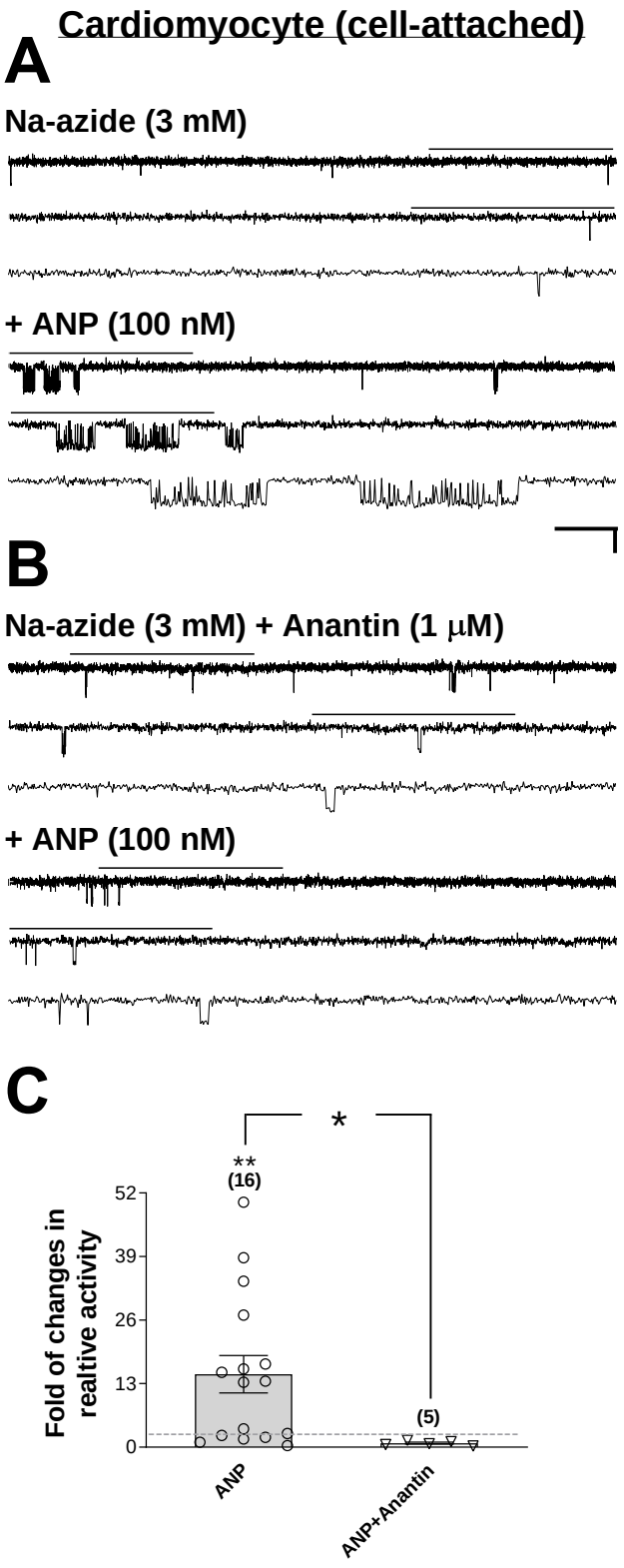

Cardiomyocyte (cell-attached)
A
Na-azide (3 mM)
+ ANP (100 nM)
B
Na-azide (3 mM) + Anantin (1 μM)
+ ANP (100 nM)
C
*
**
(16)
(5)
Fold of changes in realtive activity
52
39
26
13
0
ANP
ANP+Anantin

Figure 2

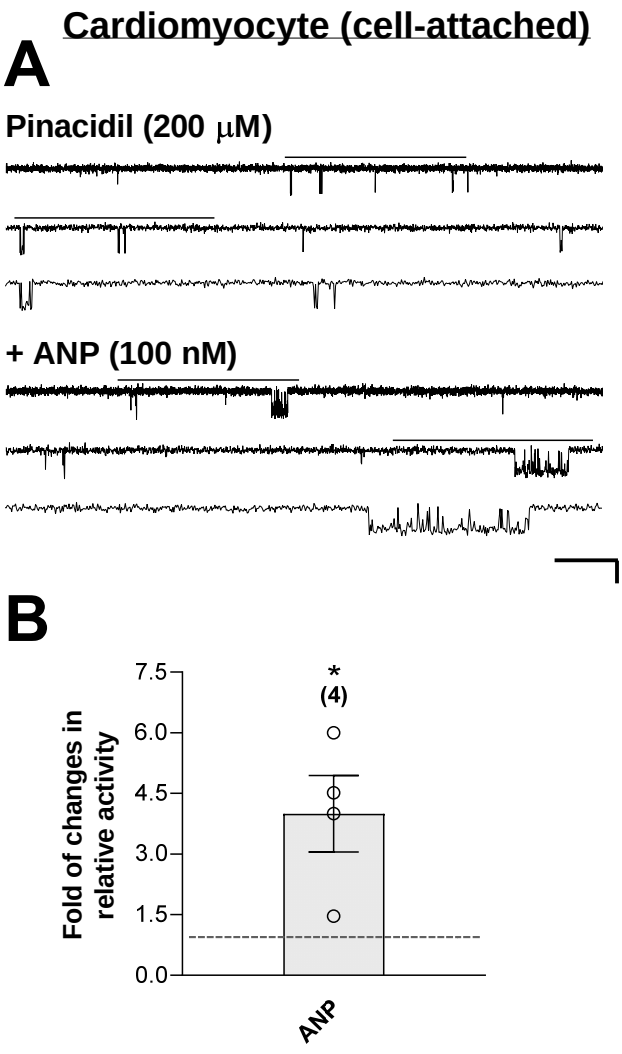

Cardiomyocyte (cell-attached)
A
Pinacidil (200 μM)
+ ANP (100 nM)
B
Fold of changes in relative activity
7.5
6.0
4.5
3.0
1.5
0.0
*
(4)
ANP

Figure 3

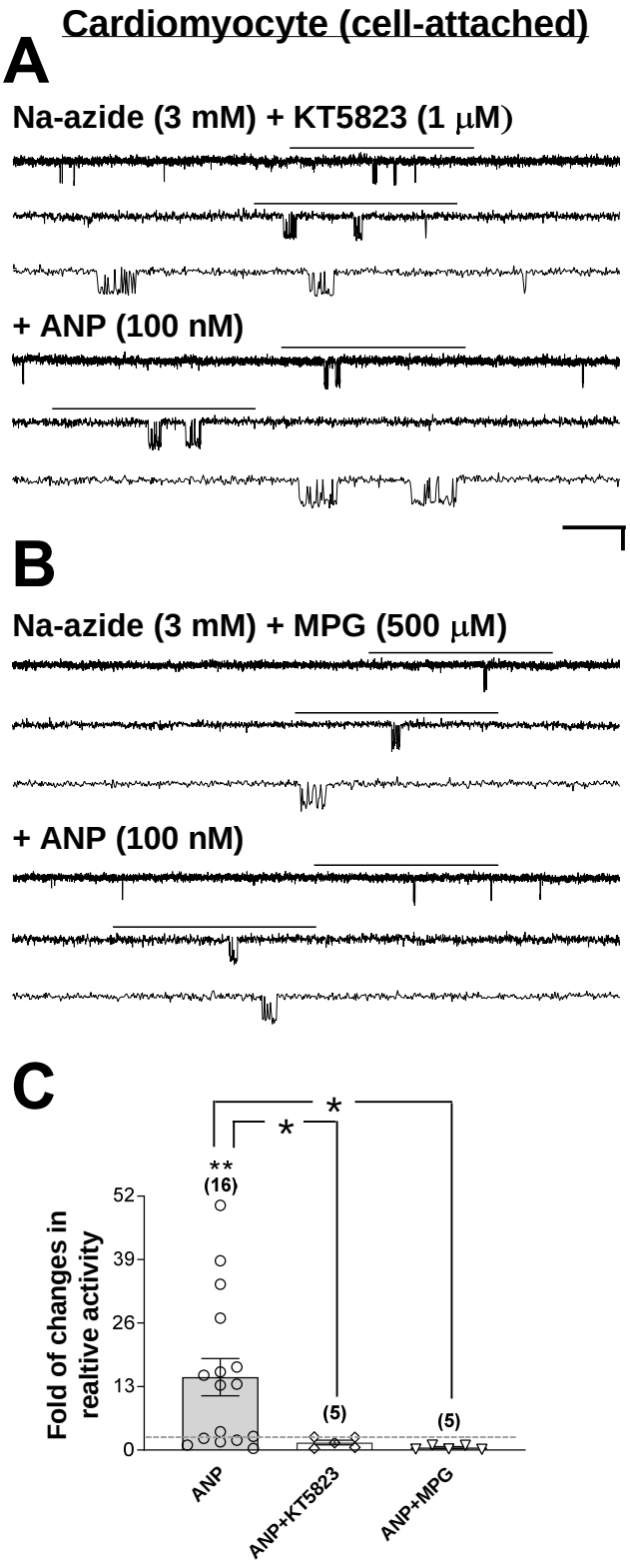

Cardiomyocyte (cell-attached)
A
Na-azide (3 mM) + KT5823 (1 μM)
+ ANP (100 nM)
B
Na-azide (3 mM) + MPG (500 μM)
+ ANP (100 nM)
C
Fold of changes in realtive activity
52
39
26
13
0
**
(16)
(5)
(5)
*
*
ANP
ANP+KT5823
ANP+MPG

Figure 4

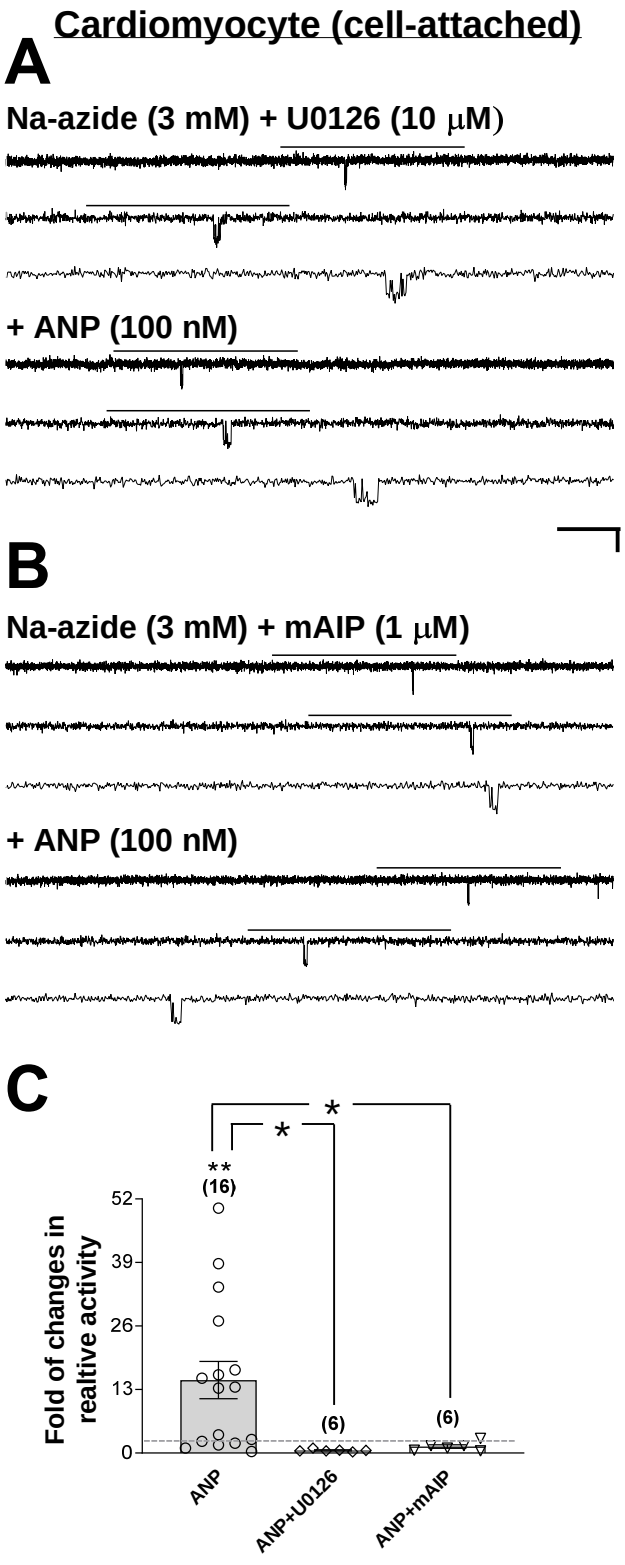

Cardiomyocyte (cell-attached)
A
Na-azide (3 mM) + U0126 (10 μM)
+ ANP (100 nM)
B
Na-azide (3 mM) + mAIP (1 μM)
+ ANP (100 nM)
C
Fold of changes in realtive activity
52
39
26
13
0
**
(16)
(6)
(6)
*
*
ANP
ANP+U0126
ANP+mAIP

Figure 5

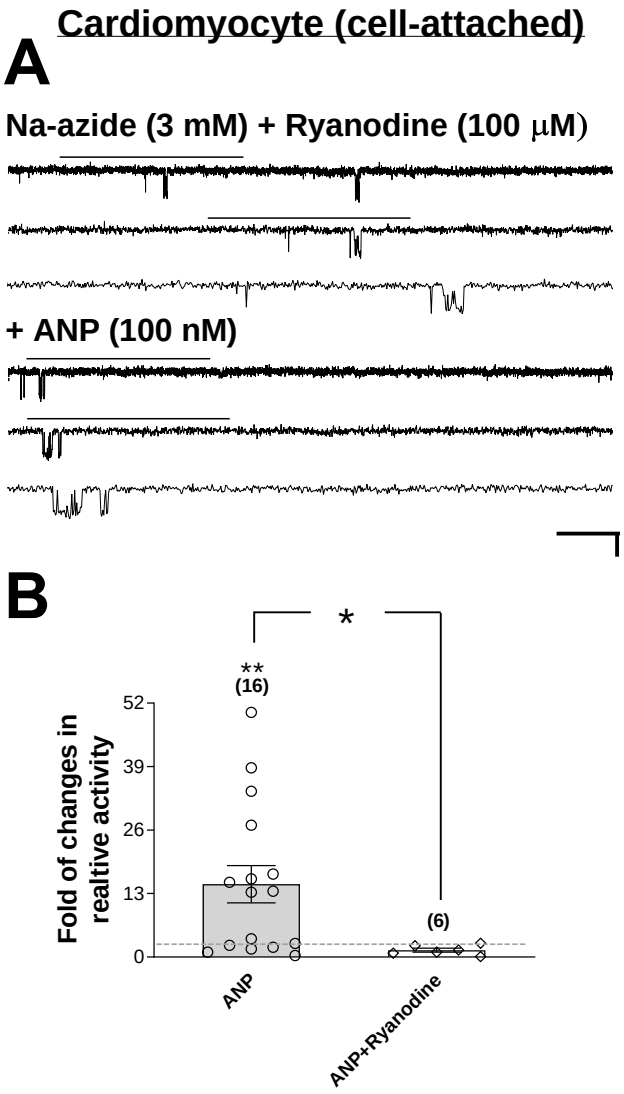

Cardiomyocyte (cell-attached)
A
Na-azide (3 mM) + Ryanodine (100 μM)
+ ANP (100 nM)
B
*
**
(16)
(6)
Fold of changes in realtive activity
52
39
26
13
0
ANP
ANP+Ryanodine

Figure 6

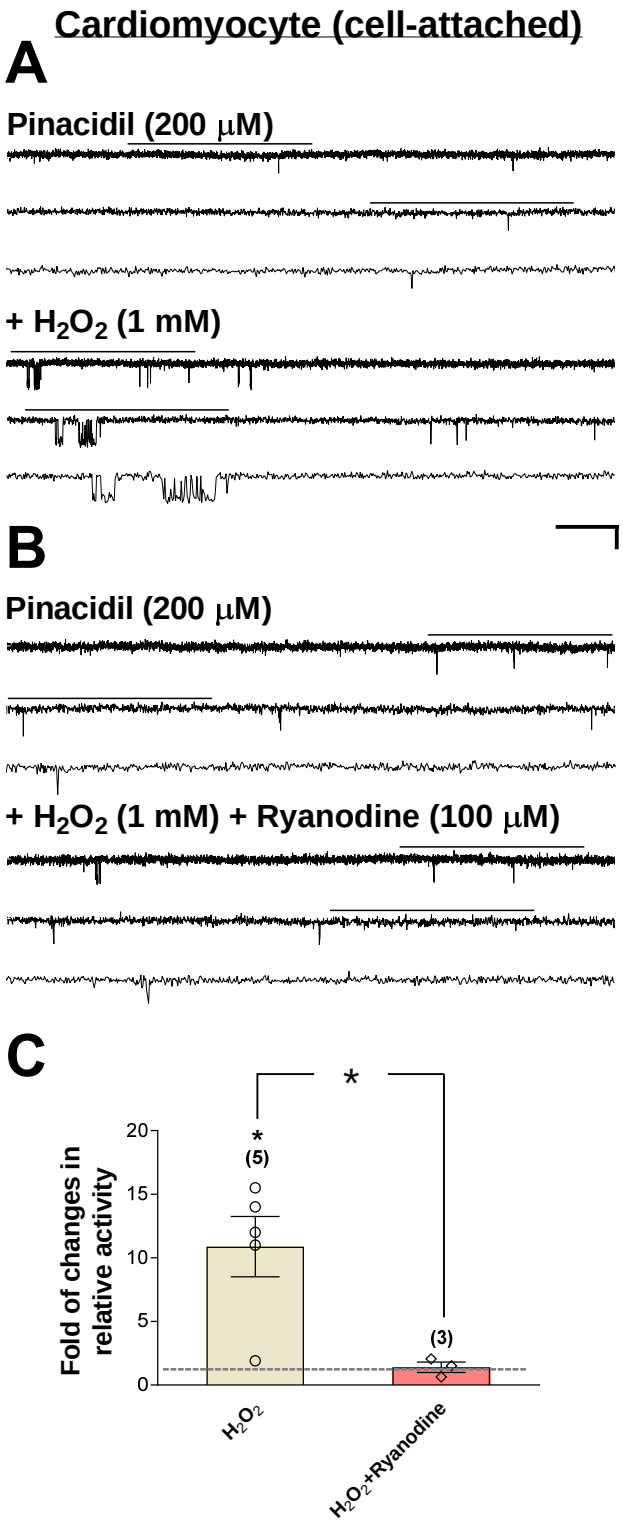

Cardiomyocyte (cell-attached)
A
Pinacidil (200 μM)
+ H2O2 (1 mM)
B
Pinacidil (200 μM)
+ H2O2 (1 mM) + Ryanodine (100 μM)
C
Fold of changes in relative activity
20
15
10
5
0
*
*
(5)
(3)
H2O2
H2O2+Ryanodine

Figure 7

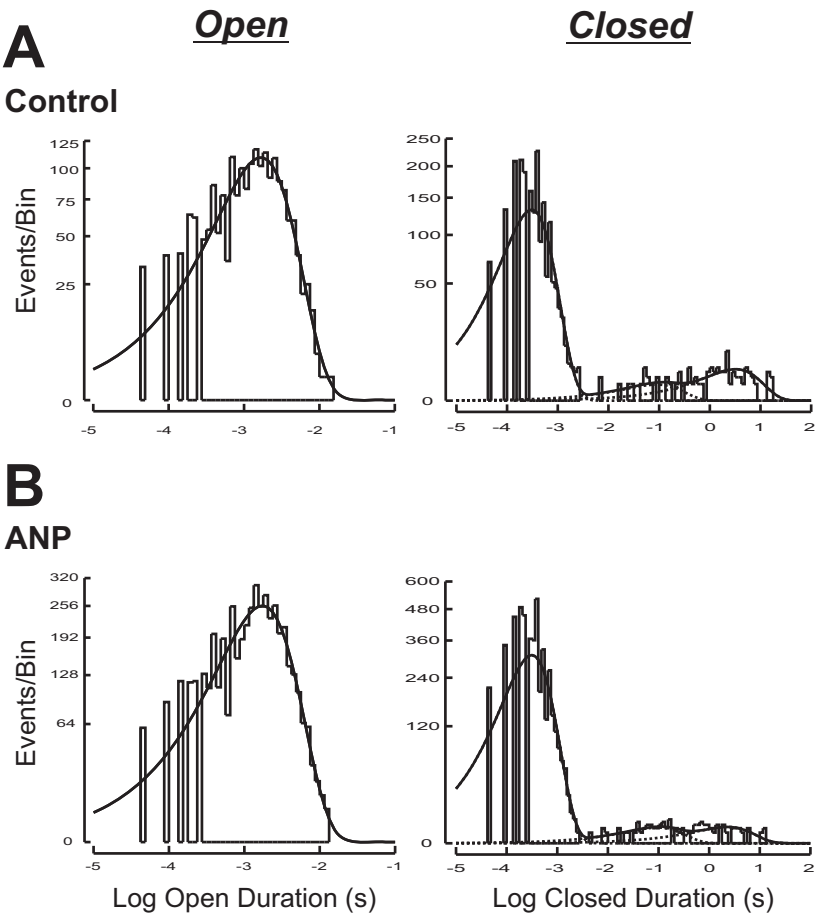
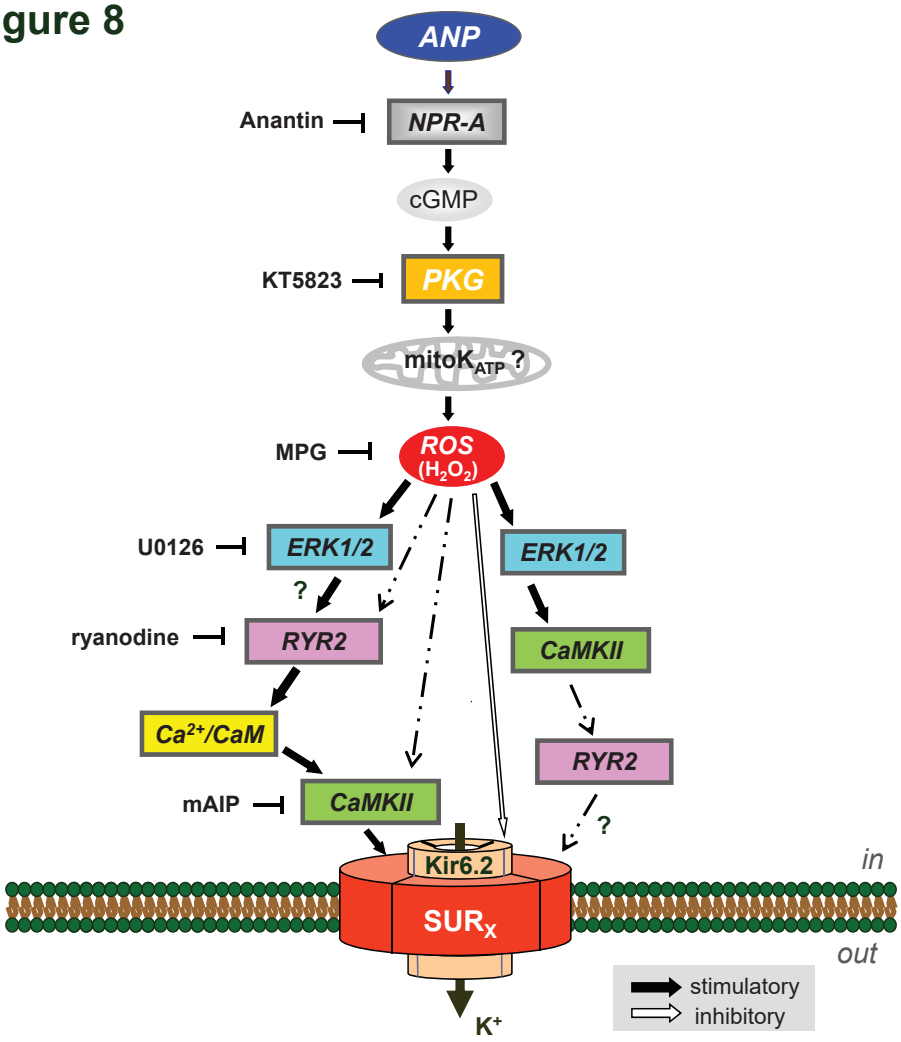
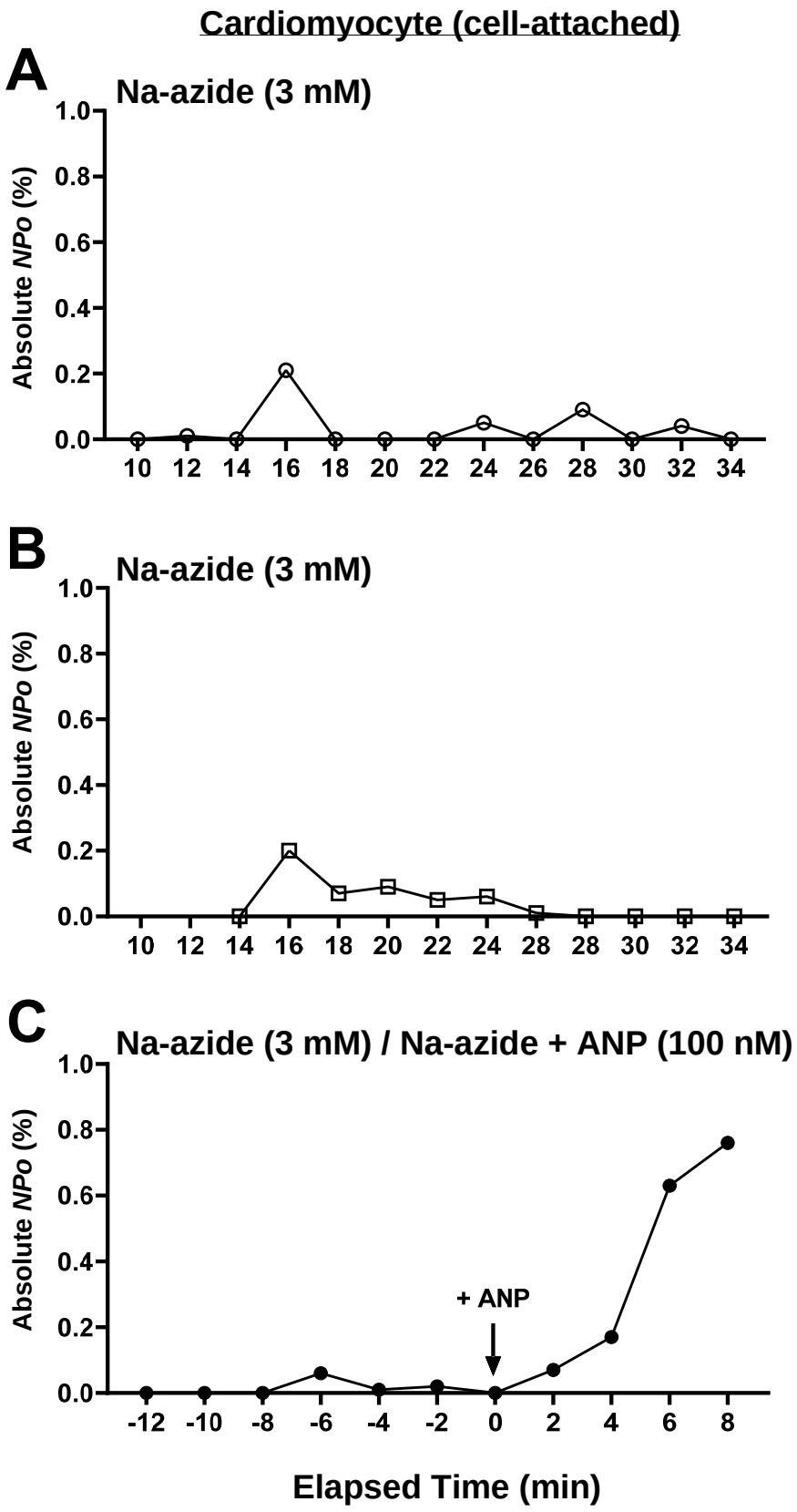

A
Open
Closed
Control
Events/Bin
125
100
75
50
25
0
250
200
150
100
50
0
-5
-4
-3
-2
-1
0
1
2
B
ANP
320
256
192
128
64
0
600
480
360
240
120
Log Open Duration (s)
Log Closed Duration (s)

Figure 8

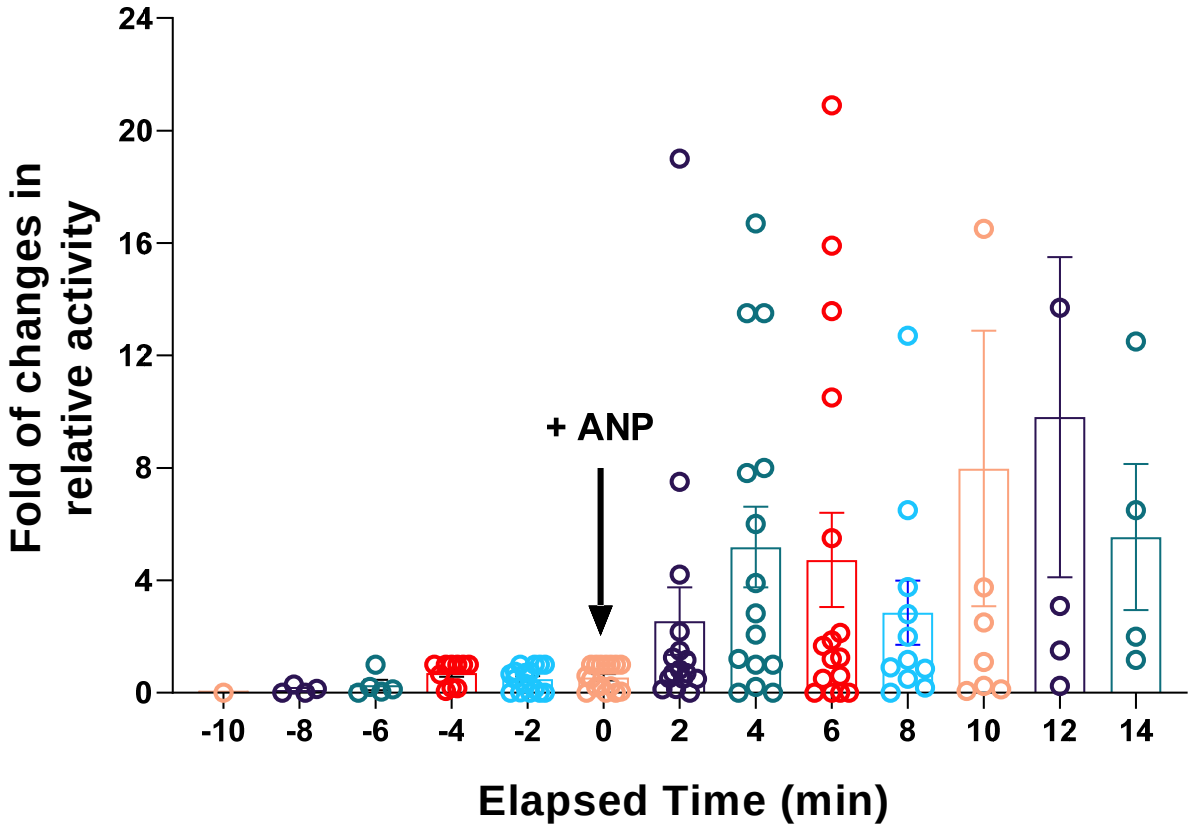

ANP
Anantin
NPR-A
cGMP
KT5823
PKG
mitoK$_{ATP}$?
MPG
ROS
(H_2O_2)
U0126
ERK1/2
?
ryanodine
RYR2
Ca^{2+}/CaM
mAIP
CaMKII
ERK1/2
CaMKII
RYR2
?
Kir6.2
SUR_X
in
out
K^+
stimulatory
inhibitory

Suppl. Figure 1



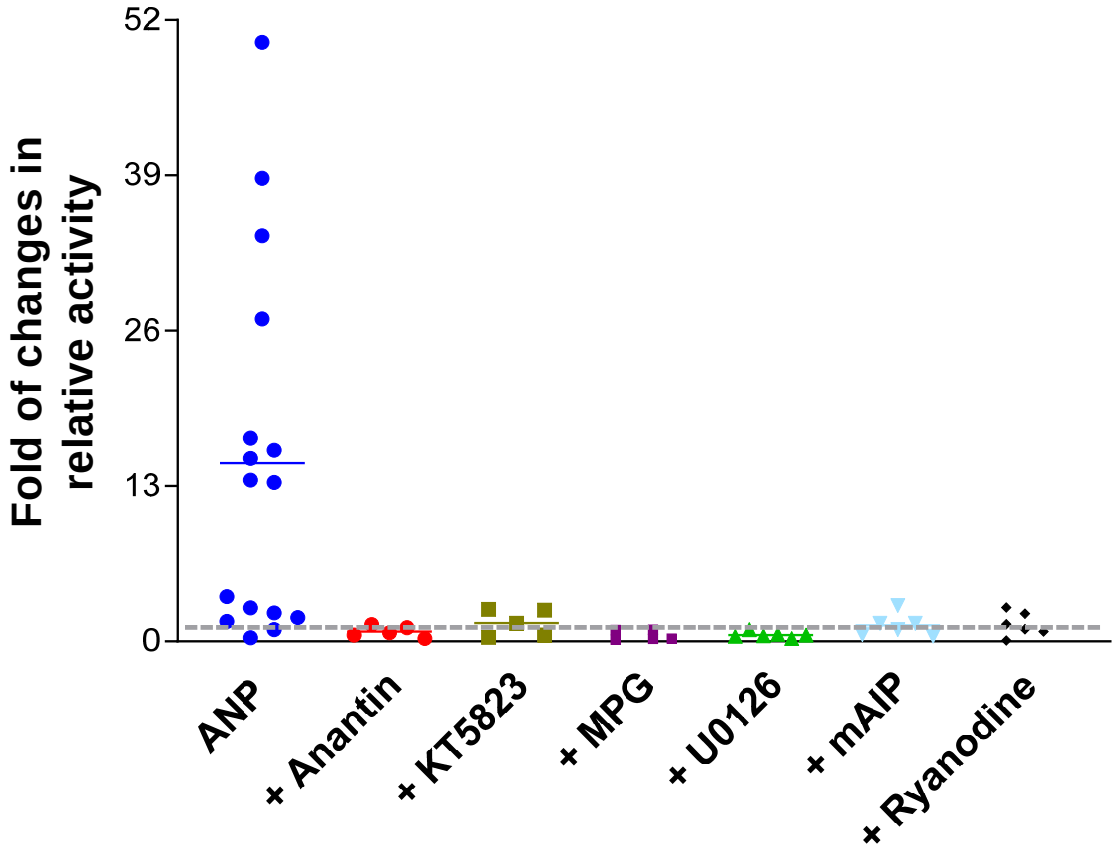
Supplemental Fig. 1. Time courses of changes in the absolute K_{ATP} channel activity induced by sodium azide in intact ventricular cardiomyocytes, with or without subsequent addition of ANP. Cell-attached patches were obtained from adult rabbit ventricular cardiomyocytes. Absolute channel activity (absolute *NPo*; without normalization) of sarcK_{ATP} channels acquired respectively from three representative cell-attached patches is displayed as a function of time during application of the metabolic inhibitor sodium azide (3 mM) alone (*A* and *B*), or during application of sodium azide (3 mM) followed by addition of ANP (100 nM) (*C*). The horizontal axis in *A* and *B* represents the elapsed time during sodium azide application, and in *C* the elapsed time relative to the time point at which ANP perfusion began (corresponding to the 28-min time point in sodium azide for this patch). In these and other cell-attached patches examined in the present study, K_{ATP} channel activity was not detected before application of sodium azide (or the channel opener pinacidil). In patches that were responsive to sodium azide treatment, channel openings usually appeared after sodium azide was administered for about 15-30 min, and it typically required another 10-15 min or longer in continuous sodium azide treatment to have the elicited baseline channel activity become relatively stable (i.e., no more upward changes by azide), ready for the subsequent addition of ANP (in the continuous presence of sodium azide). These time course plots indicate that the baseline channel activity (quantified as absolute *NPo*) induced by sodium azide was increased by addition of ANP in intact cells, whereas no comparable increase in the baseline channel activity was observed in the absence of ANP. These observations corroborate with findings described in the main text of the present study (see Fig. 1A and C) that ANP potentiates the activity of sarcK_{ATP} channels in intact ventricular cardiomyocytes.

Suppl. Figure 2



Supplemental Fig. 2. A scatter plot illustrating temporal changes in the relative activity of ventricular sarcK_{ATP} channels in response to ANP treatment. Effects of ANP (100 nM) on the relative channel activity of sarcK_{ATP} channels elicited by sodium azide (3 mM) were shown as a function of time in individual cell-attached patches obtained from adult rabbit ventricular cardiomyocytes. Sodium azide was administered first to induce baseline channel activity followed by addition of ANP in the continuous presence of sodium azide in individual patches to allow determination of ANP-induced changes at a same-patch basis. The relative channel activity values were obtained by normalizing the absolute *NPo* detected at each time point to the peak control acquired in sodium azide from the same patch (taken as 1). In this scatter plot, each symbol represents the relative activity value determined at respective time point before or during ANP application. The averaged relative channel activity values at each time point (relative to the start time point of ANP application) are displayed for reference (n = 4-16). The temporal distribution of the relative channel activity values indicates that ANP effectively augmented the baseline activity of ventricular sarcK_{ATP} channels induced by sodium azide. These results are supportive of the findings described earlier (Fig. 1 and Supplemental Figs. 1 and 2) that ANP enhances the function of ventricular sarcK_{ATP} channels in intact cardiomyocytes.

Suppl. Figure 3



Supplemental Fig. 3. Blockade of NPR-A, PKG, ROS, ERK1/2, CaMKII and RyR2 abolishes ANP modulation of K_{ATP} channels in individual cell-attached patches: *A scatter plot.* Effects of ANP signaling on the relative channel activity of rabbit ventricular sarcK_{ATP} channels preactivated by sodium azide in individual cell-attached patches obtained from adult rabbit ventricular cardiomyocytes are shown. ANP was administered by bath perfusion in the absence or presence of each of the following inhibitors: the specific NPR-A antagonist anantin, the selective PKG inhibitor KT5823, the ROS scavenger MPG, the selective MEK1/2 inhibitor U0126, the CaMKII inhibitory peptide mAIP, or ryanodine in individual groups of cell-attached patches. In this scatter plot, each symbol represents the *NPo* value obtained from the same cell-attached patch during application of ANP (100 nM) that has been normalized to the value obtained before ANP application (taken as 1) to denote changes in relative channel activity. The median relative channel activity value at 13.4 (depicted as a horizontal bar) in the ANP group (receiving no treatment of blockers for potential signaling partners) is also displayed. ANP elicited an increase in the single-channel activity of ventricular sarcK_{ATP} channels in intact cells, which effect was prevented in patches where the activity of NPR-A, PKG, ROS (generation), ERK1/2, CaMKII or RyR2 were pharmacologically blocked. The distribution of the individual values of relative channel activity obtained from patches within each drug treatment group validated the interpretation of data (Figs. 1-4 in the main text) that ANP enhances the function of ventricular sarcK_{ATP} channels via intracellular signaling mediated by NPR-A, PKG, ROS, ERK1/2, CaMKII and RYRs.