Heterogeneity of $I_{K1}$ in the mouse heart

Brian K. Panama, Meredith McLerie, and Anatoli N. Lopatin
Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan
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Panama BK, McLerie M, Lopatin AN. Heterogeneity of $I_{K1}$ in the mouse heart. Am J Physiol Heart Circ Physiol 293: H3558–H3567, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00419.2007.—Previous studies have shown that cardiac inward rectifier potassium current ($I_{K1}$) channels are heteromers of distinct Kir2 subunits and suggested that species- and tissue-dependent expression of these subunits may underlie variability of $I_{K1}$. In this study, we investigated the contribution of the slowly activating Kir2.3 subunit and free intracellular polyamines (PAs) to variability of $I_{K1}$ in the mouse heart. The kinetics of activation was measured in Kir2 concatemeric tetramers with known subunit stoichiometry. Inclusion of only one Kir2.3 subunit to a Kir2.1 channel led to an approximate threefold slowing of activation kinetics, with greater slowing on subsequent additions of Kir2.3 subunits. Activation kinetics of $I_{K1}$ in both ventricles and both atria was found to correspond to fast-activating Kir2.1/Kir2.2 channels, suggesting no major contribution of Kir2.3 subunits. In contrast, $I_{K1}$ displayed significant variation in both the current density and inward rectification, suggesting involvement of intracellular PAs. The total levels of PAs were similar across the mouse heart. Measurements of the free intracellular PAs in isolated myocytes, using transgenically expressed Kir2.1 channels as PA sensors, revealed “microheterogeneity” of $I_{K1}$ rectification as well as lower levels of free PAs in atrial myocytes compared with ventricular cells. These findings provide a quantitative explanation for the regional heterogeneity of $I_{K1}$.

IN CARDIAC MYOCYTES, the inward rectifier potassium current, $I_{K1}$, regulates the late phase of action potential (AP) repolarization and stabilizes the resting membrane potential. In most species, the inward current density of atrial $I_{K1}$ is significantly smaller than that of ventricles (4, 9, 24). The mouse heart is probably the only exception, with right atrial (RA) $I_{K1}$ as large as $I_{K1}$ in the ventricles and left atrial (LA) $I_{K1}$ even larger than that in the RA (14). Regional differences in ventricular $I_{K1}$ have also been reported, showing that both the inward current density (2) and also the strength of rectification (33) vary in different regions of the heart. While the critical role of $I_{K1}$ in cardiac excitability becomes more evident (29, 32, 36), the origin for $I_{K1}$ heterogeneity and its physiological role remain largely unknown.

$I_{K1}$ channels are believed to be homo- and/or heterotetramers of Kir2.1, Kir2.2, and Kir2.3 subunits from the Kir2 family of inward rectifier potassium channels (17, 27). A number of studies consistently indicated that the species-dependent (4) and tissue-specific expression (34) of different Kir2 subunits may contribute, at least in part, to its variability. The issue, however, remains highly controversial. For example, Liu et al. (13) showed by using single-channel analysis that, in guinea pig ventricle, Kir2.1 and Kir2.2 isoforms are the major contributors to $I_{K1}$. In contrast, Dhamoon et al. (4) found no Kir2.2 mRNA and protein; instead, a strong signal for Kir2.3 was observed. There is evidence that, in mouse ventricles, Kir2.1 is the major isoform with a significant contribution of Kir2.2, although knockout of both genes revealed the presence of another slowly activating component characteristic of Kir2.3 subunits (41).

It has also been suggested that variation in the concentration of free intracellular polyamines (PAs), the key determinants of rectification in $I_{K1}$ and Kir2 channels (15), may well be another factor. For example, Yan et al. (39) provided evidence that different concentrations of free PAs may underlie differences between atrial and ventricular $I_{K1}$ in the guinea pig heart (39). We have also previously observed that the strength of rectification varies greatly in individual mouse ventricular myocytes, but whether varying subunit composition of $I_{K1}$ or heterogeneity of free PAs underlies this phenomenon has not been established (20).

Presently, the interpretation of electrophysiological data primarily rests on the premise that the composition of heteromeric $I_{K1}$ and Kir2 channels can be derived from the known properties of underlying subunits and generally assumes the invariant concentration of free intracellular PAs and other determinants of rectification (e.g., Mg$^{2+}$). Nevertheless, although it is firmly established that Kir2 subunits may form heteromers in exogenously expressing systems (31), and likely in myocytes (35, 42), their specific contribution to the properties of the channel is largely unknown. To address this issue, we have created Kir2 concatemeric tetramers with known stoichiometry and subunit arrangement with the goal to understand the contribution of the slowly activating Kir2.3 subunits to channel kinetics. Most of the previous studies, including our own early work (15, 19), where $I_{K1}$ or Kir2 current activation was quantified in some manner, show that this activation is simply fast and thus have not exploited the idea that quantitative differences in activation kinetics may be useful in understanding the subunit composition of $I_{K1}$. Recently, this approach has been successfully implemented by Yan et al. (39) who showed that, in guinea pig heart, activation rates of $I_{K1}$ in both ventricles and atria match that of “fast” Kir2.1 channels. In this study, we have applied this approach to mouse heart and also expanded analysis to major regions of the ventricles. We took advantage of transgenic mice overexpressing Kir2.1 channels and used them as PA sensors to understand the contribution of free intracellular PAs to the regional variability of $I_{K1}$.

MATERIALS AND METHODS

Animals. Transgenic mice overexpressing a Kir2.1-green fluorescent protein (GFP) fusion construct (TG-Kir2.1-GFP) were previously produced and characterized (12). All experiments with mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy, First published September 21, 2007; doi:10.1152/ajpheart.00419.2007.

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1996) and were approved by the University of Michigan Committee on Use and Care of Animals.

**Isolation of mouse ventricular cardiomyocytes.** Ventricular cardiomyocytes were isolated from the hearts of adult wild-type and transgenic mice (2–5 mo old) of either sex anesthetized with Avertin, using collagenase treatment as described previously (25). Minor modifications included the use of 10 μM EGTA in the Ca2+-free solution. The myocytes were stored at room temperature in modified Tyrode (see Solutions, below) supplemented with 150 μM CaCl2 and 0.5% BSA and used in experiments within 1–5 h postisolation.

**Isolation of mouse atrial cardiomyocytes.** To isolate atrial myocytes, the freshly excised heart was washed in ice-cold modified Tyrode containing 0.1 mg/ml heparin (Sigma) and mounted on a Langendorff apparatus where it was perfused retrogradely with a Ca2+-free modified Tyrode solution at room temperature for 4 min. The atria were removed and washed separately in ~5 ml of the Ca2+-free solution three times to remove traces of blood. Atria were then incubated at 37°C for 45 min in 2–3 ml of modified Tyrode solution containing 10 μM CaCl2. Collagenase Type II (0.6 mg/ml; Worthington), protease (0.21 mg/ml; Sigma, Type XIV), and hyaluronidase (0.28 mg/ml; Sigma). Digested atrial tissue was triturated in KB solution (see Solutions) to yield isolated myocytes. Myocytes were used in experiments within 1–5 h postisolation.

**Cloning of Kir2.x channels.** Kir2.x subunits were cloned from mouse genomic DNA using a PCR-based technique (28) and then subcloned into either a pRES-EGFP vector (Clontech) for GFP coexpression studies or into a pBlueScript SK(−) vector (Stratagene) for further molecular operations.

**Creating concatameric Kir2.x constructs.** Kir2 tetrameric concatamers were made using a PCR-based technique and pBluescript SK(−) as a cloning vector. Four subunits were linked in tandem NH2 to COOH termini using 8–10 amino acid (primarily glutamines) linkers, each containing a specific restriction site. Master constructs of homomorphic Kir2.1 and Kir2.3 concatamers were created, each having identical restriction sites at four equivalent positions: XhoI-(1)-ClaI-(2)-EcoRV-(3)-PvuII-(4)-BamHI (1, 2, 3, and 4 are for respective positions of Kir2.x subunits in the construct). The PvuII and ClaI restriction sites in Kir2.2 and Kir2.3 coding sequences were eliminated by silent mutations to make them available for easy “cut-and-paste” cloning operations. From these master constructs, any specific arrangement of Kir2.x subunits in a heteroconcatemer is easily obtained by cutting out a desired subunit at any position and placing it into an equivalent position in another concatemer using the same restriction enzymes. The final concatemer is then subcloned into a pRES-EGFP plasmid using XhoI and BamHI restriction sites.

**HEK293 cell transfection.** HEK293 cells were cultured in DMEM supplemented with 10% calf serum and 1% antibiotic/antimycotic liquid (Invitrogen) at 37°C and 5% CO2. Cells were plated on 22 × 22-mm untreated glass coverslips in 35-mm petri dishes ~30 h before transfection. Cells were transfected with DNA constructs (1–4 μg) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. After the 4–6 h required for DNA uptake into the cells, the transfection solution was replaced with fresh media, and 5 mM sodium butyrate (Sigma) was added for 12 h to increase expression (26). The cells were used for recordings 1–3 days post-transfection.

**Electrophysiology.** Ionic currents were recorded in whole cell and patch configurations (10), using an Axopatch 200B amplifier, Digi- data 1322A, and pClamp 8.2 software (Axon Instruments). Patch pipettes (Kimble glass; no. 73813) were pulled on a horizontal puller (Sutter Instruments). The electrophysiological setup was as previously described (23). Pipette resistance varied from 1 to 2 MΩ. Applied voltages were not corrected for a liquid junction potential of ~3 mV.

**Whole cell cardiomyocyte recordings.** Isolated myocytes were bathed in modified Tyrode supplemented with 300 μM CaCl2, 2% BSA, and 2 μM nicardipine to block L-type Ca2+ channels. Pipettes were filled with KINT-ATP solution (see Solutions, below). Only noncontracting rod-shaped myocytes that had a clear striated pattern were utilized. In all recordings, the series resistance (Rs) was compensated >80%, and cells with an uncompensated Rs >4 MΩ were discarded from analysis. Recordings began within 1–3 min after establishing the whole cell configuration to minimize the washout of endogenous PAs and Mg2+. Steady-state IK1 was measured at the end of 180-ms voltage steps from a holding potential (HP) of ~30 mV to potentials between 0 and ~130 mV at 5-mV increments (0.4- to 0.5-s delay between sweeps). IK1 was isolated by subtraction of the current remaining after application of 500 μM BaCl2 from the total current. Alternatively, a zero K+ extracellular solution was used to isolate IK1 (both methods produced identical results). Kinetics of IK1 activation was measured in response to 10- to 125-ms hyperpolarizing steps from an HP of ~30 mV down to ~125 mV with 5-mV increments. The P5 procedure with a subsweep HP between ~30 and +10 mV was used to subtract the capacitative and leak currents in most experiments. In early experiments, zero K+ or 500 μM BaCl2 subtractions were performed as well. All mentioned techniques produced indistinguishable results, and therefore all relevant data were pooled together. Acetylcholine-sensitive K+ currents (IKach) were recorded at ~100 mV HP and were activated by applying 10 μM acetylcholine (Sigma) to the bath solution. For IKach experiments, 0.2 mM GTP was included in the pipette solution (KINT-ATP).

**Whole cell HEK293 cell recordings.** Whole cell recordings in HEK293 cells were similar to those in cardiomyocytes with the following exceptions: 1) FVPP solution (see Solutions, below) supplemented with 300 μM spermine (Spm) was used as the pipette solution, and 2) no BSA or nicardipine was included in the bath solution.

**Patch recordings in TG-Kir2.1-GFP transgenic cardiomyocytes.** Cardiomyocytes were bathed in FVPP-ATP solution, and pipettes were filled with KINT solution. Myocytes were kept in the recording chamber no longer than 10 min to minimize their exposure to the high-K+ FVPP-ATP. IK1 was recorded using a voltage ramp or a series of 150-ms voltage steps to potentials ranging from ~100 mV (~90 mV) to +70 mV, with an HP of 0 mV. Voltages for half block (V1/2; see Fig. 3A) were then estimated as described in Data analysis, below. To construct a dose-response curve for Spm block (Fig. 4B), transgenically expressed TG-Kir2.1-GFP channels (see Fig. 4B) were first excised into PA-free FVPP-ATP solution to wash out endogenous PAs, and then FVPP-ATP solutions supplemented with 100 and 300 μM total Spm were applied. FVPP-ATP solution dramatically slows down rundown of IK1 and Kir2 channels, and added ATP completely blocks ATP-sensitive K+ channels present in cardiac myocytes. FVPP-ATP solution contains components that strongly bind PAs (ATP, pyrophosphate), and therefore concentration of free Spm is lower than total concentration. Additional dose-response curves for Spm block were constructed using stable patches of Kir2.1-GFP channels expressed in HEK293 cells and exposed to KINT solution with a known concentration of free Spm (KINT does not have PA binding components) and FVPP-ATP solution with a known total concentration of Spm.

**Sample preparation for PA assay.** Mice (1–4 mo old) of either sex were used for PA analysis by HPLC. Hearts from anesthetized animals were rapidly excised and perfused retrogradely through the aorta with PBS for 5 min. The ventricles and atria were sectioned out, carefully blotted out of PBS on fine paper towel, weighed (wet), and frozen in liquid nitrogen until further use. For mouse atria, four to five atria were pooled and weighed together to reduce the weighing error because of their small mass. About 20–30 mg of the tissue were homogenized in 50 ml/g tissue of 5% HCl-5% trichloracetic acid, and 10 μl of internal standard of 0.1 mM diaminoheptane (DAH; Sigma) was added. The sample was stored overnight at 4°C. The homogenate was then centrifuged at 11,500 g for 30 min at 4°C. The supernatant was extracted three times with two volumes of ethyl acetate, lyophilized, and reconstituted in 200 μl of sterile deionized...
water. The primary and secondary amine groups on the PA molecules in the sample were derivatized with 6-amino-quinolinyl-N-hydroxysuccinimidy1 carbamate (Waters AccQ-Fluor reagent kit) (3) to produce a highly stable urea derivative that strongly fluoresces at 395 nm (3) and thus can be used for quantitative analysis (5, 25).

**HPLC.** Derivatized Spm, spermidine (Spd), and putrescine (Put) were separated and quantified using reverse-phase liquid chromatography (Waters, Xterra MS C18 4.6 × 150 mm, 3.5-mm analytical column and equivalent in a 3.9 × 20-mm guard column at 36°C). The column temperature was controlled by an HPLC integrated column heating system (Eppendorf Scientific, model no. CH-500). A two-pump gradient system (Waters Solvent Delivery System, model no. 510) delivered 10 mM sodium acetate, pH 5 (mobile phase A; MPA), and 60 mM acetonitrile in HPLC-grade water (mobile phase B; MPB) in the following gradient (%MPA/%MPB) at 1 ml/min: 0–5 min, 90/10 to 70/30; 5–33 min, 70/30 to 40/60; 33–34 min, 0/100; 34–37 min, 0/100; 37–38 min, 90/10; and 38–48 min, 90/10. Blanks (no PAs), standards, and samples in a 10-μl volume were injected automatically (Waters Intelligent Sample Processor, model no. 712), and fluorescent peak height and area (250-nm excitation, 395-nm emission; Waters Scanning Fluorescence Detector, model no. 474) were calculated. PA standards in the range of 2–50 μM were used to determine appropriate run times for specific PAs as well as to construct calibration curves and determine the limit of detection. Waters Millennium Chromatographic Manager software was used for acquisition and analysis. The PA amount (nmol/g wet tissue) was calculated by comparison of the PA-to-DAH peak area ratio in the sample to that in a standard containing 10 μM each PA and the same amount of DAH. The data were corrected for differences in extracellular space (ECS) using values obtained in rabbit by Poole-Wilson and Cameron (30), who estimated both atrial and ventricular ECS in the same study: 18.8% (left ventricle; LV), 22.4% (right ventricle; RV), and 28.6% (atria). We could not locate ECS values for the mouse heart, and only data for estimated both atrial and ventricular ECS in the same study: 18.8% (left ventricle; LV), 22.4% (right ventricle; RV), and 28.6% (atria). We could not locate ECS values for the mouse heart, and only data for

**Solutions.** Solutions are as follows: 1) modified Tyrode (in mM), 137 NaCl, 5.4 KCl, 0.5 MgCl2, 0.3 CaCl2, 0.16 NaH2PO4, 3 NaHCO3, 5 HEPES, 5 glucose, pH = 7.35 with NaOH; 2) KINT (in mM), 140 KCl, 1 EGTA, 10 HEPES, pH = 7.35 with KOH; 3) KINT-ATP (in mM), KINT + 5 K2ATP, pH = 7.35 with KOH; 4) FVPP (in mM), 95 KCl, 0.1 Na2VO4, 10 K2PO4, 5 KF, 10 HEPES, 1 EGTA, pH = 7.35 with KOH; 5) FVPP-ATP (in mM), FVPP + 2 K2ATP; and 6) KB (in mM), 75 KCl, 5 NaCl, 5 MgSO4, 30 K2PO4, 20 glucose, 0.5 EGTA, 5 K2ATP, 5 sodium pyruvate, 5 creatine, 20 taurine, 5 hydroxybutyrate, 1 mg/ml BSA, pH = 7.35 with KOH.

Data analysis. Data analysis was performed using Microsoft Excel (2000) and Clampfit 8.2 (Axon Instruments, Foster City, CA). To quantify the PA block, the relative current (I_{REL}) was determined as a ratio of the current in the presence of PAs and the current estimated using a linear approximation: I_{REL} = \lambda(V_{M} - V_{REV}), where V_{M} is membrane potential, V_{REV} is reversal potential, and the slope conductance (\lambda) is equal to that of corresponding chord conductance at membrane potential of −100 or −80 mV (18). In experiments with cell-attached patches, I_{REL} was fit with the sum of two Boltzmann functions

I_{REL} = \frac{A_{1}}{1 + \exp\left[\frac{Z_{1}(V_{M} - V_{1/2})}{RT}\right]} + \frac{A_{2}}{1 + \exp\left[\frac{Z_{2}(V_{M} - V_{1/2})}{RT}\right]},

where A_{1}, Z_{1}, and V_{1/2} are amplitudes, effective valences, and voltages of half block for the steep (more positive V_{1/2}) and shallow (more negative V_{1/2}) components of the block, respectively, R, F, and T have their usual values. In experiments using whole cell configuration, a fit with a single Boltzmann function was sufficient because of a significantly smaller range of applied potentials negative to the reversal potential. Accordingly, the V_{1/2} value for the steep component of I_{REL} was labeled V_{1/2}^{A}, V_{1/2}^{C}, and V_{1/2}^{D} for whole cell, cell-attached, and inside-out recordings, respectively. Fitting of the data was limited to potentials negative to V_{REV}.

Values are reported as means ± SE. A two-tailed unpaired t-test with equal variances was used for determinations of statistical significance when comparing two means. A one-way ANOVA followed by a Bonferroni posttest was used for multiple comparisons.

**RESULTS**

Kinetic properties of the Kir2.3 subunit are not dominant. We wanted to utilize the unique slow-activation kinetics of the Kir2.3 subunit as a marker for its presence in native IK1 channels. Although homomeric Kir2.3 channels activate severalfold more slowly than Kir2.1 and Kir2.2 channels (28, 39), to employ this property of the Kir2.3 subunit, one needs to know whether and to what degree the Kir2.3 subunit contributes to channel kinetics.

To precisely control the subunit composition of Kir2 channels, we created Kir2 concatemeric tetramers by linking Kir2 subunits in various combinations. This enabled us not only to control the stoichiometry of channel assembly but also to control the specific arrangement of Kir2 subunits within the channel tetramer. Activation kinetics was measured in the whole cell configuration at a physiological extracellular K−.

Under these conditions, channel activation observed at hyperpolarized potentials displays a characteristic pseudoinstantaneous phase (11, 28) followed by a much slower monoeXponential current increase (Fig. 1A). All tested constructs displayed a pseudoinstantaneous component of various degree. The nature of this phenomenon is not clear, and thus only slow exponential activation, determined primarily by intracellular Spm, was analyzed. Additionally, inactivation of inward currents, commonly observed in cardiac myocytes at far hyperpolarized membrane potentials (Fig. 2), was absent.

First, we confirmed that covalent linking does not affect the channel kinetics. Figure 1B shows that the activation kinetics between monomeric Kir2.1 channels (2.1-mono) and homomorphic Kir2.1 concatemers (1-1-1-1) are indistinguishable. Additionally, no differences were found between 2.2-mono channels and 2-2-2-2 concatemers (not shown). We have recently shown that activation kinetics of Kir2.1-mono and Kir2.2 mono channels are indistinguishable (28). Unfortunately, the activation kinetics of a 3-3-3-3 concatemer could not be measured reliably because of a very low level of expression (also characteristic for Kir2.3 monomeric subunits).

Figure 1B shows that the contribution of Kir2.3 subunits to channel kinetics is neither dominant nor recessive. Activation becomes slower as the number of Kir2.3 subunits is increased in a Kir2.1/Kir2.3 concatemer, although not "proportionally" (Fig. 1B). For example, the activation of channels containing three Kir2.3 subunits (3-3-3-1) is nearly the same a that in channels with only two Kir2.3 subunits (3-3-1-1). We also compared the activation kinetics in 3-1-1-1 vs. 1-1-1-3 concatemers and found that the positioning of the Kir2.3 subunit at either the NH3 or COOH terminus makes no difference. Concatemers with the same stoichiometry but with different symmetry (e.g., 3-3-1-1 vs. 3-1-3-1) showed identical activation kinetics as well.
Kinetics of $I_{K1}$ activation is fast throughout the mouse heart. To investigate whether Kir2.3 subunits may contribute to $I_{K1}$ in a region-dependent manner, we measured activation kinetics in cardiac myocytes isolated from LV, RV, RA, and LA tissues. Figure 2A shows representative $I_{K1}$ recorded from ventricular and atrial myocytes. Similar to the exogenously expressed Kir2 channels, cardiac $I_{K1}$ also displayed a pseudoinstantaneous component of activation (not fit). In ventricular myocytes, inward $I_{K1}$ at far hyperpolarized potentials displayed pronounced inactivation, which was nearly absent in atrial myocytes. To account for this inactivation, which primarily arises from depletion of K$^+$ in the narrow t-tubular space (1), currents were fit with two exponentials and the activation tau at $-115$ mV plotted in Fig. 2B. Consistent with the low level of Kir2.3 mRNA in the mouse ventricular tissue (41) (no data are available for atria), the kinetics of $I_{K1}$ activation resembled closely those of Kir2.1 and Kir2.2 channels in all tested tissues. An $\sim25\%$ slower activation time in RA vs. LA was not statistically significant.

Variation of $I_{K1}$ density and strength of rectification. There was no difference between the reversal potential of $I_{K1}$ (Ba$^{2+}$-sensitive current) in LA and RA myocytes, although a small difference of $\sim0.6$ mV between LV and RV myocytes was found. Surprisingly, the data show that the reversal potential of $I_{K1}$ in ventricular and atrial myocytes differs by 5.3 mV: $-70.3 \pm 0.3$ mV ($n = 33$) and $-75.6 \pm 0.8$ mV ($n = 26$; $P < 0.001$), respectively (the data from LV and RV ventricular myocytes as well as from LA and RA myocytes were pooled together). Thus, to compare the $I_{K1}$ densities in these tissues, the current amplitudes of inward $I_{K1}$ were measured at potentials $-30$ mV negative to the respective resting potentials. The steady-state current densities of inward currents were not significantly different between ventricles; however, they were, for example, 55 and 157% higher in RA and LA, respectively, compared with LV (14).

Accordingly, the amplitudes of outward $I_{K1}$ were measured 20 mV positive to the respective resting membrane potentials. $I_{K1}$ densities were not significantly different between ventricles; however, they were, for example, 55 and 157% higher in RA and LA, respectively, compared with LV (14).

![Fig. 1. Activation kinetics in heteromeric Kir2 channels. A: representative whole cell recordings from HEK293 cells, at physiological K$^+$, expressing 1-1-1-1 and 3-3-1-1 Kir2 concatemeric channels. Currents are recorded in the presence of 5.4 mM extracellular K$^+$ in response to voltage steps from $-30$ mV holding potential to $-125$ mV in 10-mV increments (see MATERIALS AND METHODS). Monoexponential fits are superimposed with the current traces and extended at the start of the activation to highlight the quasi-instantaneous phase of activation (dashes at left of recordings). B: activation tau (τ) for Kir2.1/Kir2.3 concatemeric channels of different stoichiometry and subunit arrangement was obtained from currents recorded at $-100$ mV. Currents from a 3-3-3-3 Kir2 concatemer could not be reliably measured because of a low expression level. n = 3–6 for individual constructs. Mono, monomeric.](image1)

![Fig. 2. Activation kinetics of inward rectifier potassium current ($I_{K1}$). A: representative whole cell current recordings of $I_{K1}$ from ventricular and atrial myocytes in the presence of physiological K$^+$. Currents were recorded and fit with a 2-exponential function to account for the slow inactivation of $I_{K1}$ at far negative potentials (*) (see MATERIALS AND METHODS). Fits may not be clearly visible because of overlap with raw traces. Selected traces starting at $-125$ mV down to depolarized potentials using 10-mV increments are shown. B: the activation tau (τ) measured at $-115$ mV in myocytes isolated from different regions of the heart and that of cloned mouse Kir2.1 channels are not significantly different. n = 12 (left ventricle; LV), 8 (right ventricle; RV), 7 (right atrium; RA), and 7 (left atrium; LA).](image2)
Rectification ratio \([RR = I_{K1}(−100)/I_{K1}(−50)]\) was similar between LA and RA. However, in parallel with increased outward current densities in both atria, RR was decreased by \(-26\text{–}32\%\) compared with that in ventricles (Table 1), indicating weaker rectification. The averaged RR varied only moderately between different regions of the heart, in sharp contrast to the significantly larger differences observed among individual myocytes isolated from any specific region of the heart. For example, the pooled data from all analyzed myocytes show that \(I_{K1}\) densities at \(-100\text{ mV}\) varied manifold from 4.9 to 46.8 pA/pF \((n = 59\text{ cells})\), and RR ranged from about \(-5\) to \(-50\).

There are several factors that may potentially contribute to the estimation of \(I_{K1}\) rectification in different regions of the heart. In particular, \(I_{KACa}\) may contaminate currents recorded in atrial cells, and different procedures used for isolation of ventricular and atrial myocytes may affect the balance of PAs or some other relevant parameter. Detailed quantitative analysis of these issues can be found in the Supplemental Materials (supplemental data are available at the online version of this article).

Concentration of free PAs varies greatly in individual intact myocytes. Direct measurements of total PA content in individual cells by use of conventional technologies are presently not feasible. In addition, there is presently no practical assay for direct measurements of free PAs in tissues and cells. The only practical way to measure free PA concentrations in cells is to use Kir2 channels as “PA sensors.” Therefore, to implement this idea, we have taken advantage of TG-Kir2.1-GFP mice overexpressing Kir2.1-GFP subunits in the heart (12). In these mice, the \(I_{K1}\) is carried primarily through transgenic Kir2.1-GFP channels, and the channel density is high enough such that the properties of rectification can easily be quantified in both cell-attached and inside-out configurations where currents reach up to \(1\text{–}5\text{ nA}\) (at \(-100\text{ mV}\), high extracellular K\(^+\); Figs. 3 and 4). This allows for precise analysis of \(I_{K1}\) properties under native intracellular conditions (cell-attached configuration).

Table 1. Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LV ((n = 20))</th>
<th>RV ((n = 13))</th>
<th>LA ((n = 12))</th>
<th>RA ((n = 14))</th>
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<tr>
<td>(I_{K1}(−100)^{§})</td>
<td>19.5±1.6</td>
<td>23.0±0.1</td>
<td>29.7±3.5* vs. LV</td>
<td>19.8±2.1* vs. LA</td>
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<tr>
<td>(I_{K1}(−50)^{§})</td>
<td>0.62±0.05</td>
<td>0.70±0.05</td>
<td>1.60±0.28* vs. LV/RV</td>
<td>0.97±0.12* vs. LA</td>
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<tr>
<td>(RR^{‡})</td>
<td>(-31.8\pm1.8)</td>
<td>(-34.5\pm2.4)</td>
<td>(-23.5\pm3.5* vs. RV</td>
<td>(-23.5\pm2.7* vs. RV</td>
</tr>
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</table>

Values are means ± SE. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; \(I_{K1}\), inward rectifier potassium current; RR, rectification ratio. §Inward and outward current amplitudes in pA/pF were measured at potentials \(-30\text{ mV}\) negative and \(20\text{ mV}\) positive to the respective \(I_{K1}\) reversal potentials, which approximately correspond to \(-100\text{ mV}\) and \(-50\text{ mV}\), respectively. ‡RR = \(I_{K1}(−100)/I_{K1}(−50)\). Statistics were by 1-way ANOVA followed by a Bonferroni posttest. \(*P < 0.05\) and \(^{‡}P < 0.01\).

Fig. 3. Variability of \(V_{1/2}\) values in individual myocytes. A, top: cell-attached (CA) current-voltage \((I–V)\) relations from 2 different ventricular TG-Kir2.1-GFP myocytes. The high level of Kir2.1-GFP expression in TG-Kir2.1-GFP mice allows for measuring of transgenic \(I_{K1}\) in the cell-attached configuration, thus permitting the use of Kir2.1-GFP channels as polyamine (PA) sensors. A, bottom: corresponding relative currents \(I_{REL}\) were analyzed as described in MATERIALS AND METHODS to obtain voltages for half block \((V_{1/2})\) for the steep component of rectification \((V_{1/2}^{CA})\) and \(V_{1/2}^{WA}\), where superscript is for the cell no.). B: \(V_{CA}\), calculated as the difference between \(V_{1/2}\) and corresponding reversal potential \((V_{CA}^{REV} = V_{1/2} − V_{REV})\), varies greatly in individual myocytes, reflecting variability of free intracellular PAs. In B, the data from RV and LV myocytes were pooled together. C, left: mean values for \(V_{1/2}^{CA}\) (as defined in B) were not significantly different between LV and RV or between LA and RA, and thus the data for ventricular \((V)\) and atrial \((A)\) myocytes were pooled together. Mean \(V_{1/2}^{CA}\) value in ventricular myocytes is \(-10\text{ mV}\) more negative than in atrial cells. C, right: similar results were obtained in wild-type myocytes using whole cell (WC) recordings. \(V_{1/2}^{WC}\) was calculated as the difference between \(V_{1/2}\) and corresponding reversal potential \((V_{1/2}^{WC} = V_{1/2} − V_{REV})\). Statistics were by t-test (see MATERIALS AND METHODS). **\(P < 0.01\).
tion), which can then be followed by patch excision and exposure of the patch to known total Spm concentrations in a defined solution (routinely FVPP-ATP; Fig. 4).

Experimentally, $I_{\text{K1}}$ recorded in cell-attached patches was converted into $I_{\text{REL}}$ and fit with a double-Boltzmann function (see MATERIALS AND METHODS). Figure 3A shows an example of cell-attached currents from two separate ventricular myocytes (Fig. 3A, top) and corresponding $I_{\text{REL}}$ fit with a double-Boltzmann function (Fig. 3A, bottom). The value of $V_{1/2}^{CA}$ ($V_{1/2}^{CA}$ for cell-attached patch relative to $V_{\text{REV}}$) corresponds to the steep PA-dependent component of the steady-state rectification (Fig. 3A, bottom) and was used as a readout of the underlying free Spm concentration. Previous studies have shown that the $V_{1/2}$ potential is nearly directly proportional (on log scale) to the concentration of PAs, primarily Spm (15).

We found that $V_{1/2}^{CA}$ varies significantly in individual myocytes isolated from every region of the mouse heart. In particular, in ventricular myocytes (RV and LV data pooled together), $V_{1/2}^{CA}$ ranged from $-6 \text{ mV}$ to $-32 \text{ mV}$ (Fig. 3B), while the variation in $V_{1/2}$ due to experimental error in inside-out patches was $<2.5 \text{ mV}$. Consistent with the significant regional variation of RR (Table 1) and $V_{\text{REV}}$ potentials (Fig. 3C), mean values for $V_{1/2}^{CA}$ in ventricular myocytes were $-18.6 \pm 1.4 \text{ mV}$ vs. $-8.7 \pm 0.9 \text{ mV}$ in atrial myocytes, a nearly 10-mV difference ($P < 0.0001$; Fig. 3C). Importantly, the mean values for $V_{\text{REV}}$ potentials were similar in atrial and ventricular myocytes ($2.7 \pm 0.18$ and $3.5 \pm 0.16 \text{ mV}$, respectively; $<1 \text{ mV}$ difference), thus essentially eliminating the $V_{\text{REV}}$ parameter from the equation and strongly suggesting that differences in $V_{1/2}^{CA}$ are due to free intracellular PAs. Since experiments demonstrated that exposure of myocytes to KB solution (used for isolation of atrial myocytes) leads to only minor PA leakage (characterized by a $1.7-\text{mV}$ decrease in $V_{1/2}$ value) (see supplemental materials), smaller $V_{1/2}^{CA}$ values in TG-Kir2.1-GFP atrial myocytes strongly support the hypothesis of a significantly smaller concentration of free intracellular PAs in atria.

Furthermore, in 15 (of 24) ventricular cells, the effective valency of the steep component of block in the cell-attached (native) configuration was weaker ($Z_{CA}^{CA} \sim 4$) than that in inside-out patches ($Z_{IO}^{IO} \sim 5$) (see below), suggesting a measurable contribution of Spd and/or Mg$^{2+}$ ions to $I_{\text{K1}}$ rectification. However, the generally high average values for $Z_{CA}^{CA}$ (4.53 ± 0.24 in ventricle and 5.39 ± 0.27 in atria) indicate that Spm is the primary blocker in both of these regions.

To convert $V_{1/2}^{CA}$ values to concentrations of free intracellular PAs, a “calibration” curve was constructed using Kir2.1-GFP channels expressed in HEK293 cells (Fig. 4C). In these experiments, inside-out patches were exposed to the KINT solution with known concentrations of Spm. KINT solution does not contain PA binding components, and thus total concentration of Spm corresponds to its free concentration.

It might be suggested that the PA sensitivity of Kir2.1-GFP channels can be different in HEK293 cells and cardiac myocytes because of, for example, a potential protein modification or a different composition of the membrane, thus invalidating the approach. To test this hypothesis, Kir2.1-GFP channels in inside-out patches from HEK293 cells and TG-Kir2.1-GFP myocytes were exposed to FVPP-ATP solution containing a known amount of total Spm. It should be noted that FVPP-ATP solution contains components, namely ATP and pyrophosphate, that strongly bind PAs, and thus concentration of free

![Fig. 4. Calibration of Kir2.1 "PA sensor." A, top: example of I-V relations for an inside-out patch excised from TG-Kir2.1-GFP myocyte after washout (WO) of endogenous PAs, followed by exposure to 100 and 300 $\mu$M total spermine (Spm) in FVPP-ATP solution (for details on this solution and others, see MATERIALS AND METHODS, Solutions). (Note: FVPP-ATP solution contains PA binding components such as ATP and pyrophosphate, and thus the concentration of free Spm is less than total concentration.) A, bottom: calibration of $I_{\text{REL}}$ was analyzed as described in MATERIALS AND METHODS to obtain $V_{1/2}$ for the steep component of rectification. B: $V_{1/2}^{CA}$, calculated as the difference between $V_{1/2}$ and corresponding reversal potential ($V_{1/2}^{CA} = V_{1/2} - V_{\text{REV}}$), was obtained for Kir2.1-GFP channels at varying concentrations of total Spm in 3 different conditions, as indicated. Shaded diamond represents calibration curve for Kir2.1-GFP channels expressed in HEK293 cells and exposed to KINT solution. (Note: KINT solution does not contain Spm chelators, and thus the concentration of free Spm in this solution is equal to the total concentration.) Calibration curves obtained in FVPP-ATP solution for Kir2.1-GFP channels transgenically expressed in cardiac myocytes (a) and in HEK293 cells (c) are indistinguishable. The data show that PA sensitivity of Kir2.1-GFP channels does not depend on the cell type, and therefore the concentration of free Spm inside the cells can be estimated by comparing the $V_{1/2}^{CA}$ (from myocyte cell-attached patches) with the calibration curve (shaded diamond) obtained for Kir2.1-GFP channels expressed in HEK293 cells. n = 3–7 patches for each point. SE bars are smaller than symbols and therefore are not visible.

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Spm is not known precisely. The use of FVPP-ATP solution is necessary in cardiac myocytes to significantly slow rundown of \(I_{K1}\) channels and to block ATP-sensitive K\(^+\) channels.

Figure 4A shows an example of current-voltage and \(I_{REL}\) relations (fit with the double-Boltzmann function) from a single patch excised from a TG-Kir2.1-GFP myocyte that was first washed out (WO) of endogenous PAs and then exposed to 100 and 300 \(\mu\)M total Spm in FVPP-ATP solution. Similar experiments were carried out with Kir2.1-GFP channels expressed in HEK293 cells. The data in Fig. 4B show that the sensitivities of Kir2.1-GFP channels to Spm in both expression systems are indistinguishable. It follows from the data in Figs. 3B and 4B that the variation of \(V_{1/2}\) in ventricular myocytes corresponds to the range of free Spm concentrations (from a few \(\mu\)M to a few hundred \(\mu\)M). The mean concentrations of free Spm in ventricular and atrial myocytes were estimated to be \(\sim 20\) and \(\sim 4\) \(\mu\)M, respectively.

Total PA content does not correlate with the reduced strength of rectification in atrial tissue. The differences in rectification between specific regions of the heart may also be explained by differences in the level of total intracellular PAs. Nevertheless, we have found that the total Spm and Spd content did not vary significantly along the heart (Fig. 5), with the exception of RV, where total Spm and Spd content was lower than in the other regions. The level of Put was below the limit of detection (\(\sim 5 \text{ nmol/g}\)). It follows from the data in Fig. 5 and Table 1 that the differences in PA content do not correlate with the differences in the strength of rectification among the regions of the heart (see DISCUSSION).

DISCUSSION

The overall goal of this study was to understand whether the variability of mouse \(I_{K1}\) (1) arises from differential expression of underlying Kir2 subunits or 2) is due to variation of free intracellular PAs, the key molecules underlying \(I_{K1}\) rectification (7, 8, 16). Tissue- and species-dependent expression of different subunits could potentially be the sole reason underlying \(I_{K1}\) variability. Thus several previous studies have employed subunit-specific markers of various kinds to decipher the composition of \(I_{K1}\). For example, Kir2 channels display various distinct properties, including different single-channel conductances (13), activation kinetics (39), and sensitivity to intracellular PAs (28). However, in vivo the situation is complicated by the experimental fact that Kir2 subunits can form heteromers (31), although the effects of such heteromerization on channel properties remain highly controversial. For example, Liu et al. (13) showed using single-channel analysis that, in guinea pig myocytes, Kir2.2 is the major Kir subunit, yet Dhamoon et al. (4) did not detect either Kir2.2 mRNA or Kir2.2 protein. Coexpression of various Kir2 subunits has also led to conflicting results. Preisig-Muller et al. (31) showed that coexpressed Kir2.1 and Kir2.3 channels possess an intermediate Ba\(^{2+}\) sensitivity, between that of homomeric Kir2.1 and Kir2.3 channels. In contrast, Schram et al. (35) found that coexpression of Kir2.1 and Kir2.3 leads to channels with a Ba\(^{2+}\) sensitivity significantly higher than that in the individual channels.

Most previous studies employed coexpression of different Kir2 subunits, where the level of membrane expression of specific subunits cannot be controlled and the exact subunit composition of the heteromers is also unknown. In particular, the expression of a Kir2.3 subunit in mammalian cell lines (e.g., HEK293, COS) is significantly lower than that of Kir2.1 channels (unpublished observation). In addition, in every specific case, it remains largely unknown whether the specific property of an individual subunit is dominant or recessive in a heteromeric channel. For example, Dhamoon et al. (4) provided evidence that the rectification properties of Kir2.1 are dominant in Kir2.1/Kir2.3 heteromers. Thus, to employ any specific marker of a Kir2 subunit, it is necessary to know its exact contribution in a heteromer of any specific subunit composition.

We focused on the Kir2.3 channel primarily because of its unique slow kinetics of activation, many-fold slower than for Kir2.1 and Kir2.2 channels (28). In addition, Kir2.3 channels also display a distinct “shallow” rectification profile, which potentially might underlie the observed larger outward currents in atrial myocytes in the mouse (28). It has been shown in the study by Zariisky et al. (41) that, although Kir2.1 and Kir2.2 are the major isoforms in the mouse heart, Kir2.3 mRNA was also detected. Importantly, experiments using elevated extracellular K\(^+\) in double Kir2.1\(^{--}\)/Kir2.2\(^{--}\) knockout myocytes revealed a small slowly activating K\(^+\) current accounting for \(\sim 15\%\) of that found in wild-type littermates (41), suggesting that it may be carried through Kir2.3 channels. Since measurements of Kir2 mRNAs in the above study were carried out using the whole mouse heart, it then remains a possibility that a small Kir2.3 message might originate from specific cardiac tissue such as atria, conduction system, or some other region, thus contributing to \(I_{K1}\) variability.

The data in Fig. 1 validate the usefulness of Kir2.3 kinetics as a strong marker for the presence of this subunit in a heteromer. It should also be noted that kinetics of activation (PA unblock) is essentially invariant of PA concentration (15) and thus presents a marker that is the least subjected to the variations in intracellular composition of the cell. The data show that even the presence of only one Kir2.3 subunit leads to a nearly threefold slowing of the kinetics in a Kir2.1/Kir2.3 heteromer. Importantly, kinetics becomes progressively slower as the number of Kir2.3 subunits is increased, irrespective of the exact subunit arrangement. This excludes a situation where some naturally occurring Kir2.1/Kir2.3 subunit arrangements
may produce fast kinetics like that found in Kir2.1 or Kir2.2 channels, thus leading to false conclusions.

One potential caveat to a concatemeric approach is that tetrameric constructs may not fold correctly. For example, some parts of the concatemer (e.g., COOH or NH2 terminus subunits) may contribute to another concatemer, thus making the subunit arrangement undefined. Fortunately, experimental evidence is against this possibility. In particular, early studies with Kir2.1 channels convincingly showed that 1) the properties of concatemeric channels are not affected by coexpression of excess mutant Kir2.1 monomers (40), and 2) only one mutated nonfunctional subunit in a tetrameric concatemer eliminates nearly all Kir2 current (21), suggesting a primarily intramolecular assembly.

With the reliable Kir2.3 marker, we have thus measured the kinetics of $I_{K1}$ activation in a number of distinct regions of the heart and found it to be as fast as in homomeric Kir2.1 or Kir2.2 channels. The data strongly suggest at first approximation that, in the mouse, there is no major contribution of Kir2.3 subunit to ventricular and atrial $I_{K1}$ (Fig. 2). Specifically, a decreased RR (Table 1), larger relative outward currents, and more positive $V_{1/2}$ potential in atria cannot be attributed to the contribution of Kir2.3 to atrial $I_{K1}$.

Once the major contribution of Kir2.3 to total $I_{K1}$ is excluded, the significant variability of $I_{K1}$ inward current density (Table 1) can be explained by a variable level of expression of major Kir2.1 and Kir2.2 subunits, both regionally and within the same specific cardiac tissue. Nevertheless, equally large variation of rectification (RR; Table 1) does not fit this simple explanation. Variation of rectification cannot be ascribed to differential expression of Kir2.1 and Kir2.2, since they possess very similar rectification properties (28). Thus the alternative hypothesis that the variability of $I_{K1}$ originates from the variability of free intracellular PAs, the inwardly rectifying factors themselves, may well be the most probable explanation of the phenomenon.

This idea has recently been supported in a study by Yan et al. (39) who showed that different concentrations of free intracellular PAs may underlie the differences between atrial and ventricular $I_{K1}$ in guinea pig. Presently, there is no technology available for measuring free PAs inside the cell, and in the above study, the levels of free PAs were only calculated using the concentrations of total PAs and the measured concentrations of known PA binding molecules (ATP, RNA, etc.) and corresponding binding constants (38).

We have also measured total PA levels in different regions of the mouse heart, and although some minor differences were indeed observed, they did not correlate with the differences in the strength of inward rectification. Thus the levels of total PAs do not reflect or explain the regional variation in $I_{K1}$.

To measure free intracellular PAs directly, in our study we have implemented a novel approach utilizing transgenically overexpressed Kir2.1-GFP channels such that large cell-attached currents (1–5 nA) can be easily measured in intact cells (impractical with wild-type myocytes). Overexpression of Kir2.1-GFP overrides the variable expression of native Kir2 subunits and thus allows us to use transgenic Kir2.1-GFP channels as sensors of free PAs. The voltage of half block for the steep part of rectification ($V_{1/2}^{CA}$; Fig. 3), which is primarily determined by free intracellular Spm, can be measured in intact cells, and cell-attached data then can be converted to concentrations of free Spm using calibration curves (Fig. 4B).

The first important observation is that $V_{1/2}^{CA}$, and thus free Spm (see RESULTS; Fig. 3), varies greatly in individual myocytes, a phenomenon we call “microheterogeneity.” Second, when the averaged data from ventricular and atrial myocytes were compared, a clear difference between $V_{1/2}^{CA}$ in corresponding tissues was revealed (Fig. 3C). Recalculation of $V_{1/2}^{CA}$ values to an equivalent concentration of free Spm using calibration curves (Fig. 4B) yields ~20 and ~4 μM in ventricular and atrial tissue, respectively. In contrast to total PAs, lower levels of free Spm in atria correspond to the reduced rectification of $I_{K1}$ in this tissue (Table 1). Third, it can be seen that the excision of the patch containing transgenic Kir2.1-GFP channels also leads to an increase in the amplitude of inward current at hyperpolarized potentials, far from the voltages where the steep component of Spm or Spd block of the channel occurs. Nevertheless, since the shallow component of the PA block in Kir2.1 (and Kir2.2) channels extends to far negative potentials (28), it is plausible that, at least in part, the variability of the density of inward $I_{K1}$ may also arise from variability of intracellular PAs. An alternative suggestion is that some other intracellular blocking molecules (e.g., Mg2+), other unknown factors, or specific regulation of the channel are being lost on patch excision. We have not tested the above hypothesis, since the origin of that blocking factor and the mechanism of its action are unknown. However, this is not a major concern, since the primary, and most potent, $I_{K1}$ blocker is Spm, as evidenced by the high effective valence in both atria and ventricle. The final piece of evidence is that $V_{1/2}^{CA}$ values in both atria and ventricle were comparable with those obtained in cell-attached patches ($V_{1/2}^{CA}$) overexpressing Kir2.1-GFP channels. This finding not only provides further corroboration for our main conclusion but also shows that wild-type and TG-Kir2.1 myocytes have comparable levels of endogenous PAs.

Questions remain regarding the origin of variability in PAs between individual myocytes. For example, this variability could potentially arise because of differences in cell health after the quite long procedure of collagenase-based isolation. In particular, part of the PAs may be lost by diffusion through a leaky membrane of some cells, especially through the intercalated disc membrane containing gap junctions, which may provide substantial permeability to intracellular PAs (6). Although only high-quality myocytes were used in our study, such possibilities cannot be totally excluded. In the Supplemental Materials, we have analyzed a number of other factors that may contribute and explain, at least in part, the observed variation in $I_{K1}$ rectification. Among them are differences in $V_{REV}^{CA}$ potentials (likely due to differences in intracellular $K^+$) and background $I_{Kacb}$. As we showed, when contributions from all potential factors are summed together, they may, for example, account for the essential part of the difference between rectification of ventricular and atrial $I_{K1}$. Nevertheless, experiments with TG-Kir2.1-GFP myocytes significantly diminished the above possibility. In particular, in TG-Kir2.1-GFP myocytes, differences in $V_{REV}^{CA}$ potentials between atrial and ventricular myocytes are negligible. Although some individual quantitative aspects of variation of $I_{K1}$ rectification will remain incompletely addressed until better or new techniques are developed for the estimation of free PAs, the data, taken

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together, are strongly supportive of a PA-dependent origin of variation of \( I_{K1} \) rectification.

Whether microheterogeneity of rectification exists in intact tissue also remains to be answered. In particular, it can be argued that a cell-to-cell gradient in PA concentration should be dissipated, or significantly reduced, by cell coupling through gap junctions. Unfortunately, there are presently no tools available to address this issue quantitatively; progress can be made, for example, when PA-sensitive microelectrodes are invented to allow for measurements of free PAs in intact hearts.

One potential cellular mechanism for true variability in free PAs could be due to differences in concentrations of PA binding factors such as nucleotides, DNA, RNA, and other phosphate-rich compounds (38) in cells originating from multiple, different, smaller anatomic regions (trabeculi, papillary muscles, Purkinje cells, etc.). The hypothesis of microanatomical (or functional) origin of heterogeneity seems more reasonable than the one based on cell-to-cell differences in intact tissue.

In addition to microheterogeneity or regional variability, the variation of \( I_{K1} \) across animal species can also be explained by differential regulation of free PAs. It is common knowledge that the outward current density of guinea pig \( I_{K1} \) is significantly larger than that in mouse, but the origin of this difference remains unclear. Although differential expression of distinct Kir2 subunits would seem a likely explanation, results of this study suggest an alternative hypothesis, that the reduced rectification in guinea pig ventricles is very likely due to a lower level of free intracellular PAs. Indeed, Yan et al. (39) estimated that free intracellular Spm concentration in guinea pig ventricular myocytes is \( \sim 5-10 \) \( \mu M \), which is significantly lower than the \( \sim 20 \) \( \mu M \) in the mouse ventricular cells measured in our study.

Overall, this study shows that, in the mouse heart, Kir2.3 subunits do not contribute to \( I_{K1} \) in any significant way. The data reveal significant variation in \( I_{K1} \) rectification and support the view that the origin of this phenomenon arises in large degree from the variation in the concentration of free intracellular PAs.

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