G Protein-Activated (GIRK) Current in Rat Ventricular Myocytes is Masked by Constitutive Inward Rectifier Current (I_{K1})

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Key Words

Cardiac myocytes • Inward rectifier • Kir2, Kir3 • GIRK • siRNA • Gene transfer • K^+ channel

Abstract

Inwardly-rectifying K^+ channel subunits are not homogeneously expressed in different cardiac tissues. In ventricular myocytes (VM) the background current-voltage relation is dominated by I_{K1} , carried by channels composed of Kir2.x subunits, which is less important in atrial myocytes (AM). On the other hand in AM a large G protein gated current carried by Kir3.1/3.4 complexes can be activated by stimulation of muscarinic M_2 receptors ($I_{K(ACh)}$), which is assumed to be marginal in VM. Recent evidence suggests that total current carried by cardiac inward-rectifiers ($I_{K(ATP)}$, $I_{K(ACh)}$, I_{K1}) in both, AM and VM is limited, due to K^+ accumulation/depletion. This lead us to hypothesize that in conventional whole cell recordings $I_{K(ACh)}$ in VM is underestimated as a consequence of constitutive I_{K1} . In that case a reduction in density of I_{K1} should be paralleled by an increase in density of $I_{K(ACh)}$. Three different experimental strategies have been used to test for this hypothesis: (i) Adenovirus-driven expression of a dominant-negative mutant of Kir2.1, one of

the subunits supposed to form I_{K1} channels, in VM caused a reduction in I_{K1} -density by about 80 %. In those cells $I_{K(ACh)}$ was increased about 4 fold. (ii) A comparable increase in $I_{K(ACh)}$ was observed upon reduction of I_{K1} caused by adenovirus-mediated RNA interference. (iii) Ba^{2+} in a concentration of 2 μM blocks I_{K1} in VM by about 60 % without affecting atrial $I_{K(ACh)}$. The reduction in I_{K1} by 2 μM Ba^{2+} is paralleled by a reversible increase in $I_{K(ACh)}$ by about 100%. These data demonstrate that the increase in K^+ conductance underlying ventricular $I_{K(ACh)}$ is largely underestimated, suggesting that it might be of greater physiological relevance than previously thought.

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Introduction

Inwardly-rectifying K^+ (Kir) channel currents are important for stabilizing resting potential, for action potential repolarization and for modulation of electrical excitability [1-4]. Different types of Kir channels or their subunits are differentially expressed in the heart chambers. As a rule, ventricular myocytes are characterized by a

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substantial constitutive strongly inward-rectifying conductance, representing homotetrameric or heterotetrameric channels of the Kir2.0 family [5-7]. The macroscopic current identified in cardiac muscle nearly half a century ago has been termed I_{K1} [8]. Constitutive I_{K1} is less prominent or virtually absent in atrial myocytes [5]. On the other hand, stimulation of appropriate G protein coupled receptors, such as the paradigmatic muscarinic M_2 acetylcholine (ACh) receptor in AM and pacemaker cells results in a substantial increase in inward rectifying current carried by G protein gated K^+ (GIRK; Kir3.x) channels [9, 10], whereas this current is much less pronounced in VM. Despite the small density or virtual absence of ventricular GIRK current, substantial direct effects of ACh on shape and duration of ventricular action potentials in different species have been described [11, 12]. Moreover strong evidence for a significant role of GIRK channels in electrophysiological properties of the intact mouse ventricular myocardium has been presented based on studies on GIRK4 knockout mice [13]. Using standard conditions for measuring inward K^+ currents in myocytes isolated from adult rats we determined a density of inward $I_{K(ACh)}$ in VM of <10 % of atrial $I_{K(ACh)}$.

Reducing constitutive I_{K1} in VM by $\geq 80\%$, either by adenovirus driven expression of a dominant-negative mutant of Kir2.1 or by silencing Kir2.1 using an adenoviral construct encoding for a short hairpin RNA (shRNA) was paralleled by an increase in $I_{K(ACh)}$. A reversible increase in $I_{K(ACh)}$ was also observed, when I_{K1} was acutely reduced by Ba^{2+} (2 μM), a concentration that did not affect atrial $I_{K(ACh)}$. These data suggest that ventricular $I_{K(ACh)}$ is partially masked by the large constitutive current I_{K1} . In line with recent publications [14, 15] this masking effect is likely to result from saturation of total K^+ current due to K^+ accumulation / depletion and a corresponding reduction in steady-state driving force.

Materials and Methods

Isolation and culture of atrial myocytes

Rats were killed following protocols approved by the local authorities for the regulation of animal welfare (Regierungspräsident) in accordance with the guidelines of the European Community (86/609/EEC). Wistar Kyoto rats of either sex, aged between 8 and 10 weeks, were anaesthetized by i.v. injection of urethane (1 g/kg). The chest was opened and the heart was removed and mounted on the cannula of a sterile Langendorff apparatus for coronary perfusion at constant flow. The method of enzymatic isolation of rat myocytes and serum-free culture conditions have been described in detail elsewhere [16]. To improve adherence and

thus long-term survival of VM, culture dishes were incubated with medium containing 5 % fetal calf serum (FCS) overnight. Before plating FCS was washed off by several rinses with serum-free culture medium (HEPES-buffered M199, PAA Laboratories, Pösching, Austria) supplemented with ITS (insulin / transferrin / selenium), Gentamycin (Sigma, 1.4 $\mu g/ml$) and Kanamycin (Sigma, 0.7 $\mu g/ml$).

Solutions and chemicals

For whole cell measurements of membrane currents an extracellular solution of the following composition was used (mM): NaCl 120; KCl 20; $CaCl_2$ 0.5; $MgCl_2$ 1.0; HEPES/NaOH 10.0, pH 7.4. The pipette solution contained (mM): K-aspartate 110; KCl 20; NaCl 5.0, $MgCl_2$ 1.0; Na_2ATP 2.0; EGTA 2.0; GTP 0.01; HEPES/KOH 10.0, pH 7.4. The K^+ reversal potential under these condition was calculated as -48 mV. Standard chemicals were from Merck (Darmstadt, Germany). EGTA, HEPES, MgATP, GTP and acetylcholine-chloride, were from Sigma. Tertiapin Q (T-Q) was from Tocris (Ellisville MI, USA).

Current measurement

Membrane currents were measured at ambient temperature (22-24°C) using whole-cell patch clamp as described in detail previously [16]. Cells were voltage-clamped at a holding potential of -90 mV, i.e. negative to E_K , resulting in inward K^+ currents. Rapid exposure to agonist-containing solutions was performed by means of a custom-made solenoid-operated flow system permitting a change of solution around an individual cell with a half time of about 100 ms. Only elongated VM showing cross striation were selected for the current measurements.

Site-directed mutagenesis of Kir2.1

Rat cDNA encoding for Kir2.1 (kindly provided by Dr. A. Karschin, Würzburg, Germany) was used in an overlap-extension PCR to substitute the pore-forming motif GYG by AAA, yielding Kir2.1-dn. All PCR-steps were performed using Pfu-polymerase and the following primers:

5'-CCC AAG CTT ATG GGC AGT GTG AGA ACC A-3' and 5'-GCG ATA TCT CAT ATC TCC GAT TCT CGC-3' as flanking primers, and 5'-CTG TAA CAC ACC TGA ATG CGG CAG CGA TGG TTG TCT GGG TC-3' and 5'-GAC CCA GAC AAC CAT CGC TGC CGC ATT CAG GTG TGT TAC AG-3' as mutagenic primers. The PCR product of Kir2.1-dn was directly subcloned into the shuttle vector pAdTrack-CMV using Kpn I and EcoRV restriction sites to obtain pAdTrack-Kir2.1-dn.

RNA interference

A detailed description of the experimental conditions and methodology for RNAi in adult cardiac myocytes using adenovirus driven transcription of RNA hairpins has been published elsewhere [17]. Briefly, pShuttle was used to insert the murine U6 promoter and the anti-Kir2.1 directed siRNA as double stranded oligonucleotide using XhoI and KpnI restriction sites. The vector pmU6pro containing the murine U6snRNA promoter, which served as template, was kindly provided by Dr. D. G. Turner (University of Michigan, Ann Arbor, USA).

To produce siRNA-hairpin-encoding vectors the U6 promotor and the hairpin construct were fused using an

universal 5'U6 primer (5'-GGAAGA TCT GAT CCG ACG CCG CCA TC-3') [18] and a 3' primer including the siRNA (bold) and complementary U6 promoter sequences (italic).

siRNA-Kir2.1

5'-GGG GTA CCA AAA AAG GAA TGA GAC TCT GGT CTT TCT CTT GAAAAG ACC AGA GTC TCA TTC CAA ACA AGG CTT TTC TCC AAG-3'.

This sequence has been previously identified and confirmed as efficient in silencing Kir2.1 [17, 19]. A randomised sequence based on the above sequence served as control. The recombinant Adenovirus were generated by homologous recombination between pAd-siKir2.1/1-2 and pAd-Easy-1 in *E. coli*.

Statistical analysis

Wherever possible, data are presented as mean $\pm$ SEM and were analyzed using Student's unpaired *t* test. $P < 0.05$ was considered significant.

Results

Comparison of background inward rectifier current (I_{K1}) and $I_{K(ACh)}$ in rat atrial and ventricular myocytes

Background inward rectifier current defined as current blocked by 2 mM Ba^{2+} and $I_{K(ACh)}$ activated by 10 μ M ACh in VM and AM are compared under identical experimental conditions in figure 1. In line with previous studies, AM are characterized by a small Ba^{2+} -sensitive background current. Rapid exposure to a saturating concentration of ACh induced an inward current ($I_{K(ACh)}$) that on average exceeded the background current by a factor of 11.5. In a representative VM a large background inward current was recorded at holding potential that was almost entirely blocked by 2 mM Ba^{2+} . The I/V curve of this current was characterized by strong inward rectification, typical of ventricular I_{K1} (not shown). Exposure to 10 μ M ACh in VM caused little additional inward current. The summarized data confirm that differences in density of Ba^{2+} -sensitive background current and $I_{K(ACh)}$ between VM and AM are highly significant (Figure 1 C). On average density of ventricular $I_{K(ACh)}$ amounted to 6.6 % of the current determined in AM under identical experimental conditions.

We have recently shown that macroscopic currents through different inward rectifier channels (I_{K1} , $I_{K(ACh)}$, $I_{K(ATP)}$) in VM and AM are not independent of each other. This has been interpreted in terms of K^+ accumulation / depletion affecting steady-state K^+ -driving force [14, 15], in line with publications demonstrating changes in E_K in isolated cells caused by large K^+ currents [20-22]. Based

Fig. 1. Ba^{2+} -sensitive background current and ACh-activated current in representative atrial (A) and ventricular (B) myocytes: Solutions containing ACh (10 μ M) or $BaCl_2$ (2 mM) were applied as indicated by the horizontal bars. Dotted lines in this and subsequent figures denote zero current levels. The rapid deflections in these and subsequent figures represent changes in membrane current caused by voltage ramps from -120 mV to $+60$ mV within 500 ms that were superimposed at 0.1 s $^{-1}$. Positive and negative current peaks may be distorted at the time resolution chosen. C, summarized data comparing densities of $I_{K(ACh)}$ (left) and I_{K1} , probed by 2 mM Ba^{2+} (right) in AM and VM. Myocytes used in this series of experiments were between about 24 h and 6 d *in vitro*; $n = 8$ to 12.

Fig. 2. Time in culture has little effect on I_{K1} and $I_{K(ACh)}$ in ventricular myocytes: Currents were quantified using either 2 mM Ba^{2+} (I_{K1}) or 10 μ M ACh ($I_{K(ACh)}$) as illustrated in figure 1. Current densities have been plotted vs. time after plating. Only rod-shaped myocytes with visible cross striation were selected. (n= 6 for each data point). Data on d zero were obtained between about 6 h and 10 h after myocyte isolation.

on these data we hypothesized that the amplitude or density, respectively of ventricular $I_{K(ACh)}$ might not only be limited by functional expression of one of the relevant signaling components or channel proteins [23, 24] but to some extent by current flow via the I_{K1} pathway. A prediction from this hypothesis is that reduction of I_{K1} in ventricular myocytes will result in an increase in $I_{K(ACh)}$, analogous to the finding that overexpression of Kir2.1 in AM not only results in a large constitutive inward-rectifier current but simultaneously causes a substantial reduction in $I_{K(ACh)}$ [14].

Dominant-negative knockdown of I_{K1} and silencing of Kir2.1 by RNA interference cause an increase in ventricular $I_{K(ACh)}$

Efficient reduction in ventricular I_{K1} , resulting in a change of the electrical phenotype has been described in myocytes expressing dominant negative mutants of Kir2.1 [1, 2, 14].

Manipulation of functional expression of a protein following adenovirus-driven transfer of a cDNA or a silencing construct in vitro requires a period of several days.

Fig. 3. Dominant-negative Kir2.1 and siRNA targeting Kir2.1 cause an increase in ventricular $I_{K(ACh)}$: Representative recordings of membrane current from myocytes transduced with empty virus (A – control), AdKir2.1-dn (B) and Ad-siKir2.1 (C) D, summarized current densities of I_{K1} and $I_{K(ACh)}$ (E) (current densities at -90 mV as in previous figures). Measurements were performed between d 6 and d 8 after virus exposure, n= 12 for each set of data). E. Superimposed background-subtracted current-voltage relations marked by arrowheads in A. Currents were scaled to match at -100 mV.

Previous studies demonstrated a substantial reduction in I_{K1} within few days in vitro in VM from rabbit hearts either by reduction of the expression level [25] and/or due to a loss of T-tubular membrane [26]. As illustrated

in figure 2, in rat VM cultured under the conditions described above, for a period of time between about 6 h after isolation (day zero) and 18 days in vitro no significant changes in densities of I_{K1} and $I_{K(ACh)}$ were found. This is not contradictory to the loss in T-tubules that has been described to occur within less than 24 h [27]. Our experimental protocol to maintain VM in a highly differentiated state in long term culture [17] requires a period of slow Ca^{2+} adaptation and washings that lasts for about 5 h (37°C) before plating. Within this period of time T-tubular membrane connected functionally to the surface membrane was strongly reduced.

Representative current recordings from time-matched reporter-positive myocytes infected with either the empty virus or AdKir2.1-dn or Ad-siKir2.1 are shown in figure 3 A-C. In Ad-EGFP-transduced cells I_{K1} and $I_{K(ACh)}$ were comparable to native myocytes (figure 3 A). In myocytes transduced by Ad-Kir2.1-dn, constitutive inward rectifier current was strongly reduced (B). In line with our hypothesis, reduction in I_{K1} was paralleled by an increase in $I_{K(ACh)}$ amounting to about 400%. Comparable changes in the two inwardly-rectifying currents were observed in myocytes transduced with the virus containing the shRNA cassette directed against Kir2.1 (C). No differences were found between time-matched native myocytes and myocytes transduced with Ad-EGFP or a virus containing the scrambled siRNA sequence [17]. The summarized data presented in figure 3 D and E demonstrate that reducing density of functional I_{K1} channels in VM results in a significant increase in $I_{K(ACh)}$, independent of the molecular nature of the channel knockdown. The current voltage relations of I_{K1} in the control myocyte and $I_{K(ACh)}$ in the myocytes with reduced I_{K1} are characterized by strong inward rectification, and upon scaling are almost superimposable (F).

Although these results are in support of the notion that current flow through I_{K1} channels reduces current carried by GIRK channels, it cannot be excluded that the increase in $I_{K(ACh)}$ reflects some compensatory up-regulation of functional expression of Kir3 subunits. As described previously, VM with reduced density of I_{K1} have a significantly reduced membrane resting potential and / or show pacemaker-like electrical activity [17, 28]. This is accompanied by a loss in viable cells as illustrated in figure 4 for a typical Ad-siKir3.2- treated culture. The fraction of viable cells identified by rod shape, cross striation, and a smooth membrane appearance was reduced by about 80 % (day 6) in Ad-siKir2.1 and Ad-Kir2.1dn infected myocytes. Comparable observations were made in three cultures infected with Ad-siKir2.1 and

Fig. 4. Cell viability is reduced by silencing Kir2.1: Representative photographs taken from sister cultures of VM taken on d 6 after infection with virus containing scrambled siRNA sequence (A) or Ad-siKir2.1 (B). For clarity living cells have been encircled.

Ad dn-Kir2.1 respectively.

The damaging effect of suppressing functional Kir2 channel complexes does not raise a problem for measurements of membrane current on individual cells selected on morphological criteria. Quantitative assays of message using real time RT-PCR, however, are impaired by a variable bias caused by material from dead cells, resulting in a large variability between assays from different myocyte culture.

Increase in $I_{K(ACh)}$ by acute inhibition of I_{K1}

If the interference between the inward rectifiers under study is due to K^+ current flow, an increase in $I_{K(ACh)}$ should be observed not only after reduction of surface expression of Kir2.x channels but also upon acute inhibition of I_{K1} . A potential compensatory increase in functional expression of GIRK channels could be ruled out by such an experiment. Since there are no specific inhibitors of I_{K1} , we tested if Ba^{2+} , an unspecific blocker of inwardly rectifying K^+ channels, could be used for partially blocking I_{K1} without a significant block of $I_{K(ACh)}$. Figure 5 shows concentration-response curves for the inhibitory effect of Ba^{2+} on ventricular I_{K1} and on atrial $I_{K(ACh)}$. In line with a previous study, $I_{K(ACh)}$ was less sensitive to Ba^{2+} than I_{K1} [29]. The EC_{50} for I_{K1} was determined as 1.2×10^{-6} M and as 2.6×10^{-5} M for $I_{K(ACh)}$. It should be noted, that these figures apply to the present experimental conditions, namely an extracellular K^+ -concentration of 20 mM and a membrane potential of -90 mV. Apparent

Fig. 5. Different sensitivities of I_{K1} and $I_{K(ACh)}$ to Ba^{2+} . A, inhibition of atrial $I_{K(ACh)}$ by Ba^{2+} . Note reduction in background current at 1 mM Ba^{2+} . The response to ACh is devoid of fast desensitization (comp. Figure 1), since the flow rate of the perfusion system was reduced, to minimize leakage from the Ba^{2+} -containing barrels. B, inhibition of ventricular constitutive background current by Ba^{2+} . C, summarized concentration response curves. Each symbol represent the mean $\pm$ SEM from 6 to 8 measurements. Curves were calculated using standard saturation kinetics. EC_{50} values are $1.2 \cdot 10^{-6}$ M (I_{K1}) and $2.6 \cdot 10^{-5}$ M ($I_{K(ACh)}$). The double-headed arrow indicates that 2 μM is a concentration that can be used for selective inhibition of I_{K1} .

Fig. 6. Ba^{2+} at 2 μM results in an increase in ventricular $I_{K(ACh)}$. A, current recording from a representative myocyte. ACh (10 μM) and Ba^{2+} (2 μM) were applied as indicated. B, summarized current densities from ($n=12$ each). Representative recording demonstrating that T-Q (200 nM) does not affect I_{K1} , but completely blocks the current activated by 10 μM ACh in the presence of 2 μM Ba^{2+} .

affinities of Ba^{2+} to various inward rectifier channels composed of different Kir subunits are strongly affected by these parameters. The concentration response curves (Figure 5 C) suggest that Ba^{2+} at a concentration of 2 μM blocks I_{K1} by about 60 % but causes little (< 5 %) inhibition of $I_{K(ACh)}$ as indicated by the double-headed arrow.

Figure 6 A illustrates the effect of 2 μM Ba^{2+} on I_{K1} and $I_{K(ACh)}$ in a representative ventricular myocyte. Total background current was reduced from 2.1 nA to 1.1 nA by 2 μM of the blocking ion. Paradoxically, but in line with our hypothesis, the current activated by ACh was increased by a factor of 2.2 in this cell by the blocking

Fig. 7. 2 μM Ba^{2+} causes an increase in $I_{\text{K(ACh)}}$ only in native ventricular myocytes and atrial myocytes overexpressing Kir2.1: A. effect of 2 μM Ba^{2+} on background current and $I_{\text{K(ACh)}}$ in a representative atrial myocyte infected with Ad-Kir2.1dn (A) and empty virus (B). Measurements were performed on d 6 after infection. C. Summarized data illustrating the effects of 2 μM Ba^{2+} on $I_{\text{K(ACh)}}$ in mock infected (control) and Ad-Kir2.1 infected myocytes (right panel; $n=6$ for each experimental condition). The left panel recapitulates that 2 μM Ba^{2+} increases $I_{\text{K(ACh)}}$ in native VM but not in VM with a strongly reduced I_{K1} caused by expression of the dominant negative Kir2.1 mutant.

ion. On average in this series of experiments $I_{\text{K(ACh)}}$ was significantly increased about 2-fold by 2 μM Ba^{2+} (Figure 6B). The current augmented by 2 μM Ba^{2+} was completely blocked by T-Q (200 nM), a blocking peptide with high selectivity for Kir3 channels. This concentration had no effect on I_{K1} , as shown in Figure 6 C for a representative myocyte. The I/V relation of the background-subtracted ACh-activated current in the presence of 2 μM Ba^{2+} had a reversal potential of -46.6 ± 2.8 mV ($n=10$) which is not significantly different from the reversal potential of native atrial $I_{\text{K(ACh)}}$ (-47.9 ± 1.2 mV, $n=20$) and is close to the calculated E_{k} of the present experimental conditions (-48 mV).

In a previous study we have shown that overexpression of Kir2.1 in AM not only resulted in a large constitutive inward rectifying current but also caused a significant reduction in $I_{\text{K(ACh)}}$ [14]. Figure 7 A illustrates that this could be partially rescued by 2 μM Ba^{2+} , which in that cell blocked a background current of about 1.5 nA. In the presence of 2 μM of the blocking ion, $I_{\text{K(ACh)}}$ was increased by a factor of two, comparable to the effect observed in VM. In control or mock-infected AM, which are characterized by a small background inward-rectifier current, in line with concentration-response curve shown in figure 5 $I_{\text{K(ACh)}}$, i.e the current change elicited by ACh, was not affected by 2 μM Ba^{2+} (Figure 7 B). The summarized data in figure 7 C demonstrate that in myocytes with a small background K^+ current (native AM, VM infected with AdKir2.1-dn) 2 μM Ba^{2+} had no significant effect on the amplitude of $I_{\text{K(ACh)}}$, whereas in native VM and AdKir2.1 (wt) infected AM, both featuring a substantial I_{K1} , the blocking ion caused a significant increase in $I_{\text{K(ACh)}}$. This clearly shows that the paradoxical effect of Ba^{2+} on ventricular GIRK current is related to constitutive I_{K1} and does not reflect a difference in Ba^{2+} action on atrial and ventricular Kir3.x channel complexes.

Discussion

The major result of the present study is the uncovering of a sizable GIRK current in VM by experimental maneuvers that reduce constitutive inward rectifier current (I_{K1}). This can be observed in the presence of Ba^{2+} at a concentration (2 μM) that only marginally block GIRK channel current in AM, whereas it blocked I_{K1} by about 60 %. Stronger suppression of I_{K1} in VM following either expression of a dominant-negative Kir2.1 mutant or transcription of siRNA hairpins directed against Kir2.1 resulted in a more pronounced increase in density of $I_{\text{K(ACh)}}$.

It is commonly accepted that GIRK currents are functionally expressed in the heart and in various types of neurons in the CNS and in endocrine cells [30]. More

recently, evidence for functional GIRK channels has been provided also for other types of cells, such as smooth muscle [31] and blood platelets [32], where their function is unknown. It is supposed that GIRK channels can be activated by stimulation of all receptor species that couple to pertussis-sensitive G proteins of the $G_{i/o}$ class [33, 34]. G_s -mediated activation has been described e.g. upon overexpression of receptors coupled to that species [35, 36]. In the heart the prototypic $G_{i/o}$ -coupled GPCR is the muscarinic M_2 subtype. Previous studies related to GIRK current in VM clearly demonstrated effects of acetylcholine on action potential duration and related parameters, such as contraction or cell shortening. These effects were direct, i.e. unrelated to inhibition of the cAMP signaling pathway [11, 37, 12]. The size or density respectively of GIRK current strongly depends on experimental parameters, such as ionic conditions, holding potential or temperature, but is also affected by other factors such as species and age of the donor animals [38]. In ventricular myocytes isolated from rats of similar weight as those used in the present study, McMorn et al. [37] found that outward $I_{K(ACh)}$ at 5.4 mM extracellular K^+ concentration was smaller by a factor of 6.3 as compared to atrial myocytes. Taking into account the difference in surface area between VM and AM of a factor of 4 to 5, current density in VM in that study was smaller by a factor of > 20 as compared to AM. Nevertheless, functional effects related to activation of $I_{K(ACh)}$ (decrease in action potential evoked cell shortening) differed only by a factor of < 3 , supporting the notion that $I_{K(ACh)}$ or the underlying change in conductance respectively is substantially underestimated when it is determined on the background of constitutive I_{K1} .

The observation that ventricular GIRK current is not negligible raises the question of its physiological function. Takahashi et al. [39] demonstrated that infusion of ACh in paced *in situ* Langendorff preparations (rat) caused effects in line with activation of ventricular GIRK current. Analogous effects, however, were not observed upon vagal stimulation, suggesting that there is little or no parasympathetic input to the ventricles, at least in this species. This would be in line with recent morphological data from guinea-pig heart [40]. These authors found that cholinergic innervation of the ventricular walls is extremely sparse as compared to supraventricular tissue and came to the conclusion that vagal efferent input has little or no direct influence on ventricular contractile function in that species.

Activation of GIRK channel current by receptors other than the muscarinic M_2 species has been described in atrial cells, such as the purinergic A_1 receptor [41] and a sphingolipid receptor [42], presumably the $S1P_3$ subtype [43]. For the A_1 receptor and the sphingolipid receptor we could demonstrate functional coupling to GIRK channels also in rat ventricular myocytes (unpublished data). The roles of these receptors in a physiological context to date are unknown. The same applies to $GABA_B$ receptors, that have been demonstrated to activate GIRK channels in rat ventricular myocytes [44]. Apart from these receptor species, a number of receptors are expressed by cardiac myocytes that couple to $G_{i/o}$, whose potency for activating GIRK channels thus far has not been studied in detail, such as adrenergic β_2 and β_3 receptors and opioid receptors. Thus, despite a sparse or even marginal parasympathetic innervation of the ventricles as compared to the atria and the pacemaker [40], [there are various other $G_{i/o}$ -coupled receptors that converge on GIRK channels that might become relevant to regulation of electrical parameters in VM such as excitability or action potential duration. This could contribute to the cardioprotective effect of these receptors against ischemic and hypoxic injury [45-47].

Our understanding of the normal cardiac action potential and of arrhythmogenesis have been continuously advanced since the 1960's by computational models (e.g. [48, 49]). Optimizing model calculations of cardiac action potentials under physiological and pathophysiological condition requires a detailed knowledge of the ion channel species that are differentially expressed in the heart, their functional expression levels, and their regulation by various factors including the autonomous nerves system. The demonstration of a G protein activated K^+ current or conductance respectively in ventricular myocytes that can amount to about 25 % of the constitutive I_{K1} , clearly suggests that GIRK channels ought to be integrated in simulations of ventricular electrophysiology.

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