Regulation of the Inward Rectifying Properties of G-protein-activated Inwardly Rectifying K$^+$ (GIRK)
Channels by G$\beta$$\gamma$ Subunits*

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G$\beta$$\gamma$ subunits are known to bind to and activate G-protein-activated inwardly rectifying K$^+$ (GIRK) by regulating their open probability and bursting behavior. Studying G-protein regulation of either native GIRK ($I_{K_{AC}}$) channels in feline atrial myocytes or heterologously expressed GIRK1/4 channels in Chinese hamster ovary cells and HEK 293 cells uncovered a novel G$\beta$$\gamma$ subunit mediated regulation of the inwardly rectifying properties of these channels. $I_{K_{AC}}$ activated by submaximal concentrations of acetylcholine exhibited a ~2.5-fold stronger inward rectification than $I_{K_{AC}}$ activated by saturating concentrations of acetylcholine. Similarly, the inward rectification of currents through GIRK1/4 channels expressed in HEK cells was substantially weakened upon maximal stimulation with co-expressed G$\beta$$\gamma$ subunits. Analysis of the outward current block underlying inward rectification demonstrated that the fraction of instantaneously blocked channels was reduced when G$\beta$$\gamma$ was over-expressed. The G$\beta$$\gamma$ induced weakening of inward rectification was associated with reduced potencies for Ba$^{2+}$ and Cs$^+$ to block channels from the extracellular side. Based on these results we propose that saturation of the channel with G$\beta$$\gamma$ subunit mediated regulation of the inwardly rectifying properties of these channels. I$_{K_{AC}}$ activated by G$\beta$$\gamma$ subunits to GIRK channels was mapped primarily to the C terminus of GIRK1 and GIRK4 (4–8). Cross-linking studies have demonstrated that the heterotetrameric channel can bind up to 4 G$\beta$$\gamma$ subunits (9). However, despite much experimental effort the mechanism by which G$\beta$$\gamma$ activates these channels is not well understood.

GIRK channels belong to the family of strong inwardly rectifying K$^+$ channels, which are characterized by their strong inwardly rectifying current-voltage relationships. The inward rectification has been linked to the presence of intracellular Mg$^{2+}$ and polymamines (10–12). These positively charged cytoplasmic ions are thought to block outward K$^+$ currents by blocking the pore of channels from the inside (10–13); however, for a related inwardly rectifying channel Kir2.1 this hypothesis has recently been questioned (14). Inward rectification of K$^+$ channels is not only voltage-dependent but also dependent on the extracellular K$^+$ concentration (11). The inward rectification of these K$^+$ channels is closely related to their function in the heart as well as in many neuronal tissues. In cardiac myocytes activation of inwardly rectifying K$^+$ channels such as $I_{K_{AC}}$ causes the cell membrane to hyperpolarize between action potentials because the conductivity for K$^+$ generated by these channels is high at membrane potentials close to EK. This hyperpolarization induced by $I_{K_{AC}}$ appears to be at least partially responsible for the negative chronotropic effect induced by vagal activity (1, 2, 15). During action potentials, however, the conductivity of $I_{K_{AC}}$ for K$^+$ declines several-fold with the rise of voltage enabling the myocyte to generate prolonged action potentials, which are critically important for cardiac function (16).

The initial observation that led to the study presented here was the discovery that the agonist-induced $I_{K_{AC}}$ in cardiac myocytes were quite variable in their degree of inward rectification, indicating that the modulation of the open probability of these channels (15) by ACh may not be the only property of these channels that is regulated by ACh. It seemed possible that, in addition, inward rectification of these channels may be modulated as well by ACh. The present experiments have tested this possibility.

EXPERIMENTAL PROCEDURES

Preparation of Feline Atrial Myocytes—Isolation of feline atrial myocytes was performed as described (17). Animal procedures used were in accordance with guidelines of the Animal Care and Use Committee of Northwestern University. Briefly, adult cats were first anesthetized with pentobarbital sodium (70 mg/kg body weight, intraperitoneally). The heart was quickly removed and retrograde perfused with Krebs-Henseleit buffer. It was digested by perfusion with collagenase-containing solution. After 10–15 min of digestion the atria were collected and cut into small pieces, followed by a 5-min incubation with fresh enzyme solution. Isolated atrial myocytes were collected, placed in M199 (In-
vitro), and plated in cell culture dishes. The cells were kept at 37 °C under 7% CO₂ until further use. Cell culture and Transfection—Chinese hamster ovary (CHO-K1) cells were grown in Ham’s F-12 medium (Invitrogen). The media were supplemented with 10% fetal bovine serum and streptomycin/penicillin (100 units each). Cells were grown under 7% CO₂ at 37 °C. In all transfactions for electrophysiological studies the CD8 reporter gene system was used to visualize transfected cells (15). Dynabeads coated with anti-CD8-antibodies were purchased from Dynal. CHO-K1 cells were transfected using adenovirus-mediated gene transfer (19) using the following amounts of endotoxin-free cDNAs (Qiagen)/6 cm dish: human CD8 (in naï; 0.15 μg; gift from Dr. G. Yellen); mouse GIRK1 (in pC1, 0.3 μg) and mouse GIRK4 (in pCDNA1, 0.3 μg; gifts from Drs. F. Lederkremer and M. Laszdzunski); human A1-adenosine receptors (in CLDN10B, 0.2 μg; gift from Dr. J. Linden); human M₄-mACHR (0.8 μg, in pcDNA3; gift from Dr. E. Peralta); human Gβ1 (in pCMV5, 0.3 μg) and human Gγ2 (in pCDNA1, 0.3 μg; gift from Dr. H. A. Bourne). Empty pcDNA3 was used to balance the total amount of DNA used for transfection to 2–2.35 mg/6 cm. All assays were performed 48–72 h post transfection if not otherwise mentioned. HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, streptomycin/penicillin (100 units each), and 1% glutamine. Cells were grown under 7% CO₂ at 37 °C. In cells stably expressing GIRK1/4, the media was supplemented with 200 μg/ml G418. To visualize transfected cells the CD8 reporter gene system was used as described before. Transfection was performed using the Effectene transfection kit (Qiagen) according to the manufacturer’s protocol using the following amounts of endotoxin-free cDNAs (Qiagen)/6 cm dish. Experiments described in Fig. 4: 0–1.25 μg empty pcDNA3. 0.05 μg CD8, 0.5 μg human αₛ-adrenergic receptor (AR)-G₄₃₁ fusion protein (in pcDNA3, kindly provided by Dr. G. Milligan), and GIRK1, GIRK4, Gβ1, and Gγ2 as indicated in the figure. For experiments shown in Figs. 5–7: HEK cells stably expressing GIRK1 and GIRK4 channels were transfected with 0.7 μg αₛ-AR-G₄₃₁, 1.4 μg each of human Gβ1 and Gγ2 (in pCDNA3), and CD8 (in naï, 0.2 μg). Experiments were performed 40–50 h post transfection.

Solutions—For the measurement of K⁺ currents an extracellular solution of the following composition was used (mM): NaCl, 120; KCl, 20; CaCl₂, 2; MgCl₂, 1; Hepes-NaOH, 10, pH 7.3. The internal (pipette) solution contained (mM): potassium aspartate, 100; KCl, 40; MgATP, 5; Hepes-KOH, 10; NaCl, 5; EGTA, 2; MgCl₂, 1; GTP, 0.01; pH 7.3. All standard salts as well as ACH and adenosine (Ado) were purchased either from Sigma or from Merek.

Measurement of Membrane Currents—Membrane currents were recorded under voltage-clamp conditions, using conventional whole cell patch clamp techniques (20). Patch-pipettes were fabricated from borosilicate glass capillaries, (GF-150 6M Silicate Glass, Sutter Instruments) and sealed with a P-97 puller (Narishige) to a tip resistance of 3–5 MΩ. Membrane currents were recorded using either a patch-clamp amplifier (Axopatch 200, Axon Instruments) or an EPC 9 (HEKA Instruments) as described previously (21, 22). Signals were analog-filtered using a lowpass Bessel filter (1–3 kHz corner frequency). Data were digitally stored using either a Mac (Centron 640 with pulse software) or an IBM compatible PC equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control, data acquisition, and data evaluation. IKACh was measured as an inward current using a holding potential of −90 mV as described (23). Voltage ramps (from −120 mV to +60 mV in 500 ms, every 10 s) were used to determine current-voltage (I-V) relationships. All measurements were performed at room temperature. Summarized results are presented as mean values ± S.E. Student’s t tests (two population) were performed to test for significance of differences between groups of data.

RESULTS

The atrial muscarinic K⁺ current (IKACh) is regulated by muscarinic receptors, and the underlying pathway has been studied in detail by many groups (15). The inwardly rectifying properties of this channel have been the topic of many detailed studies that provided interesting insights into the mechanisms of inward rectification (10, 11). However, so far no physiological modulation of the inward rectification of this current has been reported. The following study was based on the surprising observation that the inward rectification of ACh-activated K⁺ currents in feline atrial myocytes varied as a function of the agonist concentration.

The Inward Rectification of Feline Atrial IKACh Was Modulated by Stimulus Strength—IKACh in isolated feline atrial myocytes was measured in response to two different concentrations of ACh either in the inward or outward direction using the whole cell patch technique. The membrane potential was clamped to either −90 mV or +60 mV in the presence of 20 mM extracellular K⁺. When the holding potential was negative (-90 mV) to the potassium equilibrium potential (EK) (Fig. 1A), superfusion of the cell with 0.1 μM ACh gave rise to inward currents that were about 70% in amplitude compared with currents activated by 10 μM ACh. In contrast, at +60 mV outward currents induced by 0.1 μM ACh were barely detectable and were only about 10% in amplitude compared with currents activated by 10 μM ACh. I-V curves of ACh-induced currents (Fig. 1B) were determined by subtracting currents measured in the absence of agonist from currents measured in the presence of agonist in response to linear voltage ramps from −120 mV to +60 mV. I-V curves of the currents elicited by 0.1 μM ACh or by 10 μM ACh exhibited inward rectification and identical reversal potentials close to the EK as indicated (A). Current-voltage curves of ACh induced currents as shown in B were calculated after subtraction of background currents. The voltage dependencies of IKACh conductance in the presence of low or high agonist concentrations are plotted in C. These results were representative for similar experiments performed in four different atrial myocytes obtained from two different myocyte preparations.
HEK 293 cells using M2-mAChR, A1 adenosine receptors and with Gβγ subunits (C, D, E, F, indicated as +Gβγ) or without additional exogenous G-protein subunits (A, B, E, F, indicated as agonist). GIRK currents activated via A1 adenosine receptors or by co-expression with Gβγ subunits were measured using whole cell voltage clamp recording similar as described in the legend to Fig. 1. GIRK current-voltage curves were calculated by subtracting either background currents in the absence of agonist (B) or currents insensitive to 1 mM Ba2+ (D). Normalized GIRK current conductance in cells co-transfected with or without Gβγ subunits were plotted against voltage (E). To quantify the degree of inward rectification, an inward rectification factor was defined (F
\[F = I_{rev} - 50 \text{ mV} / (E_{rev} + 50 \text{ mV})\]) (F). Summarized data were compared for adenosine-evoked currents in the absence of exogenous Gβγ (agonist) and Ba2+-sensitive currents evoked by heterologous expression of Gβγ (F) (n = 9 each, the two groups were significantly different at p < 0.05).

Currents increased as a function of agonist concentration (Fig. 2A), no stimulus-dependent change in inward rectification of GIRK currents was observed (Fig. 2B). Indeed, agonist-induced currents all exhibited strong inward rectification very similar to atrial IKACH, activated with low doses of agonist (compare Figs. 2B and LB). Similar results were observed in transiently transfected HEK 293 cells using M2-mACHR, A1 adenosine receptors, or αβ2 adrenergic receptors (data not shown and Refs. 21 and 22).

Heterologously Expressed GIRK Currents Exhibited Weakened Inward Rectification When Activated via Co-expressed Gβγ—Agonist-induced GIRK currents obtained from cells heterologously transfected with GRIK1/4 were activated via endogenous G-proteins (Fig. 2, A and B). It seemed likely that the pool of endogenous G-proteins might have been limiting for the extent of maximal GIRK current activation. Therefore, Gβγ subunits were co-expressed with GIRK channels. GIRK currents were constitutively active due to Gβγ subunits. The amplitude of GIRK currents was determined via inhibition by Ba2+ (Fig. 2C). In most cases, activation of co-expressed A1 adenosine receptors by 10 μM Ado induced no further stimulation of Ba2+-sensitive GIRK currents, indicating a maximal stimulation of GIRK channels by Gβγ. Under these conditions, total Ba2+-sensitive GIRK currents compared with control conditions (activation via receptor and endogenous G-proteins) were about 2-fold larger in amplitude (147 ± 12.7 pA/pF with co-expressed Gβγ versus 71.2 ± 20.7 pA/pF activated via A1 adenosine receptors and endogenous G-proteins) and exhibited a weaker inward rectification (Fig. 2D versus Fig. 2B). Comparing the conductance-voltage relationship revealed a shift to more positive voltages for GIRK currents activated by heterologously expressed Gβγ subunits compared with GIRK currents activated by agonist only (Fig. 2E). To quantify the relative inward rectification of GIRK currents the ratio of GIRK current conductance in outward versus inward direction (Ba2+-sensitive GIRK currents at reversal potential (E
\[E_{rev}\]) ± 50 mV) was calculated. The ratio of outward/inward currents of Ba2+-sensitive GIRK currents activated by heterologously expressed Gβγ was significantly increased compared with Ba2+-sensitive GIRK currents activated via A1 adenosine receptors and endogenous G-proteins (0.39 ± 0.11, n = 9 versus 0.14 ± 0.05, n = 8) (Fig. 2F). The voltage-dependence of GIRK currents maximally activated by heterologous expression of Gβγ was comparable with atrial IKACH, activated by saturating concentrations of ACh (10 μM), whereas agonist-induced GIRK currents activated via endogenous G-proteins exhibited similar strong inward rectification as submaximally activated atrial IKACH. This result indicated that the inwardly rectifying properties of GIRK channels were modulated depending on the internal Gβγ concentration.

In a minority of cells transfected with Gβγ subunits addition of adenosine to stimulate A1 adenosine receptors resulted in a further increase in GIRK currents (Fig. 3A), indicating submaximal stimulation of GIRK channels by heterologously expressed Gβγ. Under these circumstances, basal Gβγ-induced GIRK currents exhibited strong inward rectification, whereas addition of adenosine resulted in a pronounced weakening of inward rectification (Fig. 3B), demonstrating that inward rectification of heterologously expressed GIRK currents can be modulated via stimulation of G-protein-coupled receptors similar to atrial myocytes. Taken together, these results suggested that Gβγ at submaximal concentrations induces strong inwardly rectifying GIRK currents, whereas at maximal concent-
tractions, Gβγ-evoked GIRK currents exhibited weakened inward rectification.

The Ratio of Gβγ to GIRK Channel Expression Is Critical for Regulation of Inward Rectification. HEK 293 cells were transiently transfected with indicated amounts of eDNAs encoding for GIRK1, GIRK4, Gβ1, and Gγ as well as constant amounts of eDNA of a α2A-AR-Gα1-fusion protein and the CD8-reporter gene. 40–48 h post transfection whole cell currents were recorded in response to voltage ramps either in the presence of 10 μM norepinephrine (maximal GIRK activation) or 1 mM Ba2+ (to specifically block GIRK currents). Summarized data for the degree of GIRK current inward rectification (Fγ = I(Emax, + 50 mV)/I(Emin, - 50 mV)) obtained under the indicated conditions is illustrated in the upper panel (n = 5–9, of 2–3 transfections). Corresponding maximal GIRK current densities measured in inward direction (−90 mV holding potential) are shown in the lower panel. Differences from the results shown in the first column (0.1 μg eDNA of GIRK1/4; 0.5 μg eDNA for Gβ1,Gγ) that reached significance are indicated (*, p < 0.05; **, p < 0.01).

nous G-proteins by prolonging the time after transfection and found a significant increase in the ratio of outward to inward currents from day 3 to 4 post-transfection in transiently transfected CHO cells (Iout/Iin; 0.22 ± 0.06 d.4 versus 0.095 ± 0.025 d.3) accompanied by a small reduction in GIRK current density determined at −90 mV (43 ± 13 pA/pF, d.4 n = 12 compared with 64 ± 10 pA/pF, d.3 n = 6). This widening of inward rectification of GIRK currents reflected most likely a decrease in GIRK channel expression in the individual cells, resulting in an increase of the ratio of G-proteins versus GIRK channels.

The Weakened Inward Rectification Was Not Accompanied by Changes in Slow Blocking Kinetics of Outward GIRK Currents Associated to Polyamine-induced Inward Rectification. For further analysis experimental conditions were chosen to consistently induce either strong inward rectifying currents (control) or weak inward rectifying currents (Gβγ-induced) in HEK cells stably expressing GIRK1 and 4. Strong inward rectifying currents were induced via agonist stimulation of α2A adrenergic receptors in the absence of exogenous Gβγ, whereas weak inward rectifying currents were evoked by additional co-transfection of Gβ1,Gγ. As described above the current model of the inward rectifying mechanism is a voltage-dependent open channel block by internal Mg2+ and polyamines such as spermine and spermidine. To test whether an alteration of the polyamine- and Mg2+-induced open channel block was the cause for the observed weakening of inward rectification, blocking and unblocking kinetics were determined using whole cell recording. According to Refs. 13, 27, and 28, the polyamine block is responsible for the time-dependent (slow) activation and inactivation of K+ currents through GIRK channels (or other inward rectifier channels) in response to voltage steps, whereas current block induced by internal Mg2+ occurs almost instantaneously. Therefore, whole cell currents resulting from voltage steps (−120 mV to 60 mV; 60 mV to −120 mV) were measured to determine the time constants of polyamine block onset and offset. In case inward rectification was weakened due to lowered polyamine block affinity, a faster polyamine unbinding from the channel and/or a slower-polyamine binding to the channel should be observed. In contrast, if Mg2+ block was altered, the fraction of channels blocked instantaneously in outward direction should be decreased, whereas changes in blocking and unblocking kinetics should not be observed. Background currents were determined by inhibiting GIRK channels via Ba2+ and subtracted from each measured whole cell current. A second-order exponential function was used to fit the current curves and determine time constants. Comparison of currents measured under control (strong inward rectification) and Gβγ over-expressed (weak inward rectification) conditions showed no striking alteration of the slow blocking kinetics (Fig. 5A). As expected, the unblocking appeared to be faster (Fig. 5B), however, this effect did not reach statistical significance (1.28 ms ± 0.1 versus 1.05 ms ± 0.46; 10 ms ± 0.95 versus 9.1 ms ± 2.8). In contrast to the proposition, blocking of the channel in the outward direction (reflecting binding of polyamines) was faster, too (4.9 ± 0.97 ms versus 2.75 ± 0.62 ms; 58.8 ± 16 ms versus 36 ± 9.3 ms). Normalizing to the maximum inward current revealed that the probability of channel opening at voltages positive to EK was increased under weak inward rectifying conditions compared with control conditions. Normalizing to the outward maximum current demonstrated that the same percentage of channels underwent a slow blockade under control as well as under weak inward rectifying conditions. Because the fraction of channels instantaneously blocked in the outward direction was lower when Gβγ was over-expressed the potency of internal Mg2+ to block the channels might have been reduced. Therefore, we increased internal Mg2+ up to 20 mM to...
exponential decay. Summarized data for the resulting time constants for the slow component of outward current block is not altered by co-expression of Gβγ subunits. Illustrated are representative current recordings measured in response to voltage steps (−120 mV, 60 mV, and −120 mV, as indicated) from cells, which did (red) or did not express exogenous Gβγ (black). Currents were normalized to the maximal inward (upper panel) and outward (lower left panel) currents, and the time course of the onset of outward current block (lower left panel) as well as the recovery from outward current block (lower right panel) was fitted best by a biexponential decay. Summarized data for the resulting time constants are illustrated in the figure (n = 5–7).

compensate for a reduced potency of Mg2+ to block GIRK channels, however, no change in inward rectification was observed (data not shown).

Affinity for Ba2+ Block Was Reduced under Weak InwardRectifying Conditions—A hallmark for strong inward rectifier potassium channels is a high affinity block by external Ba2+. Studies using crystal structures of the bacterial KcsA channel complexed with Ba2+ have located a single Ba2+-binding site on the cytosolic side of the selectivity filter (29, 30). In close proximity to this site are some of the residues that have been implicated to be critical for strong inward rectification (11, 28). To test if Gβγ mediates a conformational change of the GIRK channel that causes weakening of inward rectification by altering structures close to the selectivity filter, we questioned whether or not GIRK channel block by Ba2+ was affected by Gβγ. Whole cell currents at a holding potential of −90 mV were measured in the presence of 1 μM, 10 μM, 40 μM, 140 μM, 1 mM, and 2 mM extracellular Ba2+ under strong and weak inward rectifying conditions (Fig. 6). Ba2+ effectively inhibited GIRK currents under both conditions, however, the potency of Ba2+ to block GIRK currents was substantially decreased when channels were maximally activated by Gβγ (IC50; 73 μM versus 20 μM; Hill coefficient: n = 1.14 versus n = 2). These results strongly suggested that interaction with Gβγ subunits induced conformational changes of GIRK channel structures close to the Ba2+-binding site.

Cs+-induced Block of GIRK Channels Was Attenuated underWeak InwardRectifying Conditions—Inwardly rectifying K+ channels can be blocked efficiently by external Cs+. This block is highly voltage-dependent and most prominent at negative potentials (11, 31). Binding sites for Cs+ in the channel have been mapped to pore-lining residues of transmembrane domain 2 (M2) (32) and to a site close to selectivity filter (32). Therefore, possible Gβγ-dependent modulation of GIRK current block by external Cs+ (3 mM) was studied (Fig. 7). At a membrane potential of −90 mV, whole cell GIRK currents were inhibited under control (strong inward rectifying) conditions by 85 ± 2.6%, whereas whole cell currents in the presence of heterologously expressed Gβγ (weak inward rectifying conditions) were inhibited only by 28 ± 4% (Fig. 7, A–C). To verify whether or not the attenuation of the Cs+ block by co-expression of Gβγ was correlated to the Gβγ-mediated weakening of inward rectification, the degree of inward rectification (defined as Fir = I(Erev) − 50 mV/I(Erev) + 50 mV) was plotted against the potency of Cs+ to block GIRK channels. We obtained a close inverse correlation of the degree of inward rectification and the ability of Cs+ to block GIRK currents (Fig. 7D). This result suggested that a Gβγ-mediated conformational change of GIRK channels caused the reduced inward rectification and was mechanistically coupled to a reduction of the Cs+ block. We further analyzed the voltage-dependencies of the Cs+ block by comparing GIRK currents activated via endogenous G-proteins and selected GIRK currents activated via co-expressed Gβγ subunits, but exhibiting a different degree of inward rectification (most likely due to different expression levels of Gβγ subunits). Background-subtracted, current-voltage relationships of strong inward rectifying (Fir = 0.10; no Gβγ co-transfected) and medium and weakly inward rectifying currents (Fir = 0.14, Fir = 0.20; both with co-expression of Gβγ) were determined in the presence or absence of 3 mM external Cs+ and fitted according to the Woodhull model (33, 34) (Eq. 7E).

I(E) = I0(E)\left\{\frac{1}{1 + \exp\left[-(E/E_0 = 50 mV)/(E - E_{block}/2)\right]}\right\}

(Eq. 1)

The half-blocking voltage E_blockVs was shifted in the negative direction by up to −30 mV by Gβγ (E_blockVs = −64 mV for Fir = 0.10; E_blockVs = −79 mV for Fir = 0.14; E_blockVs = −93 mV for Fir = 0.20). Interestingly, the apparent voltage dependence of the Cs+-induced current block as indicated by the electrical distance δ was up to 3-fold steeper under weak inward rectifying conditions (δ = 2.2 for Fir = 0.10; δ = 3.0 for Fir = 0.14; δ = 6.9 for Fir = 0.20), suggesting a deeper penetration of Cs+ into the pore or a change in voltage-dependent binding parameters for Cs+ within the pore. This result strongly suggests that Gβγ induced a significant conformational change within the GIRK channel pore.
Myocytes Is Due to Binding of Gβγ—3-fold weakening of the inward rectification of IKACh. The were modulated in their inward rectifying properties by co-devoid of any other measurable inward rectifying currents the fact that GIRK channels heterologously expressed in cell lines of inward rectification in cells expressing or not expressing exogenous GIRK currents were recorded in the presence or absence of 3 mM Cs+ for strong (Fig. 2) and maximal concentrations Gβγ increased the open probability of GIRK channels (as demonstrated before (15, 36)), whereas at saturating concentrations Gβγ weakened inward rectification of GIRK channels giving rise to a substantial increase in outward K+ current conductance.

The Physiological Role of Weakened Inward Rectification—The inward rectification of IKACh channels is important for their physiological function to stabilize the membrane potential at negative voltages but not for blocking the generation of the plateau phase of action potentials (16). Because under physiological conditions net-potassium flux through this channel will always be in outward direction, one would predict that 2–3-fold increases in potassium outward currents, due to weakening of the inward rectification as observed in this study, will have a great impact on the shape and duration of supraventricular action potentials. It seems likely that the local in vivo concentration of ACh in the synaptic cleft can reach levels high enough, at least for very short periods, to cause weakening of inward rectification of atrial IKACh, because high frequency stimulation of the vagal nerves can induce a hyperpolarization in atrial tissue similar in amplitude as if directly evoked by ACh in the low μM range (37).

The Weakening of Inward Rectification Is Not Due to a Reduced Polyamine Affinity—It has been shown that open channel block by polyamines and Mg2+ ions contributes to inward rectification in GIRK channels. Therefore, changing the inward rectification in the observed way may be related to polyamine and/or Mg2+-binding properties to the channel. Mg2+ is known to block instantaneously, whereas polyamine block exhibits slow voltage-dependent blocking and unblocking kinetics (13, 27, 28). In whole cell patch clamp experiments the polyamine block is found to be responsible for the slow inactivation/activation of GIRK currents measured resulting from voltage steps (28). Our investigation of polyamine block revealed no striking alteration of the blocking/unblocking time constants in the presence of Gβγ over-expression. If a decrease of the polyamine affinity had been the cause for weakened inward rectification, a major increase in the blocking time constants and/or a major decrease in the unblocking time constant should have been observed. However, we found the contrary. Under weak inwardly rectifying conditions blocking time constants were slightly decreased and no major differences in unblocking time constants was observed. The observed weakening of inward rectification could be attributed to a decrease of the fraction of channels that were blocked instantaneously in outward direction (Fig. 3), pointing to attenuation of either the Mg2+-induced channel blockade or some yet unknown intrinsic outward current block (14). However, no change in inward rectification was observed when increasing internal Mg2+ up to 20
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mA to compensate for a possibly reduced potency. So far, there is no direct experimental evidence to attribute the weakening of inward rectification to altered binding properties of polyamines or Mγ2+ to the channels. However, we cannot exclude that Mγ2+-induced outward current block was completely impaired in weak inwardly rectifying GIRK channels.

The Weakening of Inward Rectification Is Associated with a Reduction of Ba2+ and Cs+ Affinity—In Kir2.1 channels there exists an overlap between sites important for inward rectification and blocking by external cations such as Cs+ and Ba2+ (11, 38). Therefore, Gβγ-induced reduction of the affinity of Ba2+ to block GIRK currents supports the assumption that weakening of inward rectification is induced by conformational changes in the pore region of GIRK channels. Extracellular Cs+ is known to block strongly inwardly rectifying K+ channels in a highly voltage-dependent manner. The Cs+-binding site is also located within the channel pore, probably deeper in the channel than the blocking site for Ba2+. Similarly to Ba2+-induced GIRK channel block, Cs+-induced block was attenuated under weak inwardly rectifying conditions and the weakening of inward rectification correlated to weakening of Cs+-induced current block. Gβγ-induced weakening of inward rectification was correlated as well with a stronger voltage dependence of Cs+-block and a shift to more negative potentials. These Gβγ-mediated changes in the pore blocking properties of GIRK channels compare well to the differences of the pore blocking properties of an ion channel. Furthermore, we demonstrate that binding of Gβγ subunits to the channel alters the conformation at known cation-binding sites within the channel pore, supporting the hypothesis that Gβγ might gate the channel at the selectivity filter rather than at a cytoplasmic gate.

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