Overexpression of Monomeric and Multimeric GIRK4 Subunits in Rat Atrial Myocytes Removes Fast Desensitization and Reduces Inward Rectification of Muscarinic K\(^{+}\) Current (I\(_{K(ACh)}\))

EVIDENCE FOR FUNCTIONAL HOMOMERIC GIRK4 CHANNELS*

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K\(^{+}\) channels composed of G-protein-coupled inwardly rectifying K\(^{+}\) channel (GIRK) (Kir3.0) subunits are expressed in cardiac, neuronal, and various endocrine tissues. They are involved in inhibiting excitability and contribute to regulating important physiological functions such as cardiac frequency and secretion of hormones. The functional cardiac (I\(_{K(ACh)}\)) channel activated by G\(_{i}/G_{o}\)-coupled receptors such as muscarinic M\(_{2}\) or purinergic A\(_{1}\) receptors is supposed to be composed of the subunits GIRK1 and GIRK4 in a heterotetrameric (2:2) fashion. In the present study, we have manipulated the subunit composition of the K\(_{ACh}\) channels in cultured atrial myocytes from hearts of adult rats by transient transfection of vectors encoding for GIRK1 or GIRK4 subunits or GIRK4 concatemeric constructs and investigated the effects on properties of macroscopic I\(_{K(ACh)}\). Transfection with a GIRK1 vector did not cause any measurable effect on properties of I\(_{K(ACh)}\), whereas transfection with a GIRK4 vector resulted in a complete loss in desensitization, a reduction of inward rectification, and a slowing of activation. Transfection of myocytes with a construct encoding for a concatemeric GIRK\(_{4}\) subunit had similar effects on desensitization and inward rectification. Following transfection of a tetramermeric construct (GIRK\(_{4}\)), these changes in properties of I\(_{K(ACh)}\) were still observed but were less pronounced. Heterologous expression in Chinese hamster ovary cells and human embryonic kidney 293 cells of monomeric, dimeric, and tetramereric GIRK4 resulted in robust currents activated by co-expressed A\(_{1}\) and M\(_{2}\) receptors, respectively. These data provide strong evidence that homomeric GIRK4 complexes form functional G\(_{\beta\gamma}\)-gated ion channels and that kinetic properties of GIRK channels, such as activation rate, desensitization, and inward rectification, depend on subunit composition.

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GIRK\(^{1}\) channels contribute to parasympathetic reduction of cardiac frequency and reduce excitability of central neurons and various endocrine cells (for reviews, see Refs. 1–4). The cardiac channel complex is supposed to be composed of GIRK1 (Kir3.1) and GIRK4 (Kir3.4) subunits in a heterotetrameric (2:2) fashion (5), whereas neuronal channels contain, apart from GIRK1, the subunits GIRK2 or GIRK3. Recent evidence suggests, however, that GIRK4 is also expressed in the brain (6). According to the initial concept, GIRK1 subunits without co-expressed GIRK2, GIRK3, or GIRK4 subunits do not co-assemble and are not translocated to the membrane, whereas GIRK2, GIRK3, and GIRK4 are necessary for subunit assembly and translocation but do not form functional homomeric channel without GIRK1 (7). However, more recently, it has been shown that in atrial myocytes, a large fraction of GIRK4 subunits exist as homomultimers (8). Moreover, in neurons, GIRK2 and GIRK3 subunits have been localized without GIRK1 protein (9, 10), suggesting that monomeric complexes devoid of GIRK1 may form functional channels.

Cardiac GIRK channels are activated by various heptahelical receptors coupled to heterotrimeric G-proteins of the pertussis toxin-sensitive class (G\(_{i}/G_{o}\)), of which M\(_{2}\)AChR is the paradigmatic example. Receptor activation results in dissociation of the heterotrimeric G-protein complex into its G\(_{\alpha}\) and G\(_{\beta\gamma}\) subunits. In turn, the G\(_{\beta\gamma}\) subunits interact with the GIRK subunits in a membrane-delimited fashion, causing an increase in open-state probability of the channel complex.

Following activation by exposure to ACh, atrial I\(_{K(ACh)}\) shows a peculiar type of desensitization, i.e. a partial decay in current with a half-time of a few seconds (11–13), usually referred to as “acute” or “fast” desensitization. This component of desensitization is assumed to be localized downstream of the receptor. The mechanism(s) underlying this acute desensitization, however, so far has not been resolved.

In the present study, GIRK4 subunits and GIRK4 concatemeric constructs were overexpressed in cultured adult rat atrial myocytes by transient transfection. Overexpression of GIRK4 resulted in ACh-activated currents that completely lacked fast desensitization. Strong inward rectification, a key property of GIRK currents, resulting from a block of outward current flow by intracellular cations, particularly polyamines, was reduced in GIRK4-transfected myocytes as compared with native cells. Qualitatively, this was confirmed by expressing GIRK4 subunits in CHO cells and HEK293 cells, which are assumed to be devoid of intrinsic GIRK1 subunits. These find-
ings support the notion that important physiological properties, such as inward rectification and desensitization, depend on the subunit composition of the channel complex. Moreover, in atrial myocytes GIRK channel complexes with subunit compositions different from the GIRK4-GIRK1 stoichiometry might contribute to macroscopic \( I_{\text{KAC}} \).

**EXPERIMENTAL PROCEDURES**

### Isolation and Culture of Atrial Myocytes—Experiments were performed with local ethics committee approval. Wistar Kyoto rats of either sex (around 200 g) were anesthetized by i.v. injection of urethane (1 g/kg). The chest was opened, and a heart was dissected and mounted on the cannula of a sterile Langendorff apparatus for coronary perfusion at constant flow. The method of enzymatic isolation of atrial myocytes has been described elsewhere (e.g. Ref. 12). The culture medium was fetal calf serum-free bicarbonate-buffered M199 (Life Technologies, Inc., Karlsruhe, Germany) containing gentamycin (25 \( \mu \)g/ml, Sigma), and kanamycin (25 \( \mu \)g/ml, Sigma). Cells were plated at a low density (several thousand cells per dish) on 36-mm culture dishes. Medium was changed 24 h after plating and then every second day. Myocytes were used experimentally from day 0 until day 5 after isolation. No effects of time in culture were found for the key experiments.

### Solutions and Chemicals—For the patch clamp measurements, an extracellular solution of the following composition was used: 120 mM NaCl, 20 mM KCl, 1.0 mM MgCl\(_2\), 1.0 mM Hepes/NaOH, pH 7.4. The solution for filling the patch-clamp pipettes for whole cell voltage clamp experiments contained 110 mM potassium-aspartate, 20 mM KCl, 5.0 mM NaCl, 1.0 mM MgCl\(_2\), 2.0 mM Na\(_2\)ATP, 2.0 mM EGTA, 0.01 mM GTP, 10.0 mM Hepes/ROH, pH 7.4. Standard chemicals were from Merck (Darmstadt, Germany), EGTA, Hepes, MgATP, Ado GTP, and ACh-chloride were from Sigma.

### Current Measurement—Membrane currents were measured using whole-cell patch clamp. Pipettes were fabricated from borosilicate glass and were filled with the solution listed above (direct current resistance, 4–6 M\( \Omega \)). Currents were measured by means of a patch clamp amplifier (List LMF/ECF 7, Darmstadt, Germany). Signals were analog filtered (corner frequency, 1–3 KHz), digitally sampled at 5 KHz and stored on a computer equipped with a hardware/software package (ISO2, MFK, Frankfurt/Main, Germany) for voltage control and data acquisition. Experiments were performed at ambient temperature (22–24 °C). Cells were voltage-clamped at \(-90\) mV, i.e. negative to \(E_g\), resulting in inward \( K^+ \) currents. Current-voltage relations were determined by means of voltage ramps between \(-120\) and \(-20\) mV. Rapid superposition of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system that permitted switching between up to six different solutions (\(t_{\text{sw}} \leq 100\) ms). Performance of this system was dependent on the positioning of the outlet tube in relation to the cell studied. This was routinely optimized by measuring the time course of the blocking action of Ba\(^{2+}\) on \(I_{\text{g}}\).

### Rat GIRK4 Constructs Encoding for Dimeric and Tetrameric Subunits—To obtain the different GIRK4 constructs, we amplified the rat cDNA using different polymerase chain reaction primers to attach restriction sites for further coupling. The following constructs were amplified: A, Kpnl-I-GIRK4-(Met\(^1\)-Met\(^{419}\))-(CAA\(_x\))\(_3\)-EcoRV; B, EcoRV-(CAAX\(_x\))-rGIRK4-(Met\(^1\)-Met\(^{419}\))-(CAAX\(_x\))\(_3\)Xhol; C, Xhol-(CAAX\(_x\))\(_3\)-rGIRK4-(Met\(^1\)-Met\(^{419}\))-(CAAX\(_x\))\(_3\)EcoRV; D, EcoRV-(CAAX\(_x\))\(_3\)-rGIRK4-(Met\(^1\)-Met\(^{419}\))-(CAAX\(_x\))\(_3\)Xhol; E, Xhol-(CAAX\(_x\))\(_3\)-rGIRK4-(Met\(^1\)-Met\(^{419}\))-(CAAX\(_x\))\(_3\)EcoRV; F, EcoRV-(CAAX\(_x\))\(_3\)-rGIRK4-(Met\(^1\)-Met\(^{419}\))-(CAAX\(_x\))\(_3\)Xhol. To construct the GIRK4 dimer, GIRK4-A was cut using the restriction enzymes Kpnl and EcoRV to ligate in the vector pcDNA3 (Invitrogen) using the same sites and opened after ligation with EcoRV and Apal. This was ligated into this vector using the corresponding restriction sites. The resulting tandem clone has the following amino acid sequence: kir3.4\(^{1-419}\)-QQQQQDI-QQQQQ-kir3.4\(^{1-419}\), (pGIRK4). To obtain the GIRK4 tetramer, pcDNA3-GIRK4-A-E was digested using EcoRV and Xhol, and GIRK4-B was inserted. pcDNA3-GIRK4-A-B-E was further digested with Xhol and XbaI, and GIRK4-L was ligated using the corresponding restriction sites. The amino acid sequence of the tetramer pcDNA3-GIRK4-A-B-C-E was GIRK4\(^{1-419}\)-QQQQQDI-QQQQQGIRK4\(^{1-419}\)-QQQQQLE-QQQQQ-kir3.4\(^{1-419}\)-QQQQQQ-SRQQQQQGIRK4\(^{1-419}\), (pcDNA3-GIRK4). All constructs were sequenced to verify the nucleotide sequence.

### Transfection of CHO and HEK293 Cells—One day after the inoculation of HEK293 cells or CHO cells, 3 \( \mu \)g of each GIRK clone was transfectioned into the cells on a Petri dish (9-cm diameter) with LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. CHO cells were co-transfected with a pSV-SPORT-1-R vector encoding for a rat brain A\(_{1}\)aDor (kindly provided by Dr. A. Rarsch, Göttingen, Germany). HEK293 cells were co-transfected with a pcDNA3-M\(_{1}\)AChR vector, encoding a human \( M_{1}\)AChR.

### Immunoblot Detection of GIRK4 Monomers and Dimers in HEK293 Cells—After 2 days, the cells were rinsed twice with 10 ml of phosphate-buffered saline and collected with 1 ml of a preparation buffer (20 mM Hepes/NaOH (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 150 mM NaCl, 2% (w/v) Triton X-100, 1% (w/v) cholate, 1 mM phenylmethylsulfonyl fluoride, and 5 \( \mu \)g/ml each of pepstatin, leupeptin, and chymostatin). The cell suspension was sonicated using a TOMY ultrasonic disruptor (UD-201, Tokyo, Japan) and centrifuged at 1000 \( \times \) g for 5 min. The supernatant (3 \( \mu \)l) was loaded onto SDS-polyacrylamide (11%) gels and transferred to a polyvinylidene difluoride membrane. Immunoblotting was carried out as described previously (9). Briefly, the membrane was incubated with a primary antibody against GIRK4 (aG4N-10) raised in rabbit against a synthetic peptide, DSRNAMNQDMEIGV, corresponding to the amino acids 4–17 of GIRK4 (14) at a concentration of 0.5 \( \mu \)g/ml at 4 °C overnight. After extensive washing, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:1000) for 1 h at room temperature. The immunoreactive signals were developed with a SuperSignal chemiluminescent substrate (Pierce) and exposed to Hyperfilm ECL for 5 s (Amersham Pharmacia Biotech).

### Transfection of Atrial Myocytes—Following isolation, myocytes were cultured overnight to allow for attachment. For transfection of atrial myocytes the following vectors were used: the reporter pIREs-EGFP vector (CLONTECH, 1.0 \( \mu \)g/plate), pcDNA-GIRK1, pcDNA-GIRK4, pcDNA-GIRK4\(_2\), pcDNA-GIRK4\(_4\), and pSV-SPORT1-A R (0.4 \( \mu \)g/plate). Transfection was performed by means of LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Electrophysiological recordings were made on days 3 and 4 after transfection. Transfected cells were identified using epifluorescence of EGFP (excitation wavelength, 470 nm). Time-matched EGFP-positive cells transfected with the reporter vector only served as controls.

### Statistical Analysis—Student’s t test was applied for the analysis of the results; differences at \( p < 0.05 \) were considered statistically significant.

### RESULTS

#### Immunoblot Detection of GIRK4 Tandem Constructs in HEK293 Cells—Lysates of transfected HEK293 cells were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted. Antibodies against amino acids 4–17 of rat GIRK4 recognized proteins of \( \approx 45, \approx 90, \) and \( \approx 180 \) kDa in cells transfected with pcDNA-GIRK4\(_4\), pcDNA-GIRK4\(_2\), and pcDNA-GIRK4\(_2\), respectively (Fig. 1). Because transfection rates using LipofectAMINE methodology in atrial myocytes in terms of EGFP-positive cells were usually less than 5%, corresponding blots to verify expression of these proteins in myocyte cultures could not be produced.

#### Transfection of Atrial Myocytes with GIRK4 Removes Rapid Desensitization of \( I_{\text{g}} \)—In native atrial myocytes, \( I_{\text{g}} \) upon activation by rapid exposure to \( ACh \) at concentrations \( \approx 1 \) \( \mu \)M, shows various components of desensitization (12). The acute component, not related to the activating receptor has a half-time \( t_{1/2} \) of magnitude of 5 s and is heterogeneous (15). Its magnitude varies in individual cells, and it is affected by the experimental conditions, such as rise time of the agonist concentration, which depends on the superfusion device, temperature, or density of functional receptors (12,13,16). In order to separate fast desensitization from receptor desensitization, exposures to \( ACh \) were usually limited to \( \approx 60 \) s. For simplicity, in the following experiments, the current level reached after 30 s was considered as quasi-steady-state current (15). As shown in that study, complete recovery from fast desensitiza-
tion following washout of ACh takes less than 30 s. Thus, apart from its fast onset, acute desensitization is defined by its rapid reversibility and by its heterologous nature (cf. Fig. 4).

Fig. 2 compares representative sample traces of ACh-induced (10 μM) inward currents recorded from a control (EGFP-positive) myocyte (Fig. 2A), a myocyte transfected with pcDNA-GIRK1 (Fig. 2B), a myocyte co-transfected with pcDNA-GIRK1/pcDNA-GIRK4 (Fig. 2C), and a myocyte transfected with the pcDNA-GIRK4 vector (Fig. 2D). Whereas GIRK1 and GIRK1/GIRK4 expression did not seem to affect the kinetic properties of IK(ACh), in the GIRK4-transfected cell, the current throughout exposure to ACh remained constant, with no sign of desensitization. To qualitatively assess the amount of fast desensitization, quasi-steady-state currents (at 30 s after changing to ACh-containing solution) normalized to peak inward current (for control myocytes) or current level at t=1 s in the case of GIRK4-transfected myocytes have been compared. The summarized data in Fig. 2E demonstrate that fast desensitization of IK(ACh) was completely abolished in myocytes transfected with the GIRK4 vector, whereas no significant difference was found between controls and myocytes overexpressing GIRK1 or GIRK1/GIRK4, respectively. Surprisingly, current densities of IK(ACh) were not significantly different in the groups of myocytes subject to the different transfection protocols.

Fig. 3 illustrates that apart from the removal of acute desensitization, GIRK4 overexpression resulted in a slowing of activation upon fast agonist application. The mean time constant of activation in this series of experiments was increased from about 300 ms to 750 ms. Because a slowing of the rise time of...
IK(ACh) per se results in a decrease or blunting of the fast desensitizing component (13, 16), it is conceivable that the absence of desensitization in GIRK4-overexpressing cells reflects a consequence of the slower rise time. Although the mechanism(s) underlying fast desensitization in the system under study is not understood, there is strong evidence that it reflects a phenomenon related to a signaling element downstream of the receptor, rather than the receptor itself. The major arguments against receptor desensitization come from the heterologous nature of fast desensitization. Two experimental protocols demonstrating the independence of desensitization and its removal by GIRK4 overexpression on the species of the activating receptor are illustrated in Figs. 4 and 5.

**Fig. 4. Absence of heterologous desensitization in GIRK4-transfected myocytes.** Saturating concentrations of ACh (20 μM) and Ado (100 μM) were superfused as indicated. Representative current recordings from a control (A) and a GIRK4-transfected myocyte (B). C, summarized data from six time-matched myocytes for each group. The bars indicate the ratios of peak current induced by Ado plus ACh (average of three consecutive responses from individual traces, as shown in A and B) divided by peak ACh-induced current in the absence of Ado.

**Fig. 5. Removal of desensitization by GIRK4 overexpression is not limited to activation via M2AChR.** Representative traces showing inward IK(ACh) activated by ACh (20 μM) and Ado (100 μM). Panel A, control myocyte transfected with the EGFP vector only; panel B, myocyte transfected with pSV-SPORT-A1R; panel C, myocyte transfected with pSV-SPORT-A1R plus pcDNA-GIRK4.
underscoring independence of desensitization and its removal by GIRK4 overexpression on receptor species.

Previously, it has been shown that the speed and amount of acute desensitization are increased at positive membrane potentials (19), which can be considered as additional evidence in support of the notion that it represents a phenomenon related to the channel. As shown in Fig. 6A, in a control myocyte, desensitization is more pronounced for outward as compared with inward IK(ACh) (see legend for experimental details), whereas in GIRK4-transfected myocytes, desensitization was lacking at both membrane potentials (Fig. 6B). This observation, which is representative of five time-matched GIRK4-transfected and control myocytes, demonstrates that desensitization is genuinely removed rather than altered in its voltage dependence.

**Inward Rectification of Atrial IK(ACh) Is Reduced by GIRK4 Transfection**—GIRK channels are characterized by their strong inward-rectifying properties. Inward rectification of these channels reflects a block by endogenous intracellular cations, in particular by polyamines (spermine and spermidine) (20, 21). A comparison of current-voltage relations obtained by voltage ramps from –120 to +60 mV reveals a reduction in inward rectification in GIRK4-transfected as compared with control myocytes (Fig. 7, A and B). To statistically compare inward rectification in the two groups of myocytes, ratios of current at 0 and –100 mV were calculated from I/V curves of individual cells and summarized in Fig. 7C. This qualitative assessment yields a highly significant difference in inward-rectifying properties between the two groups. No difference was found if data from native (i.e. nontransfected) myocytes and myocytes transfected with the EGFP vector only were compared (not shown).

Effects of GIRK4 Transfection on Atrial IK(ACh) Are Mimicked by Concatemeric GIRK42 and GIRK44—The data presented so far demonstrate that transfection of atrial myocytes with a vector encoding for the GIRK4 subunit affects key properties of macroscopic IK(ACh), suggesting that functional channel complexes with a subunit composition different from the native GIRK channel population are formed. To obtain further information on this issue, myocytes were transfected with concatemeric GIRK4 constructs (GIRK42 and GIRK44). The results are

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**Fig. 6.** GIRK4 overexpression removes desensitization of inward and outward IK(ACh). *A,* representative recordings from a control myocyte at two different extracellular K*⁺* concentrations and holding potentials: 20 mM/90 mV/EK = –49 mV (*a*), and 5 mM/–40 mV/EK = –84 mV (*b*). ACh (20 μM) was applied as indicated. The trace labeled 5 K*⁺* has been inverted and scaled up in c to match the peak of inward IK(ACh), at 20 mM K*⁺* and –90 mV holding potential. *B,* representative recordings from a GIRK4-transfected myocyte. Panels a–c have the same meaning as in *A*.

**Fig. 7.** Inward rectification is reduced in GIRK4-overexpressing myocytes. *A* and *B* represent difference current-voltage relations obtained by electronic subtraction of background current from ACh-induced current (voltage ramps from –120 to +60 mV). *A,* control; *B,* GIRK4-transfected myocyte. *C,* summarized data from 12 cells each. Inward rectification was expressed as ratio of current at 0 mV divided by current at –100 mV.
Fig. 8. Properties of $(I_{K_{ACH}})$ in myocytes transfected with GIRK4 and GIRK4 concatemers. Representative recordings of inward $I_{K_{ACH}}$ evoked by 20 μM ACh from a myocyte transfected with a GIRK42 (A) and a GIRK44 (B) construct. C, summarized data on fast desensitization, as in Fig. 2E. The quotient was significantly different from controls for the GIRK42 group but not different from those for the GIRK44 group (n = 6). Summarized data on inward rectification are as in Fig. 5. The bars representing the control and GIRK4 groups C and D are the same as in Figs. 1E and 4C, respectively.

The principle question to be addressed by this series of experiments was whether expression of monomeric or tetrameric GIRK4 results in receptor-activated GIRK currents in a cell line devoid of intrinsic GIRK subunits. To provide a receptor for activation of the signaling pathway, in CHO cells an A1 receptor was co-expressed with vectors encoding for GIRK1 and A1AdoR (data not shown). Thus, at least with regard to inward rectification, receptor-activated GIRK currents in the two cell lines mimic those currents observed in myocytes transfected with the same subunits. This supports the idea that the changes in properties of $I_{K_{ACH}}$, caused by GIRK4 overexpression are due to formation of homomeric channels rather than an indirect effect at some stage of the signaling pathway.

**DISCUSSION**

The cardiac $K_{ACH1}$ channel that is expressed predominantly in supraventricular tissue of the heart but also, at a lower level, in ventricular myocytes (23) is supposed to represent a heterotetrameric complex of GIRK1 and GIRK4 (24). Evidence has been provided that binding of $G_{bg}$ to the carboxyl terminus of the GIRK4 subunit is essential for channel activation, whereas the requirement for interaction of $G_{bg}$ with GIRK1 remained unclear (25–29). In a very recent study, it was demonstrated that both heterotetrameric GIRK1/GIRK4 complexes and GIRK4 homotetramers exhibit a 1:1 subunit-$G_{bg}$ binding stoichiometry (30).

Initially, it was assumed that homomorphic GIRK1 channels represented the atrial $K_{ACH1}$ channel (31). This resulted from an intrinsic GIRK subunit (XIR), homologous to GIRK4 of the...
According to the current concept, GIRK1 subunits do not assemble to form functional channels (7). Assembly and membrane translocation require the expression of GIRK4 or a related neuronal type of subunit. Moreover, in GIRK4 knockout mice, a concomitant loss of GIRK1 protein has been observed (7), suggesting a role of GIRK4 in controlling expression of GIRK1. Only small currents were measured in Xenopus oocytes expressing wild type GIRK4 alone, whereas large currents could be recorded if the GIRK4 subunit contained a point mutation (S143F) (33). Other authors described robust macroscopic Gbg-activated currents in Xenopus oocytes injected with GIRK4 mRNA (34, 35). In a mammalian expression system, macroscopic currents carried by homomeric GIRK4 channels so far have not been identified. Single channel currents carried by GIRK4 homomers expressed in oocytes and CHO cells have extremely short open times, which renders them inaccessible to an analysis of their basic properties (24).

In bovine atria, a substantial fraction of GIRK4 protein exists as homotetrameric complex (8). The physiological significance of this finding, however, remained unknown. Because the present data clearly demonstrate sizable whole cell currents in CHO cells expressing monomeric, dimeric, and tetrameric GIRK4 and a suitable receptor, intrinsic GIRK4 homotetramers are likely to contribute to macroscopic IK(ACh) in atrial myocytes.

Fast desensitization is a key property of atrial IK(ACh). Its heterologous nature between various receptors (11, 12, 17) provided the major argument that this component of agonist-dependent decay in current is unlikely to reflect receptor desensitization or down-regulation, which is common to many, if not all, G-protein-coupled receptors (36–38). Desensitization of the M2AChR in the system studied here requires much longer periods of exposure to an agonist than were used in the present study. Moreover, reversibility is much slower (16). Various mechanisms underlying fast desensitization have been proposed so far, such as a dephosphorylation of the channel (39) or a non-identified component of the signaling pathway (40). More recently, it was proposed to reflect depletion of phosphatidylinositol 4,5-bisphosphate due to simultaneous activation of a Gq-coupled M3 receptor activating phospholipase C (41). This is contradictory to the heterologous nature of fast desensitization. Moreover, recent evidence suggested that depletion of phosphatidylinositol 4,5-bisphosphate following stimulation of intrinsic PLC-coupled receptors results in inhibition of IK(ACh), that is slower than fast desensitization by at least one order of magnitude (15). Some of the properties of fast desensitization can be accounted for by the kinetics of the G-protein cycle (42). The present data, however, support the notion that fast desensitization reflects a property of the channel complex. Either it is genuinely absent in GIRK4 homomeric channel complexes or, alternatively, desensitization of the current carried by these complexes proceeds during the activation phase, which is significantly slowed as compared with the native current (compare Fig. 3). This can be formally modeled...

**FIG. 9.** Current-voltage relations of GIRK currents induced by activation of A1 receptors in CHO cells. Difference I/V-curves of currents evoked by 100 μM adenosine (background subtracted) representative of CHO cells transfected with GIRK1 (A), GIRK1 plus GIRK4 (B), GIRK4 (C), and GIRK4 (D). E, summarized data on inward rectification (quotients of current at 0 mV divided by current at –100 mV).

**FIG. 10. Simulation of desensitization properties of receptor-activated current at two different rates of activation.** A, normalized activation with time constants of 125 ms (a) and 400 ms (b). Trace c represents an inactivation process with a time constant of 500 ms and a steady-state amplitude of 50% of steady-state activation. Traces in B represent simulated normalized currents combining inactivation with fast activation (a) and slow activation (b).
by a scheme in which desensitization is linked to activation of the channel complex by G$_{Y_2}$, as illustrated in Fig. 10. In this simulation, agonist-induced activation of I$_{I\text{K}(\text{ACh})}$ was modeled for simplicity as a $\beta_2$-induced activation. Inactivation or desensitization was modeled using first order kinetics with a time constant of 500 ms (Fig. 10A). The simulated normalized current traces in Fig. 10B yield a rapidly activating current with a distinct desensitizing component, whereas the current activating with the slower rate apparently lacks desensitization (compare Fig. 3A). Although this simple model does not support any particular mechanism of GIRK channel-associated desensitization, it would be in line with a lower affinity of the carboxyl-terminal binding site of GIRK4 as compared with GIRK1 to $\beta_2$-subunits reported in Ref. 43.

Our data suggest that GIRK4 homotetrameric complexes might contribute to macroscopic I$_{I\text{K}(\text{ACh})}$ with a nondesensitizing component, whereas GIRK1/GIRK4 heteromeric channels show desensitization. This hypothesis, however, does not explain why, in GIRK4- or GIRK42-transfected myocytes, the fast desensitizing component was always completely lost, because endogenous GIRK1 should still be able to associate with GIRK4. We assume that on the background of a high expression level of monomeric or dimeric GIRK4, the low probability of formation of heteromeric complexes results in whole cell currents that are highly dominated by the properties of homomeric GIRK4 channels. This would be in line with the observation that in myocytes transfected with the tetrameric construct, I$_{I\text{K}(\text{ACh})}$ had intermediate properties, because intrinsic GIRK1/GIRK4 complexes should still exist.

An alternative explanation for the loss of contribution of heteromeric channels would be a competition of the channel complexes for a limited number of putative anchoring domains required for functional membrane targeting. This would also explain why we did not find significant changes in current densities even in myocytes transfected with both the GIRK1 and GIRK4 encoding vectors. An anchoring protein interacting with GIRK4, however, so far has not been identified. Alternatively, the total current might be limited by the expression level of endogenous G-proteins.

Inward rectification of Kir channels reflects a block by intracellular cations, such as Mg$^{2+}$ and spermine (see Refs. 3 and 21 for reviews). In the situation of a whole cell patch clamp experiment, also exogenous constituents of the pipette filling solution, such as organic buffers, might contribute to inward rectification, also exogenous constituents of the pipette filling solution. In the situation of a whole cell patch clamp experiment, also exogenous constituents of the pipette filling solution. In the situation of a whole cell patch clamp experiment, also exogenous constituents of the pipette filling solution. In the situation of a whole cell patch clamp experiment, also exogenous constituents of the pipette filling solution. In the situation of a whole cell patch clamp experiment, also exogenous constituents of the pipette filling solution.

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