The muscarinic-gated atrial potassium (IKACH) channel contributes to the heart rate decrease triggered by the parasympathetic nervous system. IKACH is a heteromultimeric complex formed by Kir3.1 and Kir3.4 subunits, although Kir3.4 homomultimers have also been proposed to contribute to this conductance. While Kir3.4 homomultimers evince many properties of IKACH, the contribution of Kir3.1 to IKACH is less well understood. Here, we explored the significance of Kir3.1 using knock-out mice. Kir3.1 knock-out mice were viable and appeared normal. The loss of Kir3.1 did not affect the level of atrial Kir3.4 protein but was correlated with a loss of carbachol-induced current in atrial myocytes. Low level channel activity resembling recombinant Kir3.4 homomultimers was observed in 40% of the cell-attached patches from Kir3.1 knock-out myocytes. Channel activity typically ran down quickly, however, and was not recovered in the inside-out configuration despite the addition of GTP and ATP to the bath. Both Kir3.1 knock-out and Kir3.4 knock-out mice exhibited mild resting tachycardias and blunted responses to pharmacological manipulation intended to activate IKACH. We conclude that Kir3.1 confers properties to IKACH that enhance channel activity and that Kir3.4 homomultimers do not contribute significantly to the muscarinic-gated potassium current.

The heart rate decrease mediated by the parasympathetic branch of the autonomic nervous system involves the release of acetylcholine from post-ganglionic cholinergic neurons onto atrial myocytes and sinoatrial and atrioventricular nodal cells. Acetylcholine binds M2 muscarinic receptors on these cells, triggering the activation of pertussis toxin-sensitive G proteins. The activated Gα and Gβγ subunits in turn modulate the function of multiple enzymes and ion channels, including the cardiac G protein-gated, inwardly rectifying potassium channel I_K(A)Ch (1). I_K(A)Ch is one of the most well characterized G protein-regulated ion channels, exhibiting potent activation by Gβγ subunits (2–4). I_K(A)Ch is thought to be a heterotetrameric complex formed by the homologous Kir3.1/GIRK1 and Kir3.4/GIRK4 potassium channel subunits (5–10). Kir3.1 was proposed initially to constitute an integral subunit of both neuronal and cardiac G protein-gated potassium channels (7, 11, 12). Recent studies, however, have presented evidence for the existence of native G protein-gated potassium channels that do not contain Kir3.1 (13–15). Indeed, Kir3.4 homotetrameric complexes have been identified in heart atrial tissue and were proposed to contribute significantly to macroscopic IKACH current (15, 16).

Studies in Xenopus oocyte and mammalian cell expression systems have offered some insight into the functional contribution of the Kir3 subunits to IKACH function. Recombinant Kir3.4 homomultimers manifest several key functional properties of IKACH, including coupling to G protein-coupled receptors, gating by Gβγ subunits, inward rectification, and potassium selectivity (7, 16–18). Furthermore, transfection of cultured rat atrial myocytes with monomeric, dimeric, and tetrameric Kir3.4 expression constructs lead to a loss of acute desensitization of the muscarinic-gated K⁺ current, a reduction in inward rectification, and a slowing of current activation (16). A comparison of the functional properties of native IKACH and recombinant Kir3.4 homomultimers suggests that Kir3.1 impacts the gating, conductance, and ATP-dependent modulation of the Kir3.1/3.4 heteromultimer (4, 7, 16, 17). The failure of recombinant Kir3.1 complexes to achieve surface membrane expression has precluded a rigorous examination of their functional properties (7, 19).

The significance of IKACH to heart rate regulation was demonstrated recently using a mouse knock-out strategy. Kir3.4 knock-out mice lacked cardiac IKACH and exhibited blunted heart rate decreases in response to indirect vagal stimulation and A1 adenosine receptor activation (20). The study indicated that IKACH is responsible for a significant fraction of the heart rate decrease associated with these manipulations. Interestingly, Kir3.4 knock-out mice were also unable to alter heart rate significantly on a beat-to-beat time scale, reflected in decreased heart rate variability, and were resistant to atrial fibrillation caused by vagal stimulation (21).

In this study, we sought to determine the significance of Kir3.1 to the formation and function of cardiac IKACH. We describe the generation of Kir3.1 knock-out mice and examine the effects of Kir3.1 ablation on Kir3.4 expression, IKACH function, and heart rate regulation. Our findings indicate that Kir3.1 is required for the effective functioning of this cardiac ion channel and argue that Kir3.4 homomultimeric complexes contribute little to the heart rate decrease associated with vagal and A1 adenosine receptor activation.

**EXPERIMENTAL PROCEDURES**

*Generation of Kir3.1 Knock-out Mice—* A Cre recombinase-based gene targeting strategy was developed to permit the generation of tissue-specific and/or conditional Kir3.1 knock-out lines. This study, however, describes only the generation of the constitutive null Kir3.1 mutant line. Suitable 5' and 3' Kir3.1 homology arms were subcloned into a pBluescript-based plasmid containing a neomycin resistance gene (NEO) driven by the mouse PGK promoter (kindly provided by M. Picciotto). The NEO cassette was flanked by Cre recombinase recogni-
129SvJ embryonic stem cells at passage 11 (Genome Systems, St. Louis, MO) were transplanted with the linearized Kir3.1 targeting vector as described (23), and 692 colonies surviving G418 selection (200 μg/ml active constituent for 10 days) were picked, amplified, and screened by PCR and Southern blotting for the appropriate homologous recombination event. A single embryonic stem cell clone (1/892 = 0.14%) harboring the targeted allele was amplified and transfected with a plasmid containing the Cre recombinase cDNA driven by the herpes simplex virus thymidine kinase promoter (kindly provided by L. Nitschke) to promote the conditional null genotype. Brains were dissected from homozygous Kir3.1−/− mice at embryonic day 16.5 (E16.5) and E17, and the diphtheria toxin (DT) was injected intraperitoneally on postnatal days 2 and 4. Animals were scored for the presence of 3×Kir3.1+ erythroid cells in bone marrow as described (24).

Western Blotting of Atrial Membrane Proteins—Adult (8–12-week) mice were sacrificed by CO2 asphyxiation. Hearts were extracted and the left atrial appendage was dissected on ice. Tissue was homogenized in 1 ml of 2% SDS buffer containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 14,000 × g for 30 min at 4°C. The crude membrane fraction was pelleted by centrifugation at 100,000 × g for 30 min. Pellets were resuspended in 1 ml of a 2% SDS solution (pre-warmed to 37°C) containing 1 mM DTT and PIC. Samples were centrifuged for 5 min at 50,000 × g to remove insoluble contents. Protein concentrations were determined using the Lowry assay followed by SDS/PAGE and transferred to nitrocellulose membranes using the semi-dry apparatus (Bio-Rad). Nonspecific binding sites were blocked for 1 h using a 5% milk solution. Atrial membrane samples were probed with antibodies to Kir3.1 (1:2000), Kir3.4 (1:2000), Kir3.5, Kir3.6, Kir2.1, Kir2.2, Kir2.3, Kir2.4, CaV1.1, CaV1.2, CaV1.3, CaV2.1, CaV2.2, CaV2.3, and CaV2.4. In some experiments, 1–10 μg of atrial membrane protein per well were loaded onto 4%–12% Bis-Tris gradient gels. Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences) under reducing conditions. Membranes were blocked for 1 h using a 5% milk solution. Anti-Kir3.1 (Alomone Laboratories; Jerusalem, Israel) and anti-mu2 muscarinic receptor (Sigma) antibodies were used at 1:100 and 1:200 dilutions, respectively. The anti-Kir3.1 antibody was raised against the carboxyl terminal 1–200 residues of the Kir3.1 subunit. The anti-mu2 muscarinic antibody recognizes a single band at 78 kDa, corresponding to the full-length G protein-coupled receptor. Membranes were incubated in primary antibody at 4°C overnight, washed with TBST (Tris-buffered saline containing 0.01% Tween 20), exposed to horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:6000; Pierce) for 1 h at room temperature, and washed with TBST. Protein bands were revealed using ECL Western blotting detection reagents according to manufacturer’s specifications (Amersham Biosciences) using BIOMAX MR x-ray film (Eastman Kodak Co.; Rochester, NY).

Preparation of Primary Atrial Myocyte Cultures—Breeding pairs containing Kir3.1+/− and Kir3.1−/− Si mice, Kir3.1 knock-out, Kir3.1+/-, and normal litter mates were established to generate litters of mice of defined genotype. Genotype was verified by PCR of tail samples as described (20). Atrial auricles from four-six neonatal mice (postnatal day 2–4) were micro dissected from total heart tissue. Myocytes were isolated using the neonatal rat cardiac myocyte isolation kit (Worthington Biochemical, Lakewood, NJ) with minor modifications to the manufacturer’s protocol designed to accommodate the smaller amount of starting tissue. Briefly, atrial tissue was incubated overnight at 4°C in 5 ml of trypsin solution (25 μg/ml). The next morning, 500 μg/ml of trypsin inhibitor and 75 units/ml of purified collagenase were added, and the samples were incubated at 37°C for 30 min with gentle shaking. Subsequently, cells were filtered through a strainer to remove undigested tissue and then counted. Cells were seeded at 50–100 × 10⁶ for 5 min and resuspended in L-15 media containing 10% fetal bovine serum and penicillin/streptomycin. Isolated cells were plated at a density of 400,000 cells/ml and incubated at 37°C/5% CO2 for 24–48 h prior to electrophysiological testing.

Electrophysiology—For whole-cell recordings, patch pipettes (3–5 MΩ) were filled with a solution containing the following (in mM): 130 KCl, 10 NaCl, 1 EGTA/KOH (pH 7.2), 0.5 MgCl2, 10 HEPES/KOH (pH 7.2), 2 Na-ATP, 5 phosphocreatine, 0.2 NaGTP. The low K+ bath solution consisted of the following (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.5 n-glucose, 2 HEPES/NaOH (pH 7.4). The high K+ bath solution was formed by replacing NaCl with KCl. The bath drug solutions were applied rapidly with an SF-77B Perfusion Fast-Step system (Warner Instruments, Inc., Hamden, CT). Cells were visualized using an inverted Olympus IX70 microscope. Whole-cell currents were detected with an Axopatch-200B amplifier (Axon Instruments, Inc., Union City, CA), low-pass-filtered at 1 kHz, and sampled at 2 kHz with pCLAMP, version 8.0 software. CCh-induced currents in the low K+ bath solution were measured in voltage-clamp mode with the membrane potential held at −90 or −50 mV to observe inward and outward currents, respectively. CCh-induced currents in the high K+ bath solution were measured in voltage-clamp mode with the membrane potential held at −90 mV. Peak currents evoked by consecutive applications of CCh (separated by a 15–30-s washout) were averaged to obtain the CCh-induced current. Current-voltage plots of CCh-induced currents were obtained by subtracting baseline traces from CCh-induced currents evoked by a voltage pulse protocol (−120 to +80 mV in 20-mV increments, 300 ms per step). The holding potential for current-voltage determinations was −80 mV.

For single channel recordings, patch pipettes (4–8 MΩ) were filled with a solution containing the following (in mM): 150 KCl, 1 EGTA/KOH (pH 7.2), 1 MgCl2, 5 HEPES/KOH (pH 7.2), and 20 μM CCh. The bath solution contained the following (in mM): 150 KCl, 1 MgCl2, 5.5 n-glucose, 5 HEPES/KOH (pH 7.2). The bath solution was partially perfused with either 6–12 mM GTP or 0.2 mM GTP plus 1 mM K+ ATP to examine the G protein dependence of measured currents and the possible regulation by ATP-dependent processes, respectively. In some experiments, 1–10 μg guanosine 5′-O-(thio)triphosphate replaced the 0.2 mM GTP in the bath. Single channel currents were low-pass-filtered at 5 kHz and stored directly onto videotape using an Instrutech VR-10B digital data recorder (Instrutech Corporation; Long Island, NY). Single channel currents were sampled at 10 kHz and stored on computer hard drive for subsequent analysis of conductance, open time, and open probability using pCLAMP, version 8.0 software.

Electrocardiogram Telemetry—Implantable PhysioTel TA100EA-P20 radiotelemetry transmitters (Data Sciences International; St. Paul, MN) were used for the ECG telemetry monitoring as described previously (20). Briefly, transmitters were implanted under ketamine/xylazine anesthesia (30–50 mg/kg intraperitoneally) according to the manufacturer’s recommendations. ECG leads were sutured to the thoracic muscles in lead II position. Prolene 5–0 was used to close the incisions. Mice were allowed to recover for 7 days prior to measuring resting heart rate. For resting heart rate determination, 6 h of baseline ECG recording began in the morning of day 8 (1000–1600). On day 9, heart rate was monitored for 15 min prior to intraperitoneal injection of 6 mg/kg methoxamine (Sigma) and for 2 h after injection. On day 10, heart rate was monitored for 15 min prior to intraperitoneal injection of 0.3 mg/kg scopolamine (CCPA; resorcinol cyclic ether) or 1 μg/kg atropine (Sigma) and for 2 h following injection. Animals were sacrificed by CO2 asphyxiation, and transmitters were expelled and reused after cleaning and sterilization with 2% glutaraldehyde.

Statistical Analysis—All electrophysiological and electrocardiogram data are presented as the mean ± S.E. Statistical comparisons were made using one-way analysis of variance, followed by Tukey’s HSD post-hoc test for pairwise comparisons. The level of significance was considered as p < 0.05.

RESULTS

We reported recently the structure of the mouse Kir3.1 gene (24). A Cre/oxyP-based targeting strategy involving the third exon of Kir3.1 was utilized to generate constitutive null Kir3.1 mutant mice (Fig. 1A) (25). Exon 3 was chosen for targeting as it contains a protein-coding sequence for most of the key functional domains of the Kir3.1 subunit, including the pore and

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1 The abbreviations used are: DTP, dithiotreitol; BisTris, 2-[bis(2-hydroxyethyl)aminio]-2-(hydroxyethyl)propane-1,3-diol; CCH, carbachol; ECG, electrocardiogram; CCPA, 2-chloro,N6-cyclopentyl adenosine; bpm, beats per min; HEK, human embryonic kidney.
In Kir3.1 knockout samples, most of the Kir3.4 immunofraction of Kir3.4 that exists as homomultimers in heart tissue been reported previously and was interpreted to represent a mass (amount of immunoreactivity observed at a high molecular was observed predominantly as a 45-kDa band, with a small fraction observed in a band with an electrophoretic mobility of ~200 kDa, shown previously to represent Kir3.4 homotetrameric complexes (15). In Kir3.1 knockout samples, the predominant Kir3.4 immunoreactivity was found in the ~200-kDa band, with bands of lesser intensity observed at ~90 and 45 kDa, consistent with Kir3.4 dimeric and monomeric forms, respectively (n = 4). The protein sample from Kir3.4 knockout mice demonstrated the selectivity of the anti-Kir3.4 antibody. Right panel, incubating the protein samples at 100 °C in the presence of 100 mM DTT resulted in the conversion of the higher molecular mass Kir3.4 forms into the monomeric form (45 kDa). The levels of Kir3.4 were not different in atrial membrane protein samples from wild-type and Kir3.1 knockout mice (n = 5).

membrane-spanning domains, the entire amino terminus, and the translation initiation codon (Fig. 1B). A single targeted embryonic stem cell clone was identified (1/692 = 0.14% targeting efficiency) and was transfected subsequently with a Cre recombinase expression construct to isolate clonal derivatives harboring a Kir3.1 null mutant allele. Vertical arrowheads indicate the positions of loxp sites. Abbreviations are as follows: S, SacI restriction enzyme site; NEO, neomycin resistance gene cassette; DTA, diphtheria toxin gene cassette. B, membrane topology of the Kir3.1 subunit. The domain of black circles reflects the coding sequence absent in the constitutive null Kir3.1 gene. C, Southern analysis of tail biopsies from siblings obtained from a cross of heterozygous Kir3.1 mutant parents. Genomic DNA was digested with SacI and probed with a 32P-radiolabeled fragment corresponding to the gray rectangle shown in A as described (20).

Western blots of crude atrial membrane extracts from wild-type and Kir3.1 knockout mice confirmed the success of the gene targeting (n = 5; see Fig. 2, left panel). All Kir3.1 immunoreactivity was absent in samples from Kir3.1 knockout mice. Interestingly, the pattern of Kir3.4 immunoreactivity was altered in samples from Kir3.1 knockout mice (n = 4; see Fig. 2, middle panel). In wild-type samples, Kir3.4 immunoreactivity was observed predominantly as a 45-kDa band, with a small amount of immunoreactivity observed at a high molecular mass (>200 kDa). This pattern of Kir3.4 immunoreactivity has been reported previously and was interpreted to represent a fraction of Kir3.4 that exists as homomultimers in heart tissue (15). In Kir3.1 knockout samples, most of the Kir3.4 immuno-reactivity was observed at higher molecular masses (~90 kDa, >200 kDa), consistent with the observation that Kir3.4 homomultimeric complexes are more resistant to denaturing gel electrophoresis than heteromultimeric complexes containing both Kir3.1 and Kir3.4 (15). Prolonged incubation of the samples in 100 mM DTT at 100 °C, however, converted all Kir3.4 immunoreactivity into a single band corresponding to monomeric Kir3.4 (n = 2; see Fig. 2, right panel). The total level of Kir3.4 protein in heart tissue from Kir3.1 knockout mice was unchanged relative to the level observed in wild-type atrial tissue.

Resting membrane potentials and CCh-induced currents were measured in primary cultures of atrial myocytes isolated from wild-type and Kir3.1 knockout mice to determine whether the loss of Kir3.1, and the presence of a homogeneous population of Kir3.4 homomultimeric complexes, correlated with altered electrophysiology. The average resting membrane potential of wild-type atrial myocytes was −62 ± 3 mV (n = 21). In comparison, atrial myocytes from Kir3.1 knockout (−54 ± 3 mV, n = 18) and Kir3.4 knockout (−53 ± 4 mV, n = 10) mice were slightly depolarized at rest. There was, however, no statistically significant difference between the resting membrane potentials of myocytes from wild-type, Kir3.1 knockout (p = 0.13), and Kir3.4 knockout (p = 0.19) mice (Table 1).

In a physiological extracellular K+ (5.4 mM) bath solution, 20 μM CCh relied evoked small outward currents (2.5 ± 0.3 pA/pF, n = 18; Vhold = −50 mV) and larger inward currents (−4.7 ± 0.7 pA/pF, n = 20; Vhold = −90 mV) from wild-type atrial myocytes (see Table I and Fig. 3, A, C, and D). In contrast, CCh did not elicit comparable whole-cell currents under these conditions in Kir3.1 knockout atrial myocytes (see Table I and Fig. 3, B and D). Indeed, in nine of ten experiments, CCh...
Data represent the mean ± S.E. for (n) experiments. RMP, resting membrane potential; \( V_{\text{hold}} \), holding potential; \([K^+]_{\text{out}}\), bath potassium concentration. Resting membrane potentials were corrected for liquid junction potential. There were no significant differences between the CCh-induced currents measured in Kir3.1 knock-out and Kir3.4 knock-out mice under these conditions (\( p < 0.001 \)). In contrast, CCh did not induce comparable currents in either Kir3.1 (0.63 ± 0.4 pA/pF, \( n = 23 \); \( p < 0.001 \)) or Kir3.4 (-0.01 ± 0.3, \( n = 13 \); \( p < 0.001 \)) knock-out atrial myocytes. There was no significant difference between the CCh-induced currents observed in myocytes from Kir3.1 knock-out and Kir3.4 knock-out mice under these conditions (\( p = 0.99 \)).

Because the level of Kir3.4 protein was unaltered in Kir3.1 knock-out atrial tissue (Fig. 2), we speculated that the failure to measure significant whole-cell current in Kir3.1 knock-out atrial myocytes was because of the perfusion of critical intracellular elements. Thus, we examined CCh-induced single channel activity in cell-attached patches from wild-type and Kir3 knock-out myocytes. Robust \( I_{K\text{ACH}} \)-like channel activity was observed in Kir3 knock-out myocytes, indicating a complete loss of cardiac \( I_{K\text{ACH}} \).

FIG. 4. Whole-cell, CCh-induced currents measured in a high extracellular K' bath (25.4 mM) in wild-type and Kir3.1 knock-out atrial myocytes. A, current evoked by 20 \( \mu \)M CCh in a typical wild-type atrial myocyte at a holding potential of -50 mV (top trace) and -90 mV (bottom trace). The solid bars indicate the beginning and end of CCh perfusion. B, 20 \( \mu \)M CCh failed to evoke significant current at holding potentials of either -50 (top trace) or -90 mV (bottom trace) in an atrial myocyte from a Kir3.1 knock-out mouse. C, current-voltage relationship of the CCh-induced current in a typical wild-type atrial myocyte. The figure was generated by subtracting the trace obtained prior to the addition of CCh from the traces obtained during CCh perfusion. D, average CCh-induced current densities measured in myocytes from wild-type (open squares), Kir3.1 knock-out (open circles), and Kir3.4 knock-out (black circles) mice. *, \( p < 0.001 \), wild-type versus Kir3 knock-out.

Altered \( I_{K\text{ACH}} \) in Kir3.1 Knock-out Mice

TABLE I

Summary of whole-cell, electrophysiological properties of atrial myocytes from wild-type, Kir3.1 knock-out, and Kir3.4 knock-out mice

| Genotype       | RMP   | CCh-induced current density | \( V_{\text{hold}} \) = -50 mV | \( V_{\text{hold}} \) = -90 mV | \( V_{\text{hold}} \) = -90 mV |
|----------------|-------|-----------------------------|--------------------------------|--------------------------------|--------------------------------|
|                | mV    | \( (\text{pA/pF}) \)       | \( ([K^+]_{\text{out}} = 5.4 \text{ mM}) \) | \( ([K^+]_{\text{out}} = 5.4 \text{ mM}) \) | \( ([K^+]_{\text{out}} = 25.4 \text{ mM}) \) |
| Wild-type      | -62 ± 3 (21) | 2.5 ± 0.3 (18) | -4.7 ± 0.7 (20) | -52.0 ± 7.8 (12) |
| Kir3.1 ko      | -54 ± 3 (18) | 0.1 ± 0.1" (10) | -0.1 ± 0.1" (8) | 0.6 ± 0.4" (23) |
| Kir3.4 ko      | -53 ± 4 (10) | -0.01 ± 0.02" (10) | -0.07 ± 0.03" (9) | -0.01 ± 0.3" (13) |

\( *p < 0.001 \), wild-type vs. Kir3 knock-out.

**FIG. 3.** Whole-cell, carbachol-induced currents measured in a physiological extracellular K' bath (5.4 mM) in wild-type and Kir3 knock-out atrial myocytes. A, currents evoked by 20 \( \mu \)M CCh in a typical wild-type atrial myocyte at holding potentials of -50 (top trace) and -90 mV (bottom trace). The small arrow represents the zero-current level for this experiment. B, 20 \( \mu \)M CCh failed to evoke significant current at holding potentials of either -50 (top trace) or -90 mV (bottom trace) in an atrial myocyte from a Kir3.1 knock-out mouse. C, current-voltage relationship of the CCh-induced current in a typical wild-type atrial myocyte. The figure was generated by subtracting the trace obtained prior to the addition of CCh from the traces obtained during CCh perfusion. D, average CCh-induced current densities measured in myocytes from wild-type (open squares), Kir3.1 knock-out (open circles), and Kir3.4 knock-out (black circles) mice. *, \( p < 0.001 \), wild-type versus Kir3 knock-out.

failed to evoke measurable current or induced small changes in holding current that did not reverse upon agonist withdrawal and/or were not reproducible. In one experiment, however, small (<5 pA) whole-cell currents of appropriate sign were evoked repeatedly by CCh at both holding potentials (data not shown). No measurable CCh-induced current was observed in Kir3.4 knock-out myocytes (\( n = 10 \); see Table I and Fig. 3D), consistent with the single channel analysis of Kir3.4 knock-out atrial myocytes that indicated a complete loss of cardiac \( I_{K\text{ACH}} \) in this mutant mouse line (20).

CCh-induced, whole-cell currents were measured in a high extracellular K' (25.4 mM) bath solution to facilitate the observation of small inward potassium currents. CCh reliably induced large inward currents (\(-52 ± 7 \text{ pA/pF; } V_{\text{hold}} = -90 \text{ mV} \)) in wild-type atrial myocytes (\( n = 12 \); see Table I and Fig. 4A). Voltage pulse protocols revealed the strong inward rectification of the CCh-induced current (Fig. 4, C and D). In contrast, CCh did not induce comparable currents in either Kir3.1 (0.63 ± 0.4 pA/pF, \( n = 23 \); \( p < 0.001 \)) or Kir3.4 (-0.01 ± 0.3, \( n = 13 \); \( p < 0.001 \)) knock-out atrial myocytes. There was no significant difference between the CCh-induced currents observed in myocytes from Kir3.1 knock-out and Kir3.4 knock-out mice under these conditions (\( p = 0.99 \)).

Because the level of Kir3.4 protein was unaltered in Kir3.1 knock-out atrial tissue (Fig. 2), we speculated that the failure to measure significant whole-cell current in Kir3.1 knock-out atrial myocytes was because of the perfusion of critical intracellular elements. Thus, we examined CCh-induced single channel activity in cell-attached patches from wild-type and Kir3 knock-out myocytes. Robust \( I_{K\text{ACH}} \)-like channel activity...
induced by 20 μM CCh was observed in 17 of 19 cell-attached patches from wild-type atrial myocytes (Fig. 5A). These channels exhibited inward rectification, a 1.0 ± 0.1-ms mean open time, and a 35 ± 12% decrease in open probability over a 1-min interval. Upon formation of the inside-out patch, GTP-dependent gating was observed readily, and single channel conductance (36 ± 1 pS) and channel mean open time (1.1 ± 0.1 ms) were consistent with previous studies of rodent I_{KACa} (4, 20).

In contrast, I_{KACa}-like channels were not observed in cell-attached patches from Kir3.1 knock-out myocytes (n = 24). In nine of 24 cell-attached patches from Kir3.1 knock-out mice, however, channels with the distinctive gating and conductance profile of recombinant Kir3.4 homomultimers were observed (Fig. 5B) (7, 17). Comparable channel activity was not observed in cell-attached patches from Kir3.4 knock-out myocytes (n = 14), nor was this activity reported in a previous study of Kir3.4 knock-out myocytes (20). The open probability of the residual channel observed in Kir3.1 knock-out myocytes was very low (P_o < 0.001), and channel activity typically ran down within 1 min of gigaseal formation. Furthermore, we were unable to recover reliably channel activity in the inside-out configuration despite the addition of 0.2 mM GTP (n = 8) or 0.2 mM GTP + 1 mM ATP (n = 7) to the bath. The presence of an active, small conductance, non-rectifying channel in >50% of all patches tested (both wild-type and Kir3 knock-out myocytes) made it difficult to analyze rigorously the single channel properties of the residual channel. In one instance, however, we did observe persistent channel activity in the inside-out configuration, and the activity was dependent upon GTP. In this experiment, single channel conductance was determined to be 17 ± 4 pS, and mean open time was 0.6 ± 0.1 ms, consistent with the properties of the recombinant Kir3.4 homomultimer (7, 17).

We next used ECG telemetry to determine the impact of Kir3.1 subunit ablation on resting heart rate, as well as heart rate responses to pharmacological manipulation (20). Resting heart rates were higher in both Kir3.1 knock-out (623 ± 13 bpm, n = 7; p = 0.06) and Kir3.4 knock-out mice (640 ± 9 bpm, n = 7; p = 0.005), relative to the average resting heart rate of wild-type mice (588 ± 9 bpm, n = 10; see Fig. 6). Following the intraperitoneal administration of 6 mg/kg methoxamine, an α₁-adrenergic receptor agonist that triggers the baroreflex, on the heart rates of wild-type, Kir3.1 knock-out, and Kir3.4 knock-out mice. C, the effect of an intraperitoneal injection of 0.3 mg/kg CCPA on the heart rates of wild-type, Kir3.1 knock-out, and Kir3.4 knock-out mice. *, p < 0.05, wild-type versus Kir3 knock-out. There were no significant differences between Kir3.1 and Kir3.4 knock-out mice with respect to resting heart rate or heart rate decrease in response to pharmacological manipulation.

DISCUSSION

In this study, we report the generation and preliminary characterization of Kir3.1 knock-out mice. These mice are via-
ble and appear normal. Despite the normal expression levels of Kir3.4 protein in atrial tissue from Kir3.1 knock-out mice, atrial myocytes from these animals displayed a severe reduction in G protein-gated potassium current. The small amount of residual channel activity exhibited properties reminiscent of recombinant Kir3.4 homomultimers, studied previously in heterologous expression systems. Indeed, the lack of similar channel activity in Kir3.4 knock-out myocytes argues strongly that the channels observed in Kir3.1 knock-out myocytes were Kir3.4 homomultimers. Consequences of Kir3.1 ablation were also observed at the whole organ level. Both Kir3.1 knock-out and Kir3.4 knock-out mice exhibited a modest resting tachycardia, consistent with the loss of an inhibitory influence on heart rate. In addition, Kir3.1 knock-out mice displayed blunted responses to both indirect vagal activation and direct adenosine A1 receptor activation.

Previously, we reported that the resting heart rates of wild-type and Kir3.4 knock-out mice were similar (20). In this study, we observed that both Kir3.1 knock-out and Kir3.4 knock-out mice exhibited slightly elevated resting heart rates compared with the wild-type control group. The discrepancy reflects the lower resting heart rate observed in wild-type mice for this study (588 bpm), as the resting heart rates measured for Kir3.4 knock-out mice were comparable in both studies (640 versus 647 bpm). In the previous study, the effect of propranolol administration on heart rate suggested that the animals were experiencing a high degree of sympathetic tone (20). Indeed, a study involving ECG telemetry in mice demonstrated that resting heart rates decreased between 4 and 7 days following surgery, presumably reflecting a gradual decline in animal stress and/or sympathetic tone (26). Accordingly, for this study we allowed the animals 7 days for recovery following surgery prior to measuring resting heart rates, in contrast to the 4-day recovery period used in the previous study. In addition, ECG transmitters were implanted in the peritoneal cavity rather than under the skin of the back, and experiments were performed on older and larger animals better able to tolerate the physical demands associated with a relatively large implant. As a result, our measured resting heart rate values for wild-type mice were consistent with those from other studies, including studies involving cannulation or tethering approaches (27–29).

Early studies suggested that Kir3.1 was an integral component of native G protein-gated potassium channels and that the functional properties of G protein-gated potassium channels were largely dependent on subunit composition (5–7, 11, 12, 31, 32). Indeed, channels formed by Kir3.1 and Kir3.2, Kir3.3, or Kir3.4 exhibited largely indistinguishable properties (33). Recent studies, however, have offered biochemical evidence for the existence of Kir3.2 homomultimers in the substantia nigra (13), Kir3.2/Kir3.3 heteromultimers in brain (14), and Kir3.4 homomultimers in heart (15). The significance of these G protein-gated potassium channels is largely unknown. Our findings argue, however, that Kir3.4 homomultimers cannot support significant levels of muscarinic-gated or adenosine A1 receptor activation in heart atria. As such, the presence of a population of Kir3.4 homomultimers in wild-type atria (15) may simply reflect the random nature of Kir3.1/3.4 channel assembly in this tissue. We cannot rule out the possibility, however, that native cardiac Kir3.4 homomultimers couple efficiently to a signaling pathway unexplored in this study.

Interestingly, the residual channel activity observed in Kir3.1 knock-out myocytes typically ran down in less than 1 min in cell-attached patches. Desensitization mechanisms targeting the muscarinic receptor cannot explain the rundown phenomenon completely, as CCh-induced IKACh activity in wild-type myocytes was relatively stable over the course of the experiments. Although the mechanism underlying the rundown of Kir3.4 homomultimers in Kir3.1 knock-out myocytes is unknown at present, our findings do suggest that Kir3 channels of distinct subunit composition can be affected in different ways by intracellular regulatory pathways.

The reduced potassium efflux and apparent enhancement in the function of Kir3.1 and Kir3.4 homomultimers in atrial tissue from Kir3.1 knock-out mice exhibited a modest resting tachycardia, consistent with the loss of an inhibitory influence on heart rate. In addition, Kir3.1 knock-out mice displayed blunted responses to both indirect vagal activation and direct adenosine A1 receptor activation.

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