Molecular Properties of Neuronal G-protein-activated Inwardly Rectifying K\(^+\) Channels*

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Four cDNA-encoding G-activated inwardly rectifying K\(^+\) channels have been cloned recently (Kubo, Y., Reuveny, E., Slesinger, P. A., and Jan, L. Y. (1993) Nature 364, 802-806; Leugue, E., Duprat, F., Fink, M., Guillemaure, E., Coppola, T., Lazdunski, M., and Hugnot, J. P. (1994) FEBS Lett. 353, 37-42; Krapivinsky, G., Gordon, E. A., Wickman, K., Valmirevic, B., Krapivinsky, L., and Clapham, D. E. (1995) Nature 374, 135-141). We report the cloning of a mouse GIRK2 splice variant, noted mGIRK2A. Both channel proteins are functionally expressed in Xenopus oocytes upon injection of their cRNA, alone or in combination with the GIRK1 cRNA. Three GIRK channels, mGIRK1-3, are shown to be present in the brain. Colocalization in the same neurons of mGIRK1 and mGIRK2 supports the hypothesis that native channels are made by an heteromeric subunit assembly. GIRK3 channels have not been expressed successfully, even in the presence of the other types of subunits. However, GIRK3 chimeras with the amino- and carboxyl-terminal of GIRK2 are functionally expressed in the presence of GIRK1. The expressed mGIRK2 and mGIRK1-2 currents are blocked by Ba\(^2+\) and Cs\(^+\) ions. They are not regulated by protein kinase A and protein kinase C. Channel activity runs down in inside-out excised patches, and ATP is required to prevent this rundown. Since the nonhydrolyzable ATP anolog AMP-PCP is also active and since addition of kinases A and C as well as alkaline phosphatase does not modify the ATP effect, it is concluded that ATP hydrolysis is not required. An ATP binding process appears to be essential for maintaining a functional state of the neuronal inward rectifier K\(^+\) channel. A Na\(^+\) binding site on the cytoplasmic face of the membrane acts in synergy with the ATP binding site to stabilize channel activity.

Inward rectifier K\(^+\) channels were first described in skeletal muscle and egg-cell membranes (1, 2). They are now found in many cell types and are characterized by the following properties: (i) an activation by hyperpolarization negative to the resting potential for K\(^+\) (E\(_K\)), (ii) an activation potential shifting with E\(_K\), (iii) a blockade by Cs\(^+\) and Ba\(^2+\) (3).

A class of inward rectifier K\(^+\) channels is gated via G-proteins (GIRK). In atrial cells, acetylcholine released by stimulation of the vagal nerve causes the opening of a GIRK channel (I\(_{KACH}\)) via the activation of a m2-muscarinic receptor. The induced hyperpolarization results in a slowing of cardiac frequency (4, 5). GIRK channels also exist in a variety of neuronal cells and the modulation of such channels generates slow synaptic potentials (6, 7). They are coupled to various neurotransmitter receptors such as the muscarinic cholinergic, \(\mu\), \(\delta\), and \(\kappa\) opioid, \(\alpha_2\)-adrenergic, somatostatin, substance P, and GABA\(_B\) receptors (8-10).

A GIRK channel, termed GIRK1 (11) or KGA (12), was cloned from rat heart, and two structural homologs were cloned from mouse brain, mGIRK2 and mGIRK3 (13). Another close structural parent of the GIRK family (rcKATP) was described initially as an ATP-sensitive K\(^+\) channel (14), i.e. a channel for which activity is controlled by intracellular ATP (15). However, it has been shown recently that the functional I\(_{KACH}\) channel stimulated by the G-protein \(\beta\gamma\) subunits is a heteromultimer composed of two GIRK subunits, GIRK1 and CIR, a channel subunit which is nearly identical with rcKATP (16).

A mouse analog of rcKATP/CIR that we have cloned, and designed in this paper as mGIRK4, presents the characteristic features of a G-protein-activated inward rectifier K\(^+\) channel. In contrast to mGIRK1, which is present both in heart and brain, mGIRK4/CIR is specifically localized in the heart, whereas mGIRK2 and mGIRK3 are expressed mainly in the brain. By using different strategies, co-localization of transcripts, immunoprecipitation, and electrophysiology, we present evidence for a heterologous GIRK subunit assembly in the brain. The paper also describes the main electrophysiological properties and the modulation by ATP and Na\(^+\) of the currents expressed by mGIRK2 and the mGIRK1 + mGIRK2 combination in Xenopus oocytes.

EXPERIMENTAL PROCEDURES

Isolation of mGIRK2A and mGIRK4/CIR Clones and mGIRK2/3/2 Chimera Construction—The mGIRK2A clone was isolated by screening a mouse brain library with a GIRK1 probe as described (13). The mouse GIRK4/CIR clone was amplified by polymerase chain reaction (PCR)\(^1\) using primers corresponding to the published rat sequence (16) and subcloned into the pEXO plasmid (17). cDNA clones were sequenced on both strands by using the dye terminator method on an automatic Sequencer (Applied Biosystems model 373A).

To construct the chimeric mGIRK2/3/2, the mGIRK3 sequence was mutated at positions 151 (the A of the initiation codon taken as base 1) and 1012 to introduce MunI and NheI restriction sites, respectively, without modification of the amino-acid coded sequence. Site-directed mutagenesis was performed using oligonucleotide primers according to

\(^1\)The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione S-transferase; AMP-PCP, adenosine 5'-[y-32P]-triphosphate.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^1\)/EMBL Data Bank with accession number(s) U33631.

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the manufacturer’s protocol (Promega). The central mGIRK3 sequence between these two sites was exchanged with the corresponding mGIRK2 sequence in which a Muni site was created at position 255. The Nhdi site is found naturally in the mGIRK2 sequence.

Northern Blot Analysis—Poly(A)+ RNAs were isolated from adult mouse heart and brain and blotted onto nylon membranes as described previously (20). The blots were probed with the 32P-labeled pBS-GIRK1, pBS-GIRK2, pBS-GIRK3 (13), and pEPO-GIRK4 in 50% formamide, 5× SSPE (0.9 M sodium chloride, 50 mM sodium phosphate, 5 mM EDTA, pH 7.4), 0.1% SDS, 5× Denhardt’s solution, 20 mM potassium phosphate (pH 6.5), and 200 μg/ml denatured salmon sperm DNA at 55 °C for 18 h and washed stepwise to a final stringency of 0.5× SSC (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0, 0.3% SDS at 55 °C.

In Situ Hybridization—All experiments were performed on 10- to 12-week-old (250–300 g) male Wistar rats (Charles River Laboratories), by using standard procedures (19). Antisense RNA probes were generated by in vitro transcription, using (α-33P)UTP (1000 Ci/mmol, Amersham) from linearized plasmids containing a 82-base pair HindII fragment of GIRK1 cDNA in the 3′-coding sequence, and a 244-base pair BamHI fragment of GIRK2 cDNA in the 5′-untranslated sequence. Sections (10 μm) were treated and probed as described (19) and exposed to Amerham 8-max Hyperfilm. Selected slides were dipped in Amersham LMI photographic emulsion and exposed for 2 weeks at 4 °C and then developed in Kodak D-39 for 4 min. All slides were counterstained with Cresyl violet. For control experiments, adjacent sections were hybridized with sense probe or digested with RNAase before hybridization.

Antibody Preparations, Immunoprecipitations of Transfected GIRK Channels—DNA fragments corresponding to the carboxyl termini of mGIRK2 (44 amino acids) and mGIRK4/CIR (91 amino acids) were amplified by PCR and subcloned into the pGEX3 plasmid behind the glutathione S-transferase (GST) coding sequence. GST-GIRK fusion proteins were prepared and purified according to the manufacturer’s protocol (Pharmacia Biotech Inc.). Antibodies directed against these proteins were raised in rabbits and guinea pigs using routine immunization protocols, boosting and bleeding being performed every 4 weeks. Polyclonal rabbit (R) and guinea pig (GP) antibodies, noted RuGIRK2 and GIGIRK2 respectively, to mGIRK2 and GIRK3 and GIRK4 and GIRK4a, were first depleted of the anti-GST antibodies by repeated absorption against strips of nitrocellulose saturated with the GST protein. The depleted sera were then affinity-purified against their respective antigen fixed on nitrocellulose. Specificity of antibodies was verified by Western blotting and immunoprecipitation assays on transfected cell microsomes. TSA202 cells were transfected with mGIRK2 and mGIRK4/CIR subcloned into the expression vector pcDNA (Invitrogen) by the calcium transfection method, boosting and bleeding being performed every 4 weeks. Altemative immunoprecipitation and Western blotting were performed as described (20). For patch-clamp experiments, adjacent sections were hybridized with sense probe or digested with RNAase before hybridization.

Electrophysiology—The two-microelectrode electrophysiological measurements were performed as described (20). For patch-clamp experiments, devitellinized oocytes were placed in a bath solution containing 140 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, 5 mM HEPES at pH 7.4 with KOH. Pipettes were filled with a high K+ solution containing 140 mM KCl, 100 mM potassium methanesulfonate, 1.8 mM CaCl2, 2 mM MgCl2, and 5 mM HEPES adjusted to pH 7.4 with KOH. 100 μM GdCl3 was added to the pipette solution to inhibit the activity of the stretch-activated channels. Inside-out patches were perfused with a solution containing 140 mM KCl, 10 mM MgCl2, 5 mM HEPES adjusted at pH 7.2 with KOH and 5 mM EGTA added daily. Single-channel signals were filtered at 3.5 kHz and analyzed with the Biopac software (BioLogic).

RESULTS

Cloning of a Splice Variant of mGIRK2—Screening a mouse brain cDNA library at low stringency with a GIRK1 probe resulted in the isolation of the done mGIRK2 (13). During this screening, a splice variant of mGIRK2, noted herein as mGIRK2A, was also isolated. This clone had an overall size of 2.7 kilobases, identical with mGIRK2. It also displayed exactly the same 5′ sequence up to the GGG glycine codon located just upstream of the TGA stop codon of mGIRK2. From that point, the mGIRK2A sequence totally diverged, leading to an extra 1,240 bp starting 3′ of the TGA stop codon. The entire 3′-untranslated sequence was then found to be unique as judged by sequence determination of its 300 first nucleotides and by analysis of its restriction map (not shown). It is likely that the two mGIRK2 clones are alternatively spliced products of the same gene. The amplification by reverse transcription-PCR of DNA fragments specific for each splice variant from mouse brain messenger RNA excluded the possibil...
expression patterns of GIRK1, GIRK2, and GIRK3 are widely distributed in the brain and are very similar (21, 22) and results not shown. The highest expression levels appeared in the neo- and allocortical regions, hippocampus, olfactory bulb, and cerebellum. The mGIRK4/CIR expression was very low in the adult rat brain (not shown). To determine the potential significance of heteromultimeric formation in the brain, a high-resolution study obtained by microscopic analysis of emulsion-dipped sections has been performed. Fig. 2b shows an example of the high degree of overlap of the expression patterns of mGIRK1 and mGIRK2 transcripts. More than 80% of the neurons are labeled with the mGIRK1 and mGIRK2 probes in the CA3 pyramidal cell layer of the hippocampus (Fig. 2b). Coexpression of both transcripts in the same neuron type was also observed in most of the other strongly labeled central nervous system areas and was particularly evident in the granule cells of the dentate gyrus, in granular layers of the cerebellum, and in the mitral cells in the olfactory bulb (not shown). No area expressing only one GIRK mRNA could be clearly detected in the whole brain.

Properties of Macroscopic mGIRK Currents Expressed in Xenopus Oocytes—Both Northern blot analysis and in situ hybridization have shown that mGIRK1, mGIRK2, and mGIRK3 are highly represented in brain while mGIRK4/CIR is not. Thus, it is unlikely that the mGIRK1 + mGIRK4/CIR (mGIRK1-4) combination that has been shown to form the $I_{K_{ACh}}$ channel in atrial cells (16) is an abundant GIRK channel in the brain. The most probable combinations are made of the assembly of the three other subunits. All attempts to express the mGIRK3 subunit have been unsuccessful, either alone or in combination with mGIRK1, mGIRK2, mGIRK4/CIR, or mGIRK1-4 (8, 7, 4, and 4 independent batches of oocytes for the combinations mGIRK1-3, mGIRK2-3, mGIRK3-4, and mGIRK1-3-4, respectively, with at least 3 oocytes per batch). Consequently, the electrophysiological study was restricted to currents expressed by the mGIRK1, mGIRK2, and mGIRK4/CIR cDNAs.

Similarly to $I_{K_{ACh}}$ (23), expressed mGIRK channels are stimulated by the G-protein $\beta\gamma_{2}$ dimer. Although the requirement for these G-protein subunits was not systematically observed for heteromultimeric channels (not shown), $\beta\gamma_{2}$ were always co-injected in the following part of the work. Injections of mGIRK2 or mGIRK1 + mGIRK2 (mGIRK1-2) into Xenopus oocytes resulted in the expression of inwardly rectifying currents. The expression of mGIRK2 in the absence of other mGIRK subunits was successful only in 35% of the oocyte batches tested. This low expression frequency was nevertheless sufficient to allow a detailed characterization of the biophysical properties of the current. The GIRK current expression frequency reached 100% with combined injections of mGIRK1 and mGIRK2. Fig. 3, a and b, shows superimposed current traces evoked by voltage steps ranging from $-135$ to $+45$ mV in 30-mV increments from a holding potential of 0 mV ($K^+$/ equilibrium potential). mGIRK2 (Fig. 3a) and mGIRK1-2 (Fig. 3b) currents in response to hyperpolarizing voltage steps display different kinetics. mGIRK2 currents activate rapidly, in less than 5 ms, and then partially inactivate with a time constant of $243 \pm 15$ ms ($n = 8$) at $-130$ mV. Activation/inactivation kinetics of mGIRK1-2 currents were very different, with a slower time constant ($81 \pm 5$ ms at $-130$ mV, $n = 14$) and no inactivation.

As expected for a $K^+$-selective inward rectifier (24), the activation potential of mGIRK2 became more negative as external $K^+$ concentration decreased and the amount of shift ($52.6 \pm 0.8$ mV, $n = 6$, for a 10-fold change in external $K^+$) was close to the $K^+$ equilibrium value (59 mV) estimated from the Nernst equation (Fig. 3, c and d). The shifts in the threshold of
activation for mGIRK1,-2 and mGIRK2 + mGIRK4/CIR (mGIRK2,-4) were, respectively, 50.9 ± 2.3 mV (n = 4) and 50.6 ± 3.3 mV (n = 3) for a 10-fold change in external [K+] (not shown), consistent with a predominant K⁺ selectivity for these channels.

As for the majority of K⁺ selective channels, external application of Ba²⁺ or Cs⁺ blocked mGIRK2 currents in a concentration-dependent manner (Fig. 3, e-h). The Cs⁺ block was voltage-dependent giving rise to typical bell-shaped I/V curves for potential values negative to −50 mV (Fig. 3f). The mechanism of Ba²⁺ block, for concentrations less than 1 mM, is probably of the "open channel block" type (24) as suggested by the pronounced fast inactivation component of the resulting current (Fig. 3g). The IC₅₀ values for the Cs⁺ inhibition were 94.2 ± 16 μM (n = 3), 94.5 ± 7.6 μM (n = 5), and 94.3 ± 1.3 μM (n = 3) for mGIRK2, mGIRK1,-2, and mGIRK2,-4, respectively, while the IC₅₀ values for the Ba²⁺ inhibition were 94.2 ± 38 μM (n = 3), 105.7 ± 6.9 μM (n = 6), and 97.9 ± 1.9 μM (n = 3) for mGIRK2, mGIRK1,-2, and mGIRK2,-4, respectively. Other K⁺ channels blockers, including tetraethylammonium (3 mV), 4-aminopyridine (100 μM), diltiazem (33 μM), tetradiam (50 μM), RP 98886 (30 μM), glibenclamide (10 μM), or K⁺ channel openers (15), such as pinacidil and P1060, both at 100 μM, were without effect on mGIRK2, mGIRK1,-2, and mGIRK2,-4 currents. On the other hand, verapamil and bepridil, two L-type Ca²⁺ channel blockers, partially inhibited these currents, up to 60% and 40%, respectively, at 100 μM (not shown).

Finally, activation of protein kinase C by the phorbol 12-myristate 13-acetate (30 nm), the diacylglycerol analog, OAG (100 μM), or arachidonate (100 μM), and activation of protein kinase A by forskolin or 8-chloro-cAMP (3 and 300 μM) were without effect on GIRK currents (not shown).

Single-channel Analysis and Rectification Properties—Single-channel properties of the two splice variants, mGIRK2 and mGIRK2A co-expressed with mGIRK1 in Xenopus oocytes (mGIRK1,-2 and mGIRK1,-2A), were compared by examining the dependences on internal Mg²⁺ concentration of their inward rectification and by measuring their unitary conductances and their open-time distributions (Fig. 4, a-f). In the presence of 10 mM Mg²⁺, the mGIRK1,-2 and mGIRK1,-2A channels recorded in inside-out patches showed similar inward rectification which could be removed in Mg²⁺-free internal solution. The unitary conductances of mGIRK1,-2 and mGIRK1,-2A were 37 ± 8 pS (n = 5) and 39 ± 6 pS (n = 5), respectively. The open-time distribution in steady-state conditions for mGIRK1,-2 and mGIRK1,-2A at −80 mV was fitted by a single exponential characterized by a time constant of 0.21 ms and 0.16 ms, respectively (Fig. 4, c and f). In conclusion, no difference in the channel properties of the two forms of mGIRK2 transcripts was detected.

Since a highly voltage-dependent block both by intracellular Mg²⁺ and by the polyamine spermine have been shown to underlie strong inward rectification in cloned inward rectifiers (25–27), we tested the internal spermine dependence of the inward rectification of our expressed channels. In the experi-
ment illustrated in Fig. 4, g and h, inside-out patches containing mGIRK1,-2 channels maintained at +80 mV were first perfused with a Mg2+-free internal solution leading to an immediate removal of the inward rectification. Then, application of 100 μM spermine led to a complete blockade of the outward current which promptly reappeared after spermine removal. The bar graph (Fig. 4i) shows that the spermine block was dose-dependent with an IC50 of about 10 μM. Essentially the same spermine effects were obtained on oocytes co-expressing mGIRK1 and the splice variant mGIRK2A (not shown).

Aspartic acid, a negatively charged amino acid present in the second transmembrane domain of inward rectifiers which are not regulated by G-proteins such as IRK1 and BIR10 (positions 172 and 158) has been shown to be implicated in their Mg2+ and spermine sensitivities (25, 27–29). The corresponding residue is an aspartate in position 173 in mGIRK1 and a neutral asparagine in position 185 in mGIRK2. To evaluate the importance of the charge at this position for the rectification characteristics of the heteropolymeric G-protein-activated channel mGIRK1,-2, we took advantage of the presence of an asparagine (instead of an aspartate) residue in the corresponding position in mGIRK4/CIR (position 180) as in mGIRK2 (13). The mGIRK2,-4 channel has no negative charge in the positions that have been considered as crucial for Mg2+- and polyamine-induced inward rectification in IRK channels. Similarly to mGIRK1,-2, mGIRK2,-4 presents an inward rectification in the presence of 10 mm Mg2+ (Fig. 5a, b and c). In addition, Fig. 5d and e shows that the outward current recorded in a Mg2+-free solution at +80 mV was totally abolished in the presence of 100 μM spermine. In symmetrical 140 mM K+, the unitary conductance was 39 ± 5 pS (n = 5), and the time constant of the open-time distribution in steady-state conditions at −80 mV was 0.51 ms (Fig. 5c).

Immunochromatographic Demonstration that mGIRK Proteins Form Heteromultimers—To demonstrate biochemically the effective association of mGIRK2 and mGIRK4/CIR proteins in a multimeric complex, specific antibodies were raised against these two subunits and used in immunoprecipitation-immunoblot studies. The RgGIRK4 antibodies are specific for the mGIRK4/CIR subunit as they do not immunoprecipitate the mGIRK2 subunit in mGIRK2-TsA transfected cells. However, the two proteins were coprecipitated by RgGIRK4 in cells cotransfected with both plasmids (Fig. 5f, RK4 immunoprecipitated). The two subunits did not coprecipitate when they were expressed in separate cells and mixed afterward during the solubilization process, demonstrating that mGIRK2 and mGIRK4/CIR subunits cannot co-aggregate during the immunoprecipitation reaction. These data strongly suggest that the observed coprecipitation is indeed due to the biosynthetic formation of heteromultimeric channels. The specificity of the guinea pig revealing antibodies (GPαGIRK2 and GPαGIRK4) was demonstrated by using a combination of cells transfected with the different GIRK plasmids (Fig. 5f, Microsomes).

ATP Prevents the Rundown of mGIRK Channel Activity—In the cell-attached conformation, the expression of the mGIRK currents was stable for periods of time as long as 1 h. However, when patches were excised in ATP-free internal solution, channel activities quickly ran down. The presence of an internal solution containing ATP but not ADP partially prevented this rundown. Fig. 6 illustrates the effect of 10 mM disodium ATP (ATP/2Na) on the cytoplasmic face of the patch excised from an oocyte expressing the mGIRK1,2 channel. The final Na+ concentration of the internal solution was kept constant at 20 mM. Fig. 6a and b, shows that the channel activities were strongly dependent on the presence of ATP. Because the internal solution contained 10 mM MgCl2, it appeared that ATP was probably mainly associated with Mg2+, suggesting a possible involvement of a kinase in the rundown process. However, in the presence of 10 mM ATP, the perfusion of 40 units/ml protein kinase A catalytic subunit did not modify channel activity and did not reverse or prevent the slow rundown (Fig. 6c). Moreover, neither the protein kinase C inhibition with protein kinase inhibitor PKI (20 μM), nor the protein kinase C activation with OAG (100 μM) modified the rundown and/or the effect of ATP (not shown). Alkaline phosphatase (100 units/ml) did not prevent the effect of ATP (Fig. 6d). Finally, the nonhydrolyzable ATP analog (AMP-PCP) was as effective as ATP itself in reversing the rundown (Fig. 6e). These experiments suggest that the activity of the mGIRK1,-2 channel does not require a phosphorylation/dephosphorylation process but rather the binding of ATP without hydrolysis. In the experiment shown in Fig. 6f, the time constants of the open-time distribution of mGIRK1,-2 channel activities were not modified significantly in the ATP-free solution (0.5 ms), in the presence of ATP (0.3 ms), or in the presence of AMP-PCP (0.4 ms).

Surprisingly, channel activities were maximal when disodium ATP (ATP/2Na) was used instead of MgATP. Fig. 7a shows that mGIRK1,-2 channel activity could be partly re-
stored by application of a 20 mM NaCl in an ATP-free internal solution. To reach maximal channel activity, the simultaneous presence of ATP and Na\(^+\) ions was required. Channel activity was only 20% of the maximal activity on application of 10 mM Mg-ATP in Na\(^+\)-free solution (Fig. 7b). Fig. 7c presents mean results from 5 experiments and clearly shows the synergy of effects of 10 mM ATP and 10 mM ATP + 20 mM Na\(^+\). After the Na\(^+\) removal, note the instantaneous reduction of activity to the level reached in the presence of ATP alone. c, bar graph (n = 5) indicating the respective increase of GIRK1,-2 activities in the presence of Na\(^+\), ATP, and ATP + Na\(^+\) (taken arbitrarily as 100% in each experiment), d, effects of 20 mM Li\(^+\) followed by 20 mM Na\(^+\) in the presence of 10 mM ATP.

**DISCUSSION**

Four proteins with structures corresponding to G-protein-gated inward rectifier (11–14, 16) have been cloned to date. They are designated as mGIRK1, mGIRK2, mGIRK3, and mGIRK4/CIR. mGIRK4/CIR seems to be specific to the heart, and it is not detected in the brain. Conversely, mGIRK2 and mGIRK3 transcripts are specifically present in the brain. mGIRK1 is present at similar levels in heart and brain. In situ hybridization experiments have shown that the distribution of the three mGIRKs is very similar if not identical. Moreover, the colocalization of distinct GIRK transcripts in the same neuronal cells is in agreement with the hypothesis of heteromeric formation. This hypothesis is strongly supported by the tremendous increase of functional expression of GIRK channels...
when they are co-injected in the same oocyte as compared to single injections.

K+ channels expressed after the injection of the mGIRK2 cRNA alone or in combination with mGIRK1 cRNA (mGIRK1,-2) or with mGIRK4/CIR cRNA (mGIRK2,-4) in the presence of βγγγ had the hallmarks of inward rectifier channels: (i) an activation by hyperpolarization negative to the reversal potential for K+ (EK), (ii) an activation potential shifting with EK, and (iii) a blockade by Cs+ and Ba2+. However, in voltage-clamp conditions, there were some differences between expressions of mGIRK2 and mGIRK1,-2. mGIRK2 currents displayed a rapid activation (<5 ms) and partial inactivation, whereas mGIRK1,-2 channels had a slow activation and did not inactivate. Single-channel analysis of mGIRK2, mGIRK1,-2, and mGIRK2,-4 currents clearly demonstrated that there was only one population of channels with very similar properties characterized by a unitary conductance of about 40 pS and a flickering activity with a mean open time duration of less than 1 ms. The single-channel parameters of mGIRK2 and mGIRK1,-2 were very similar although their activation kinetics at the whole oocyte level were distinct.

A splice variant of mGIRK2 (mGIRK2A) has also been cloned. It has the same sequence as mGIRK2 but contains 11 additional amino acids in the carboxyl-terminal end. mGIRK2A transcripts are also specifically located in the brain. Electrophysiological results have not shown any significant difference between the two forms. Therefore, it is not easy to suggest any specific new function for mGIRK2A. A first possibility would be that the mGIRK2A subunit could associate with other mGIRKs which are not yet discovered. Another possibility is that the different carboxyl-terminal sequences could serve to impose different cellular localizations. Interestingly, the mGIRK2A terminal sequence SKV is very similar to the microbody targeting signal motif SKL (33).

How do expressed neuronal GIRK channels compare with "native" channels? Native GIRK channels recorded in different neuronal cell types have unitary conductances varying from 38 to 55 pS and a time constant of their open-time distribution which is of the order of 2 ms (7, 34). It then appears that their conductances are similar, but flickering is more rapid for the cloned channels expressed in Xenopus oocytes. However, it should be noted that detailed literature describing neuronal GIRK channel properties at the single-channel level is not yet available. One possibility is that flickering GIRK channels are difficult to record in neuronal membranes where numerous other K+ channel activities might coexist. Another likely possibility is that some subunit which normally slows down the gating kinetics in native channels is still missing in cloned heteromultimeric channels.

It has been shown previously that the functional cardiac G-protein-activated inward rectifier is in fact composed of an assembly of rat GIRK1 and GIRK4/CIR (16). The K+ current expression described above suggests that mGIRK2 can also form heteromultimeric assemblies with mGIRK1 and mGIRK4/CIR. This was actually directly demonstrated by immunoprecipitation studies in the case of the mGIRK2,-4 complex. Experiments using coexpression of mGIRK1 with chimeras of mGIRK3 (which do not express alone or co-injected with mGIRK1, mGIRK2, or mGIRK4/CIR) with the amino- and carboxyl-terminal sequences of mGIRK2 also tend to lead to the same conclusion. The apparent co-localization of mGIRK1 and mGIRK2 in the brain, particularly in CA3 pyramidal cells, is a strong indication that the mGIRK1,-2 complex is a major neuronal GIRK channel. The case of mGIRK3 is not clear. Its lack of expression suggests that it might need a partner that still has to be discovered. One possible partner is the sulfonylurea receptor (35). ATP-sensitive K+ channels are present in the brain (36–38). They have inward-rectifying properties (23), are regulated by G-proteins (39, 40), and may be constituted by the assembly of the protein that binds antidiabetic sulfonylureas (35) and an inward rectifier-type K+ channel.

After this work was submitted, it was published that the GIRK3 subunit can assemble with GIRK1 and with GIRK2 to either increase (GIRK1) or decrease (GIRK2) their activities (41). These effects were never seen in our own experiments. These apparently conflicting observations might be explained by assuming that a third, not yet identified, subunit is endogenously present in oocytes and confers the expression properties observed by Kofuji et al. (41). This component would not be present in our oocytes.

The inward rectification in cloned inward rectifiers (25–27) is due to a highly voltage-dependent block by intracellular Mg2+ and by polyamines. Mutagenesis experiments have strongly suggested that aspartic acid in position 172 in the inward rectifier IRK1 is pivotal for the effects of Mg2+ and spermine on the inward rectification (25–27). This Asp residue is present at corresponding positions in sequences of a number of cloned inward rectifier such as IRK1, mGIRK1, and BIR10 (29), but this residue is replaced by an asparagine in mGIRK2, mGIRK4/CIR, and also in ROMK1 (42). Although they lack this Asp residue, both mGIRK2 and mGIRK4/CIR, when they are expressed independently or when they co-expressed, possess all the hallmarks of inward rectifiers, contrary to ROMK1 which also has an Asn in the corresponding position 171 and which presents a quasilinear I-V relationship. The fact that replacement of Asn-171 by Asp in ROMK1 results in the appearance of a Mg2+-dependent inward rectification (43) would tend to confirm the important role of an Asp for Mg2+-dependent inward rectification. However, the fact that the expression of GIRK2,-4, with Asn in the sequences instead of Asp, also leads to a Mg2+-dependent inward rectifier K+ channel pleads for the importance of other residues and questions the unique role of this Asp for inducing this inward rectification.

One particularly interesting observation is the requirement of a high concentration of internal ATP (10 mM) in excised patches to prevent a fast rundown of both mGIRK1,-2 and mGIRK2,-4 activities. This ATP dependence would immediately suggest an important role of phosphorylation. However, results presented in this paper show that a kinase activity involving ATP hydrolysis is not implicated as it is for IRK1 and ROMK1 channels (44, 45). Treatments capable of activating or inhibiting protein kinase A or protein kinase C activity were without effect on the rundown and/or the reactivating action of ATP. Moreover, alkaline phosphatase which would produce a dephosphorylation did not modify the response to ATP. Finally, the activating effects of the nonhydrolyzable ATP analog AMP-PCP on channel activity were similar to if not identical with those of ATP. All these results taken together show that mGIRK1,-2 and mGIRK2,-4 channels are ATP-regulated channels. They require ATP binding to be functional, but ATP hydrolysis is not necessary. ATP binding might occur at the nucleotide-binding site represented by the consensus Walker type A sequence G(X)\(_{X}\)GK(X)\(_{i}\)V. This exact motif is missing in mGIRK sequences, but two motives that share similarities with the Walker A consensus sequence are present in the carboxyl-terminal extremities of mGIRK1, mGIRK2, and mGIRK3 subunits. The I(X)\(_{X}\)GK(X)\(_{i}\)V motif is present in mGIRK1 and mGIRK2, the V(X)\(_{X}\)GR(X)\(_{i}\)V sequence is present in mGIRK3. It has been suggested that similar motives could be implicated in ATP binding (44). The mGIRK4/CIR sequence does not possess such an ATP consensus sequence.

This paper also shows that internal Na+ is a regulator of the
neuronal mGIRK1,-2 channel activity. This type of property has in fact been observed before with the inward rectifier K⁺ channel which is present in starfish eggs (46). ATP and Na⁺ are synergistic in their activating effects. The ATP and Na⁺ channel which is present in starfish eggs (46). ATP and Na⁺ has in fact been observed before with the inward rectifier K⁺ channel activity. This type of property might be an important component in a cascade of events leading to very deleterious effects.

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