Characterization of Heteromultimeric G Protein-coupled Inwardly Rectifying Potassium Channels of the Tunicate Tadpole with a Unique Pore Property*

Yoshimichi Murata‡, Haruo Okado§, and Yoshihiro Kubo¶¶

Unique Pore Property*

From the ‡Department of Physiology, Tokyo Medical and Dental University, Graduate School and Faculty of Medicine, Bunkyo, Tokyo 113-8519, Japan and the Departments of ¶Neurobiology and ¶¶Neurophysiology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183-8526, Japan

Two cDNAs that encode the G protein-coupled inwardly rectifying K⁺ channel (GIRK, Kir3) of tunicate tadpoles (tunicate G protein-coupled inwardly rectifying K⁺ channel-A and -B; TuGIRK-A and -B) have been isolated. The deduced amino acid sequences showed ~60% identity with the mammalian Kir3 family. Detected by whole mount in situ hybridization, both TuGIRK-A and -B were expressed similarly in the neural cells of the head and neck region from the tail bud stage to the young tadpole stage. By co-injecting cRNAs of TuGIRK-A and G protein β1/γ1 subunits (Gbg) in Xenopus oocytes, an inwardly rectifying K⁺ current was expressed. In contrast, co-injection of TuGIRK-B with Gbg did not express any current. When both TuGIRK-A and -B were co-expressed together with Gbg, an inwardly rectifying K⁺ current was also detected. The properties of this current clearly differed from those of TuGIRK-A current, since it displayed a characteristic decline of the macroscopic conductance at strongly hyperpolarized potentials. TuGIRK-A/B current also differed from TuGIRK-A current in terms of the lower sensitivity to the Ba²⁺ block, the higher sensitivity to the Cs⁺ block, and the smaller single channel conductance. Taken together, we concluded that TuGIRK-A and -B form functional heteromultimeric G protein-coupled inwardly rectifying K⁺ channels in the neural cells of the tunicate tadpole. By introducing a mutation of Lys161 to Thr in TuGIRK-B, TuGIRK-A/B channels acquired a higher sensitivity to the Ba²⁺ block and a slightly lower sensitivity to the Cs⁺ block, and the decrease in the macroscopic conductance at hyperpolarized potentials was no longer observed. Thus, the differences in the electrophysiological properties between TuGIRK-A and TuGIRK-A/B channels were shown to be, at least partly, due to the presence of Lys161 at the external mouth of the pore of the TuGIRK-B subunit.

The physiological significance of the G protein-coupled inwardly rectifying K⁺ (GIRK, Kir3) channels in the regulation of the heartbeat as well as neuronal excitability has been well known (1). Upon stimulation of Gαi/o-coupled receptors by various neurotransmitters, these channels open and induce hyperpolarization (1). Thus, it has been accepted that these channels function as a basic mechanism for an inhibitory modulation of neuronal excitability.

Since the first cDNA cloning of a subunit of G protein-coupled inwardly rectifying K⁺ channels (GIRK1/KGA, Kir3.1) (2, 3), some members of this channel family have been subsequently isolated and classified as a Kir3.x subfamily of two transmembrane type Kir K⁺ channels (4–6). The members of the Kir3 subfamily were shown both in vitro and in vivo to multimerize, namely to comprise functional heteromultimers. It is well known that the muscarinic K⁺ channel in the heart is the heteromultimer of Kir3.1 and Kir3.4 (5) and that the majority of the G protein-coupled inward rectifiers in the brain are heteromultimers of Kir3.1 and Kir3.2 (7).

Tunicate is classified into protochordates and regarded as one of the closest relatives of the vertebrates. Tunicate embryos form a neural tube through a folding of the neural plate consisting of the dorsal ectoderm underlined with the dorsal mesoderm (8). Since this process is highly similar to that occurred in vertebrates, the tunicate embryo is regarded as a prototype of neural development of mammals (8). Using a very simple system of cleavage-arrested tunicate embryos, the developmental changes of the excitable membrane and the mechanism of neural induction have been studied intensively (9–13). In those studies, it was shown that the expression level of the simple inward rectifier K⁺ channel is correlated with the cell fate selection of neural/epidermal cells (13). However, the existence of G protein-coupled inwardly rectifying K⁺ channels in tunicate has not yet been reported. It is thus of great interest to know whether the G protein-coupled regulation of excitability through the control of the inwardly rectifying K⁺ channels is conserved or not in the prototype of vertebrates.

In the present work, we, for the first time, have isolated cDNAs of two G protein-coupled inwardly rectifying K⁺ channel subunits from the tunicate (Halocynthia roretzi) young tadpole. These cDNAs were designated as tunicate G protein-coupled inwardly rectifying K⁺ channel-A and -B (TuGIRK-A and TuGIRK-B).

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¶ Supported by CREST from the Science and Technology Corporation of Japan. To whom correspondence should be addressed: Dept. of Physiology, Tokyo Medical and Dental University, Graduate School and Faculty of Medicine, D566, Yushima 1-5-45, Bunkyo, Tokyo 113-8519, Japan. Tel.: 81-3-5803-5156; Fax: 81-3-5803-5156; E-mail: ykubo.phy2@med.tmd.ac.jp.

1 The abbreviations used are: G protein, GTP-binding protein; GIRK, G protein-coupled inwardly rectifying potassium channel; TuGIRK-A, tunicate G protein-coupled inwardly rectifying potassium channel-A; TuGIRK-B, tunicate G protein-coupled inwardly rectifying potassium channel-B; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; Gbg, G protein β1/γ1 subunits; TuGIRK-A*, TuGIRK-A T173K mutant; TuGIRK-B*, TuGIRK-B K161T mutant.
and -B), respectively. We have shown that these two subunits form heteromultimers whose electrophysiological properties are different from the TuGIRK-A homomultimers. The macroscopic conductance of the TuGIRK-A/B heteromultimer showed a characteristic decline at strongly hyperpolarized potentials. Furthermore, to reveal the structural determinant for this property, we have performed a mutagenesis study and identified a positively charged amino acid at the external mouth of the channel pore (H5) as a critical residue that was found uniquely in the TuGIRK-B subunit.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning**—Unidirectional cDNA library of tunicate (H. roretzi) young tadpoles was constructed using a ZAP-cDNA synthesis kit (Stratagene) with oligo(dT)-linker primers. ZAP-cDNA was purified using the Wizard Lambda Preps DNA purification system (Promega). It was used as a template for PCR screening with a degenerative oligonucleotides, which were designed based on the conserved sequences of the Kir family, QTTIGYG (P1: 5' -CA(A/G)ACIACIAT(T/C/A)GGI TA(T/C)GG-3') and PKKRAET (P2: 5' -IGT(T/C)TCIGCICG(T/C)TT(T/C)TIGG-3').

**Fig. 1.** The primary structures of TuGIRK-A and TuGIRK-B and comparison with members of the GIRK family. A, the alignment of the deduced amino acid sequences of the TuGIRK-A and -B channels with mammalian Kir3.1 (2, 3) and 3.2 (4). The putative two-transmembrane regions (M1, M2) and H5 pore region are indicated by the boxes. Amino acids identical in four channels are marked by asterisks. B, an oligodendrogram to show the relationship of members of the mammalian GIRK channel families and TuGIRK-A and -B. The sequences used were Kir3.1 (2, 3), Kir3.2 (4), Kir3.3 (35), and Kir3.4 (5).
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pairs in length were subcloned, and the sequences were determined. For obtaining full-length cDNA clones, the same cDNA library was screened by the plaque hybridization method using the two subcloned PCR products as probes. Inserts of positive phages were excised by plasmid rescue of pBluescript SK− (Stratagene) and R408 helper phage. The obtained clones were sequenced using the PRISM sequence reaction kit (Applied Biosystems) and a DNA sequencer (Applied Biosystems model 377-18).

For the electrophysiological analysis, cRNAs were transcribed from Xhol-digested cDNA using methylated cap analog (Amersham Pharmacia Biotech) and T3 RNA polymerase (Stratagene). The mutation was confirmed by sequencing made using the Sculptor Kit (Amersham Pharmacia Biotech) or Quick-Change Kit (Stratagene). Point mutants were sequenced using the PRISM sequence reaction kit (Applied BioSystems).

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Change Kit (Stratagene). Point mutants were made using the Sculptor Kit (Amersham Pharmacia Biotech) or Quick-Change Kit (Stratagene). The mutation was confirmed by sequencing the primer and the surrounding regions.

RT-PCR—Unfertilized eggs or young tadpoles were rinsed once in sea water and then transferred to microtubes containing guanidine isothiocyanate solution. After incubation at room temperature for 2 h, RNA was extracted using the acid guanidium-phenol chloroform method (14). 5 μg of the purified RNA was reverse transcribed by Superscript 2 (Life Technologies, Inc.), and one-twentieth of the obtained cDNA solution was used as a template for PCR. TuGIRK-A or TuGIRK-B-specific PCR primer sets were as follows: for TuGIRK-A, 5′-CGTCAAGGATTATCCG-3′ (upstream) and 5′-ATCTGGGCTTGTCGATATGC-3′ (downstream); for TuGIRK-B, 5′-CCACTTGCGTCAGTTACGC-3′ (upstream) and 5′-CGATTTGACTATCTTTGCGCCG-3′ (downstream).

Amplifications were performed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated on agarose gels and visualized with ethidium bromide.

Whole Mount in Situ Hybridization—Whole mount in situ hybridization was carried out as described previously (15, 16). The plasmids containing the inserts were linearized with either Xhol (sense probe) or NotI (antisense probe) for in vitro synthesis of RNA probes.

two-electrode Voltage Clamp—Oocytes were treated with type 1 collagenase (2 mg/ml; Sigma) for 2 h at room temperature to remove follicle cells and injected with 50 nl of in vitro transcribed cRNA solution. For the analysis of TuGIRK-A/B heteromultimers, the concentrations of cRNA were ~50 ng/μl (TuGIRK-A) and 500 ng/μl (TuGIRK-B), respectively. This concentration ratio was used to avoid the formation of TuGIRK-A homomultimers. Since TuGIRK-B homomultimers were confirmed to be nonfunctional, this ratio of TuGIRK-A/B cRNAs enabled us to analyze a channel pool that consisted mostly of TuGIRK-A/B heteromultimers. Gβγ-cRNAs were used at a concentration of 500 ng/μl to achieve maximal expression level.

Electrophysiological recordings were carried out 2–3 days later under two-electrode voltage clamp (OC-725B-HV; Warner Co.) at 23 ± 2°C. Data acquisition and analysis were done on an 80486-based computer using Digidata 1200 and pCLAMP program (Axon Instruments). Intracellular glass microelectrodes were filled with 3 M potassium acetate supplemented with 10 mM KCl, and the resistance ranged from 0.2 to 0.8 MΩ. The bath solution contained 90 mM KCl, 3 mM MgCl₂, and 5 mM Hepes (pH 7.4). To decrease the K⁺ concentration, KCl was replaced with N-methyl-D-glucamine chloride. The chord conductance (see Figs. 5, C and F, 9C, and 10, C and F) was initially calculated based on the EK values assuming intracellular K⁺ to be 80 mV. However, the exact values of [K⁺]i and EK of each oocyte are unknown. Therefore, EK used for the calculation of the chord conductance was adjusted in the range of ±3 mV to yield a continuous conductance-voltage plot. In the experiments using blockers, Ba²⁺ or Cs⁺ was added simply to the bath solution. Fractions of the remaining currents were calculated at each potential in various concentrations of blockers. By fitting the plot of the dose-block relationship, the half-block concentration (IC₅₀) was calculated at each potential. The IC₅₀ values, which reflect the depth of the blocking site in the electric field, were calculated as described previously (18).

Patch Clamp—Single channel recordings were carried out in the cell-attached configuration of the patch clamp (Axopatch-1D; Axon Instruments) at 22–25°C. Data were filtered at 1 kHz by a built-in eight-pole bessel filter, sampled at 5 kHz through the Digidata 1200 (Axon Instruments), and stored in a computer with the pCLAMP program (Axon Instruments). The resistance of the patch pipettes ranged from 3 to 6 MΩ. The patch pipette (extracellular) solution contained 140 mM KCl, 3 mM MgCl₂, and 5 mM Hepes (pH 7.4). The bath (extracellular) solution contained 140 mM KCl, 3 mM MgCl₂, and 5 mM Hepes (pH 7.4). Single channel current amplitudes at various potentials and mean open times were determined by the pCLAMP software. The slope conductance was calculated from a single line fitted by eye for the plot of the single channel current-voltage relationship.

FIG. 2. Analysis of transcription of TuGIRK-A and -B in unfertilized eggs and in young tadpoles by RT-PCR. PCR fragments for TuGIRK-A (A) and TuGIRK-B (B) were analyzed on agarose gels, respectively. The expression of neither TuGIRK-A nor TuGIRK-B was detected in unfertilized eggs. Both of them were expressed in the young tadpole larvae. M, size markers; lane 1, unfertilized egg; lane 2, young tadpole; lane 3, negative control (without reverse transcription) of lane 1; lane 4, negative control (without reverse transcription) of lane 2; lane 5, negative control (without RNA); lane 6, positive control using a cloned cDNA as a template.
RESULTS

Primary Structures—We aimed at the isolation of cDNA for inwardly rectifying K\textsubscript{1} channels (Kir) from tunicates (\textit{H. roretzi}) and screened a cDNA library of young tadpole by PCR using Kir-specific degenerative oligonucleotide primers. We obtained two kinds of 159-base pair DNA fragments, whose sequences showed \( \geq 60\% \) amino acid identity with G protein-coupled inwardly rectifying K\textsubscript{1} channels of the Kir3 subfamily. These PCR fragments were subcloned and used as probes to isolate full-length cDNA clones from the same cDNA library. Finally, we isolated two cDNA clones, which were designated as TuGIRK-A and -B. TuGIRK-A and -B were \( \approx 2.4 \) and \( \approx 2.0 \) kilobase pairs in size, encoding 612 and 399 amino acid residues, respectively (Fig. 1A). Both the TuGIRK-A and -B proteins had two putative transmembrane segments (M1, M2) and an H5 pore region, similar to mammalian Kir channels (17, 18). The amino acid sequence of TuGIRK-A exhibited an identity of \( \approx 60\% \) with murine Kir3.2 or Kir3.4 (4) channels. The identity of TuGIRK-B and mammalian Kir3.2 or Kir3.4 was \( \approx 55\% \). The results of an oligodendrogram analysis based on the Unweighted Pair-Group Method using Arithmetic averages package are shown in Fig. 1B, demonstrating the relationship of two TuGIRKs and members of the mammalian Kir3 channel families.

Members of the Kir family have a conserved sequence GY(or FGXR in the H5 pore region. GY(or FG is shown as the K\textsuperscript{+} ion-selective filter (19, 20), and a positively charged Arg at the external mouth of the pore is known to have a critical role for permeation and block (21–25). Both TuGIRK-A and -B had the GYXR sequence in the H5 pore. The sequence of TuGIRK-B (GYXR) was very unusual in that it had two positively charged amino acids in tandem. It was expected that this unique Lys residue at 161 might affect the channel properties of TuGIRK-B.

Developmental Changes of the Expression Patterns—The distributions of mRNA expression of TuGIRK-A and -B were studied by RT-PCR and whole mount \textit{in situ} hybridization. The RT-PCR analysis was done using \textit{H. roretzi} unfertilized eggs and young tadpoles. Neither mRNA of TuGIRK-A nor TuGIRK-B could be detected in unfertilized eggs, but both of them were expressed in the young tadpoles (Fig. 2). As a next step, we performed the whole mount \textit{in situ} hybridization analysis in the embryos of various developmental stages, 64-, 110-, and 117-cell, gastrula, neurula, tail bud, and young tadpole stages. The expression patterns of TuGIRK-A and -B highly resembled each other (Fig. 3). They were not expressed up to the gastrula stage (Fig. 3, A and B (a, 64-cell stage; b, gastrula stage)). The transcription of TuGIRK-A and -B mRNA started at the neurula stage in the putative neural cells of the head region (Fig. 3, A and B (c)). From the tail bud stage, the
expression was observed additionally in the neural cells of the neck region (Fig. 3, A and B (d)). These signals were also observed in young tadpoles (Fig. 3, A and B (e)).

**Electrophysiological Properties—**Electrophysiological properties of TuGIRK channels were analyzed under two-electrode voltage clamp using the *Xenopus* oocyte expression system. Mammalian Kir3 channels are known to be activated by Gbg, which is released upon receptor stimulation (1, 26, 27). When TuGIRK-A or -B cRNAs were injected alone, they did not express any detectable current (Fig. 4, A (upper panel) and B), as is generally observed for Kir3 channels (2–5). As a next step, we co-injected them with Gbg cRNAs. TuGIRK-A coexpressed with Gbg showed inwardly rectifying K+ current (TuGIRK-A current), but TuGIRK-B with Gbg did not (Fig. 4, A (lower panel) and B). When TuGIRK-B was co-expressed with TuGIRK-A and Gbg, an inwardly rectifying K+ current, whose electrophysiological properties were different from those of the TuGIRK-A current as shown as follows, was observed (Fig. 4, A (lower panel, middle, TuGIRK-A/B current) and B). These results demonstrate that TuGIRK-A forms functional homomultimeric G protein-coupled inwardly rectifying K+ channels and that TuGIRK-A and -B form functional heteromultimeric G protein-coupled inwardly rectifying K+ channels. In the case of the Kir3.1 channel, the small expressed current was attributed not to homomultimer formation but to heteromultimerization with the endogenous Kir subunit of *Xenopus* oocytes (6). However, the expression level of TuGIRK-A is extremely high (Fig. 4B), in clear contrast with that of Kir3.1, suggesting that TuGIRK-A forms a functional homomultimer. In various concentrations of extracellular K+, both TuGIRK-A and TuGIRK-A/B channels were active below the equilibrium potential of K+ (E_K) (Fig. 5), as is observed in mammalian Kir channels. TuGIRK-A/B current differed from TuGIRK-A current in the following ways. (a) The macroscopic chord conductance of TuGIRK-A/B channels remarkably decreased at strongly hyperpolarized potentials (Fig. 5, D–F). This property was apparently different from that of TuGIRK-A channels (Fig. 5, A–C). (b) TuGIRK-A/B current was less sensitive to the block by extracellular Ba2+ (Fig. 6, D–F) than TuGIRK-A current (Fig. 6, A–C). The values of mean and S.D. of K_i at −120 mV were 52.4 ± 12.5 μM (n = 4) (TuGIRK-A) and 628.5 ± 89.3 μM (n = 3) (TuGIRK-A/B). (c) TuGIRK-A/B current was more sensitive to the block by extracellular Cs+ (Fig. 7, D–F) than TuGIRK-A current (Fig. 7, A–C). The values of mean and S.D. of K_i at −120 mV of TuGIRK-A/B were 188.4 ± 88.2 μM (n = 3). In the case of TuGIRK-A, due to the very low sensitivity, the data point range was insufficient for reliable curve fitting to obtain K_i values. The K_i value at −120 mV was estimated to be approximately 2532.2 ± 916.3 μM (n = 3) and was obviously no less than 1 mM. It is apparent that the K_i value of TuGIRK-A is significantly larger than that of TuGIRK-A/B. Thus, it was confirmed that the TuGIRK-B subunit forms a functional heteromultimer with TuGIRK-A, whose electrophysiological properties are distinguishable from those of TuGIRK-A homomultimer.

We examined the single channel properties of TuGIRK-A channels and TuGIRK-A/B channels expressed in *Xenopus* oocytes by the cell-attached patch clamp method with 140 mM K+...
respectively. The recordings were obtained in 0 mM extracellular Ba\(^{2+}\) channels to the block by Ba\(^{2+}\) D–F.

Functional Significance of Lys161 Residue of TuGIRK-B Subunit—To determine the possibility that Lys\(^{161}\) of TuGIRK-B, an additional positively charged amino acid at the external mouth of the pore, is the structural determinant for the difference between the electrophysiological properties of the pore of TuGIRK-A homomultimeric channels and TuGIRK-A/B heteromultimeric channels at the single channel level.

**FIG. 6.** The sensitivity of the TuGIRK-A homomultimer and TuGIRK-A/B heteromultimer to the block by extracellular Ba\(^{2+}\). Channels were expressed in Xenopus oocytes and recorded under two electrode voltage clamp in 90 mM K\(^+\). Shown are the current traces (A and D), the current-voltage relationships (B and E), and the dose-block relationships (C and F) of TuGIRK-A (A–C) and TuGIRK-A/B channels (D–F), respectively. The recordings were obtained in 0 mM (filled circles), 3 mM (open circles), 30 mM (filled squares), 300 mM (open squares), and 3 mM (filled triangles) extracellular Ba\(^{2+}\). In C and F, the data points and fitted lines indicate the data from −70 mV (far right) to −160 mV (far left).

To confirm further the functional significance of an additional positively charged amino acid at the external mouth of the pore, we introduced a reverse mutation, T173K, in the TuGIRK-A subunit (TuGIRK-A*). TuGIRK-A* carries two positively charged amino acids at the external mouth of the pore, similar to the TuGIRK-B subunit. In TuGIRK-A homotetramers (eight charges), a slight decrease in the macroscopic conductance at hyperpolarized potential was observed (Fig. 11, A–C), which was not at all seen in the TuGIRK-A/B heteromultimer (four charges) (Fig. 5, A–C). In comparison with the TuGIRK-A/B heteromultimer (5–7 charges at the pore mouth) (Fig. 5, D–F), the decrease in the macroscopic conductance at hyperpolarized potential was more enhanced in TuGIRK-A*/B heteromultimer (eight charges) (Fig. 11, D–F). The values of the ratio of the conductance at −160 mV and the peak conductance were 65.8 ± 2.8% (n = 3) (TuGIRK-A*/B) and 78.9 ± 9% (n = 3) (TuGIRK-A/B). Taken together, the effect of positively charged amino acid at the external mouth of the pore to the macroscopic conductance was further supported by T173K mutation of TuGIRK-A. In addition, we unexpectedly observed that TuGIRK-A* caused a decrease in the intensity of inward rectification (Fig. 11), suggesting a difference in the contribution of this site between TuGIRK-A and -B subunits.
DISCUSSION

We have isolated two types of cDNA, TuGIRK-A and TuGIRK-B, from the young tadpole larvae of the tunicate (H. roretzi), which belong to the Kir3 subfamily. In Xenopus oocytes, TuGIRK-A showed inwardly rectifying K⁺ current when coexpressed with Gbg. In contrast, TuGIRK-B did not show detectable current even in the presence of Gbg. When TuGIRK-A and -B together with Gbg were coexpressed, an inwardly rectifying K⁺ current was observed whose electrophysiological properties were different from those of Tu-GIRK-A. Thus, it was demonstrated that TuGIRK-A but not -B can form functional homomultimeric G protein-coupled channels and that -A and -B can form functional heteromultimeric G protein-coupled channels.

Corresponding Subunit of Murine Kir—Among the members of the Kir3 subfamily, TuGIRK-A exhibited a higher similarity (60%) with murine Kir3.2, -3.3, and -3.4 than with Kir3.1, as shown in the dendrogram of Fig. 1B. From this sequence homology and its neuron-specific expression pattern, TuGIRK-A was speculated to correspond to the murine Kir3.2 subunit. The fact that the homomultimeric channel is functional also supported this speculation.

TuGIRK-B was rather distant from Kir3.2, -3.3, and -3.4 and was closer to Kir3.1 (Fig. 1B). In the H5 pore region of the Kir3.1 subunit, there is a unique Phe at 137, where all other members have a conserved Ser. This unique Phe of Kir3.1 was reported to have an important role in the control of the channel activity (28, 29). In TuGIRK-B, Tyr, which has similarity with Phe, is present at the corresponding site. TuGIRK-B is similar to Kir3.1 also in that the homomultimeric channel is not functional (5, 7). Taken together, TuGIRK-B was speculated to correspond to the murine Kir3.1 subunit.

Significance in the Tunicate Embryo—Since a tunicate tadpole is a simple prototype of vertebrates, it has been used for various studies of developmental biology. The developmental changes of ion channel expression during neural differentiation were studied intensively using cleavage-arrested blastomeres (9). By these studies, the presence of multiple types of Ca²⁺ channels (30), Na⁺ channels (31), K⁺ channels (32), and a simple inward rectifier K⁺ channel (13) was demonstrated. It was also shown that the expression level of the simple inward rectifier K⁺ channel is dramatically regulated at the initial stage of neural/epidermal cell fate selection (13). Furthermore, cDNAs for voltage-dependent Na⁺ (16), K⁺ (33), and Ca²⁺ channels² were already isolated.

On the other hand, knowledge of G protein-coupled responses in tunicate is still insufficient. The presence of the G protein-coupled inwardly rectifying K⁺ channel is not demonstrated, although it would be expected to be a common cell response mechanism of animals. By molecular cloning, we, for the first time, have demonstrated the presence of G protein-coupled inwardly rectifying K⁺ channels in tunicate young tadpole. It remains to be elucidated what kind of receptors link

² Y. Okamura, personal communication.

Fig. 7. The sensitivity of the TuGIRK-A homomultimer and TuGIRK heteromultimer to the block by extracellular Cs⁺. A similar presentation to Fig. 6 is shown (A, B, D–F). The effect of Cs⁺ was examined instead of Ba²⁺. In the case of TuGIRK-A (A–C), reliable curve fittings to obtain Kᵢ values could not be done, because the data of effective doses were insufficient due to the extremely low sensitivity. In C, the ratios of the current amplitudes in 0 mM Cs⁺ and in various concentrations of Cs⁺ at each membrane potential are presented as a bar graph instead of a plot for curve fittings as in F.
FIG. 8. Single channel recordings of the TuGIRK-A homomultimer and the TuGIRK-A/B heteromultimer. Channels were expressed in Xenopus oocytes and recorded in the cell-attached configuration of the patch clamp method. The patch pipette and the bath contained 140 mM K\(^+\). A and B, single channel recordings of the TuGIRK-A homomultimer (A) and the TuGIRK-A/B heteromultimer (B). The displayed traces were low pass-filtered at 500 Hz by a digital filter of the pCLAMP software. The membrane potentials are shown on beside each recording. The zero current level is shown by thin lines. C, relationships of the single channel current amplitude and the voltage. The values of single channel conductance of the inward current range of TuGIRK-A (filled circles) and TuGIRK-A/B (open squares) were estimated to be 24.5 and 18.0 picosiemens, respectively. The lines were fitted by eye.
to the regulation of these channels, due to the lack of information of G protein-coupled receptors in tunicate.

We investigated the developmental changes of TuGIRK-A and -B mRNA expression by RT-PCR and whole mount in situ hybridization analyses (Figs. 2 and 3). TuGIRK-A and -B mRNA were observed from the neurula stage in presumptive neural cells of the head region. These expressions were continuously seen in the tail bud stage and in the young tadpole larvae. From the tail bud stage, they were additionally expressed in the neural cells of the neck region. During the entire course of development, the expression patterns of TuGIRK-A and -B were highly similar. These results show that TuGIRK-A and -B form functional heteromultimeric channels in neurons in the head and neck region. They might function to regulate the speed of the fictive locomotion of the tadpole by regulating the firing rate of neurons that innervate tail muscles.

Structure-Function Relationship—We compared the electrophysiological properties of the TuGIRK-A homomultimer and the TuGIRK-A/B heteromultimer and observed the following differences. (a) The macroscopic conductance of TuGIRK-A/B decreased at strongly hyperpolarized potential, but that of TuGIRK-A did not (Fig. 5). (b) TuGIRK-A/B was less sensitive to the block by extracellular Ba$^{2+}$ than TuGIRK-A (Fig. 6). (c) TuGIRK-A/B was more sensitive to the block by extracellular Cs$^+$ than TuGIRK-A (Fig. 7). (d) The single channel conductance of TuGIRK-A/B was smaller than TuGIRK-A (Fig. 8). What is the structural determinant for these differences?

In Kir channels, there is a highly conserved positively charged amino acid (Arg$^{148}$ of Kir2.1) after the K$^+$-selective filter at the external mouth of the pore. The only exception is the Kir7.1 subfamily (23, 24). This Arg residue is known to have an important role for permeation (21, 23, 24), Mg$^{2+}$ block (22), and ionic selectivity (25). It is speculated to serve as an electrostatic barrier for extracellular cations that regulates the...
permeation and block of the channel (22). In both TuGIRK-A and -B, the Arg residue is also conserved at the corresponding sites. Interestingly, TuGIRK-B has an additional positively charged amino acid, Lys, just before the conserved Arg. Thus, two positive charges exist at the external mouth of the pore of TuGIRK-B subunit.

We speculated that this site might be the structural determinant for the unique macroscopic conductance property of TuGIRK-A/B channel and introduced a point mutation of K161T to TuGIRK-B (TuGIRK-B*) to neutralize this additional positive charge. In the TuGIRK-A/B* channel, the plot of the current-voltage relationship was straight (Fig. 9), similar to TuGIRK-A channel (Fig. 5, A–C). This result demonstrates that Lys 161 of TuGIRK-B is, at least in part, the structural determinant for the unique macroscopic conductance property of TuGIRK-A/B channels. This point was further supported by the data of a reverse mutant of TuGIRK-A, T173K (TuGIRK-A*) (Fig. 11).

The TuGIRK-A/B* channel was more sensitive to the Ba\(^{2+}\) block and less sensitive to the Cs\(^{+}\) block than the TuGIRK-A/B channel. We analyzed the voltage dependence of $K_v$ values of Ba\(^{2+}\) block displayed in Figs. 6, C and F, and 10C. The calculated $K_v$ values (18), which reflect the depth of the binding site, differed significantly as follows: TuGIRK-A, 0.25; TuGIRK-A/B, 0.075; TuGIRK-A/B*, 0.32. From these results, it was suggested that the high sensitivity in TuGIRK-A and TuGIRK-A/B* is due to a block at a deep site in the electric field. In TuGIRK-A/B, it was speculated that the positive charge at the external mouth of the pore inhibited the access of Ba\(^{2+}\) to the deep blocking site, and the remaining low sensitivity is due to a block at another very shallow site(s). Taken together, Lys\(^{161}\) was thought to serve not as a binding site for these pore blockers but as an electrostatic barrier for the entrance of extracellular cations, similar to Arg\(^{148}\) of Kir2.1 (22).

**Significance of Heteromultimer Formation**—We showed that TuGIRK-A is sufficient to form a functional G protein-coupled inwardly rectifying K\(^{+}\) channel. Why then is TuGIRK-B subunit coexpressed in the same neuron? What is the physiological significance of the TuGIRK-B subunit?

One possibility may be that the TuGIRK-B subunit functions to determine the subcellular distribution of the heteromultimeric channel protein. It is known that all members of the murine Kir3 family have a binding motif for integrin, RGD, at the extracellular loop between the first transmembrane region and the H5 pore region (34). It is reported that the interaction of these subunits with integrin is required for appropriate localization and function of Kir3 channels. This integrin binding motif RGD was also present in the TuGIRK-B subunit (amino acids 119–121) but not in TuGIRK-A. It is possible that the TuGIRK-A homomultimer is not located appropriately or not clustered due to the lack of integrin binding domain and that the channel cannot exert its full function, even if it is fully functional in vitro when coexpressed with $\beta_3$. In contrast, the TuGIRK-A/B heteromultimer, which is supplied an integrin binding site by the TuGIRK-B subunit, might acquire physiological function by localizing at appropriate sites.

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