Inhibitory Interactions between Two Inward Rectifier K⁺ Channel Subunits Mediated by the Transmembrane Domains*

Inwardly rectifying K⁺ channel subunits may form homomeric or heteromeric channels with distinct functional properties. Hyperpolarizing commands delivered to Xenopus oocytes expressing homomeric Kir 4.1 channels evoke inwardly rectifying K⁺ currents which activate rapidly and undergo a pronounced decay at more hyperpolarized potentials. In addition, Kᵢ₄.1 subunits form heteromeric channels when coexpressed with several other inward rectifier subunits. However, coexpression of Kᵢ₃.4 with Kᵢ₉.1 causes an inhibition of the Kᵢ₄.1 current. We have investigated this inhibitory effect and show that it is mediated by interactions between the predicted transmembrane domains of the two subunit classes. Other subunits within the Kir 3.0 family also exhibit this inhibitory effect which can be used to define subgroups of the inward rectifier family. Further, the mechanism of inhibition is likely due to the formation of an "inviable complex" which becomes degraded, rather than by formation of stable nonconductive heteromeric channels. These results provide insight into the assembly and regulation of inwardly rectifying K⁺ channels and the domains which define their interactions.

Inwardly rectifying potassium channels (Kir)¹ are found in a wide variety of tissues and cell types where they are involved in the maintenance of the resting membrane potential and control of excitability (1–6). The diversity of these channels can at least in part be explained by the growing number of cloned inward rectifier subunits (7–14). In addition, as with voltage-dependent potassium channels (Kv), diversity is enhanced by the ability of inward rectifier subunits to form homomeric or heteromeric channels. For example, coexpression of Kir 3.1 (BIR10; Ref. 10) with Kir 3.1.1 (ROMK1; Ref. 9) or Kir 3.1 (BIR9) results in heteromeric channels distinct from either homomeric parental channel (15).² Also, coexpression of different members of the Kir 3.0 subfamily has profound effects. For instance, coexpression of either Kir 3.2 (GIRK2; Refs. 12 and 14), 3.3 (GIRK3; Ref. 12), or 3.4 (CIR; Refs. 16 and 17) with Kir 3.1 results in significant G-protein stimulated channel activity (16, 18, 19). Also, an inhibitory effect of Kir 3.3 upon Kir 3.2 channel activity has been reported although the mechanism has not been determined (19).

In this study, we have investigated the effects of coexpression of Kir 3.4 with Kir 4.1. In this case, the effect is neither a potentiation nor a modification of channel activity, rather an inhibitory "dominant-negative" effect upon Kir 4.1 currents. We show that this effect on Kir 4.1 is also endowed by other members of the Kir 3.0 family, and that the TMs are the structural elements which mediate the inhibitory interactions. Further analysis suggests that the inhibitory interactions occur shortly after translation and that the resulting complexes are degraded rather than processed as nonconducting complexes to the plasma membrane.

EXPERIMENTAL PROCEDURES

Electrophysiology—Xenopus laevis care and handling were in accordance with the highest standards of institutional guidelines. Frogs underwent no more than two surgeries, separated by at least 3 weeks. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Standard recording solution contained 90 mM KCl, 3 mM MgCl₂, 10 mM HEPES (pH 7.4) unless otherwise stated. Microelectrodes were filled with 3 M KCl and had resistances of 0.1–0.5 MΩ. Recordings were performed at 22 °C, 24–36 h after injection using a Geneclamp 500 amplifier (Axon Instruments) interfaced to a Macintosh Quadra 800 computer. Currents were evoked by voltage commands from a holding potential of −5 mV, delivered in −10-mV increments from 40 mV to −100 mV, unless otherwise stated. Data collection and analyses were performed using Pulse, PulseFit (Heka), and IGOR (Wavemetrics) software. Values for the average whole-cell current were obtained by measuring the steady state current at −100 mV. All data are presented as the mean ± S.E. for groups of at least six oocytes (actual numbers used in each group are indicated above the bars) and are expressed as the percentage of control current observed for an identical group of oocytes injected with an equivalent amount of Kir 4.1 mRNA. In all cases, the amount of Kir 4.1 mRNA and the volume injected per oocyte were held constant at approximately 0.1 ng in 50 nl, which would typically result in the 10–15 pg mRNA injected per oocyte. All co-injected mRNAs were varied according to the ratios described in the text, e.g. a 10-fold excess of Kir 3.4 (10:1 ratio) was obtained by conjuncting 1 ng of Kir 3.4 + 0.1 ng of Kir 4.1 mRNA per oocyte.

Molecular Biology—All channel subunits were subcloned into the oocyte expression vector pB8 (graciously provided by Dr. B. Fakler) which provides 5'- and 3'-untranslated regions from the Xenopus β-globin gene flanking a polylinker containing multiple restriction sites. In vitro mRNAs were generated using SP6 polymerase (Life Technologies, Inc.), following synthesis, mRNAs were evaluated spectrophotometrically and by ethidium bromide staining after agarose gel electrophoresis. Chimera were generated by a method described by Horton et al. (20) in which the chimeric junctions were generated by overlap extension of PCR primers which encoded the desired sequence. The subunit domains are defined by the following amino acids: Kir 3.4, N terminus, amino acids 1–82; TM1, amino acids 83–116; pore, amino acids 117–164; TM2, amino acids 165–192; C terminus, amino acids 192–419; Kir 4.1, N terminus, amino acids 1–60; TM1, amino acids 61–93; pore, amino acids 94–143; TM2, amino acids 144–170; C terminus, amino acids 171–379. To engineer the FLAG epitope onto the C terminus of Kir 4.1, an oligonucleotide which deleted the Kir 4.1 stop

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¹ The abbreviations used are: Kir, inward rectifier potassium channel; Kirᵥ, voltage-dependent potassium channel; TM, transmembrane domain.

² J. P. Adelman, submitted for publication.

³ B. Fakler, unpublished data.
Inhibition of Kir 4.1 by Kir 3.4 and Other Members of the Kir 3.0 Family—Injection of mRNA encoding Kir 3.4 into Xenopus oocytes results in hyperpolarization-activated, inward potassium currents which have a time-dependent decay at more negative potentials (Fig. 1a; Refs. 10 and 22). In contrast, hyperpolarizing commands delivered to oocytes injected with Kir 3.4 mRNA do not evoke macroscopic channel activity different from control oocytes (Fig. 1b; Ref. 16). However, expression of Kir 3.4 with Kir 4.1 results in a reduction of Kir 3.4 whole cell currents that is proportional to the ratio of Kir 3.4 to Kir 4.1 mRNA injected (Fig. 1c and d). Indeed, when Kir 3.4 mRNA is coexpressed in a 10-fold excess to Kir 4.1, all current is abolished (Fig. 1d and e). Inhibition of the Kir 3.4 current is not due to effects of excess mRNA on translation because coinjection of Kir 3.4 and Kir 4.1 mRNA with an equivalent excess of mRNA for another membrane protein, the dopamine D2 receptor, does not affect Kir 3.4 current amplitudes (Fig. 1e; Ref. 23).

The reduced Kir 3.4 currents evoked from coexpressed oocytes were not different from Kir 3.4 currents recorded from oocytes injected only with Kir 4.1 mRNA. Fitting the time-dependent component of the whole cell current trace recorded at -100 mV with a double exponential yielded time constants of $\tau_e = 57.7 \pm 2.5$ ms and $\tau_i = 366.0 \pm 11.0$ ms ($n = 6$) for oocytes injected only with Kir 4.1, and of $\tau_e = 50.0 \pm 1.2$ ms and $\tau_i = 277.2 \pm 9.9$ ms ($n = 6$) for oocytes injected with a 1:1 ratio of Kir 3.4 and Kir 4.1 mRNA. These results suggest a specific inhibitory effect of Kir 3.4 upon Kir 4.1.

To determine if other members of the Kir 3.0 subfamily had a similar effect on Kir 3.4, Kir 3.1 (8, 24), and Kir 3.2 (14, 12) were coexpressed with Kir 4.1. As shown in Fig. 2, both of these Kir 3.0 subfamily members had similar inhibitory effects upon Kir 3.4 current amplitudes. To test whether Kir 3.4 inhibits other inward rectifier subunits, Kir 1.1 (9), a subunit closely related to Kir 4.1, was coexpressed with Kir 3.4. Currents evoked following coexpression of Kir 1.1 and Kir 3.4 were reduced compared to oocytes expressing only Kir 1.1, similar to the effects on Kir 4.1 (currents reduced to <5% of controls; not shown).

Inhibition Is Mediated by the Transmembrane Domains—To localize structural elements responsible for the inhibitory interactions between members of these two inward rectifier subfamilies, a panel of chimeras between Kir 3.4 and Kir 4.1 was constructed (Fig. 3; see “Experimental Procedures”). When expressed alone, chimeras 1413, 1414, and 1415, all containing the TM/pore region of Kir 3.4, did not produce currents different from control oocytes. In contrast, chimeras 1407, 1408, and 1409 which share a common structural domain, the TM/pore region of Kir 4.1, did yield significant channel activity resembling Kir 4.1 currents (Fig. 3).

To determine which domains mediate the inhibitory effect of Kir 3.4 upon Kir 4.1, chimeras 1413, 1414, and 1415 were coexpressed in a 10-fold excess to Kir 4.1 and chimeras 1407, 1408, and 1409. The reduced Kir 4.1 currents evoked from coinjected oocytes are not different from Kir 4.1 currents recorded from oocytes injected with only Kir 4.1 mRNA (Fig. 3a). Currents evoked following injection of Kir 3.4 mRNA alone or a 10:1 ratio of Kir 3.4 to Kir 4.1 mRNAs were indistinguishable from mock-injected oocytes. Current families were evoked by 500-ms voltage steps from a holding potential of -5 mV to potentials from 40 mV to -100 mV in 10-mV increments. Averaged current amplitudes were recorded at -100 mV from oocytes injected with a constant amount of Kir 4.1 mRNA and varying ratios of Kir 3.4 or D2 receptor mRNAs. Currents were normalized relative to current amplitudes recorded from oocytes injected with only Kir 4.1 mRNA. Error bars represent ± S.E.
with a 10-fold excess of Kir 3.4 mRNA. The table in Fig. 3 shows that those chimeras with the TM/pore domains of Kir 3.4 (1413, 1414, and 1415) had an inhibitory effect on Kir 4.1, while chimeras 1407, 1408, and 1409, which contain the TM/pore region of Kir 4.1, were inhibited by coexpression with Kir 3.4. Therefore, the structural elements which mediate inhibition reside within the TM/pore domain.

To further localize the structural elements responsible for the inhibitory interactions, three more chimeras were constructed in which Kir 4.1 contained either the first, second, or both transmembrane domains of Kir 3.4 (Fig. 4a). None of these three chimeras was functional when expressed alone, and coexpression of Kir 4.1 and 1417 or 1418, the chimeras containing either one or the other of the Kir 3.4 TM s, had no significant effect upon Kir 4.1 currents. In contrast, coexpression of Kir 4.1 and 1419, the chimera with both Kir 3.4 TM domains, inhibited Kir 4.1 currents, similar to the inhibition by wild type Kir 3.4 (Fig. 4b). These results demonstrate that both TMs are necessary and sufficient for inhibition of Kir 4.1 channel activity.

Inhibition Results in Subunit Degradation—To investigate the mechanism of inhibition, the 8-amino acid FLAG epitope tag was engineered onto the C terminus of the Kir 4.1 subunit (Kir 4.1-F), permitting immunological detection with a monoclonal antibody (m2-FLAG ab). Currents recorded from oocytes injected with Kir 4.1-F mRNA were indistinguishable from those recorded from oocytes injected with wild type Kir 4.1 mRNA and, when coexpressed with Kir 3.0 mRNA, currents were reduced similar to coexpression with unmodified Kir 4.1 (not shown).

Total membranes were prepared from oocytes injected with either Kir 4.1-F mRNA alone or from oocytes coexpressed with Kir 4.1-F mRNA plus a 10-fold excess of test mRNAs. The membrane fractions were prepared as a Western blot and probed with the m2-FLAG antibody (Fig. 5). The Kir 4.1-F protein was detected by the m2-FLAG antibody as a protein of approximately 40 kDa, in close accord with its predicted molecular mass (41.1 kDa); the fainter bands of higher molecular weight likely represent aggregates of the Kir 4.1-F protein. These bands were not detected from mock injected oocytes (not shown) or oocytes coexpressed with Kir 3.4, Kir 3.1, or Kir 3.2 mRNAs, in which Kir 4.1-F currents were completely inhibited (<6% of the control current). However, the Kir 4.1-F protein was detected in oocytes coexpressed with mRNAs encoding Kir 4.1-F and the D2 receptor, Kir 1.1, or the two transmembrane chimeras which do not inhibit Kir 4.1 (1417 and 1418); currents from these oocytes were not reduced compared to control oocytes expressing Kir 4.1-F (Fig. 5).

When injected at a 10-fold excess, all members of the Kir 3.0 family tested, as well as chimera 1419, abolish the Kir 4.1 current and result in undetectable levels of the Kir 4.1-F protein, suggesting that they act through a common mechanism. Because the membrane preparations contained intracellular as well as plasma membrane compartments, it is likely that co-

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**FIG. 3.** Chimeras between Kir 4.1 and Kir 3.4 suggest the inhibitory interaction resides within the TM/pore region. Top, current families recorded from oocytes injected with mRNAs encoding chimeras 1407, 1408, and 1409; no currents different from control oocytes were detected following injection of mRNAs for chimeras 1413, 1414, or 1415 (not shown). Diagrammatic representations of the chimeric subunits are shown below. Bottom, table showing the effects of Kir 4.1 and Kir 3.4 on the current activities of the chimeras presented above when injected alone and their effects upon coexpression with Kir 4.1 or Kir 3.4 (N.D. = not determined).

**FIG. 4.** Both TMs are required for the inhibition. a, chimeras of Kir 4.1 containing either the first, second, or both putative TMs of Kir 3.4. b, averaged current amplitudes recorded at −100 mV from oocytes coexpressed with Kir 3.4 mRNA and the chimeric mRNAs in the indicated ratios.

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<th>1414</th>
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Interactions between Inward Rectifier Potassium Channel Subunits
expression of Kir 4.1 and Kir 3.4 results in degradation of heteromeric complexes. If this is the case, then temporally separating the expression of Kir 4.1 and Kir 3.4 might separate coassembly of these subunits and allow Kir 4.1 channels to reach the plasma membrane. To test this hypothesis, oocytes were injected with a 10-fold excess of Kir 4.1 mRNA either 12 h before or 12 h after injection of a constant amount of Kir 4.1 mRNA. Fig. 6 shows that if the expression of either subunit is delayed by 12 h then approximately 50% of the control Kir 4.1 current is observed. However, if the two are simultaneously coinjected, then Kir 4.1 currents are abolished. These results suggest that coassembly of Kir 4.1 and Kir 3.4 subunits occurs shortly after translation, and temporally separating translation of the two classes of mRNAs reduces the likelihood of heteromer formation.

DISCUSSION

Coexpression of members of the Kir 3.0 family with Kir 4.1 inhibits Kir 4.1 currents, an effect which is likely due to cotranslational subunit assembly and subsequent degradation of the heteromeric complexes. The structural motifs which mediate the inhibitory interactions of Kir 3.4 with Kir 4.1 reside within the putative TMs, and, while these are the only necessary structural motifs, both are required for inhibition.

The inhibition of Kir 4.1, Kir 4.1 currents by Kir 3.4 is not due to nonspecific effects on translation, because coexpression of Kir 4.1 with an equivalent amount of additional mRNA encoding another membrane protein, such as the dopamine D2 receptor, is without effect. Further, Kir 3.4 has a similar inhibitory effect upon Kir 1.1 (9), another inward rectifier which is closely related to Kir 4.1. The inhibition of Kir 4.1 and related subunits by members of the Kir 3.0 family thus provides an additional criterion for inward rectifier subunit classification (25).

The observation that both TMs are required for the inhibitory interaction suggests that when a single TM is swapped for that of another family, the subunits cannot be coassembled. Chimera 1419 which has both Kir 3.4 TMs is capable of interacting with Kir 4.1 subunits and inhibiting the current similar to Kir 3.4. Although the structural domains which mediate inhibitory interactions between members of the Kir 3.4 subfamily and Kir 4.1 are different from the domains which mediate coassembly of distinct Kir subunits, both Kir 3.0 and Kir 4.1 subunit coassembly may occur cotranslationally (26).

The mechanism of inhibition appears to act through sequestration of heteromeric complexes into a degradative pathway soon after translation and before insertion in the plasma membrane. If heteromeric channels are processed to the plasma membrane as inactive complexes or if subunit coassembly occurs by association within the plasma membrane, then the Kir 4.1-F subunit should be detected in oocytes injected with Kir 4.1-F and Kir 3.4 mRNAs. However, Kir 4.1-F subunits were not detected by Western blot, even though the preparations did not separate intracellular and plasma membrane compartments. In addition, the inhibitory interactions were uncoupled by temporally separating expression of the two different subunit types. Thus, when Kir 4.1 subunits are allowed to assemble together before interference from Kir 3.4 subunits, they are processed to the plasma membrane as functional channels, and, conversely, when Kir 3.4 subunits have already assembled together, subsequent inhibition of Kir 4.1 is reduced. However, when they are simultaneously expressed, as shown above, Kir 4.1 currents are abolished. The fact that other members of the Kir 3.0 family also had the same effect implies that the mechanism of inhibition by members of this family is the same.

There are several tissues, including the heart and brain, where members of the Kir 3.0 family and the subunits they inhibit are coexpressed. In atrial myocytes for example, Kir 3.4 and Kir 3.1 coassemble to form the channel underlying I_{K1,4} (16), but Kir 4.1 is also expressed in this tissue (10). It is possible that the inhibitory interaction described here between the Kir 3.4 and Kir 4.1 provides a way for the cell to prevent either inactive or possibly disruptive heteromeric complexes from reaching the plasma membrane, reflecting an additional physiological mechanism which regulates the array of distinct inward rectifier subtypes.

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