# Inward Rectifier Potassium Channels

## Cloning, Expression and Structure-Function Studies

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**SUMMARY**

A PCR-based cloning strategy was used to identify novel subunits of the two-transmembrane domain inward rectifier potassium channel family from rat brain, heart, and skeletal muscle. When expressed in *Xenopus* oocytes, two of these clones (Kir4.1 and Kir2.3) gave rise to inwardly rectifying potassium currents. Two-electrode voltage clamp commands to potentials negative to $E_K$ evoked inward potassium-selective currents which rapidly reached a peak amplitude and then relaxed to a steady-state level. Differences in the extent of current relaxation, the degree of rectification, and the voltage-dependent block by external cesium were detected. Two other members of this family (Kir5.1 and Kir3.4) did not produce macroscopic currents, when expressed by themselves, yet both subunits modified the currents when coexpressed with other specific members of the Kir family. Expression of chimeric subunits between Kir4.1 and either Kir5.1 or Kir3.4 suggested that the transmembrane domains determine the specificity of subunit heteropolymerization, while the C-terminal domains contribute to alterations in activation kinetics and rectification. Expression of covalently linked subunits demonstrated that the relative subunit positions, as well as stoichiometry, affect heteromeric channel activity. (Jpn Heart J 1996; 37: 651–660)

**Key words:** Polymerase chain reaction (PCR) Two-transmembrane domain potassium channel family Inward rectifier potassium channel (Kir) Heteromeric channel Two electrode voltage clamp (TEVC) Subunit positional effects

BASED on predicted structural similarities, mammalian potassium channels may be grouped into four large families. Molecular characterization of the six-transmembrane domain, voltage-dependent (Kv) potassium channel family

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began with genetic studies of the *Shaker* mutant from *Drosophila*.[1] The single-transmembrane domain, voltage-dependent minK channel, and the first two-transmembrane domain inward rectifier (Kir) potassium channels were identified using expression cloning.[2–4] The latest family described is that of the transient outward current, or TOK channels, identified by DNA database searches for homologies with the conserved potassium channel pore sequence. TOK subunits have eight putative transmembrane domains and two pore regions, topologically resembling a tandem of Kv and Kir subunits.[5] Excluding the minK protein, which shows no significant homology to any of the other families, a prevalent motif in all cloned potassium channels is the presence of a potassium-selective pore with the sequence GYG as an important structural determinant for selectivity, and two transmembrane domains flanking this pore region. Kv potassium channels contain four additional transmembrane domains, including a highly charged S4 segment, and TOK channels seem to possess characteristics of Kv as well as Kir families. Kv and Kir channels form functional channels by assembling as tetramers[6] whereas TOK channels are presumably dimers, although this has not yet been demonstrated.

Although the initial characterization of discrete potassium channel families started with little or no prior knowledge of their primary amino acid sequence, the recent characterization of TOK channels, conducted exclusively as a database search for potassium pore sequences, underscores the importance of studies relying on sequence homology. Furthermore, characterization of new members within each potassium channel family has been achieved by screening cDNA libraries using low-stringency hybridization with family-specific probes, and by use of the polymerase chain reaction.[7,8] Using both of these techniques, our laboratory has successfully characterized novel members of the Kir potassium channel family. This article summarizes our major findings, including the cloning and characterization of novel subunits, heterologous expression in *Xenopus* oocytes, and structure-function studies. A detailed description of some of the topics reviewed in this article has been presented elsewhere.[9–12]

## Cloning of Novel Kir Subunits

After the initial characterization of the first cloned inward rectifier subunits, ROMK1 (Kir1.1)[3] and IRK1 channels (Kir2.1)[4], as well as the G-protein coupled subunit, GIRK1 (or KGA; Kir3.1)[13,14] our laboratory embarked on a systematic characterization of Kir subunits by using the polymerase chain reaction (all Kir sequences are designated by the nomenclature proposed by Doupnik et al)[8]. Figure 1 is a diagramatic representation of our strategy (The original description of the first subunits cloned using this strategy has been published

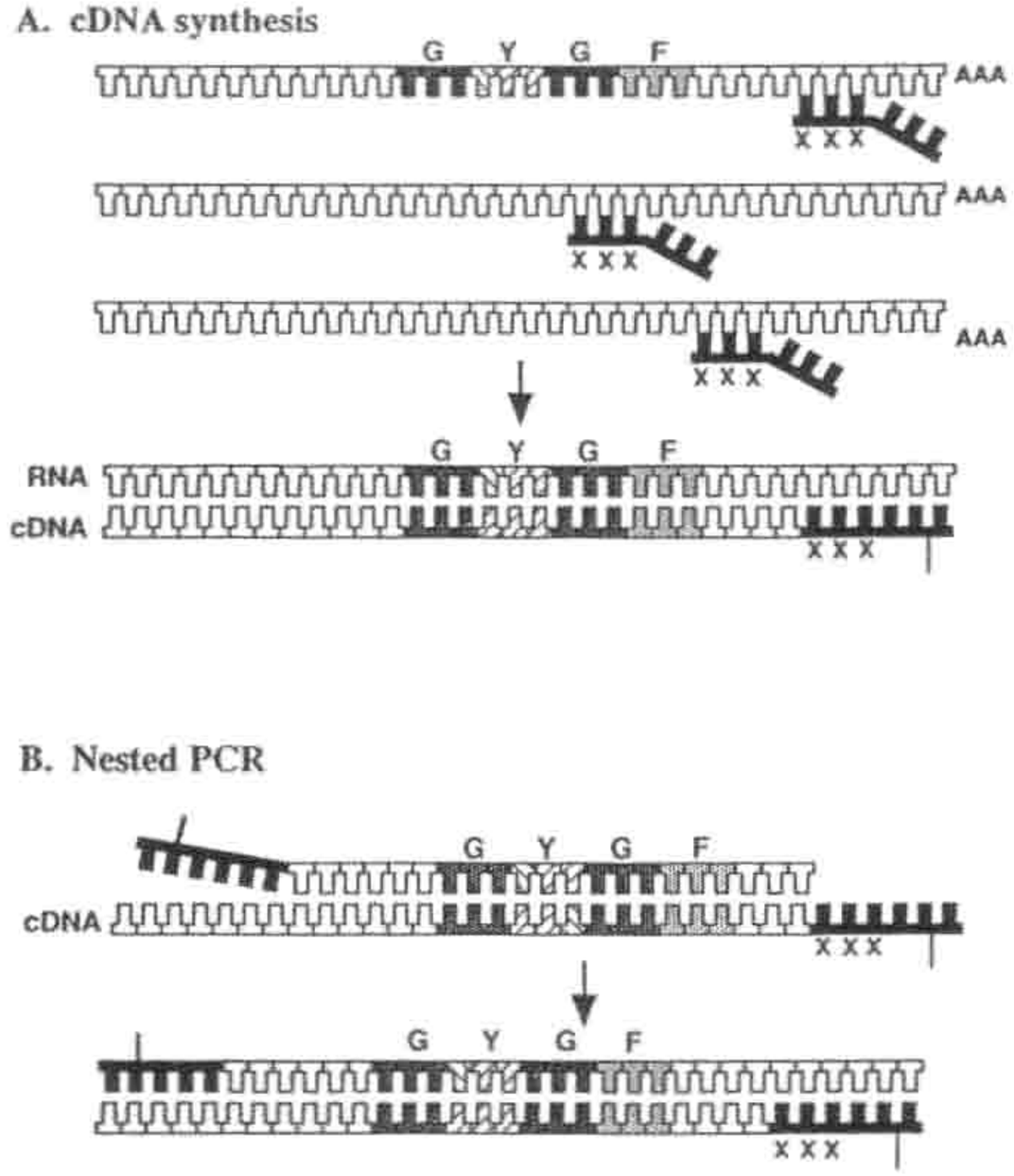


**Figure 1.** Schematic representation of PCR cloning strategy used to isolate novel Kir channel subunits. (A) cDNA synthesis was primed using a degenerate heptamer containing a 5′ tag. Every cDNA synthesized in this fashion would contain a 5′ tag. Sources for mRNA were tissues which express Kir channels like ROMK1 and IRK1. Transcripts for Kir channels would encode the "signature" sequence GYGF. (B) A degenerate oligonucleotide encoding the "signature" sequence was used as upstream primer for PCR. The downstream primer encoded the 5′ tag sequence present in all cDNAs. The double-stranded sequences amplified at the end of PCR amplification cycles would be tailed by the 5′ tags present in the priming oligonucleotides, which specify restriction sites useful for cloning.

elsewhere).[9] Our primary objective was to isolate clones encoding additional members of the Kir family. The strategy entailed hybridization of a degenerate oligonucleotide pool selective for the conserved pore sequences of IRK1, ROMK1, and GIRK1, and amplification of regions immediately downstream from this sequence, presumably encoding a hydrophobic transmembrane domain. To this end, we synthesized first-strand cDNA using random primers with a 5′ end nondegenerate tag, using polyA(+) RNA from tissues known to express inward rectifier potassium channels (Figure 1A). This cDNA served as substrate for sequential amplification with two sets of pore-specific oligonucleotide primer pairs (nested PCR). The downstream oligonucleotide primer for both rounds of

PCR coded for the nondegenerate tag present in the cDNA synthesis primer which was incorporated onto the 5′ end of all cDNA molecules. Specificity was endowed by the upstream primers. On the first round of PCR, the upstream primer (outer nested primer) was a 192-fold degenerate pool containing all possible coding combinations for the N-terminal portion of the Kir pore region. For the second round of PCR, the upstream primer (inner nested primer) was a 384-degenerate pool containing all possible coding combinations for amino acid residues TIGYGF, characteristic of the pore region of ROMK1 and IRK1 (Figure 1B). Cycling conditions for the two rounds of PCR consisted of 40 thermal cycles (denaturation: 94°C, 30 s; annealing: 53°C, 60 s; primer extension: 72°C, 30 s). The substrate for the second round of PCR was a dilution of the material amplified on the first round (up to 1:1000), eliminating substrate which had not been efficiently amplified during the first round. Amplified products were cloned using restriction endonuclease sites present in the 5′ sequences of the inner nested upstream primer and the downstream primer; the use of different restriction sites at each end allowed for directional cloning into m13 bacteriophage and efficient DNA sequencing of the PCR products. DNA sequences potentially encoding novel inward rectifier subunits were identified as open reading frames which included a hydrophobic stretch of amino acids homologous to transmembrane domain 2 of previously cloned Kir subunits. Initial attempts using rat brain and heart resulted in the isolation of 4 novel sequences. This "anchored, nested PCR" technique relies upon 1) the specifity of the upstream primers, 2) an uninterrupted amino-acid "signature" sequence for a family of related gene products, and 3) the level of degeneracy in the codons specifying this "signature" amino-acid sequence.

### Functional Expression and Structure-function Studies of Kir Subunits

Full-length sequences for new Kir subunits were obtained either by homology screens of cDNA libraries, or by rapid amplification of complementary ends (RACE: a form of anchored PCR which amplifies sequences spanning a specific region in the middle of a cDNA to its 5′ and 3′ ends.)[15] These clones were expressed in *Xenopus* oocytes and currents monitored by the two-electrode voltage clamp technique. The cloned sequences, now designated Kir4.1 and Kir2.3, gave rise to currents similar to, but distinct from those obtained by expression of IRK1 (Kir2.1). Figure 2 summarizes the major similarities and differences between Kir4.1 and Kir2.3 potassium channels.[9] Both channels displayed inwardly rectifying potassium currents, fast activation kinetics and slight time-dependent inactivation, and were blocked by micromolar concentrations of external cesium and barium. Quantitative differences between the two new channel types were noted

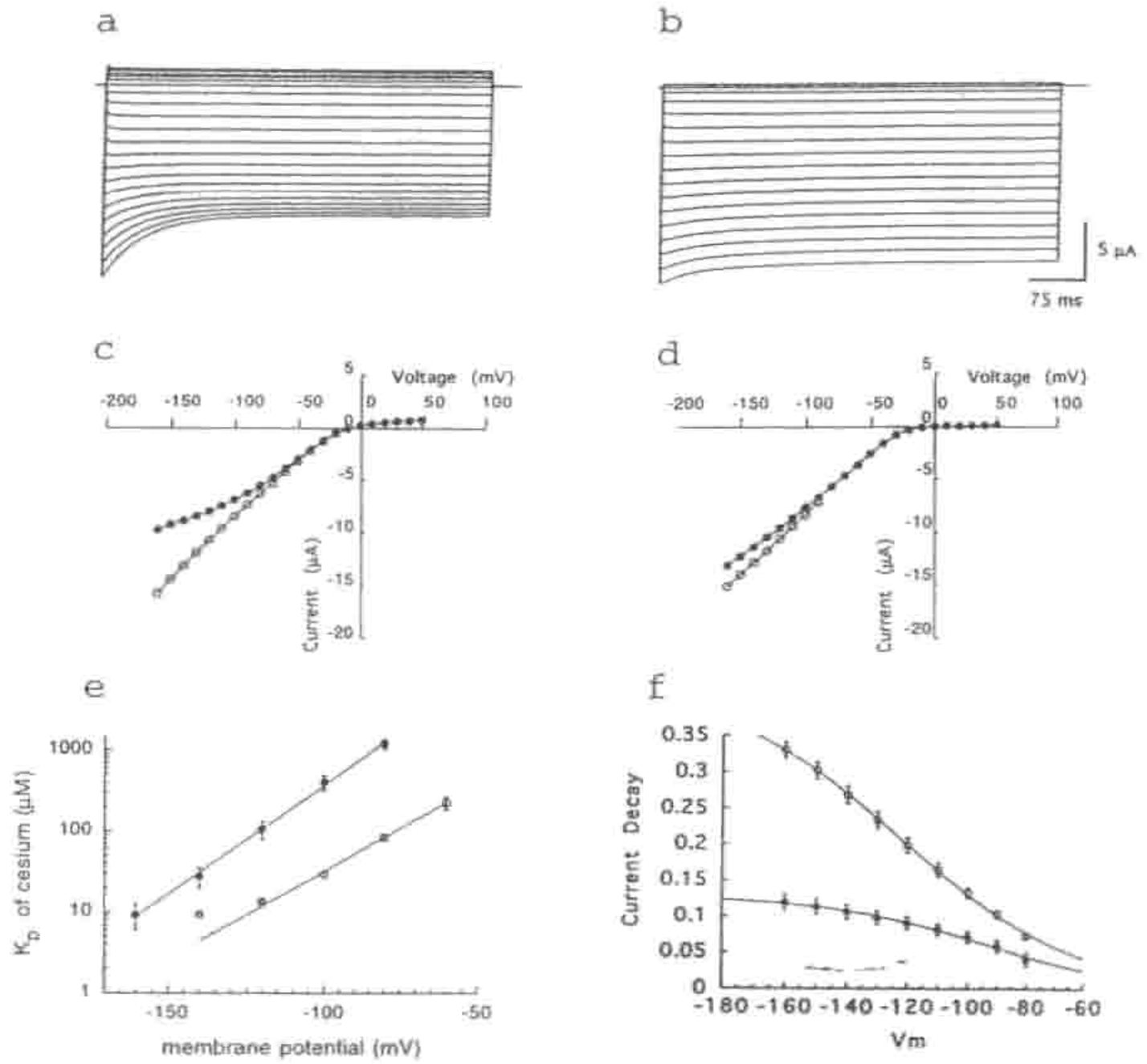


**Figure 2.** Functional differences between Kir4.1 and Kir2.3. Ionic currents were recorded in *Xenopus* oocytes expressing Kir4.1 (a) or Kir2.3 (b), using the TEVC technique. External solution contained 90 mM $K^+$. The major differences were the degree of rectification, which is steeper for Kir 2.3, and the extent of inactivation, which is larger for Kir4.1. These differences are illustrated in the middle panels, which plot peak (open circles) and steady state (closed circles) currents for Kir4.1 (c) and Kir2.3 (d). Differences were detected in the parameters of external cesium block (e): Kir2.3 (open circles) showed higher affinity, i.e. smaller dissociation constants, than Kir4.1 (closed circles), and a somewhat smaller voltage dependence of block; data were fitted by the equation $K_D = K_{D0} \exp(\delta zFE/RT)$. Differences were also detected in the voltage dependence of current decay (f): the current which inactivates at every test potential, (Ipeak-Isteady state)/Ipeak (vs Vm) was fitted to Boltzmann functions, and revealed a $V_{0.5}$ and k factor of -123 mV and 29 mV, respectively, for Kir4.1 (open circles), and – 96 mV and 27 mV, for Kir2.3 (closed circles).

in the degree of rectification, in the time-dependence of activation and inactivation, and in the voltage-dependence of ion block.

In contrast, two other novel subunits failed to produce functional channels. One of these sequences (Kir3.4) was shown by others to form part of a heteromeric complex with GIRK1 (Kir3.1), enhancing G-protein stimulated channel activity.[16] Other investigators showed that Kir4.1 subunits may

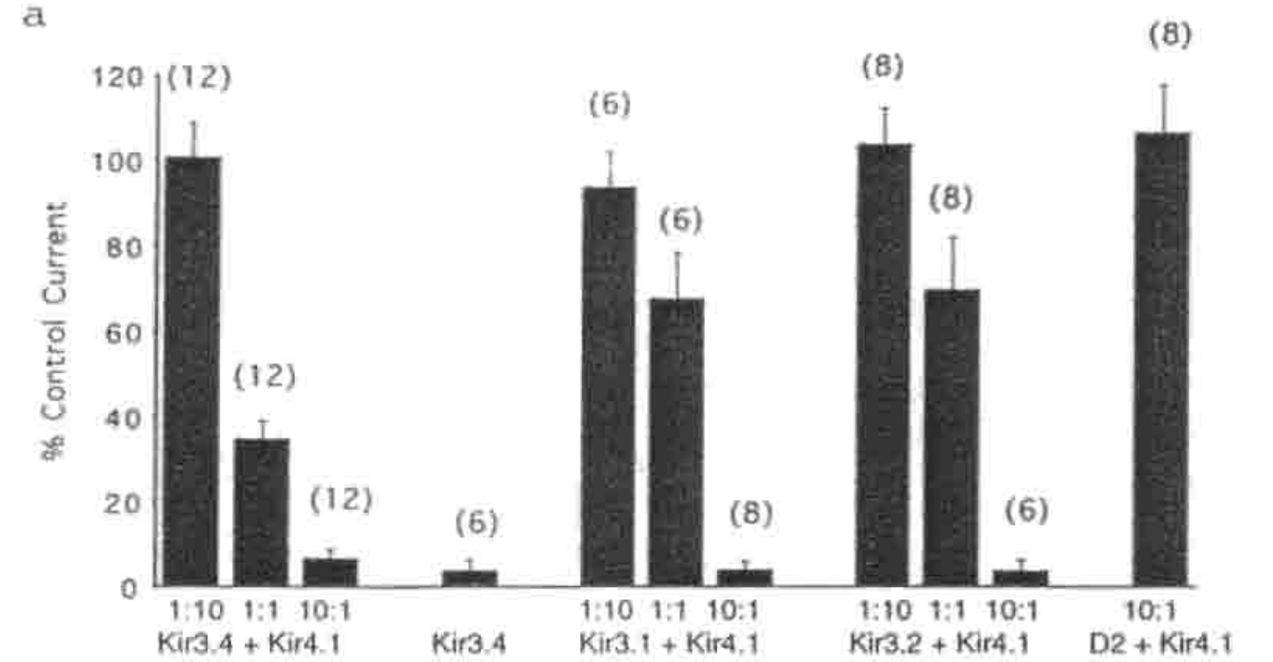


**Figure 3.** Inhibition of Kir4.1 currents by Kir3.4 and other members of the Kir3.0 subfamily (a). Averaged current amplitudes were recorded at − 100 mV from oocytes injected with a constant amount of Kir4.1 mRNA and varying ratios of Kir3.4, Kir3.1, or Kir3.2 or D2 receptor mRNA. Currents were normalized relative to current amplitudes recorded from oocytes injected with only Kir4.1 mRNA. Error bars represent ± s.e.m. Expression of Kir4.1/3.4 chimeras suggest that both TMs are required for the inhibition (b). The left panel shows a plot of averaged current amplitudes recorded at -100 mV from oocytes coexpressing Kir4.1 subunit and each of the chimeric subunits shown on the right panel, in the indicated ratios. Currents were normalized as described for (a). For all experiments shown, external solution contained 90 mM $K^+$.

coassemble with Kir1.1 subunits to form heteromeric channels with properties different from either parental homomeric channel.[17] Together, these and related observations made by several other groups, led us to propose a general hypothesis about subunit assembly and higher-order structure in the inward rectifier potassium channel family. First, we proposed that Kir subunits may assemble into either homomeric or heteromeric channels; second, that homo or heteromeric complexes may or may not reach the plasma membrane in a fully functional

state; and third, that the functional characteristics of stable heteromeric channels might be markedly different from parental homomeric channels. To test these hypotheses, we monitored the effect of Kir5.1 or Kir3.4 subunits (which on their own failed to express macroscopic currents above background) when coexpressed with Kir4.1 subunits. Here, we present in summarized form the most salient observations and general conclusions derived from these experiments.

Figure 3 illustrates the effect of coexpressing Kir4.1 with Kir3.4 or with other members of the Kir3.0 subfamily: a dose-dependent inhibition of the Kir4.1 current. In coexpressing oocytes, the current which remains is not obviously different from the currents observed by expression of Kir4.1 alone (not shown).[11] We observed similar inhibitory effects upon coexpression of any member of the Kir3.0 subfamily with Kir4.1, and showed that coinjection with similar concentrations of dopamine D2 receptor mRNA did not affect Kir4.1 current amplitudes, ruling out non-specific effects on Kir4.1 translation (Figure 3a). To investigate the structural basis of the inhibition, we constructed a panel of chimeras (Figure 3b)[11]; expression of subunits containing the TMs and pore region from Kir4.1 resulted in functional channels which failed to inhibit coexpressed Kir4.1, while subunits containing the TMs and pore region from Kir3.4 failed to form functional channels and inhibited coexpressed Kir4.1 (not shown).[11] These observations suggested that the region encompassing the transmembrane domains of Kir3.0 subfamily members mediate inhibitory interactions with Kir4.1. Additional chimeric subunits demonstrated that the inhibitory effect requires both Kir3.4 TMs (Figure 3b). Finally, using a Kir4.1 subunit tagged by a FLAG epitope, we obtained biochemical evidence that the inhibitory effects of Kir3.4 on Kir4.1 are not due to the formation of stable, non-conductive heteromeric complexes, but rather to the formation of complexes which are degraded soon after subunit protein synthesis (not shown).[11]

Figure 4 illustrates the effect of coexpressed Kir5.1 and Kir4.1 subunits, which produced whole cell currents with steeper inward rectification and slower activation kinetics than seen from expression of Kir4.1 alone. In addition, the averaged whole cell current amplitudes in coexpressing oocytes was 7–12 fold larger than for Kir4.1 alone (not shown).[12] This increase in current amplitude correlated with a unit conductance for coassembled Kir5.1 and Kir4.1 channels approximately 3 fold larger than for Kir4.1 (not shown).[12] Coexpression of Kir4.1 and Kir4.1/Kir5.1 chimeras showed that the C-terminal domains are important determinants of rectification and kinetics.[10] Moreover, expression of concatenated Kir4.1 and Kir5.1 heterotetramers suggested that the relative positions of the different kinds of subunits affect channel activity (Figure 4, c and d): tetramers in which Kir5.1 subunits were flanked by Kir4.1 subunits produced changes in rectification and kinetics similar to those seen upon coexpression of

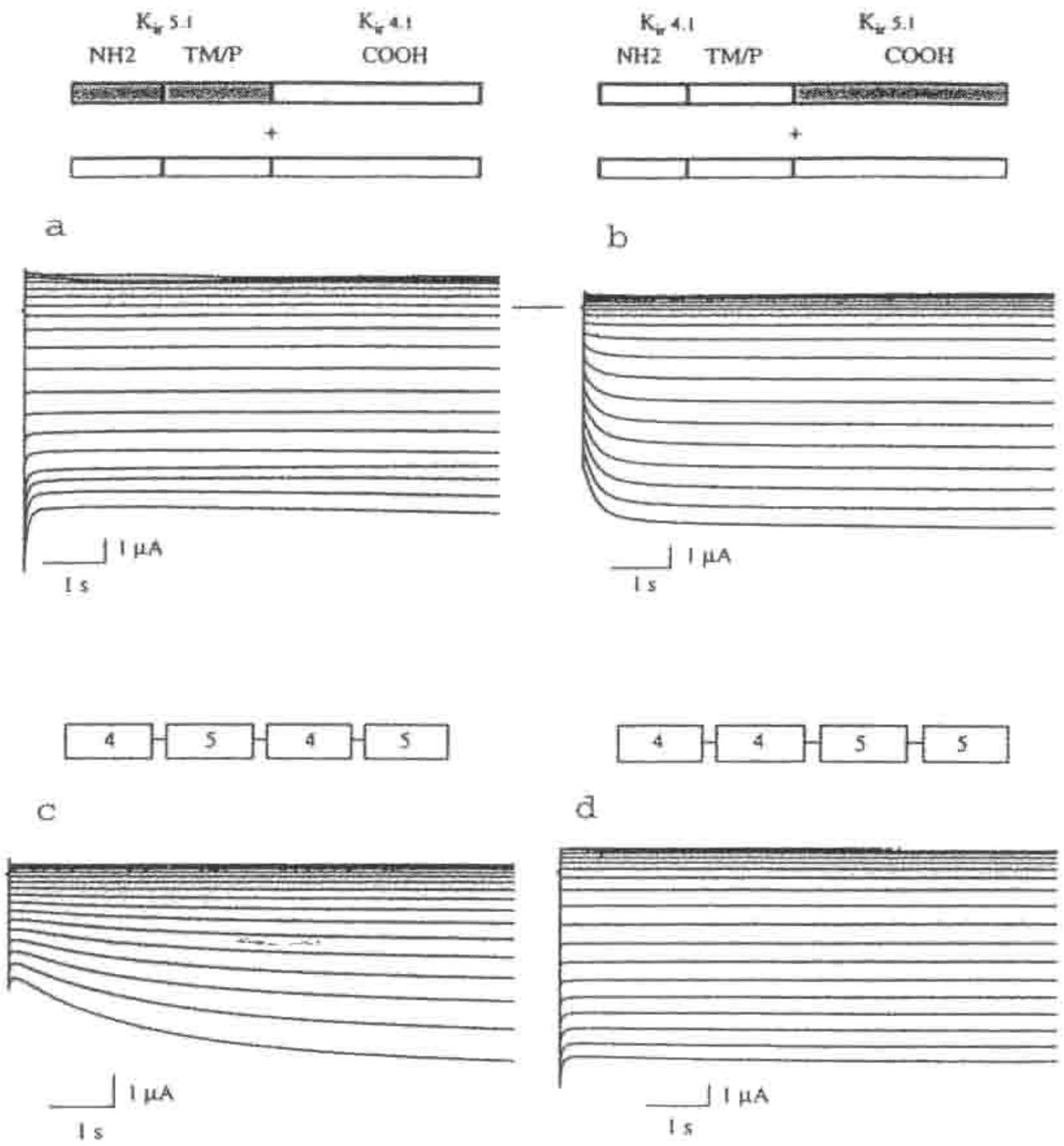


**Figure 4.** The C-terminal domain of Kir5.1 alters the rectification, activation and inactivation properties of Kir4.1/Kir5.1 heteromultimers (a and b). The chimera 5.1/4.1, containing the C-terminal tail of Kir4.1, was coinjected with Kir4.1, to produce whole cell currents indistinguishable in rectification, activation, and inactivation kinetics from those obtained by injection of Kir4.1 (a). The chimera 4.1/5.1, containing the C-terminal tail of Kir 5.1, was coinjected with Kir4.1, to produce whole cell currents with steeper rectification, and a slow time-dependent component of activation (b). This effect is similar to that seen in oocytes where Kir5.1 is coinjected with Kir4.1. Subunit positional effects are revealed by injection of concatenated tetramers (c and d). Injection of the tetramer 4–5–4–5 produced whole cell currents with steep rectification and a slow time-dependent component of activation (c). Injection of the tetramer 4–4–5–5 produced whole cell currents with rectification, activation, and inactivation kinetics similar to those obtained after injection of Kir4.1 (d). For all experiments shown, external solution contained 90 mM $K^+$.

Kir4.1 and Kir5.1 subunits, while tetramers in which Kir5.1 subunits were adjacent to each other produced currents similar to those seen from injection of Kir4.1 mRNA alone, with clearly distinct single channel conductance.[12] The effects of Kir5.1 and Kir4.1 coexpression were specific, since coexpression of

Kir5.1 with seven other cloned inward rectifier subunits did not affect the properties of these other channels (not shown).[12]

## Conclusions

Our PCR cloning strategy revealed the existence of two Kir subfamilies in addition to those characterized at the outset of the study.[3,4,13,14] Recently, screening of pancreatic cDNA libraries by low-stringency hybrization has indicated a sixth subfamily, including members which show properties similar to the native $K_{ATP}$ channel upon reconstitution with cloned sulfonylurea receptors.[18,19] Clearly, these recent findings emphasize the value of cloning methodologies based on nucleotide sequence homology in the complete characterization of the Kir family. In addition, they reveal a higher-order structure for functional Kir channels beyond homomeric complexes. In particular, the results of our experiments coexpressing Kir5.1 and Kir4.1, and from expression of concatenated tetramers, revealed not only distinct functional parameters for heteromeric channels,[16] but also that there are subunit positional effects which affect Kir channel function. Heterologous Kir polypeptides can interact in positive or negative ways, and coexpression of Kir3.0 subfamily members with Kir4.1 revealed that negative interactions may occur at early stages in subunit biosynthesis. Thus, in addition to multiple, different Kir subunits, multiple molecular mechanisms underlie the diversity of inward rectifier potassium channels.

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