The EMBO Journal vol.15 no.12 pp.2980–2987, 1996

Subunit positional effects revealed by novel heteromeric inwardly rectifying K^+ channels

Mauro Pessia[1], Stephen J.Tucker[2], Kevin Lee[3], Chris T.Bond and John P.Adelman[4]

Vollum Institute, Oregon Health Sciences University, Portland, OR 97201, USA

[1]Present address: Consorzio Mario Negri Sud, Centro di Ricerche Farmacologiche e Biomediche, 66030 S. Maria Imbaro, Chieti, Italy
[2]Present address: University Laboratory of Physiology, Oxford OXI 3PT, UK
[3]Present address: Parke Davis Research Institute, Addenbrookes Hospital, Cambridge, UK

[4]Corresponding author

M.Pessia and S.J.Tucker contributed equally to this work and are both considered first authors

K_{ir} 4.1 is an inward rectifier potassium channel subunit isolated from rat brain which forms homomeric channels when expressed in *Xenopus* oocytes; K_{ir} 5.1 is a structurally related subunit which does not. Co-injection of mRNAs encoding K_{ir} 4.1 and K_{ir} 5.1 resulted in potassium currents that (i) were much larger than those seen from expression of K_{ir} 4.1 alone, (ii) increased rather than decreased during several seconds at strongly negative potentials and (iii) had an underlying unitary conductance of 43 pS rather than the 12 pS seen with K_{ir} 4.1 alone. In contrast, the properties of K_{ir} 1.1, 2.1, 2.3, 3.1, 3.2 or 3.4 were not altered by co-expression with K_{ir} 5.1. Expression of a concatenated cDNA encoding two or four linked subunits produced currents with the properties of co-expressed K_{ir} 4.1 and K_{ir} 5.1 when the subunits were connected 4–5 or 4–5–4–5, but not when they were connected 4–4–5–5. The results indicate that K_{ir} 5.1 associates specifically with K_{ir} 4.1 to form heteromeric channels, and suggest that they do so normally in the subunit order 4–5–4–5. Further, the relative order of subunits within the channel contributes to their functional properties.

Keywords: inward rectifiers/heteromeric channels/*Xenopus*

Introduction

Inward rectifier potassium channels contribute to the maintenance of resting membrane potential and control of excitability. They have been found in a wide variety of tissues and cell types including red blood cells, nerve and glial cells and cardiac and skeletal muscle cells (Katz, 1949; Standen and Stanfield, 1978; Sakmann and Trube, 1984b; Mihara *et al.*, 1987; McKinney and Gallin, 1988; Barres, 1991; Lewis *et al.*, 1991). Potassium ions flow through inward rectifier channels when the membrane potential is negative to E_K, but at more positive potentials outward currents are inhibited. The rectifying nature of the conductance is due, at least in part, to a voltage-dependent block of the pore by intracellular cations (Matsuda *et al.*, 1987; Vandenberg, 1987; Lu and Mackinnon, 1994; Stanfield *et al.*, 1994; Wible, 1994). However, some inward rectifiers also show an apparent intrinsic gating as the channel open probability increases with hyperpolarization (Sakmann and Trube, 1984a). Interestingly, this process is coupled to the concentration of potassium, indicating an interaction between permeation, block and gating (Hagiwara and Yoshii, 1979; Leech and Stanfield, 1981; Silver and DeCoursey, 1990). Recent studies suggest a role for cytoplasmic polyamines in the latter processes (Fakler *et al.*, 1994; Lopatin *et al.*, 1994).

Many members of the inward rectifier family (K_{ir}) have now been cloned (Ho *et al.*, 1993; Kubo *et al.*, 1993a,b; Morishige *et al.*, 1993; Bond *et al.*, 1994; for nomenclature, see Doupnik *et al.*, 1995). Structural comparisons which suggest that these subunits fall into several subclasses are supported by expression studies demonstrating distinct kinetic and conductance properties. Like voltage-dependent potassium channels, inward rectifiers are tetramers (Glowatzki *et al.*, 1995; Yang *et al.*, 1995), and channel diversity may be enhanced by the ability of different K_{ir} subunits to form heteromeric as well as homomeric channels (Krapivinsky *et al.*, 1995). For example, several members of the closely related G protein-sensitive K_{ir} 3.0 subfamily can co-assemble into heteromeric channels which results in distinct channel activity and potentiation of current amplitudes compared with homomeric channels (Duprat *et al.*, 1995; Ferrer *et al.*, 1995; Krapivinsky *et al.*, 1995). Also, the closely related subunits K_{ir} 1.1 (ROMK1; Ho *et al.*, 1993) and K_{ir} 4.1 (BIR10; Bond *et al.*, 1994) are expressed in inner ear cells and, when these subunits are co-expressed in *Xenopus* oocytes, they co-assemble to form novel channels with distinct functional properties (Glowatzki *et al.*, 1995). In contrast, heteropolymerization between K_{ir} 4.1 and members of the K_{ir} 3.0 subfamily results in an inhibition of K_{ir} 4.1 channel activity (Tucker *et al.*, 1996).

Although clearly a member of the inward rectifier family, K_{ir} 5.1 is structurally distinct, being the least related of the K_{ir} subunits (36, 45, 38 and 36% identity to K_{ir} 1, 2, 3 and 4 family members, respectively; Doupnik *et al.*, 1995) and expression of K_{ir} 5.1 alone in *Xenopus* oocytes failed to produce functional channels (Bond *et al.*, 1994). We now report that co-expression of K_{ir} 5.1 and K_{ir} 4.1 results in potentiated currents and channels with novel macroscopic and single channel activity. Furthermore, linking these subunits into tetramers with conserved stoichiometry but different relative positions results in channels with distinct properties.

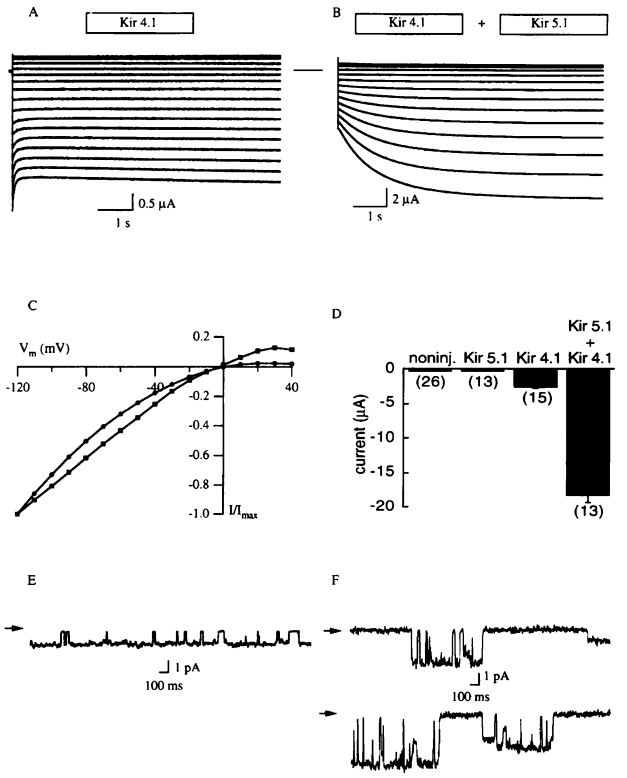

Fig. 1. Expression of K_{ir} 4.1 and K_{ir} 5.1 + K_{ir} 4.1. Representative current families from oocytes expressing (**A**) K_{ir} 4.1 or (**B**) K_{ir} 5.1 + K_{ir} 4.1. Currents were evoked by voltage commands from a holding potential of −10 mV, and were delivered in −10 mV increments from 40 to −120 mV. Horizontal line between panels indicates 0 mV potential. (**C**) Steady-state current–voltage relationship for the oocytes shown in (A) and (B). For comparison, currents from K_{ir} 4.1 (open circles) or K_{ir} 5.1 + K_{ir} 4.1 (filled squares) expressing oocytes were normalized to the current amplitude at −120 mV. (**D**) Current amplitudes recorded at −100 mV for non-injected, K_{ir} 5.1-, K_{ir} 4.1- and K_{ir} 5.1 + K_{ir} 4.1-injected oocytes. A constant amount of K_{ir} 5.1 or K_{ir} 4.1 mRNA was injected in each group. For co-injections, K_{ir} 4.1 mRNA was diluted such that, when injected alone, 0–2 μA of current were detected after 24 h. This amount was mixed with an amount of K_{ir} 5.1 mRNA such that the whole cell currents after 24 h were 5–15 μA, resulting in an excess of K_{ir} 5.1 mRNA relative to K_{ir} 4.1 mRNA: 0.1 ng K_{ir} 4.1 and 10 ng K_{ir} 5.1 per oocyte. The number of oocytes examined from each group is shown in parentheses; bars represent SEM. (**E**) Representative cell-attached patch recording at −100 mV from an oocyte injected with K_{ir} 4.1 or (**F**) K_{ir} 5.1 + K_{ir} 4.1 mRNAs. Arrows denote closed level.

Results

Co-expression of K_{ir} 5.1 changes the properties of K_{ir} 4.1

Expression of K_{ir} 4.1 in *Xenopus* oocytes results in inwardly rectifying potassium currents (Bond *et al.*, 1994). Figure 1A shows a current family evoked by 8 s voltage commands from a holding potential of −10 mV to potentials from 40 to −120 mV delivered in −10 mV increments. The currents activated rapidly, faster than could be resolved in this recording configuration (~1 ms), and underwent a time-dependent relaxation at more hyperpolarized potentials; the current–voltage relationship showed inward rectification (n = 40; Figure 1C). The relaxation of the current at −100 mV was well described by a double exponential, with time constants of τ_f = 57.7 ± 2.4 ms and τ_s = 366 ± 11 ms (A_f = 76.6 ± 1.1; n = 6).

Injection of K_{ir} 5.1 mRNA did not result in currents different from non-injected oocytes (n = 40; Figure 1D). However, hyperpolarizing commands delivered to oocytes co-injected with K_{ir} 5.1 and K_{ir} 4.1 mRNAs evoked inwardly rectifying currents different from those seen from K_{ir} 4.1 expression (n = 138; Figure 1B and C). The currents showed stronger inward rectification and the relaxation seen with K_{ir} 4.1 was lost. Instead, the instantaneous component was followed by a time-dependent increase in the current. At −100 mV, the time-dependent component was well fitted by a single exponential function which yielded a time constant of τ = 2.43 ± 0.11 s (n = 6). Co-injection of K_{ir} 5.1 and K_{ir} 4.1 mRNAs resulted in currents 7- to 12-fold larger than currents from oocytes injected with the same amount of K_{ir} 4.1 mRNA alone (Figure 1D). Channels resulting from co-expression of K_{ir} 5.1 and K_{ir} 4.1 were examined for potassium selectivity. As the external potassium concentration was altered, the reversal potential shifted in accord with the Nernst equation for a potassium-selective conductance (51.4 ± 4.3 mV/10-fold change, K^+ substituted by NaCl, n = 3–6).

Cell-attached patch recordings from oocytes injected with either K_{ir} 4.1 or K_{ir} 5.1 + K_{ir} 4.1 mRNAs exhibited channel activity different from non-injected oocytes, while patches from K_{ir} 5.1-injected oocytes (n = 23) were not different from non-injected or water-injected oocytes (n = 62). K_{ir} 4.1 channels had a unit conductance of 12.3 ± 0.3 pS (n = 9) in the inward direction, and hyperpolarizing commands evoked channel activity with few closures (Figure 1E). In contrast, patches from co-injected oocytes contained a predominant conductance level of 43.2 ± 1.7 pS (n = 9); additional, rarer conductance levels of ~35 and 15 pS were also observed. Hyperpolarizing commands evoked bursts of openings separated by long closures (Figure 1F). The increase in macroscopic current amplitude seen in co-injected oocytes (see Figure 1D) may be due, at least in part, to the observed differences in unit conductances.

K_{ir} 5.1 mRNA was co-injected with mRNAs encoding six other cloned inward rectifier subunits, K_{ir} 2.1 (IRK1; Kubo *et al.*, 1993a), K_{ir} 1.1 (ROMK1; Ho *et al.*, 1993), K_{ir} 3.2 (BIR1, GIRK2; Lesage *et al.*, 1994; Bond *et al.*, 1995), K_{ir} 3.4 (CIR; Krapivinsky *et al.*, 1995), K_{ir} 3.1 (GIRK1; Dascal *et al.*, 1993; Kubo *et al.*, 1993b) and K_{ir} 2.3 (BIR11; Bond *et al.*, 1994); in no case was either current amplitude or the shape of the current traces altered by the presence of K_{ir} 5.1 (n >6). Co-expression of cloned G-$\beta_1\gamma_2$ with either K_{ir} 5.1 (n = 10), K_{ir} 4.1 (n = 20) or K_{ir} 5.1 + K_{ir} 4.1 (n = 10) was without effect. These results demonstrate that K_{ir} 5.1 subunits specifically co-assemble with K_{ir} 4.1 subunits to form novel heteromeric channels. Compared with expression of K_{ir} 4.1 alone, whole cell currents are larger, increase rather than decrease during several seconds at strongly negative potentials, and had an underlying unitary conductance of 43 rather than 12 pS.

Subunit stoichiometry and positional effects

The whole cell and cell-attached patch recordings from oocytes injected with either K_{ir} 4.1 mRNA or K_{ir} 5.1 + K_{ir} 4.1 mRNAs provided evidence for co-assembly of the different subunits. Co-injection of separate mRNAs will result in a heterologous population of channels and, for co-injection of K_{ir} 5.1 + K_{ir} 4.1 mRNAs, the amount of

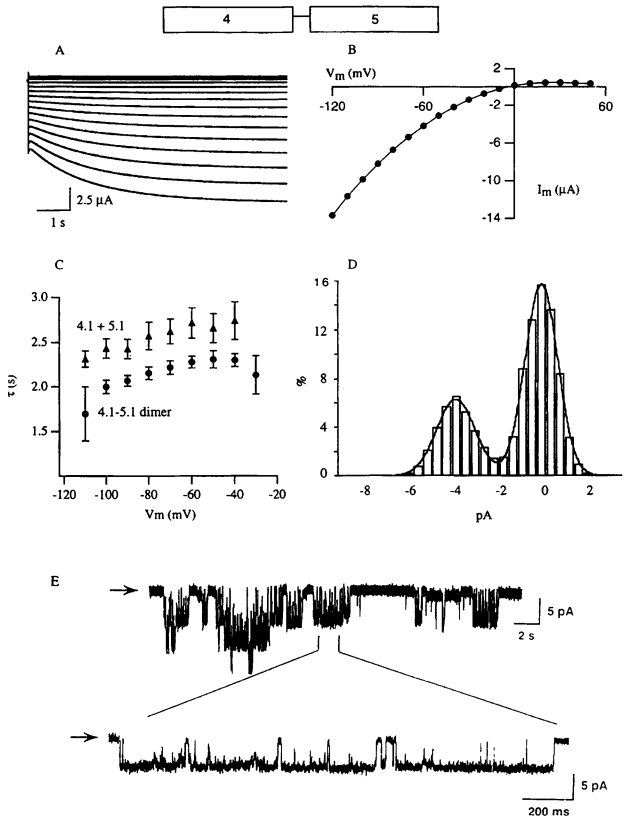

Fig. 2. Expression of the 4–5 dimer. (**A**) Representative current family from an oocyte injected with K_{ir} 4–5 dimer mRNA (0.1 ng). (**B**) The steady-state current–voltage relationship for the traces presented in (A). (**C**) Activation kinetics from oocytes expressing K_{ir} 5.1 + K_{ir} 4.1 or the 4–5 dimer. Currents were recorded between –30 and –110 mV and the time-dependent component fitted with a single exponential function from immediately following the instantaneous current to the end of the trace. The time constants are plotted as a function of the command potential. Circles, 4–5, n = 5; triangles, co-injected K_{ir} 5.1 + K_{ir} 4.1, n = 6. Bars represent SEM. (**D**) Amplitude histogram of unitary currents recorded at –100 mV from oocytes expressing the 4–5 dimer. The histogram was constructed from stretches of recordings in which openings to only a single level were detected. (**E**) Representative cell-attached recording at –100 mV from an oocyte injected with 4–5 dimer mRNA. The bottom record is shown on an expanded time scale.

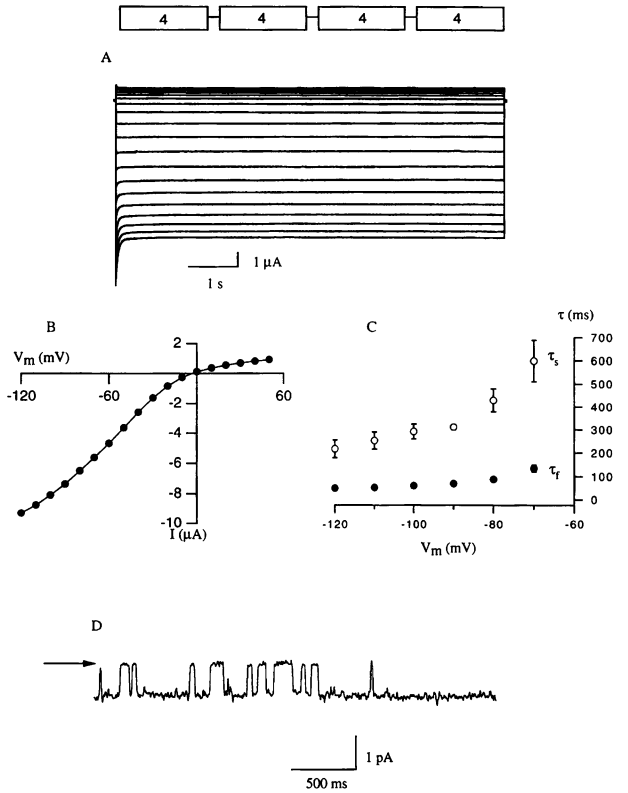

Fig. 3. Expression of the K_{ir} 4.1 tetramer (4–4–4–4). (**A**) Representative current family from an oocyte injected with 4–4–4–4 mRNA (0.5 ng). (**B**) The steady-state current–voltage relationship for the traces presented in (A). (**C**) Kinetics of the current decay from 4–4–4–4 injected oocytes. Currents were recorded between –70 and –120 mV and the time-dependent component fitted with a double exponential function from immediately following the instantaneous current to the end of the trace. The time constants are plotted as a function of the command potential; τ_f (closed circles) and τ_s (open circles). Bars represent SEM. (**D**) Representative cell-attached patch recording at –100 mV from an oocyte injected with 4–4–4–4 mRNA.

K_{ir} 4.1 mRNA was reduced 100-fold to reduce the number of homomeric K_{ir} 4.1 channels which mask the altered properties of the heteromeric channels. Therefore, to investigate the stoichiometry of heteromeric channels and to circumvent the problem of contaminating homomeric K_{ir} 4.1 channels, subunits were tandemly linked, first as dimers and then as tetramers. This approach has been employed successfully for voltage-dependent potassium channels and controls, not only for the overall stoichiometry but also the relative positions of the contributing subunits (Hurst *et al.*, 1992, 1995; Tytgat and Hess, 1992).

Initially, the K_{ir} 4.1 and K_{ir} 5.1 coding regions were linked in a dimeric K_{ir} 4–5 construct. Injection of mRNA encoding the K_{ir} 4–5 dimer produced functional potassium channels. Whole cell currents evoked by hyperpolarizing commands were similar to those observed from oocytes co-injected with K_{ir} 5.1 + K_{ir} 4.1 mRNAs (n = 89; Figure 2A and B). The channels retained selectivity for potassium over sodium (E_{rev} shifted 58.1 ± 1.4 mV/10-fold change in external potassium; n = 5) and the time constant of the time-dependent component was only slightly different from that of oocytes co-injected with K_{ir} 5.1 + K_{ir} 4.1 mRNAs (2.0 ± 0.1 s, n = 5; Figure 2C). Cell-attached patch recordings from oocytes expressing the 4–5 dimer contained bursting channel activity similar to oocytes co-injected with K_{ir} 5.1 + K_{ir} 4.1 mRNAs with a predominant 42.7 ± 1.9 pS (n = 13) conductance (Figure 2D and E); two much rarer apparent subconductance levels of ~32 and ~10 pS were also observed. Therefore, expression of the 4–5 dimer produces channels similar to those from co-injections, suggesting that the predominant channels in co-injected oocytes are comprised of two subunits of each type.

To assess whether linkage of the subunits into tetramers would have any significant effects on channel activity, a tetrameric construct of K_{ir} 4.1 subunits (4–4–4–4) was expressed. Whole cell channel activity was not different from expression of K_{ir} 4.1 (n = 36; Figure 3). Fitting the relaxation of the whole cell current traces at potentials between –70 and –120 mV with a double exponential function yielded time constants not different from those obtained from K_{ir} 4.1 expression (at –100 mV, τ_f = 59.7 ± 2.5 ms, τ_s = 293 ± 33; A_f = 82.4 ± 1.5; n = 6; Figure 3C). Cell-attached patches contained a single conductance of 15.6 ± 1.7 pS (n = 11); no bursting behavior or

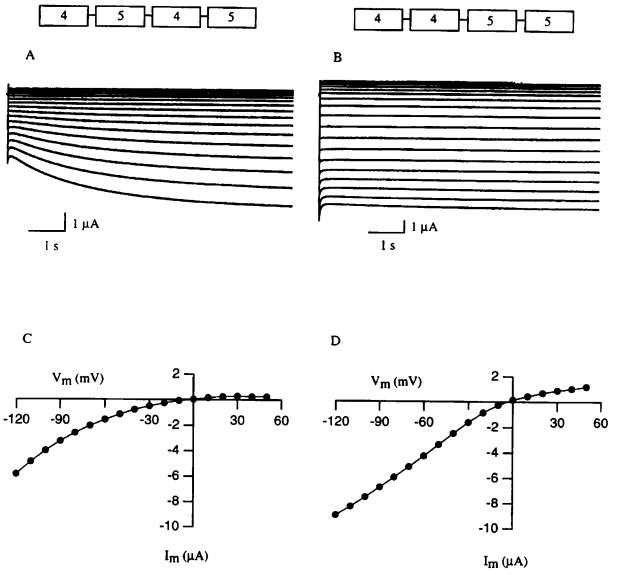

Fig. 4. Subunit positional effects. Representative whole cell current families from oocytes injected with 0.5 ng of (**A**) 4–5–4–5 or (**B**) 4–4–5–5 mRNAs. (**C**) and (**D**) The steady-state current–voltage relationships for the traces presented for 4–5–4–5 and 4–4–5–5, respectively.

additional conductance levels were observed (Figure 3D). These results indicate that tandem linkage of the subunits does not obviously alter channel function.

To investigate further the nature of the heteromeric channels formed following co-injection, K_{ir} 5.1 and K_{ir} 4.1 coding sequences were tandemly linked into tetramers. Two tetramers were constructed, each with an even number of K_{ir} 5.1 + K_{ir} 4.1 subunits but with different relative positions: 4–5–4–5 and 4–4–5–5. Injection of mRNA encoding the 4–5–4–5 tetramer yielded whole cell currents not different from the 4–5 dimer (n = 26; Figure 4A and C). Currents rectified more strongly than for 4–4–4–4, and the time constants of the time-dependent component were similar to those obtained from oocytes co-injected with K_{ir} 5.1 + K_{ir} 4.1, or 4–5 mRNAs [at –100 mV, τ = 2.72 ± 0.08 s (n = 4) and 2.0 ± 0.08 s (n = 5) for 4–5–4–5 and 4–5, respectively; see Figure 2]. Cell-attached patches from oocytes expressing 4–5–4–5 displayed a principal conductance of 43.9 ± 1.4 pS (n = 9; Figure 5A and B), similar to oocytes injected with 4–5 mRNA or co-injected with K_{ir} 4.1 + K_{ir} 5.1 mRNAs. Analysis of the intraburst open time of the channel (trace shown in Figure 5C) yielded a mean open time of 12.7 ms.

In contrast, injection of mRNA encoding the 4–4–5–5 tetramer, with the same stoichiometry as 4–5–4–5 but with different relative subunit positions, resulted in distinct whole cell and single channel activity. Whole cell currents were similar to those seen from K_{ir} 4.1 (Figure 4B and D); fitting the relaxation of the current traces with a double exponential function yielded time constants not different from those derived from K_{ir} 4.1 traces (see Figure 3C). In cell-attached patches, the channels had a unit conductance of 32.1 ± 0.7 pS (n = 12; Figure 6B), with a pattern of openings similar to K_{ir} 4.1 channels. The mean open time of the channels for the record shown was 71.5 ms (Figure 6C).

Discussion

The results show that K_{ir} 5.1 subunits specifically co-assemble with K_{ir} 4.1 subunits to produce novel heteromeric channels, and that the relative subunit positions are important determinants of rectification, kinetic and conduction properties.

Krapivinsky and co-workers (Krapivinsky *et al.*, 1995) demonstrated that another pair of inward rectifier subunits, K_{ir} 3.1 and K_{ir} 3.4 which are co-expressed in cardiac atrial cells, co-assemble to form novel heteromeric channels with potentiated current amplitudes compared with either kind of homomeric channel. Also, K_{ir} 4.1 and K_{ir} 1.1 are co-expressed in auditory hair cells (Glowatzki *et al.*, 1995) and form novel heteromeric channels. However, in these cases, the contributing subunits alone are capable of channel formation. In contrast, K_{ir} 5.1 subunits do not form functional homomeric channels, but endow significant functional alterations when co-assembled into channels with K_{ir} 4.1 subunits.

Co-injection of K_{ir} 5.1 and K_{ir} 4.1 mRNAs resulted in currents different from injection of K_{ir} 4.1 alone. At the whole cell level, the most significant distinctions are potentiation of the current amplitude, stronger rectification and the presence of a time-dependent increase in the currrent. A similar time-dependent component was seen from expression of K_{ir} 3.1 following stimulation by G proteins (Kubo *et al.*, 1993b), and from expression of a K_{ir} 4.1 chimeric channel containing the C-terminus of K_{ir} 3.1 (Pessia *et al.*, 1995). In the co-injection experiments reported here, a large excess of K_{ir} 5.1 mRNA was required because, at more equivalent mRNA ratios, homomeric K_{ir} 4.1 channels mask the altered whole cell kinetics. Unequal translational efficiencies or a preference for homomeric over heteromeric channel assembly may contribute to the predominance of homomeric K_{ir} 4.1 channels. In addition, heteromeric channel assembly does not occur randomly, as evidenced by the similarity between co-injected oocytes and those expressing the 4–5 dimer and the 4–5–4–5 tetramer; there is a preferred stoichiometry and positional orientation. The results suggest that the contributions of K_{ir} 5.1 subunits are different when K_{ir} 5.1 and K_{ir} 4.1 subunits are alternated, as for 4–5–4–5, compared with those in which they are next to each other, as for 4–4–5–5. A preferred subunit stoichiometry and positional orientation has been inferred from studies of co-expressed K_{ir} 3.1 and K_{ir} 3.4 subunits (Tucker *et al.*, 1996), also suggesting an alternating arrangement of the heterologous subunits within the tetrameric channel. This is different from what has been reported for voltage-dependent potassium channels (Hurst *et al.*, 1995), suggesting that heteromeric inward rectifier channels may not co-assemble randomly, and may have 2-fold rather than 4-fold symmetry.

Expression of the 4–5–4–5 tetramer resulted in currents with stronger rectification and a time-dependent increase in the current when held at strongly negative potentials; channel activity in cell-attached patches was bursting and channels had a unit conductance of 43.9 pS in the inward direction, with a mean open time of 12.7 ms. In contrast, expression of the 4–4–5–5 tetramer, which has the same number of K_{ir} 5.1 and K_{ir} 4.1 subunits as 4–5–4–5 but in different relative positions, resulted in significantly

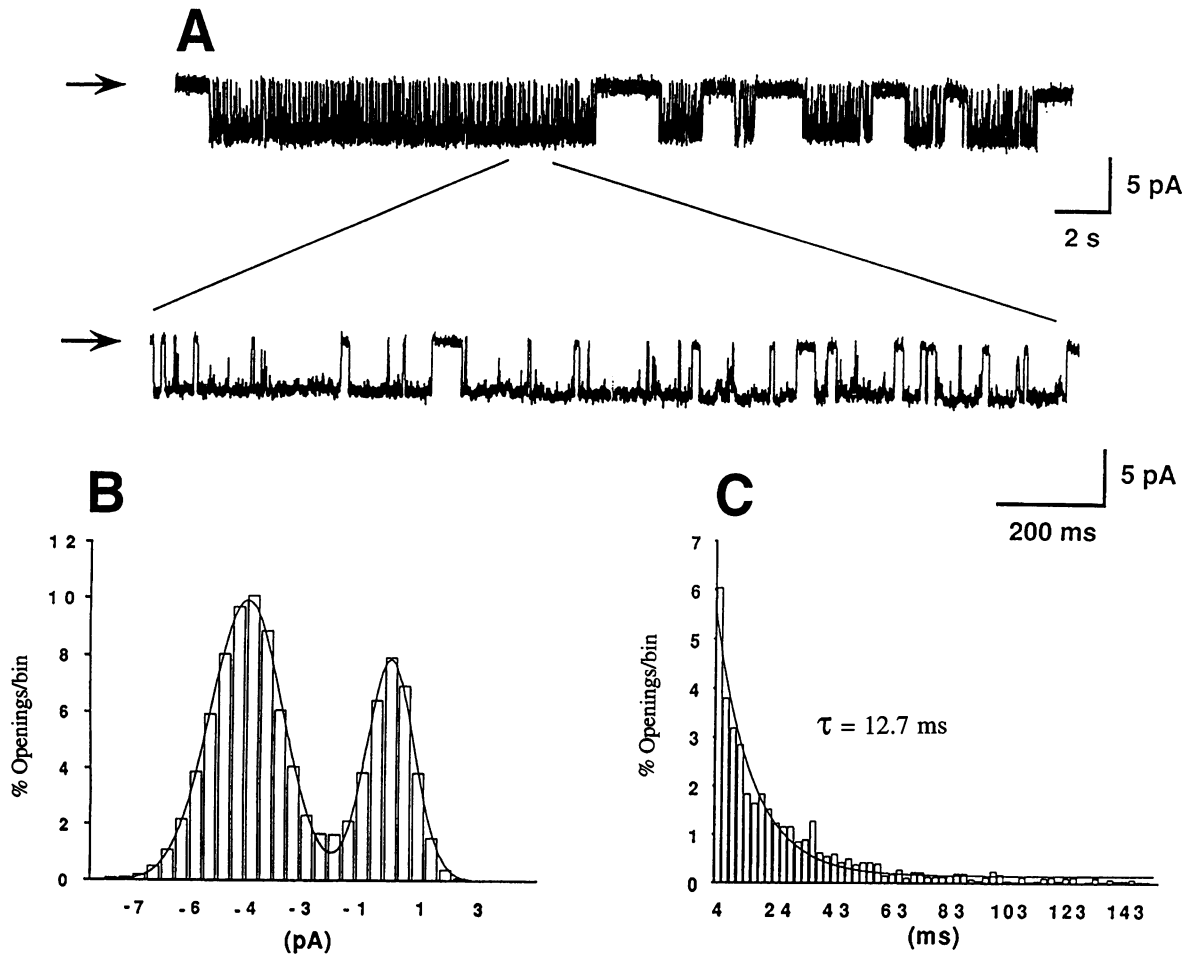

Fig. 5. (**A**) Representative cell-attached patch recording at −100 mV from an oocyte expressing the 4–5–4–5 tetramer. The bottom record is shown on an expanded time scale. (**B**) Amplitude histogram at −100 mV of unitary currents from the record shown in (A). (**C**) Intraburst open time histogram from the record shown in (A), with a mean open time of 12.7 ms. Bursts were defined as channel activity separated by >500 ms.

different channel activity. The whole cell currents were similar to K_{ir} 4.1, and channels had a unit conductance of 32.7 pS and a mean open time of 71.5 ms.

Although it is impossible to be sure, it is likely that the channels recorded following injection of tetramer mRNAs form by intramolecular assembly. If channels are formed by intermolecular assemblies, then oocytes injected with 4–4–5–5 should also contain the ~12 pS conductance representing channels formed from K_{ir} 4.1 subunits. During 72 min of cell-attached recordings at −100 mV among 12 patches, no ~15 pS openings were observed. Further validation of this approach for inward rectifier potassium channels has been provided recently by experiments which determined the subunit stoichiometry of K_{ir} 2.1 (Yang *et al.*, 1995). In addition, expression of tetrameric voltage-dependent potassium channels, constructed using the same subunit linker, showed that the fraction of channels formed by intermolecular association was <5%. When intermolecular subunit assembly accounted for the observed channel activity, current amplitudes were reduced by at least two orders of magnitude compared with channels formed by intramolecular assembly (Hurst *et al.*, 1992, 1995), which was not the case for any of the channels expressed here.

K_{ir} 5.1 is structurally distant from other cloned inward rectifier subunits, and co-assembled into functional channels only with K_{ir} 4.1 among the seven inward rectifier subunits tested. K_{ir} 5.1 and K_{ir} 4.1 initially were isolated from brain; their mRNAs have been co-localized by RT–PCR to the preoptic and superchiasmatic regions of the hypothalamus, and the cerebellum in the central nervous system, as well as in spleen, liver and kidney (Bond *et al.*, 1994). In these tissues, K_{ir} 5.1 and K_{ir} 4.1 subunits may be present within the same cells and may form heteromeric channels like those observed from heterologous co-expression.

Like voltage-dependent potassium channels, the molecular diversity of inwardly rectifying potassium channels may be expanded by the ability of specific subunits to form heteromeric channels with other inward rectifier subunits, producing channels with distinct characteristics. In addition, the relative positions of subunits within the heteromeric channel have profound effects on inward rectification, kinetics and conductance properties.

Materials and methods

Electrophysiology

Whole cell recording. Xenopus laevis care and handling were in accordance with the highest standards of institutional guidelines. Frogs under-

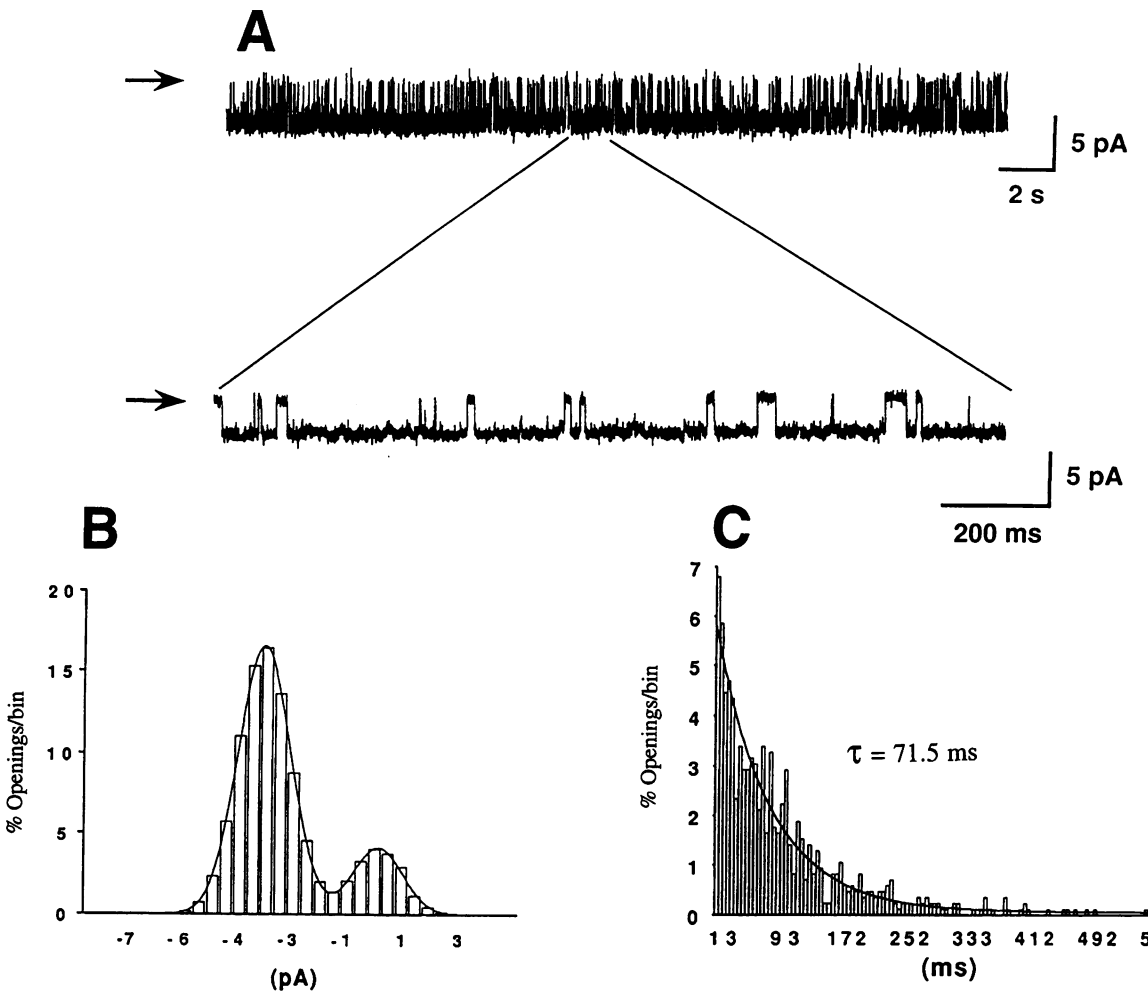

Fig. 6. (**A**) Representative cell-attached patch recording at −100 mV from an oocyte injected with 4–4–5–5 mRNA. The bottom record is shown on an expanded time scale. (**B**) Amplitude histogram at −100 mV of unitary currents from the record shown in (A). (**C**) Intraburst open time histogram from the record shown in (A), with a mean open time of 71.5 ms. Bursts were defined as channel activity separated by >500 ms.

Table I. Summary of the presumed structures, whole cell kinetics and single channel conductances for the expressed channels

Channel Structure	Whole Cell Kinetics	Single Channel Conductance (pS)	Channel Structure	Whole Cell Kinetics	Single Channel Conductance (pS)
4+5 / 5+4	Time-dep. increase	43 (~35; ~15)	4+4 / 4+4	Time-dep. relaxation	12
4–5 / 5–4	Time-dep. increase	42	4–4 / 4–4	Time-dep. relaxation	15
4–5 / 5–4	Time-dep. increase	43	4–4 / 5–5	Time-dep. relaxation	32

went 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 $MgCl_2$, 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 18–48 h after injection with a Geneclamp 500 amplifier (Axon Instruments) interfaced to a Macintosh Quadra 800 computer. Currents were evoked by voltage commands from a holding potential of −10 mV, delivered in −10 mV increments from 50 mV to −120 mV, unless otherwise stated. Data collection and analyses were performed using Pulse, PulseFit (Heka) and IGOR (Wavemetrics) software.

Patch–clamp recording. Patch–clamp recordings were performed using an Axopatch 1B amplifier (Axon Instruments). Oocytes were bathed in a solution containing 80 mM KCl, 10 mM EGTA, 10 mM HEPES and 10 μM $GdCl_3$ (pH adjusted to 7.2 with KOH, total K^+ 110 mM). Gd^{3+} was included in the pipet to suppress stretch-activated channels (Yang and Sachs, 1989). No effect of 10 or 100 μM $GdCl_3$ was observed on the whole cell currents. Recording electrodes were pulled from borosilicate glass, dipped in Sticky wax (Kerr) prior to polishing and, when filled with bath solution, had resistances of 15–25 MΩ. Patch records were obtained in the cell-attached configuration by stepping the holding potential to various test potentials for 1–10 min, and were stored on videotape. Current traces at each holding potential were filtered at 0.5–5 kHz and digitized at 5–25 kHz. Analysis was performed offline using the Dempster suite of programs, and a digital storage oscilloscope (Tektronix 2212). The mean amplitude of the unitary currents was estimated for individual patches by fitting simple Gaussians to the all-points histogram or, in some cases, by computing the mean open level directly from the digitized current trace. All points histograms were used particularly because of the apparent subconductance levels observed for some subunit combinations (see Results). Single channel current–voltage relationships were constructed from the measured single channel current amplitudes (n >9 for each voltage) and the slope conductance determined

following linear regression between –40 and –100 mV (Kaleidograph). All patch records shown contained more than one channel and were obtained at –100 mV; all-points histograms were constructed from 20–30 s stretches where openings to only one level were observed. Open times were obtained from records containing only single level openings, filtered at 5 kHz and digitized at 25 kHz, and the distributions fitted using a Levenberg–Marquardt, non-linear, least squares method (Dempster).

Molecular biology

Plasmids containing the coding sequences for G protein $\beta_1\gamma_2$ subunits were the generous gift of Drs Lily Jan and Paul Slesinger. Plasmids containing the coding sequences of K_{ir} 1.1 and K_{ir} 2.1 were kindly provided by Drs B.Fakler and P.Ruppersberg. All channel subunits were subcloned into the oocyte expression vector, pBF (courtesy of 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. To join subunits in tandem, the relevant stop codon was removed and a linker encoding 10 glutamine residues was inserted between the last codon of the 5′ subunit coding sequence and the initiator codon of the following subunit. This was achieved using a sequential PCR protocol modified from Horton (Horton *et al.*, 1989). Briefly, junctions were generated by overlap extension of PCR primers which also encoded the glutamine linkers. Tetramers were constructed by sequential ligation of linked subunits. The nucleotide sequences of all linked subunits were determined across the joined segments. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer.

Acknowledgements

We thank our colleagues Drs Jim Maylie, Armando Lagrutta and Neil Marrion for fruitful discussions and critical comments. We thank Xiao-Ming Xia for early experiments with K_{ir} 5.1. We also thank Amela Brankovic and Wei-Bin Wu for *Xenopus* handling, oocyte preparation and patience. S.J.T. and K.L. were supported by Wellcome Trust International Prize Travelling Research Fellowships. This work was supported by National Institutes of Health grants to J.P.A.

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Received on January 4, 1996; revised on March 1, 1996