Kv2.1 and electrically silent Kv6.1 potassium channel subunits combine and express a novel current

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Abstract Heteromultimer formation between Kv potassium channel subfamilies with the production of a novel current is reported for the first time. Protein–protein interactions between Kv2.1 and electrically silent Kv6.1 α-subunits were detected using two microelectrode voltage clamp and yeast two-hybrid measurements. Amino terminal portions of Kv6.1 were unable to form homomultimers but interacted specifically with amino termini of Kv2.1. *Xenopus* oocytes co-injected with Kv6.1 and Kv2.1 cRNAs exhibited a novel current with decreased rates of deactivation, decreased sensitivity to TEA block, and a hyperpolarizing shift of the half maximal activation potential when compared to Kv2.1. Our results indicate that Kv channel subfamilies can form heteromultimeric channels and, for the first time, suggest a possible functional role for the Kv6 subfamily.

Key words: Kv; Heteromultimer; Potassium channel

1. Introduction

Functional diversity among K^+ channels is generated by a variety of mechanisms. One of these is the association of different subunits within a K^+ channel subfamily to form heteromultimeric channels that express novel currents. This process has been shown to occur for the Kv1 subfamily of voltage-sensitive K^+ (Kv) channels [1–6] and for the Kir3 family of inwardly rectifying potassium channels [7]. For α-subunits from the Kv1 subfamily, recognition sites for multimerization have been identified biochemically and mapped to amino terminal domains [8,9]. Heteromultimeric channels may also include α-subunits that are electrically silent when expressed alone, as is the case for the Kir2 subfamily [10], cyclic nucleotide gated channels [12–15], and perhaps the Kv4 subfamily [11]. To date heteromultimerization with the production of novel currents has been restricted to members within, but not between, Kv subfamilies. Our study describes the first exception to this restriction and suggests a possible role for the Kv6 subfamily, which has previously been considered to be non-functional.

The sparsely populated Kv channel subfamilies Kv5 and Kv6 each contain one member, Kv5.1 and Kv6.1 ([16], referred to as IK8 and K13, respectively, in [17]). No function has yet been demonstrated for either of these proteins [17]. Comparisons of predicted amino acid sequences strongly indicate that both Kv5.1 and Kv6.1 are members of the Kv family [17], exhibiting hallmarks such as the conserved GYGD sequence in H5 [18], six hydrophobic transmembrane domains including the positively charged S4 [19,20], and amino terminal T1 [8] or N_A and N_B [17,9] domains.

The Kv subfamily to which Kv5.1 and Kv6.1 are most closely related is Kv2.1, a delayed rectifier K^+ channel [16,17]. By applying two independent approaches, one biochemical and the other electrophysiological, we have found evidence for protein–protein interactions between Kv2.1 and the electrically silent Kv6.1. Using the yeast two-hybrid assay, protein–protein interactions were detected between amino terminal fragments of Kv2.1 and Kv6.1, but not between Kv6.1 and Kv1.2. *Xenopus* oocytes co-injected with Kv2.1 and Kv6.1 cRNAs produced novel currents that exhibited decreased rates of deactivation and a greatly reduced sensitivity to TEA block.

2. Materials and methods

2.1. Oocyte preparation and two microelectrode voltage clamp

Stage V–VI *Xenopus* oocytes were defolliculated by collagenase treatment (2 mg/ml for 1.5 h) in a Ca-free buffer solution (in mM): NaCl, 82.5; KCl, 2.5; $MgCl_2$, 1; HEPES, 5 (+100 μg/ml gentamicin); pH 7.6. The defolliculated oocytes were injected with 46 nl of cRNA solution (in 0.1 M KCl) and incubated at 19°C in culture medium (in mM): NaCl, 100; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 5; pyruvic acid, 2.5 (+100 μg/ml gentamicin), pH 7.6. Two microelectrode voltage clamp (OC725B; Warner instruments) experiments were performed 2–6 days after cRNA injection. Bevel-tipped glass micropipettes were filled with a solution of 3 M KCl + 1% agar, and then backfilled with 3 M KCl, to provide sharp-tipped microelectrodes with low electrical resistance (0.2–0.4 MΩ). Under these conditions, the input resistance of oocytes impaled with two microelectrodes was in the range 0.5–1.0 MΩ (oocytes with input resistance below this level were considered to be damaged and were discarded). Linear leakage current was subtracted off-line and in the records illustrated, capacitative currents were blanked. K-Ringer bathing solution contained (in mM): KCl, 120; $MgCl_2$, 2; $CaCl_2$, 1; HEPES, 10; pH 7.2 with Tris-OH, and flowed continuously through the recording chamber at a rate of 3 ml/min. TEA dose–response relationships were obtained by the cumulative addition of TEA to the bath solution, and fractional inhibition was determined relative to the whole cell current obtained just prior to TEA addition. All electrophysiological measurements were made at room temperature (21–23°C). Data were filtered at 1 kHz and digitized at 5 kHz. Data acquisition and analysis (peak current amplitudes and time course of decay) respectively, used the Clampex and Clampfit programs of the pClamp suite (Axon Instruments), and TEA binding isotherms were analyzed using NFit (Island Products, Galveston, TX).

2.2. RNA synthesis

Kv2.1, Kv3.1, Kv1.2, and Kv6.1 (with most of the 5′-UTR removed), all in Bluescript SK− (Stratagene, La Jolla CA) were digested overnight with *Not*I (Boehringer Mannheim Biochemicals, Indianapolis, IN), Proteinase K-treated (GIBCO BRL, Grand Island, NY), phenol chloroform extracted, precipitated and resuspended in RNase-free water. Linearized cDNA was transcribed in vitro using the mMessage Machine kit (Ambion, Austin TX) and the final cRNA product was suspended in RNase-free 0.1 M KCl. The cRNA con-

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centration and integrity was determined using formaldehyde agarose gel electrophoresis. Oocytes were injected at 1 ng/μl cRNA for Kv1.2, Kv3.1, and Kv2.1. Kv2.1 and Kv6.1 were co-injected at 1 ng/μl of each cRNA. Kv1.2 and Kv6.1, and Kv3.1 and Kv6.1 were co-injected at 1 ng/μl of Kv1.2 or Kv3.1 cRNA and 10 ng/μl of Kv6.1 cRNA.

2.3. Yeast two-hybrid system and fusion protein constructs

The MATCHMAKER Two-Hybrid System was purchased from Clontech Laboratories, Inc. (Palo Alto, CA); included in this kit was the parental DNA binding domain vector pGBT9 (TRP1, amp^r) and the parental transcription activation domain vector pGAD424 (LEU2, amp^r). The yeast strain Y190 (MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2::GAL1$_{UAS}$–HIS3$_{TATA}$–HIS3, URA3::GAL1$_{UAS}$–GAL1$_{TATA}$–lacZ)), which possesses the reporter genes *lacZ* and *HIS3*, with dissimilar promoters, was used for all experiments. Oligonucleotides were synthesized with either *Eco*RI or *Sal*I overhangs (392 DNA/RNA Synthesizer, Applied Biosystems Inc., Foster City, CA). Inserts coding for the amino terminal portions of Kv1.2 (amino acids 1 through 164), Kv2.1 (amino acids 1 through 168), or Kv6.1 (amino acids 1 through 209) were: PCR amplified and cloned using the GeneAmp kit from Perkin-Elmer (Branchburg, NJ) and the TA cloning kit from Invitrogen Corp. (San Diego, CA); sequenced (Sequenase kit from United States Biochemicals; Cleveland, OH); and then shuttled into the *Eco*RI/*Sal*I sites of the yeast two-hybrid vectors. All constructs were sequenced across the yeast two-hybrid vector-insert junction. Y190 cells were transformed with the plasmids of interest (0.1–1 μg of each) using a modified lithium acetate procedure and plated on either SD −trp or SD −leu medium to select for cells harboring pGBT9 or pGAD424 based vectors, respectively; or on SD −trp/−leu media to select cells harboring both vectors or preparatory to the lacZ expression assay for interacting fusion proteins; or on SD −trp/−leu/−his + 25 mM 3-amino-1,2,4-triazole (Sigma Chemical Company, St. Louis, MO) media to select for double transformants bearing interacting fusion proteins. *LacZ* expression assays were performed by lifting Y190 colonies onto grade 410 filter paper (VWR, West Chester, PA), freeze thawing the filter paper in liquid nitrogen, and incubating the filter paper at 37°C for up to 6 h on filter paper soaked in Z buffer (in mM: Na_2HPO_4, 60; NaH_2PO_4, 40; KCl, 10; $MgSO_4$, 1; pH 7.0) to which (final concentrations) 0.27% β-mercaptoethanol and 0.33 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) had been added. All reagents used, unless otherwise stated, were purchased from Sigma Chemical Company (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or Difco Laboratories (Detroit, MI).

3. Results

3.1. Direct interaction between Kv2.1 and Kv6.1 can be detected using a yeast two-hybrid reporter assay

To test the notion that Kv2.1 and Kv6.1 channel subunits could form heteromultimeric channels we employed a yeast two-hybrid reporter assay and tested the ability of amino terminal fragments of Kv6.1 amino acids 1 through 209 (Kv6.1/1–209), Kv2.1 amino acids 1 through 168 (Kv2.1/1–168), and Kv1.2, amino acids 1 through 164 (Kv1.2/1–164) to interact. Both reporter genes, *HIS3* and *lacZ*, were assayed to allow a greater ability to resolve false-positive signals. None of the fusion proteins transformed individually into Y190 cells gave a positive result (data not shown). Fig. 1A displays Y190 cells transformed with combinations of AD and BD constructs for Kv2.1/1–168, Kv6.1/1–209, and Kv1.2/1–164, and grown on SD −trp/−leu (selection media for double plasmid uptake), SD −trp/−leu/−his (selection media for double plasmid uptake and protein–protein interaction) or assayed for *lacZ* expression. Y190 growth on −trp/−leu medium is evident for all conditions, indicating that the cells harbor both of the indicated fusion protein constructs. Y190 growth on SD −trp/−leu/−his is evident for the cells transformed with the AD and BD Kv2.1 constructs, the AD Kv2.1 and BD Kv6.1 constructs, and the AD Kv6.1 and the BD Kv2.1 constructs. In contrast, Y190 cells harboring the AD Kv6.1 and the BD Kv6.1 constructs fail to grow on SD −trp/−leu/−his media. Similar results were obtained with the assay for *lacZ* expression. Y190 cells harboring both of the Kv2.1 constructs or either combination of the Kv2.1 and Kv6.1 constructs were positive for the expression of *lacZ*. Y190 cells harboring the Kv6.1 constructs were negative.

Table 1 summarizes the results of the yeast two-hybrid experiments. The ability of Kv1.2/1–164 to interact with Kv2.1/1–168 and Kv6.1/1–209 was tested. As expected, *lacZ* expression was detected for Y190 cells transformed with the AD and BD Kv1.2/1–164 constructs [6], but not for Y190 cells transformed with any combination of Kv1.2/1–164 and either Kv2.1/1–168 or Kv6.1/1–209 constructs. These results are consistent with the notion that Kv2.1 proteins are able to interact with each other, that Kv2.1 and Kv6.1 proteins are able to interact, but that Kv6.1 proteins are unable to interact with each other or with Kv1.2 proteins. Furthermore, consistent with previous reports [21–24], the locus of the self–self interactions is the amino terminal portion of the protein.

3.2. Tail currents of Kv2.1–Kv6.1 co-injected oocytes are distinct from those of Kv2.1 injected oocytes

Two microelectrode voltage clamp (TEVC) of *Xenopus* oocytes was used to assay for functional interactions between the K^+ channel α-subunits of Kv2.1 and Kv6.1 or between Kv1.2 and Kv6.1. Fig. 2 depicts a tail current analysis of oocytes injected with either Kv2.1 cRNA or co-injected with Kv2.1 and Kv6.1 cRNA (A,C) or of oocytes injected with either Kv1.2 cRNA or co-injected with Kv1.2 and Kv6.1 cRNA (B,D). Fig. 2A,B displays representative, normalized whole cell currents. The pulse protocol is shown at the top of each graph. Stepping the clamping potential from the initial holding potential of −80 mV to +40 mV evoked outward currents mediated by Kv2.1 channels (Fig. 2A, solid trace) or by Kv1.2 channels (Fig. 2B, solid trace). The subsequent hyperpolarizing step to −100 mV elicited tail currents which rapidly decayed to the current level obtained during the initial holding potential of −80 mV. When the same pulse protocol was applied to oocytes co-injected with Kv2.1 and Kv6.1 cRNAs (dotted trace) the tail currents displayed markedly different kinetics. Upon stepping the potential to −100 mV from +40 mV the tail currents displayed a markedly decreased rate of deactivation such that, during the test pulse interval, the whole cell current did not return to the current level obtained during the initial holding potential of −80 mV. Fig. 2B presents a similar comparison of the currents evoked from oo-

Table 1
Yeast two-hybrid assay of selected Kv subfamily interactions[a]

AD Construct	BD Construct	cfu color[b]
Kv1.2/1–164	Kv1.2/1–164	blue
Kv1.2/1–164	Kv2.1/1–168	white
Kv1.2/1–164	Kv6.1/1–209	white
Kv2.1/1–168	Kv1.2/1–164	white
Kv6.1/1–209	Kv1.2/1–164	white
Kv2.1/1–168	Kv2.1/1–168	blue
Kv2.1/1–168	Kv6.1/1–209	blue
Kv6.1/1–209	Kv2.1/1–168	blue
Kv6.1/1–209	Kv6.1/1–209	white

[a]Results are typical of at least four independent transformations.
[b]cfu, colony forming unit; blue color indicates *lacZ* expression.

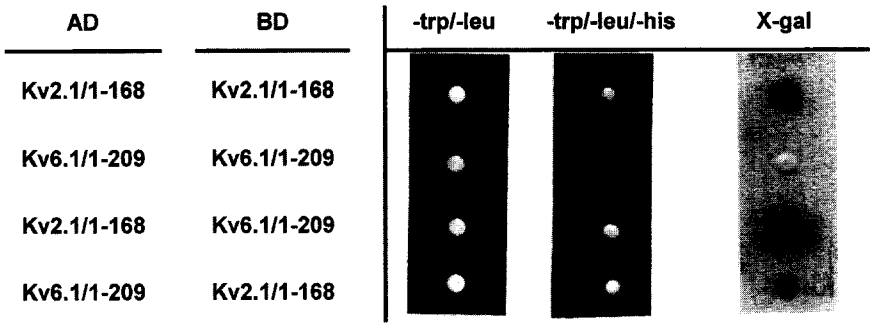

Fig. 1. Kv2.1 and Kv6.1 interactions detected by yeast two-hybrid assay. Y190 cells, transformed with the indicated fusion protein constructs, were plated on SD −trp/−leu or SD −trp/−leu/−his 25 mM 3-amino-1,2,4,-triazole selection media and then either assayed for the expression of β-galactosidase or monitored for cell growth. Fusion protein species co-transformed into Y190 cells are indicated on the left (BD, DNA binding domain fusion plasmid; AD, activation domain fusion plasmid). Growth or assay conditions are indicated along the top (−trp/−leu, cells plated on media lacking tryptophan and leucine; −trp/−leu/−his, cells plated on media lacking tryptophan, leucine, and histidine and containing 25 mM 3-amino-1,2,4-triazole; X-gal, cells lysed and assayed for β-galactosidase activity). Aliquots of the same transformation were plated for each condition. Similar results were obtained in five independent transformations. Co-transformation of the transcription activation domain and DNA binding domain fusion protein constructs for Kv2.1/1–168, and Kv2.1/1–168 and Kv6.1/1–209 led to growth on −trp/−leu/−his medium as well as a positive signal for β-galactosidase activity. In contrast, co-transformation of the Kv6.1 AD and BD constructs did not give a positive result for either the −trp/−leu/−his selective growth assay or the *lacZ* expression assay, indicating that these channel fragments do not interact with each other in this system.

cytes expressing Kv 1.2 alone (solid trace) or Kv1.2 and Kv6.1 (dotted trace). There was no discernable difference between the tail currents in the presence of Kv1.2 and either the presence or absence of Kv6.1. Additional experiments using the same pulse protocol were also performed on oocytes injected with Kv6.1 cRNA. Under these conditions no currents which could be attributed to Kv6.1 were detected, as the whole cell currents obtained were indistinguishable from those obtained from uninjected control oocytes (data not shown).

Fig. 2C,D present a more detailed analysis of the effect of Kv6.1 co-expression on the tail currents mediated by Kv2.1 and Kv1.2, respectively. Tail currents evoked by stepping the holding potential to −100 mV were plotted on a log scale versus time and the best fit to an exponential decay function calculated. The decay of Kv2.1 tail currents was fit by a single exponential function with a time constant of 5.2 ms (Fig. 2C, '+' symbols). The decay of the tail currents obtained from oocytes co-injected with Kv2.1 and Kv6.1 cRNA was a complex function and was not able to be fit by a single exponential (Fig. 2C, ●). In fact, although a double exponential function, with time constants of 19 and 153 ms provided an adequate fit to the data, this failed to separate the contribution of 'pure' Kv2.1 channels ($\tau = 5.2$ ms) from those affected by Kv6.1 co-injection. In contrast, co-injection of Kv6.1 with Kv1.2 had no effect on the rate of decay of the tail current. The time constant obtained from oocytes expressing Kv1.2 was 2.6 ms (Fig. 2D, '+' symbols) and that obtained from oocytes co-injected with Kv1.2 and Kv6.1 (with Kv6.1 in 10-fold excess) was 2.2 ms (Fig. 2D, ●).

Fig. 2E presents the time constants for tail current decay as a function of the test potential. Data for Kv2.1 (○), Kv1.2 (□), and Kv1.2 and Kv6.1 co-injections (■) were obtained by calculating a single exponential fit to the tail current decay at the indicated voltage. Data for Kv2.1 and Kv6.1 co-injections were obtained by calculating a weighted average of two single exponential fits to the tail current decay data at the indicated voltage. The time constant for tail current decay is voltage dependent for Kv2.1 and Kv1.2. Co-injection of Kv6.1 did not alter the time constant–voltage relationship for Kv1.2. In contrast, the time constant for tail current decay is greatly prolonged and much less voltage sensitive for oocytes co-injected with Kv2.1 and Kv6.1 cRNA. Over the range of test potentials the time constants for tail current decay obtained from oocytes co-injected with Kv2.1 and Kv6.1 cRNA are, at identical test potentials, 2–10 times greater than those obtained from oocytes expressing Kv2.1 alone.

The voltage for half maximal activation was shifted by −34 mV in the Kv2.1–Kv6.1 co-injected oocytes. Inspection of Fig. 2E reveals that a 70 mV shift of the Kv2.1–Kv6.1 curve would be required to superimpose this curve with that of Kv2.1; that is, the difference in the time constant–voltage relationship between Kv2.1 and Kv2.1–Kv6.1 expressing oocytes can not be accounted for by the 34 mV shift in the voltage for half maximal activation. Inspection of Fig. 2A reveals that currents from Kv2.1–Kv6.1 co-injected oocytes activated at an earlier time after the depolarizing step than currents from Kv2.1 injected oocytes. This can be accounted for by the hyperpolarizing shift in the activation curve. Taken together these results support the notion that co-injection of Kv2.1 and Kv6.1 cRNA leads to the formation of heteromultimeric channels with novel biophysical properties.

3.3. TEA sensitivity of Kv2.1–Kv6.1 co-injected oocytes is distinct from that of Kv2.1 injected oocytes

Alignment of the predicted amino acid sequences for Kv2.1, Kv3.1, and Kv6.1 indicated that the residue critical for TEA sensitivity (Y380 and Y447 in Kv2.1 and Kv3.1, respectively) [31] is replaced by a valine (V431) in Kv6.1. We reasoned that if Kv6.1 formed heteromultimeric channels with either Kv2.1 or Kv3.1 then these channels would be likely to display reduced TEA sensitivity. To enhance our ability to detect a reduction in TEA sensitivity Kv1.2, which forms TEA insensitive channels (50% inhibitory concentration > 50 mM, see [16] for review), was replaced with Kv3.1. Fig. 3 presents TEA dose–response curves obtained from oocytes injected with Kv3.1 (◇), Kv3.1 and Kv6.1 (◆), Kv2.1 (○), or Kv2.1 and Kv6.1 (■). Symbols represent data points and smooth lines are the calculated best fits of a 1:1 binding isotherm for the data obtained from oocytes injected with Kv3.1, Kv3.1 and Kv6.1, or Kv2.1 cRNA. The fit to the data obtained from

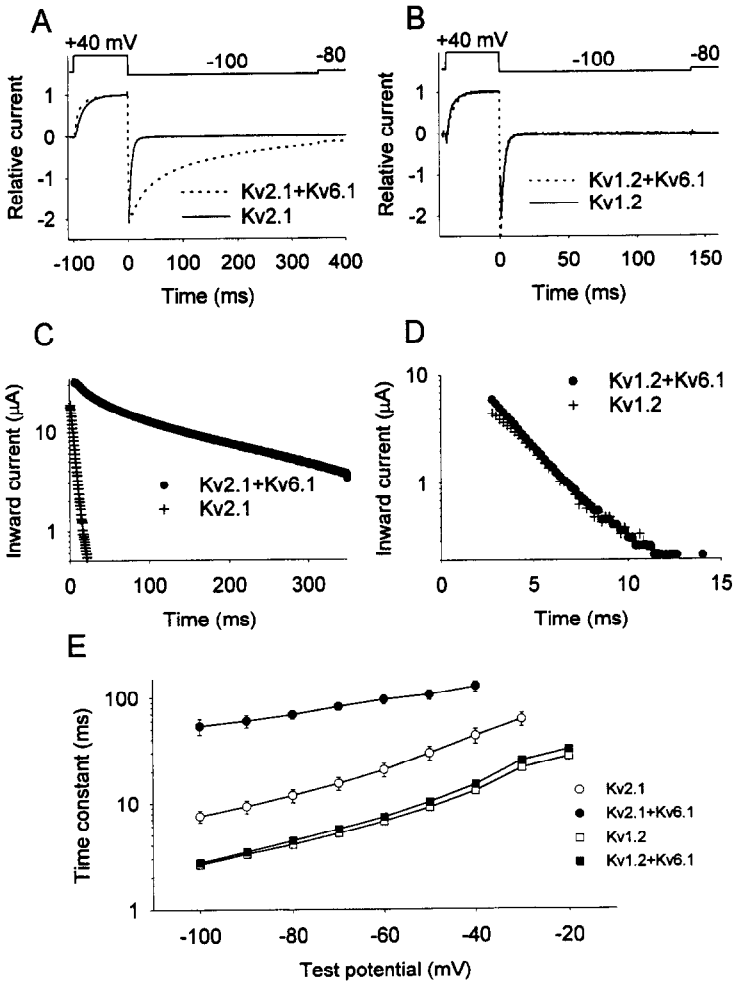

Fig. 2. Tail current analysis of Kv2.1–Kv6.1 co-expressed in oocytes. Typical tail current records (A,B) were obtained with a conditioning potential of +40 mV to fully activate the channels, followed by a test pulse to −100 mV to evaluate the rate of closing upon repolarization. Traces obtained from oocytes injected with either a single cRNA species (——: Kv2.1, A; Kv1.2, B) or in combination with Kv6.1 (· · ·) were normalized to the maximum currents obtained at +40 mV. Recording solution was K-Ringer. Panels C,D plot the decay of the tail currents. For Kv2.1 alone, the data points (+) were well fit by a single exponential decay function with time constant 5.2 ms, whereas co-injection with Kv6.1 markedly prolonged the decay such that the sum of two time constants (19 and 153 ms, with amplitudes 0.2 and 0.8, respectively) were required to fit the data. The decay of currents in the co-injected oocytes was complex such that each of the major time constants (τ) presumably contains multiple components that are not easily dissociated. Thus, the shortest time constant probably contains contributions from both pure Kv2.1 homomultimers (τ = 5 ms) as well as heteromultimers with longer time constants. Compared with Kv2.1, Kv1.2 homotetramers (B,D) have much faster tail currents (τ = 2.6 ms; note the different time scales in A,B) and co-expression of Kv6.1 (co-injected at 10-fold excess over Kv1.2 cRNA) did not alter their time course (τ = 2.2 ms, ■, D). Panel E shows mean time constants ± SEM on a logarithmic scale over a range of test potentials (−100 to −20 mV) for Kv2.1 (○, $n = 6$ oocytes), Kv1.2 (□, $n = 5$), Kv2.1 and Kv6.1 co-expressed (●, $n = 5$; each point represents the weighted average time constant obtained by fitting the decay curves with monoexponential functions ± SEM) and Kv1.2–Kv6.1 co-expressed (■, $n = 5$). In separate measurements (data not shown) the voltage dependence of steady-state activation (obtained from tail currents at −50 mV upon repolarization from conditioning steps from −100 to +80 mV, 10 mV increments; K-Ringer recording solution) was shifted −32 mV with no change in slope ($n = 7$ and 5 oocytes, respectively, for Kv2.1 and Kv2.1–Kv6.1). Co-injection of Kv1.2–Kv6.1 produced no shift when compared with Kv1.2 alone. Note that in (E) a 70 mV shift of the Kv2.1–Kv6.1 curve would be required to superimpose this curve with that of Kv2.1; that is, the difference in the time constant–voltage relationship between Kv2.1 and Kv2.1–Kv6.1 expressing oocytes can not be accounted for by the 34 mV shift in the voltage for half maximal activation.

oocytes co-injected with Kv2.1 and Kv6.1 cRNA was calculated as the sum of two isotherms. The apparent K_d for TEA binding to Kv3.1 injected oocytes was 100 μM; to Kv3.1 and Kv6.1 co-injected oocytes was 100 μM; and to Kv2.1 injected oocytes was 3.7 mM. The TEA dose–response data obtained from oocytes co-injected with Kv2.1 and Kv6.1 cRNA was fit by the sum of two isotherms. One K_d was fixed at 3.7 mM and the other K_d and the weighting factor relating the two K_ds were allowed to vary. Under these conditions the second K_d for TEA binding was calculated to be 42 mM and the weighting factor, r, was 0.5. These results suggest that the co-injection of Kv2.1 and Kv6.1 cRNAs caused the appearance of at least one additional channel population (for a Kv2.1–Kv6.1 tetramer there are five possible arrangements) which displayed a markedly reduced TEA sensitivity as compared to the channels formed by the injection of Kv2.1 alone. By contrast, co-injection of Kv3.1 and Kv6.1 cRNAs resulted in channels which did not differ in their TEA sensitivity from that of channels formed by the injection of Kv3.1 alone, suggesting that no interaction between Kv3.1 and Kv6.1 occurred.

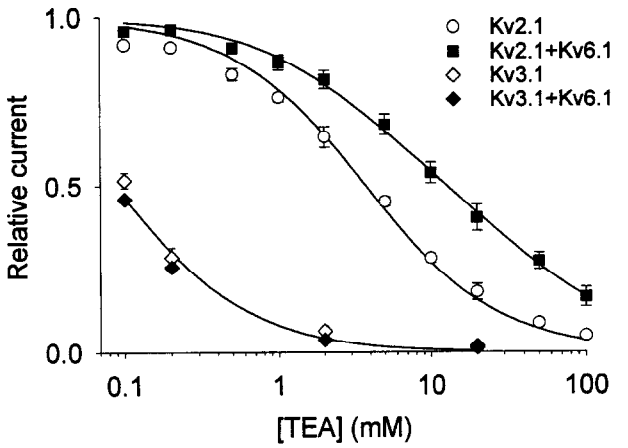

Fig. 3. Co-expression of Kv6.1 alters Kv2.1 TEA sensitivity. TEA was added cumulatively to the external solution and current amplitudes as a function of TEA concentration (mM) are plotted relative to drug-free controls. Test potential +40 mV, holding potential −80 mV. Smooth curves are best fits to 1:1 binding isotherms with apparent K_ds (mM) of 3.7, 0.1 and 0.1 for Kv2.1 (○, mean ± SEM, $n=5$ oocytes), Kv3.1 (◇, $n=5$), or Kv3.1+Kv6.1 (◆, $n=6$), respectively; or to the sum of two isotherms (Kv2.1+Kv6.1; ■, $n=5$, mean ± SEM): $1-(r*([TEA]/([TEA]+K_1))+((1-r)*([TEA]/([TEA]+K_2))))$, where r = weighting factor that relates the amplitude of each isotherm, and K_1 and K_2 are the apparent K_ds. A best fit, with K_1 fixed at 3.7 mM (TEA sensitivity of Kv2.1 homomultimers), and r and K_2 allowed to vary, was obtained with $r=0.5$ and $K_2=42$ mM. Thus, the co-injection of equal concentrations of Kv2.1 and Kv6.1 caused the appearance of a fraction of channels (roughly half) with markedly reduced sensitivity to TEA block. By contrast, co-injection of Kv3.1 with 10-fold excess Kv6.1 cRNA produced no apparent change in TEA sensitivity.

4. Discussion

In these studies we have demonstrated that Kv6.1 and Kv2.1 α-subunits can form heteromultimeric K^+ channels, and that Kv6.1 α-subunits, unlike Kv2.1 α-subunits, are unable to interact with each other through their amino terminal domains. TEVC of *Xenopus* oocytes gave results consistent with those obtained using yeast two-hybrid assays. That is oocytes expressing Kv2.1, but not Kv6.1 alone, gave rise to exogenous currents and, in the case of oocytes co-expressing Kv2.1 and Kv6.1, exhibited a novel K^+ current which displayed reduced TEA sensitivity, markedly decreased rates of deactivation, and a hyperpolarizing shift in the half maximal activation voltage. Co-injection of Kv6.1 in combination with either Kv1.2 or Kv3.1 cRNAs resulted in currents which were indistinguishable from those obtained in the absence of Kv6.1. It is important to note that Kv6.1 is electrically silent, so that the problem of distinguishing between a whole cell current which is the sum of two currents mediated by two mixed populations of homomultimeric channels or a novel current mediated by a population of heteromultimeric channels is obviated. Our results are consistent with the hypothesis that Kv2.1 and Kv6.1 α-subunits selectively associate to form heteromultimers and provide the first evidence that heteromultimers between different Kv subfamilies may express novel currents. Our results also provide the first insight into a possible function of the Kv6 channel subfamily.

Previous work has shown that protein–protein interactions between Kv channel α-subunits may be mediated by regions within the amino portion of the channel protein. These sites, termed N_A and N_B boxes [21,24] or T1 A,B, and C [23] domains, mediate attractive interactions between α-subunits from a single Kv subfamily, and also perhaps repulsive interactions between Kv subfamilies [22]. A number of studies, employing a diverse array of methodologies, have failed to detect interactions between subunits from different Kv subfamilies [24,8,5,25], although a very recent report described co-immunoprecipitation of a novel clone, Kv8.1, with Kv2.1, and suppression of Kv2.1 currents in *Xenopus* oocytes by Kv8.1 [26]. We have obtained similar results for Kv5.1 and Kv2.1 co-expressed in *Xenopus* oocytes and assayed by the yeast two-hybrid system (Post, Kirsch, and Brown, unpublished observation). TEVC studies of oocytes expressing amino terminal deletion mutants suggest that some of these amino terminal sites are not required for homomultimer formation [27]. It is interesting to note that these deletions (amino acids 1 through 139 of Kv2.1) did not exclude the C subdomain [8], therefore this domain may be important in homomultimer formation. Further studies are needed though to resolve why Kv6.1 fails to form homomultimers, since this channel subunit contains the defined amino terminal subdomains described in [24] and [8]. Because their amino termini do not interact it is tempting to speculate that the absence of Kv6.1 mediated currents results from a lack of homomultimer formation rather than from an inability of the homomultimer, once formed, to conduct current.

4.1. Implications for K^+ channel diversity

The Kv family of potassium channels encompasses at least six and possibly seven subfamilies [16,26]. Three of these, Kv5, Kv6, and Kv8, are known to be electrically silent, and no functional role has yet been suggested for these channel subunits. Our finding that Kv6.1 forms functional heteromultimeric channels specifically with Kv2.1 suggests this as one possible role for this Kv subfamily in vivo. Consistent with this notion, Kv2.1 and Kv6.1 mRNAs have been shown to colocalize to the piriform cortex, hippocampus, dentate gyrus, and olfactory tubercle in the brain [17] and to the SA node, atria and ventricle in the heart [28,29]. This suggests that Kv6.1 may be physiologically important in generating an increased number of Kv channel phenotypes even though it does not itself form functional homomultimeric channels. The function of this particular phenotype in the cell's physiology is unknown, but one possible scenario is that a Kv2.1–Kv6.1 heteromultimeric channel with a decreased rate of deactivation would bias the cell potential towards E_K and enhance afterhyperpolarization, which in turn could prolong the interval between action potentials in excitable cells.

A number of authors have looked for and failed to find interactions between the current-expressing Kv1, Kv2, Kv3, and Kv4 subfamilies [30,21,9,8]. Consequently, heteromultimerization might be considered to occur only within Kv subfamilies, but the present results show this clearly not to be the case. Thus heteromultimerization between Kv subfamilies may require, besides the current-expressing α-subunit, a member of one of the electrically silent Kv subfamilies ([26], present study). This process may be a general mechanism for generating an increased variety of cellular K^+ currents.

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