Interactions among Inactivating and Noninactivating Kvβ Subunits, and Kvα1.2, Produce Potassium Currents with Intermediate Inactivation*

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Eric A. Accili†‡§, Johann Kiehn¶, Barbara A. Wible†**, and Arthur M. Brown‡ ‡‡

From the Rammelkamp Center for Research, MetroHealth Campus, and ‡Department of Physiology and Biophysics, **Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44109-1998

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Experiments were carried out to determine whether coinjection of Kvα1.2 with inactivating and noninactivating Kvβ subunits would produce currents with intermediate kinetics and channel complexes containing a mixture of these subunits. Upon coexpression with a saturating amount of Kvβ1.2 and increasing levels of a noninactivating deletion mutant of Kvβ1.2, we show that macroscopic Kvα1.2 currents have levels of fractional inactivation and inactivation time constants that are intermediate between those obtained with either the inactivating Kvβ1.2 or the noninactivating Kvβ1.2 mutant. We also find that coexpression of Kvα1.2 with saturating amounts of Kvβ1.2 and the deletion mutant produces a population of single channels with properties intermediate to either the inactivating or noninactivating parental phenotype. Our data can best be explained by the presence of an intermediate population of heterooligomeric channels consisting of Kvα1.2 with different combinations of both types of subunits. Since Kvα1.2 subunits coexist in cells with inactivating and noninactivating Kvβ subunits, our findings suggest that heterooligomeric assembly of these subunits occurs to increase the range of K+ current kinetics and expression levels.

Biochemical data suggests dendra toxin-sensitive K+ channels exist as αβ complexes (1). Moreover, both noninactivating (Kvβ2) and inactivating Kvβ subunits (Kvβ1) have been found in complexes with Kvα1.2-α-subunits (2, 3). These observations suggest that various combinations of inactivating and noninactivating Kvβ subunits may exist in complexes with Kvα1 α-subunit tetramers. The functional consequences of Kvβ heterooligomerization on Kvα1 currents has not been fully described. A recent study showed that the inactivation of a Shaker mutant produced by Kvβ1.2 can be removed by Kvβ2 leading to currents that were either inactivating or noninactivating (4). Intermediate levels of inactivation, although anticipated, were not observed. A model was proposed in which the Kv α-subunits exist as complexes with either Kvβ1 or Kvβ2 subunits. Binding of Kvβ2 to Kvβ1 was also reported, and it was suggested that this interaction might reduce the effective concentration of inactivating Kvβ1 subunits available for binding to Kvα1 subunits.

To further investigate whether intermediate levels of inactivation might exist in the presence of both inactivating and noninactivating Kvβ subunits, we coexpressed Kvα1.2, Kvβ1.2 and a noninactivating Kvβ1.2 N-terminal deletion mutant in Xenopus oocytes (5). We used the mutant rather than Kvβ2, because differences in β-α binding could be excluded. Kvβ1 and Kvβ2 are homologous, but not identical, in the C terminus, the Kvβ region shown to bind to Kvα1 N termini (6, 7). We observed macroscopic currents with inactivation kinetics intermediate to either parental phenotype. Single channel analysis revealed a population of channels with kinetics spanning inactivating to noninactivating phenotypes. We also found that Kvβ1.2 and its deletion mutant could interact in the yeast two-hybrid assay. Thus, the deletion mutant, by reducing the effective concentration of Kvβ1.2, may have contributed to the production of intermediate inactivation as suggested by Xu and Li (4). Because noninactivating (Kvβ2) and inactivating Kvβ subunits (Kvβ1) have been found in complexes with Kvα1.2 and all three subunits may coexist cells (2, 3, 8), we suggest that our single channel results may be the functional counterparts of such complexes. Some of the data have been presented in abstract form (9).

**EXPERIMENTAL PROCEDURES**

Yeast Two-hybrid Interaction—The Kvα1.2 cDNA was a gift from O. Pongs (Zentrum für Molekulare Neurobiologie, Institut für Neurale Signalarbeitung, Hamburg, Germany). Kvβ1.2 was cloned as described previously (10). Protein-protein interactions were tested using the yeast GAL-4 matchmaker two-hybrid system from CLONTECH. In-frame EcoRI and SalI sites were incorporated into the 5′- and 3′-ends, respectively, of the coding sequences of Kvβ1.2 (amino acids 1–408) and Kvβ1.2-N520 (amino acids 21–408) by polymerase chain reaction for cloning into the yeast shuttle vectors, pGBT9 and pGAD424. The polypeptide chain reaction-amplified constructs were sequenced to confirm the correct reading frame for the yeast fusions and check that no unwanted polypeptide chain reaction mutations were introduced.

Protein-protein interactions were tested in the yeast host strain Y190 by cotransformation with pairs of pGBT9 and pGAD424 fusion constructs. Cotransformants were selected on medium lacking tryptophan (trp−) and leucine (leu−) after growth for 2–3 days at 30 °C. Representative colonies from these transformations were replated on trp−/leu− media to allow direct comparison of individual colonies. Transcription of the reporter gene, lacZ, was tested by a β-galactosidase filter assay.

In Vitro Transcription of cRNAs and Oocyte Injection—In vitro transcription of Kvα1.2, Kvβ1.2, and Kvβ1.2-N520 cRNAs and injection into Xenopus oocytes were as described previously (5). The Kvβ1.2-N520 construct was prepared as described previously (5).

Electrophysiology—The standard two-microelectrode voltage clamp
FIG. 1. Interactions between Kvβ1.2 and Kvβ1.2-NA20 using the yeast two-hybrid assay. Kvβ1.2 binds to itself as well as the NA20 deletion. The yeast host strain Y190 was cotransformed with GAL4 binding domain (pGBT9) and activation domain (pGAD424) fusion plasmids as indicated and plated on medium lacking trp and leu. Each plasmid was tested for autonomous transactivation by transforming with the appropriate vector, either pGBT9 or pGAD424, alone. All cotransformants grew on medium and were tested for activation of the reporter gene, lacZ, by a filter assay shown in the middle panel. None of the control reactions resulted in the activation of lacZ transcription, but the Kvβ subunits tested together gave a positive signal.

FIG. 2. Addition of increasing amounts of the Kvβ1.2 deletion mutant progressively reduces the inactivation of Kvα1.2 by Kvβ1.2. A, normalized current traces showing the reduction of inactivation of Kvα1.2 produced by 100 ng of Kvβ1.2 by addition of the noninactivating Kvβ1.2-NA20. Note also that activation becomes slower as inactivation is removed. B, bar plots showing the increase in the inactivation time constant at increasing concentrations of Kvβ1.2-NA20 in the presence of 100 ng of Kvβ1.2. Inactivating currents were fit with single exponential functions. The values are 5.06 ± 0.29 ms, 5.72 ± 0.15 ms, 6.30 ± 0.34 ms, and 7.34 ± 0.43 ms for 0, 1.5, 3, and 6 ng of Kvβ1.2-NA20, respectively. C, bar plots showing the reduction of inactivation calculated as the fraction of steady state to peak current levels at increasing concentrations of Kvβ1.2-NA20 in the presence of 100 ng of Kvβ1.2. The values are 0.63 ± 0.02, 0.56 ± 0.01, 0.54 ± 0.02, and 0.40 ± 0.02 for 0, 1.5, 3, and 6 ng of Kvβ1.2-NA20, respectively. Oocytes were held at −80 mV, pulsed to +70 mV for 125 ms, and pulsed back to −80 mV. The numbers above each bar represent the numbers of oocytes used.

A

B

C

FIG. 3. Kinetics of single channels formed by coexpression of Kvα1.2 with both Kvβ1.2 and the Kvβ1.2-NA20 deletion mutant. A–B, individual values for open time and open probability of single channels in patches recorded in oocytes injected with Kvα1.2 plus both Kvβ1.2 and Kvβ1.2-NA20 (n = 29 patches). For comparison, also shown are individual and mean values ± S.D for open time and open probability of single channels in patches recorded in oocytes injected with Kvα1.2 and Kvβ1.2-NA20 (open time = 3.93 ± 0.33 ms; open probability = 0.81 ± 0.13; n = 6 patches) or Kvα1.2 (open time = 0.74 ± 0.09 ms; open probability = 0.98 ± 0.03; n = 8 patches). The means ± S.D. placed above individual values and were taken from Accili et al. (5). The vertical arrows represent a distance of 2 S.D. from the mean of the Kvα1.2 plus Kvβ1.2 group (solid arrow) or the Kvα1.2-NA20 group (open arrow). Thirteen out of 29 channels recorded have values lying between the solid and open arrows for both open time and open probability. Oocytes were held at −80 mV, and 1000 traces were recorded in the cell-attached configuration with 5 ms K+ in the pipette during pulses to +70 mV of 100-ms duration at a frequency of 2 Hz. The pipette solution for the single channel recordings had the low K+ composition. The bath solution in all single channel measurements was a high K+ solution containing (in mmol/liter): 5 KCl, 100 NaCl, 1.5 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.3) or a high K+ solution containing 50 KOH, 72.5 NMDG, 122.5 MES,1 and 10 HEPES (pH 7.3). Single channel measurements were performed as described previously (5, 6). The pipette solution for the single channel recordings had the low K+ composition. The bath solution in all single channel measurements contained (in mmol/liter): 100 KCl, 1 MgCl2, and 10 HEPES (pH 7.3). All measurements were done at room temperature (20 °C).

Single channel experiments were carried out as described previously (6). I–V protocols were performed to ensure that the investigated channel had the expected activation threshold and reversal potential. Measurements with only one channel in the patch were used for further investigation. From a holding potential of −80 mV, we measured 1000 test pulses to a potential of 70 mV at a frequency of two per second. Leak subtraction was done by averaging 10 null records and subtracting it from each trace as described previously (11). Data were low-pass-filtered at 2 kHz (~3.3, 4-pole Bessel filter) before digitalization at 10 kHz. PClamp software (Axon Instruments) was used for generation of the voltage-pulse protocols and for data acquisition. All single channel measurements were leak subtracted in Fetchan and analyzed using TRANSIT software (12). This produced histograms for the open time, ensemble current traces, and values of open probability. The maximum likelihood method is utilized by TRANSIT to determine time constants from open time histograms. The data for open time from TRANSIT are presented on a log scale as described previously (12).

1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
RESULTS

Interactions between Kvβ1.2 and Kvβ1.2 N-terminal Deletion Mutants—We tested whether Kvβ1.2 and a noninactivating form of Kvβ1.2, in which the N-terminal 20 amino acids were deleted, could interact using the yeast two-hybrid assay. This deletion had no effect on the ability of Kvβ1.2 to bind to the Kvα1.2 N terminus (5). We found that Kvβ1.2 was able to interact with itself as well as with the NΔ20 deletion (Fig. 1). These results parallel the findings of Xu and Li (4) who showed that Kvβ2 can interact with itself and with Kvβ1.1. The results suggest that Kvβ1.2 and Kvβ1.2 NΔ20 may exist as heterooligomers in the absence of Kvα1.2.

Intermediate Levels of Kvα1.2 Inactivation Are Produced by Different Ratios of Kvβ1.2 and the Kvβ1.2 Deletion Mutant—We coexpressed Kvα1.2 with a saturating amount of Kvβ1.2 and amounts of Kvβ1.2 NΔ20 (5), ranging from 0 to 100 ng. The Kvβ1.2 deletion mutant was used instead of Kvβ2 to exclude differences in binding affinities between Kvβs. The Xenopus oocyte expression system also allowed the delivery of mixed cRNAs to be measured accurately. In the presence of Kvβ1.2 alone inactivating currents were measured. The maximal amount of inactivation, calculated as the ratio of peak to steady state current was approximately 0.60–0.65 and was similar to that found previously (5, 6). Addition of increasing amounts of the deletion mutant progressively removed inactivation and slowed the rate of inactivation (Fig. 2A). A 1:4 ratio of the deletion mutant:Kvβ1.2 cRNAs was sufficient to completely remove inactivation. Addition of the deletion mutant also slowed activation (Fig. 2A) and increased the rate of deactivation (not shown). Fig. 2B and C show the averaged values for rate of inactivation and fractional inactivation in the presence of increasing amounts of the deletion mutant and saturating amounts of Kvβ1.2.

We have found similar effects on both fractional inactivation and inactivation time constants by simply decreasing Kvβ1.2 in the absence of the deletion mutant. Previously, we reported that fractional inactivation of Kvα1.2 went from 0.66 (400 ng of Kvβ1.2 cRNA), to 0.57 (100 ng of Kvβ1.2 cRNA), to 0.19 (25 ng of Kvβ1.2 cRNA) (5). Here, we found that by reducing Kvβ1.2 cRNA inactivation time constants increased from 3.81 ± 0.13 ms (400 ng of Kvβ1.2 cRNA, n = 10 oocytes) to 5.16 ± 0.40 ms (100 ng of Kvβ1.2 cRNA, n = 8 oocytes), to 14.45 ± 3.54 ms (25 ng of Kvβ1.2 cRNA, n = 9 oocytes). Since we also found that Kvβ1.2 and the deletion mutant interact in the yeast two-hybrid assay, the deletion mutant may have exerted part or all of its effect on Kvα1.2 by binding and reducing the effective concentration of Kvβ1.2 in coexpression experiments.

These results show that intermediate forms of inactivation may result from a mixture of inactivating and noninactivating Kvβ subunits. The data do not distinguish among three possibilities: 1) a fully occupied mixed channel having four Kvβ subunits (Kvβ1.2 + deletion mutant = 4); 2) a partially occupied mixed channel (Kvβ1.2 + deletion mutant < 4); or 3) two populations of fully occupied channels, one having four inactivating Kvβ subunits and another having four noninactivating Kvβ subunits.

Intermediate Distinct Kvα1.2 Single Channels Result from Coinjection with Both Inactivating and Noninactivating Kvβ Subunits—To distinguish among the three possibilities presented above, we characterized the single channel properties of Kvα1.2 in the presence of saturating amounts of an equal mixture of Kvβ1.2 and Kvβ1.2 NΔ20 and compared these to single channels measured in the presence of either Kvβ1.2 or Kvβ1.2 NΔ20. Fig. 3 shows open times (A) and open probabilities (B) of single channels recorded from three groups of oocytes injected with different cRNA combinations. Out of 29 patches from eggs injected with Kvα1.2 cRNA along with both Kvβ1.2 and the deletion mutant, 12 patches contained single channels with open time and open probability values more than
2 S.D. from the mean values for channels recorded from oocytes injected with either Kvβ1.2 or the deletion mutant. Single channels from five patches had values within 2 S.D. of the mean values for channels injected with only Kvβ1.2 and another set of six channels had values within 2 S.D. of the mean values for channels injected with only the Kvβ1.2 deletion mutant. Thus, the single channels resulting from this mixture of cRNAs had kinetic parameters spanning a range from inactivating to noninactivating parental phenotypes.

When we used open time, open probability, burst duration, and closed time to distinguish between groups, 14 of 29 channels measured fell into three separate populations (Fig. 4). Fifteen channels had individual parameters that could not be classified neatly into any of the three defined groups possibly, because they possessed a combination of Kvβ subunits that altered certain kinetic parameters more than others. Fig. 5 shows three examples of channels where all four kinetic parameters lie within one of the three defined groups described above.

**DISCUSSION**

The purpose of the present experiments was to determine whether macroscopic currents with intermediate inactivation kinetics could be obtained by expression of Kvα1.2 with a mixture of inactivating and noninactivating Kvβ subunits and, if so, what the mechanisms might be. We found macroscopic currents with values of fractional inactivation and inactivation kinetics intermediate to the two phenotypes that result upon coexpression of Kvα1.2 with equal amounts of either Kvβ1.2 or its deletion mutant. Reducing the amount of Kvβ1.2, in the

**FIG. 5.** Representative kinetics of three separate groups of single channels formed by coexpression of Kvα1.2 with both Kvβ1.2 and the Kvβ1.2-NΔ20. A–C, representative single channel traces recorded in Xenopus oocytes injected with Kvα1.2 (2 ng/ml), Kvβ1.2 and Kvβ1.2-NΔ20 (both at 100 ng/ml). D–F, corresponding open time distributions. The line of fit was generated using a maximum likelihood method as described previously (3). Note that the x axis is on a log scale and most of the data are at the longer time points. The line of fit follows the data very closely at these longer times. G–I, corresponding open probabilities. Oocytes were held at −80 mV, and 1000 traces were recorded in the cell-attached configuration with 5 mM K+ in the pipette, during pulses to +70 mV of 100-ms duration at a frequency of 2 Hz. These individual channels were also representative of three separate groups based on burst duration (channel A = 16.55 ms; channel B = 3.58 ms; channel C = 1.47 ms) and closed time (channel A = 0.67 ms; channel B = 3.58 ms; channel C = 7.50 ms).

**FIG. 6.** Simplified scheme for the formation of Kvα1.2 channel complexes responsible for the range of kinetic phenotypes produced by Kvβ1.2 (Kvβ1.2) and the NΔ20 deletion mutant (Kvβ1.2-NΔ20). The model depicts two channel complexes fully occupied by Kvβ1.2 or the Kvβ1.2 deletion mutant (C and D) and homo/hetero-oligomeric β-β complexes (E) as described for Kvβ2 and Kvβ by Xu and Li (4). In addition, the model also shows two channel complexes not fully occupied by Kvβ1.2 (A and B), which could be responsible for the single channels with intermediate kinetics observed.

absence of the deletion mutant, also produced currents with intermediate kinetics. Taken together with our finding that Kvβ1.2 and the noninactivating mutant interact in the yeast two-hybrid assay, and with evidence that Kvα and Kvβ sub-
units exit in a 1:1 stoichiometry (1), the data suggest that the intermediate kinetics were produced by one, or a combination, of the following three channel complexes: 1) a fully occupied mixed channel (Kvβ1.2 + deletion mutant = 4); 2) a partially occupied mixed channel (Kvβ1.2 + deletion mutant < 4); or 3) two fully occupied populations of channels with either Kvβ1.2 or Kvβ1.2-NΔ20.

Single channel analysis showed that three groups of single channels with distinct inactivation kinetics could be distinguished when Kvα1.2 was coexpressed with equal amounts of Kvβ1.2 and the deletion mutant. Two groups had kinetics identical to those of single channels formed from the expression of Kvα1.2 with a saturating amount of either Kvβ1.2 or the deletion mutant. A third group had inactivation kinetics significantly different from those of either parental group. The presence of an intermediate group of channels rules out the possibility that the intermediate kinetics of macroscopic currents were produced solely by two populations of fully occupied nonmixed channels. The single channels with intermediate kinetics must have been produced by a fully or partially occupied mixed channel. This extends the scheme of Xu and Li (4) to include channel complexes not only fully occupied by either inactivating or noninactivating Kvβ subunits (4; Fig. 6, C and D), but also complexes fully or partially occupied by mixed combinations of α and β subunits (Fig. 6, A and B). Because both noninactivating (Kvβ2) and inactivating Kvβ subunits (Kvβ1) have been found in complexes with Kvα1.2, and all three subunits are likely to exist in the same cell (2, 3, 8), we favor the view that Kvα1.2 channel complexes fully occupied with a mixture of both inactivating and noninactivating Kvβ subunits (Fig. 6B) underlie the single channels with intermediate kinetics. However, further experiments will be required to determine precisely the subunit stoichiometry responsible for the intermediate single channel kinetics observed here.

In summary, our results clearly demonstrate the existence of a population of single channels with intermediate inactivation kinetics. This functional evidence suggests multiple combinations of inactivating and noninactivating Kvβ subunits exist in complexes with Kvα subunits. Since Kvβ subunits are also able to variably modify expression levels of Kv1 channels (5, 13, 14), the additional level of Kvβ-mediated regulation described in the present study likely contributes to the large range of K+ current kinetics and expression levels found in native tissues (15, 16).

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