We constructed tandem cDNA by linking the 5' end of a delayed rectifier-type (Kv1.2) clone to the 3' end of a transient-type (Kv1.4) K+ channel clone. Fusion genes were also constructed, consisting of Kv1.4 and mutants of Kv1.2, which have a single amino acid substitution in the S4-S5 loop. From electrophysiological characterization, it is likely that two pairs of tandem heterodimer constructs can form hybrid channels. In addition, it has been revealed that the wild-type hybrid channel shows a time constant of inactivation very similar to that observed in the homotetrameric Kv1.4 channel. Difference of inactivation kinetics between wild-type and mutant hybrid K+ channels suggests that not only the S4-S5 loop of Kv1.4 but also that of Kv1.2 can serve as the acceptor sites for the inactivation gates, and that all of four sets of loops should be functional for rapid inactivation. From these results, in the hybrid channels the structure and composition of the acceptor sites could be important factors for determining the rate of inactivation.

As K+ channels are generally considered to assemble as tetramer (16-21), a rapidly inactivating K+ channel has four sets of inactivation balls and acceptor sites. Recently, MacKinnon et al. (22) have reported that only a single inactivation gate is necessary to produce inactivation in a Shaker B K+ channel and the inactivation rate constant is dependent on the number of gates. It is still unknown, however, how many of the acceptor sites are necessary for binding the inactivation balls.

The channel formed by Kv1.4, a vertebrate homologue of Shaker K+ channel gene, shows inactivation, and that inactivation is considered to be due to “ball and chain” mechanism (23). Additionally, it has been suggested that coexpression of Shaker-related vertebrate homologues can form heteromultimeric K+ channels (16, 18, 24). Furthermore, Sheng et al. (25) have shown recently that two Shaker subfamily K+ channel subunits, Kv1.4 and Kv1.2, co-assemble and actually form a heteromultimeric K+ channel in vivo in the rat brain.

In the present study, we constructed fusion genes encoding hybrid K+ channels using a transient-type (Kv1.4) and a delayed rectifier-type (Kv1.2) K+ channel clones isolated from rat heart muscle (26, 27), and we investigated how the S4-S5 loop of the non-inactivating Kv1.2 subunits participates in inactivation of the hybrid channels. We show here that the S4-S5 loop of the non-inactivating subunits also contributes to the formation of the acceptor sites and that the wild-type hybrid channel gives rise to an inactivation rate constant very similar to that observed in the homotetrameric Kv1.4 channel.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Oligonucleotides used in this study were synthesized using the phosphoramidite method with an Applied Biosystems 391 DNA synthesizer. OPC cartridges (Applied Biosystems) were used for purification.

Oligonucleotide-directed Mutagenesis—Three distinct mutants were made in the S4-S5 loop of rat cardiac cDNA clone Kv1.2. The PstI/BamHI restriction fragment (nucleotides 845-1857) was excised from Kv1.2 and subcloned into M13mp18. Single-stranded DNA prepared from the resulting construct was used as the template DNA in the Amersham oligonucleotide-directed mutagenesis system to introduce mutations in the S4-S5. The synthesized mutant oligonucleotides with the appropriate base change(s) flanked by 9 or 10 complementary nucleotides were used. The KpnI/PstI fragment (nucleotides 707-844) and the mutated PstI/BamHI fragment were ligated back into Kv1.2 between the KpnI and BamHI sites. The mutations were confirmed by sequencing the final constructs with the BcaBEST sequencing kit (Takara, Japan). The mutant generated are named as follows, and the substituted amino acids with residue numbers are given: mKv1.2 T320A, mKv1.2 S324G, and mKv1.2 S324A.

Dimeric cDNA Construction—The cDNAs Kv1.2 and Kv1.4 were altered near the 5' translation start site or the 3' translation stop site using polymerase chain reaction (PCR). Kv1.4 cDNA coding for the first (the 5'site) subunit of the tandem dimer was altered on the 3' end by introduction of the linker sequence, which replaced the stop codon

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‡ This abbreviation is used in: PCR, polymerase chain reaction.
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(2043-24139, TGA). This linker consisted of a SpeI restriction site. PCR amplification was performed using an antisense oligonucleotide containing the SpeI restriction site and the last 23 nucleotides, and a sense oligonucleotide generated by addition of a HindIII site to the sequence, which contains a EcoRI site (nucleotide 1641). 150 pmol of each primer, 100 ng of Kv1.4 cDNA, and 2.5 units of Tag DNA polymerase (Promega) were used in a 50-µl PCR reaction. Reaction temperatures were varied using a thermal cycler (Perkin-Elmer): 94 °C, 1 min; 60 °C, 2 min; and 72 °C, 3 min for 10 cycles. The amplified fragment was digested with SpeI and HindIII (fragment I). For the second position of the tandem dimer, Kv1.2 cDNA was inserted at the 5' end by adding a SpeI restriction site. PCR amplification between a sense oligonucleotide corresponding to the SpeI restriction site and the first 23 frame nucleotides of Kv1.2 and an antisense oligonucleotide that overpassed the StuI restriction site (nucleotide 512) generated a fragment that was digested with SpeI and StuI (fragment II). Conditions used for amplification of fragment II were the same as fragment I. Sequences of all the PCR-generated fragments were verified by DNA sequence analysis with the BcaBEST™ sequencing kit. Fragment I and II were cloned into the corresponding sites of Kv1.2 and its mutants in pBluescript (Stratagene), which had been digested with HindIII and StuI. These intermediate constructs of tandem linkage and Kv1.4 in pBluescript were then digested with BstEII and BcmHI and ligated into the complete tandem constructs. These intermediate and complete tandem constructs were mapped by restriction enzyme digestion to ensure proper insertion and orientation. The tandem heterodimers generated in this manner were Kv1.4-Kv1.2, Kv1.4-m1Kv1.2, Kv1.4-m2Kv1.2, and Kv1.4-m5Kv1.2.

Expression of Dimeric Constructs and Current Recording.—The pBluescript vectors containing the dimeric constructs were linearized with EcoRI, and cRNAs were prepared from these templates with T7 RNA polymerase (Stratagene) as described (26). Transcribed RNAs were dissolved in water at a final concentration of 0.4 µg/µl for oocyte injection. The integrity of transcribed cRNAs was examined by running the samples on formaldehyde agarose gel. Xenopus oocytes (stage IV-V) were defolliculated by treatment for 2 h with 0.2% collagenase (Wako, Japan) in Barth’s medium. Defolliculated oocytes were then injected with 40-50 nl (approximately 16-20 ng) of cRNA. The injected oocytes were incubated in Barth’s medium supplemented with penicillin G (71.5 units/ml) and streptomycin (35.9 µg/ml) at 19 °C for 2-4 days before electrophysiological measurements. The K⁺ currents were recorded by a conventional two-microelectrode voltage clamp with 3 m KCl-filled electrodes as described (26, 27). The bath recording solution consisted of ND 96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5), and all electrophysiological measurements were carried out at room temperature (21 ± 1 °C). Current records were low-pass-filtered at 3 kHz.

Data Analysis.—Inactivation of the current during the depolarizing pulse was fitted by a single exponential to measure time constants of macroscopic inactivation. Curves of fraction recovery were also fitted by an exponential to estimate time constants of recovery from inactivation. The values for the voltage for half-maximal activation (Vₐ) and slope factor (α) were determined by fitting the peak conductance data with a Boltzmann distribution, G(V) = Gₐ/(1 + exp(−(V−Vₐ)/α)), where Vₐ is the membrane voltage of depolarization pulse. Normalized prepulse inactivation data were fitted with a Boltzmann distribution, Vₐ/α = (1−α)/(1+α)1+exp((V−Vₐ)/α)] + α (26) to determine half-inactivation voltage Vₐ, the slope factor α, and the non-inactivating component α. Vₐ is the prepulse voltage.

RESULTS

The proposed membrane topology of the peptides encoded by the tandem dimeric constructs and a sequence alignment of a portion of the S4-S5 loop of Kv1.2 and its mutants are shown in Fig. 1. Substitution of alanine for threonine residue at position 320 in Kv1.2 (m1Kv1.2) induced no marked changes in the currents compared with the currents generated by the wild-type Kv1.2 (data not shown). However, mutants in which glycine or alanine was substituted for serine residue at position 324 in Kv1.2 (m2, m5Kv1.2) did not lead to the expression of functional channels. Injection of Kv1.4 cRNA into Xenopus oocytes resulted in time- and voltage-dependent transient outward currents (Fig. 2A). On the other hand, oocytes injected with Kv1.2 cRNA generated large outward currents, which showed rapid activation and little inactivation during depolarizing pulses (26).

![Fig. 1. Heterodimeric constructs generated by tandem linkage of Kv1.4 and Kv1.2 or its mutants. Upper panel, proposed membrane topology of tandem dimeric polypeptide encoded by the fusion genes.](image)

![Fig. 2. Macroscopic currents obtained by two-microelectrode voltage clamp from injected Xenopus oocytes.](image)

The currents produced by the dimeric constructs in which both subunits could form functional K⁺ channels by themselves (Kv1.4-Kv1.2 and Kv1.4-m1Kv1.2) showed similar characteristics to those shown with Kv1.4 alone; they activated rapidly upon depolarization and subsequently inactivated in an expo-
Kv1.4, while the difference in time constants between Kv1.2 were fitted to a single exponential rather than multiple exponentials. Time constants of recovery from inactivation were calculated by fitting curves of fraction recovery. The values given are the means ± S.E. The number of oocytes tested is given in parentheses. Differences of the values between channels were analyzed by analysis of variance and the significance was isolated using Dunnett's test.

\[
\tau_{\text{act}} = k_{\text{act}} f \quad \text{and} \quad \tau_{\text{rec}} = k_{\text{rec}} f
\]

where \( f \) is the fraction recovery and 0 and 1 are the open and inactivated states. \( k_{\text{act}} \) and \( k_{\text{rec}} \) are the rate constant of inactivation and recovery of the microscopic current. For the above reaction model, \( \tau_{\text{inact}} = (k_{\text{inact}})^{-1} \) and \( \tau_{\text{rec}} = (k_{\text{rec}})^{-1} \).

From comparison of the time constants between channels, it is suggested that the recovery rate depends on the composition of the acceptor sites. As the time constants of macroscopic inactivation of the currents were changed upon introducing mutations in the hybrid channels, we estimated, using the measured values in Table I, the microscopic inactivation rate constant according to the equation below in order to gain insight into the inactivation process more precisely. If it is assumed that a channel recovers from inactivated state to open state and then deactivates under the conditions used, the following simple reaction rate model can be drawn.

**TABLE I**

| Channels | \( \tau_{\text{act}} \) (ms) | \( \tau_{\text{rec}} \) (ms) |
|----------|----------------|------------------|
| Kv1.4-Kv1.2 | 78.6 ± 5.3 (16) | 2.7 ± 0.3* (10) |
| Kv1.4-m2Kv1.2 | 110.6 ± 9.4 a,b (11) | 2.0 ± 0.2 a,b (5) |
| Kv1.4-m5Kv1.2 | 127.6 ± 6.5 a,b (8) | 2.3 ± 0.2 a,b (8) |
| Kv1.4-m5Kv1.2 | 182.5 ± 13.8 a,b (6) | 2.0 ± 0.1 a,b (9) |
| Kv1.4 | 71.7 ± 3.4 (10) | 8.1 ± 0.6 (9) |

* p < 0.01 versus Kv1.4.

Substituting the values of \( \tau_{\text{act}} \) and \( \tau_{\text{rec}} \) measured into the equation gives the values of \( k_{\text{act}} \) and \( k_{\text{rec}} \) for individual channels. The ratio of \( k_{\text{act}} \) for Kv1.4-Kv1.2 to Kv1.4 is close to 1. This indicates that the microscopic inactivation rate constant of the wild-type hybrid channel, Kv1.4-Kv1.2, is not different from homomeric Kv1.4 channel even though the number of the inactivation balls of the hybrid channel is half of Kv1.4 channel. When the values of \( k_{\text{act}} \) were compared between the hybrid channels, it became evident that the ratios of \( k_{\text{act}} \) for the mutant hybrid channels to wild-type channels are much less than unit; the ratios of \( k_{\text{act}} \) of Kv1.4-m1Kv1.2, Kv1.4-m2Kv1.2, and Kv1.4-m5Kv1.2 to the wild-type hybrid channel were approximately 0.7, 0.6, and 0.4, respectively. This could be attributed to the difference in the composition of the acceptor sites. Because of the hybrid channels, the same number of inactivation balls.

As inactivation properties of the hybrid channels were modified compared with homotetrameric Kv1.4 channel, the steady-state voltage dependence of their activation and inactivation was studied using two-pulse voltage-clamp protocols to determine the functional properties of those currents. The voltage dependence of normalized peak K+ conductances for the channels is shown in Fig. 4A. The average values of half-activation

**FIG. 3.** Time courses of recovery from inactivation. Recovery from inactivation was determined by recording currents using two-microelectrode voltage-clamp method. A control 400-ms depolarization from a holding potential of -80 mV to +20 mV was given. A second identical depolarization followed each control pulse after an interval of increasing duration at -80 mV. The peak amplitude of the current evoked by the second voltage pulse was expressed as a fraction of the corresponding values obtained by control depolarization. Fraction recovery was plotted as a function of the interpulse interval. Curves of fraction recovery are the means for each channel. Symbols for each channel are as follows: C, Kv1.4; O, Kv1.4-Kv1.2; □, Kv1.4-m1Kv1.2; ■, Kv1.4-m2Kv1.2; ○, Kv1.4-m5Kv1.2.
potential and the slope factor for those channels are summarized in Table II. K+ currents generated by all of the tandem constructs gave values of slope factor that were smaller than the parent Kv1.4 channel. The voltage dependence of steady-state inactivation of the expressed channels is shown in Fig. 4B. The resulting inactivation curves are sigmoidal and can be described by a Boltzmann distribution function described by Perozo et al. (28). Compared with Kv1.4, the voltage dependence of inactivation was shifted to positive membrane potential for all dimeric constructs by 10–13 mV (Table IV). Furthermore, little voltage dependence of inactivation for Kv1.4-m2Kv1.2 and Kv1.4-m5Kv1.2 was observed at membrane potentials more positive than -15 mV (Fig. 4B). A non-inactivating component was hardly observed in homotetrameric Kv1.4 channel, whereas that was evident in the hybrid channels (Table II).

**FIG. 4. Voltage dependence of peak conductance and inactivation for the homotetrameric Kv1.4 and hybrid channels.** A, normalized peak conductance-voltage relations of the channels from Kv1.4 (O), Kv1.4-Kv1.2 (●), Kv1.4-m1Kv1.2 (□), Kv1.4-m2Kv1.2 (■), and Kv1.4-m5Kv1.2 (△). The oocytes were held at -80 mV and depolarized every 30 s by 400-ms pulses to potentials ranging from -70 to +40 mV in 10-mV increments. Peak current amplitudes were measured and then converted into peak conductance (G) by the formula G = I/V - Vm, assuming a reversal potential (Vr) of -85 mV. B, the voltage dependence of steady-state inactivation was plotted as a function of prepulse potential. Oocytes were held at -80 mV and depolarized with a 400-ms prepulse to conditioning voltages ranging from -100 to +20 mV, immediately followed by a 400-ms test pulse to +20 mV. The interval between test pulses was 90 s. The peak currents elicited by the test pulse were normalized to the peak current obtained with conditioning prepulse negative to -80 mV and plotted as a function of prepulse potential. The results in A and B were best described with a Boltzmann distribution function described under "Experimental Procedures." The values of parameters are presented in Table II. Symbols in A and B represent means.

**Table II**

| Parameters of voltage-dependent activation and inactivation for the expressed channels |
|---|---|---|---|---|
|    | Activation |    | Inactivation |    |
|    | V1 | a1 |    | V1 | a1 | α |
| Kv1.4-Kv1.2 | -9.0 ± 1.3 | 13.1 ± 0.4 | (9) | -31.0 ± 0.7 | 5.3 ± 0.1 | 0.118 ± 0.009 | (10) |
| Kv1.4-m1Kv1.2 | -11.1 ± 1.6 | 13.8 ± 0.3 | (7) | -30.2 ± 1.3 | 5.3 ± 0.3 | 0.216 ± 0.016 | (6) |
| Kv1.4-m2Kv1.2 | -9.7 ± 1.2 | 14.3 ± 0.2 | (8) | -33.6 ± 1.9 | 6.4 ± 0.5 | 0.382 ± 0.027 | (8) |
| Kv1.4-m5Kv1.2 | -8.0 ± 0.9 | 15.6 ± 0.4 | (3) | -33.4 ± 1.7 | 5.5 ± 0.4 | 0.623 ± 0.004 | (3) |
| Kv1.4 | -9.9 ± 1.2 | 15.5 ± 0.3 | (9) | -43.5 ± 0.6 | 4.7 ± 0.2 | 0.566 ± 0.004 | (8) |

*p < 0.01 versus Kv1.4.

DISCUSSION

In the present study, we made tandem constructs of inactivating and non-inactivating subunits and examined the underlying mechanism of inactivation observed in the resulting hybrid K+ channels. Isaac et al. (17) have suggested that mRNA of heteromeric tandem dimers induces the formation of heteromultimeric channels in which both subunits of the dimeric construct participate in channel formation. Liman et al. (21) demonstrated that full-length polypeptides were generated by the tetrameric constructs. These lines of evidence support the notion that tandem constructs linking subunits can direct the expression of multimeric channels. The result reported by Heginbotham and MacKinnon (20) also supports that notion. However, in contrast to the results described above, it has been also suggested that the tandem linkage of Shaker subunit does not guarantee the stoichiometry of the expressed channel protein. McCormack et al. (29) demonstrated that a tandem construct gave rise to currents whose kinetics and voltage dependence were indistinguishable from those of the first subunit, and amplitude was not dramatically suppressed when the construct expressed containing non-functional truncated mutant in its second position. They have suggested that the first subunit of a dimeric construct is more likely to be incorporated into the oligomeric channel protein than the second subunit. Results obtained with dimeric constructs containing non-functional mutant of Kv1.2 give support to our conclusion that the dimeric tandem constructs in the present study can direct the expression of heteromultimeric K+ channels. The current amplitudes of those were dramatically suppressed, and their inactivation kinetics was also remarkably changed, as would be expected if the subunits in the second position, which could not express channel by themselves, were assembled stoichiometrically with the first subunit Kv1.4 to produce functional channel. Results of steady-state inactivation and recovery from inactivation further support our conclusion. The reason for the difference between the results of McCormack et al. (29) and ours is unknown. However, they suggested that since in their dimeric constructs the second subunits lack the H5 and other downstream regions, which may be an important determinant in the folding and assembly of subunits into the channel complex, they might be incapable of channel assembly.

It has been demonstrated that inactivation of Shaker K+ channel is caused by interaction of amino-terminal domains, which form tethered "balls," with cytoplasmic face of the channel (15). It is probable that inactivation of the current produced by Kv1.4 is caused by the "ball and chain" mechanism as it has
been suggested that the NH2 terminus of Kv1.4, like Shaker K+ channel, plays important roles in mediating inactivation of the channel (23). In this study, we investigated the relative contribution of the S4-S5 loop of the non-inactivating subunit to inactivation of the hybrid K+ channel. Inactivation kinetics of the mutant hybrid channels, which have a point mutation in the S4-S5 loop of the second subunit, was compared with that of wild-type hybrid channel, Kv1.4-Kv1.2. The time constant for inactivation of macroscopic current increased remarkably upon point mutation of the amino acid (Table I). The microscopic inactivation rate constants, estimated from time constants for inactivation and recovery of macroscopic current, were also increased in the mutant hybrid channels. All of the hybrid channels have two inactivation balls as the dimeric tandem constructs direct the expression of heterotetrameric channels as discussed above. Therefore, the remarkable slowing of inactivation should be attributed to the differences in the characteristics and/or the number of the putative acceptor regions for inactivation subunit. Comparison of the decay constant of inactivation for Kv1.4-Kv1.2 and Kv1.4-m1Kv1.2 suggests that the S4-S5 loop of the second subunit participates in inactivation of the evoked K+ currents. The ratio of microscopic inactivation rate constant ($k_{on}$) of Kv1.4-m1Kv1.2 to Kv1.4-Kv1.2 is estimated as approximately 0.7, suggesting that substitution of a single amino acid in that region of the second subunit could lead to a change in the structure and alter the accessibility of the inactivation balls to the acceptor sites. In the hybrid channels of which the second subunit is m2 or m5Kv1.2, the S4-S5 loop from the subunit might not work as acceptors due to unusual folding of the encoded peptide. So, the number of inactivation balls of the hybrid channel is considered to be much lower than that of wild-type hybrid channel, Kv1.4-Kv1.2 (22) have reported that in the homomeric Shaker B channel the inactivation rate constant is dependent on the number of inactivation balls. If the inactivation process were determined only by the number of the balls, the hybrid channel Kv1.4-Kv1.2 would show much slower inactivation kinetics than Kv1.4 channel. Therefore, the difference of the composition of the acceptor site between the hybrid and homomeric K+ channel could explain the above observation. While two acceptor sites derivadae from the inactivating subunits themselves, the counterparts of the non-inactivating subunits also participate in forming the acceptor for inactivation balls as discussed above. There are differences in two amino acid residues in the S4-S5 loop between Kv1.4 and Kv1.2 (Fig. 1). These differences could result in an alteration of binding interaction of inactivation balls of the hybrid K+ channel and confer fast inactivation on the hybrid channel similar to that of homomeric Kv1.4 channel. In fact, in our study, $k_{on}$ of wild-type hybrid channel was increased by 3-fold compared with Kv1.4 homotetrameric channel, indicating decreases in affinity of inactivation balls to the acceptor sites. Increase of $k_{on}$ of wild-type hybrid channel, in turn, contributes to an alteration of macroscopic inactivation of the channel. This suggests that an alteration of inactivation could be induced by even a single amino acid change in the putative acceptor region through apparent change in the three-dimensional structure of the cytoplasmic face of the channel.

As recent reports on 4-AP and TEA binding to K+ channels suggest that other regions, including S6 segment, may participate in forming the mouth of the pore (30–32), the inactivation balls might interact with those regions as one of acceptor sites. Additionally, structural differences in those regions between constituent subunits might determine the characteristics of inactivation observed in the channels. These problems should be addressed by further studies.

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