Changes in the Inactivation of Rat Kv1.4 K⁺ Channels Induced by Varying the Number of Inactivation Particles*

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Yoshihiro Hashimoto‡, Kazuo Nunoki§, Hironori Kudo, Kuniaki Ishii¶, Norio Taira¶, and Teruyuki Yanagisawa

From the Department of Pharmacology, Tohoku University School of Medicine, 2-1 Seiryomachi Aobaku, Sendai 980-8575, Japan and ¶Department of Pharmacology, Yamagata University School of Medicine, 2-2-2 Ida-nishi, Yamagata 990-8585, Japan

N-type inactivation of rat Kv1.4 channels with one, two, or four inactivation balls was investigated using homogeneous populations of channels expressed in *Xenopus* oocytes. Tandem dimeric and tetrameric constructs of Kv1.4 were made. Channels encoded by tandem cDNAs Kv1.4-Kv1.4Δ1–145 and Kv1.4-Kv1.4Δ1–145] have two or only one tethered inactivation ball, respectively, whereas Kv1.4 itself encodes channels having four inactivation balls. The time constants for inactivation of macroscopic currents were increased significantly as the number of inactivation balls was decreased, whereas the time constants for recovery from inactivation were not modified. The ratios of the rate constants of inactivation (k_inact) of Kv1.4-Kv1.4Δ1–145 and Kv1.4-Kv1.4Δ1–145] channels to that of the Kv1.4 channel were 0.65 and 0.4, respectively, whereas the ratio of the rate constant of recovery (k_rec) of these two constructs to that of Kv1.4 were almost unity. The rate constants k_inact for channels having two and four inactivation balls are smaller than those that would be expected if inactivation balls on each channel are independent, suggesting some interaction occurs between inactivation balls. Furthermore, noninactivating current became apparent as the number of inactivation balls on a channel was decreased.

Isolation of the clone encoding the *Shaker* K⁺ channel from *Drosophila* has made it possible to examine the molecular mechanism of N-type inactivation of A-type K⁺ channels. The *Shaker* K⁺ channel provided the first example of “ball and chain”-type inactivation, which was originally proposed for voltage-dependent Na⁺ channels (1). The mechanisms of fast inactivation observed in mammalian homologues of *Shaker* K⁺ channels have been proved to be the same as that of *Shaker* K⁺ channels (2–5). K⁺ channels are composed of four α-subunits (6–12), each of which is encoded by a K⁺ channel gene. Functional K⁺ channels are also formed by the assembly of different α subunits encoded by different genes within the same K⁺ channel subfamily (13–19).

In a previous study (20), we made dimeric cDNA by tandem linkage of the rapidly inactivating K⁺ channel clone Kv1.4 and the noninactivating K⁺ channel clone Kv1.2 isolated from rat heart. The dimeric cDNA encodes a hybrid channel that is composed of Kv1.4 and Kv1.2 subunits with 1:1 stoichiometry. In the hybrid channel there are two inactivation balls tethered to Kv1.4 subunits. This approach revealed that in the hybrid channel inactivation is determined not only by inactivation balls but also by the composition of the pore-forming α-subunits, of which intracellular loops form the receptor sites for inactivation. It is, however, inappropriate to use hybrid channels to examine the relationship between inactivation kinetics and the number of inactivation balls, because the heterogeneity of the receptors for inactivation has, in itself, a certain effect on inactivation kinetics.

In this study we investigated how inactivation kinetics are regulated by inactivation balls in rat Kv1.4 channels. For this purpose we made tandem dimeric and tetrameric cDNAs from a Kv1.4 clone, and homogeneous populations of K⁺ channels with one, two, or four inactivation balls were expressed in *Xenopus* oocytes. The ratio of the inactivation rate constant of the channel with one or two inactivation balls to the Kv1.4 channel with four balls was larger than expected. This suggests that in rat Kv1.4 channels some interaction may exist between inactivation balls although previous studies in *Shaker* K⁺ channels have suggested that inactivation balls are independent (21, 22).

EXPERIMENTAL PROCEDURES

Dimeric cDNA Construction—Two different dimeric cDNA constructs were generated from the cDNA clone, Kv1.4 isolated from rat cardiac muscle (23). One was generated by tandem linkage of two Kv1.4 sequences and another by linking the 3’ end of the first Kv1.4 to the 5’ end-deleted Kv1.4. 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 recognized by SpeI, which replaced the stop codon nucleotides 1963–1965. PCR amplification was performed to generate the BstEII (nucleotide 1560)/SpeI fragment (fragment 1). Fragment I was ligated back into Kv1.4 in pBluescript vector (Stratagene, La Jolla, CA, USA) (intermediate construct). For the second position of the tandem dimers, Kv1.4 cDNA was altered at the 5’ end or nucleotide 436G (N-terminal end of assembly domain) by adding the SpeI restriction site. The SpeI/MluI (nucleotide 531) fragments were amplified by PCR (fragments II and II’). Fragments II and II’ and the MluI/NotI fragment from Kv1.4 in pBluescript were digested into the corresponding sites of the 3’ end-modified intermediate construct digested with SpeI and NotI. The tandem dimers generated in this manner were Kv1.4-Kv1.4 (full dimer) and Kv1.4-Kv1.4Δ1–145 (partial dimer). The procedure for constructing tandem dimeric cDNA, Kv1.4-Kv1.4 was described in detail elsewhere (20).

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§ To whom correspondence should be addressed: Dept. of Pharmacology, Tohoku University School of Medicine, 2-1 Seiryomachi Aobaku, Sendai 980-8575, Japan. Tel.: 81-22-717-8063; Fax: 81-22-717-8065; E-mail: nu09-23@mail.cc.tohoku.ac.jp.

† Present address: Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 2-2-50, Kawagishi, Toda 335-8505, Japan.

‡ Present address: Kohnan Hospital, 4-20-1 Nagamachiminami, Taihakuku, Sendai 982-0012, Japan.

1 The abbreviation used is: PCR, polymerase chain reaction.
**RESULTS**

The schematic diagrams of the polypeptides encoded by tandem dimeric and tetrameric Kv1.4 cDNAs are shown in Fig. 1A. Polypeptide (Kv1.4-Kv1.4) encoded by the full tandem dimeric cDNA has amino acid sequence corresponding to ball and chain region in the second (3′) subunit as well as in the first subunit. On the other hand, polypeptide (Kv1.4-Kv1.4Δ1–145) from the partial tandem dimeric cDNA lacks amino acid sequence corresponding to ball and chain region in the second subunit. Channels formed by the partial tandem polypeptide of Kv1.4 have two tethered inactivation balls, whereas the full tandem dimeric cDNA encodes channels with two tethered inactivation balls and two positioned in the loops connecting the subunits (Fig. 1B, b and c). Because the polypeptide encoded by the tandem tetrameric cDNA lacks amino acid sequences preceding the assembly domain in each subunit except the first one, there is only one inactivation ball per channel (Fig. 1B, d).

The currents observed when cRNAs of the tandem dimeric and tetrameric constructs were injected into the oocytes showed similar characteristics to Kv1.4 (Fig. 2, B–E). The current-voltage relationships of Kv1.4-Kv1.4, Kv1.4-Kv1.4Δ1–145, Kv1.4-[Kv1.4Δ1–145]3, and Kv1.4-Kv1.2 (hybrid) channels were quite similar to Kv1.4. However, the currents generated by Kv1.4-Kv1.4, Kv1.4-Kv1.4Δ1–145, and Kv1.4-[Kv1.4Δ1–145]3 inactivated more slowly than Kv1.4. A comparison of the current decay for Kv1.4, Kv1.4-Kv1.4, Kv1.4-Kv1.4Δ1–145, Kv1.4-[Kv1.4Δ1–145]3, and Kv1.4-Kv1.2 at a potential of +20 mV is shown in Fig. 3. Time constants \( \tau_{\text{inact}} \) for test pulses to +20 mV are presented in Table I. The time constants of inactivation for Kv1.4-Kv1.4Δ1–145 and Kv1.4-[Kv1.4Δ1–145]3 were significantly increased compared with Kv1.4, whereas the difference in time constants between Kv1.4 and Kv1.4-Kv1.4 was not statistically significant. The time constant for Kv1.4-Kv1.2 did not differ from that for Kv1.4 as described previously (20) (Table I). In addition to the slowing of current decay, inactivation became less complete as the number of inactivation balls on a channel was decreased. It was estimated from the exponential fits of current decay that noninactivating currents generated by Kv1.4, Kv1.4-Kv1.4, and Kv1.4-[Kv1.4Δ1–145]3 were between Kv1.4 channels with different numbers of inactivation balls as described previously (20). We calculated the values of...
uted to the difference in the composition and the structure of 
smaller than that of the hybrid channel. This could be attrib-
utes to a difference in the number of inactivation balls. 

and Kv1.4-Kv1.2, respectively. Oocytes were held at -80 
voltage clamp from injected Xenopus oocytes. A, B, C, D, 
Kv1.4-Kv1.4 channel was smaller than that of the Kv1.4 chan-
E, Kv1.4-[Kv1.4–145]3, and Kv1.4-Kv1.2 were 3.7
Kv1.4-D1–145, Kv1.4-Kv1.4 channel was smaller than that of the 
Kat 0.4 (n = 30), and 5.8 ± 1.2 (n = 8) μA, respectively. Records were obtained 3–6 days after injection of oocytes with cRNAs in vitro transcribed from respective cDNAs. Calibration bars are 100 ms and 1 μA.

k_{inact} only from the oocytes in which both the time constants for inactivation and recovery from inactivation were measured (Table II). The ratio of k_{inact} for Kv1.4-Kv1.4D1–145 to Kv1.4 is 0.65. This indicates that the microscopic inactivation rate constant of the channel with two inactivation balls is half that of the channel with only one inactivation ball. Furthermore, the ratio of k_{inact} for Kv1.4-[Kv1.4–145]3 to Kv1.4 is 0.4, indicating that the microscopic inactivation rate constant of the channel with only one inactivation ball is larger than one-fourth that of Kv1.4 channel. The k_{inact} value of the Kv1.4-Kv1.4 channel was smaller than that of the hybrid channel, although both types of channels have the same number of inactivation balls. When the values of k_{inact} were compared between Kv1.4-Kv1.4D1–145 and Kv1.4-Kv1.2 channels, it became evident that k_{inact} for the Kv1.4-Kv1.4D1–145 channel is smaller than that of the hybrid channel. This could be attributed to the difference in the composition and the structure of the receptor sites for inactivation balls, because Kv1.4-Kv1.4D1–145 and the hybrid channels have the same number of inactivation balls.

To further characterize the properties of the channels encoded by the dimeric and tetrameric cDNAs, the steady-state voltage dependence of their activation and inactivation was studied. The average values of the parameters are summarized in Table III. There were no differences between Kv1.4 channels with different numbers of inactivation balls except in the noninactivating component. A noninactivating component increased as the number of inactivation balls on each channel was decreased.

**DISCUSSION**

Inactivation of Kv1.4 channel is caused by occlusion of the intracellular mouth of the pore by the inactivation ball located...
in the N terminus of the polypeptide as in the *Shaker* K⁺ channel (2, 5). The ball binds to its receptor via electrostatic and hydrophobic interactions. As polypeptides of the principle subunit of voltage-gated K⁺ channels form tetramers (6–9), it is evident that Kv1.4 has four inactivation balls on each channel when expressed alone.

Relationship between inactivation balls and rate of inactivation has been examined using *Shaker* B channel. To investigate the relationship, MacKinnon et al. (21) studied channels carrying a specific mutation in a single subunit quantitatively by monitoring scorpion toxin sensitivity. On the other hand, Gomez-Lagunas and Armstrong (22) used the approach of gradually removing inactivation with intracellular papain. However, these analytical procedures were based on the assumption that co-assembly of wild-type and mutated subunits or the predicted fraction of channels with various numbers of inactivation balls after protease treatment obeys the rules of a binomial distribution. In this study we have investigated the role of inactivation balls in the rate of inactivation by use of a different strategy than these reports. This approach seems to have the advantage that interpretation of the results does not need any assumptions about subunit stoichiometry. To constrain the number of inactivation balls in the expressed channels, dimeric and tetrameric cDNAs were constructed from rat cDNA clone Kv1.4, which encode two or four Kv1.4 subunits on a single polypeptide chain (Fig. 1A). It has been demonstrated that two polypeptides encoded by the dimeric cDNA construct can assemble to form functional channels with tetrameric structure (7, 11, 18, 24, 25). For K⁺ channels, tetrameric cDNA constructs also encode functional channels resembling, in their electrophysiological properties, those expressed from monomers (7, 11, 24). Therefore, it is probable that the channels have two or only one inactivation ball when the partial tandem dimeric or tetrameric cDNAs of Kv1.4 are expressed in *Xenopus* oocytes.

The voltage dependence of activation and inactivation of the channels with different numbers of inactivation balls is similar, suggesting that alterations in inactivation should be attributed to the difference in the number of inactivation balls. We have demonstrated directly that the channels that have only one ball can inactivate, which is consistent with the results with *Shaker* K⁺ channels described by MacKinnon et al. (21). However, inactivation of the current from the channel with only one ball was incomplete in our experimental conditions, compared with the channel with four balls. Increases in the noninactivating currents may be due to less frequent occlusion of the pore by a smaller number of particles. Transitions between open and closed states before entering the inactivated state might be another possibility.

The ratios of the inactivation rate constants from the channel with one and two balls were larger than one-fourth and one-half that of the channel with four balls, respectively. MacKinnon et al. (21) described that in *Shaker* K⁺ channels the rate constant for the channel with a single gate was one-fourth that of channels with four gates and suggested independence of each inactivation gate. We considered the following possibilities that might account for the difference. First, there may be some interaction between inactivation balls: electrostatic repulsion between balls due to positive charges on their surfaces could decrease access of inactivation balls to their receptor sites when a channel has more than a single ball. Second, slow recovery of Kv1.4 may result in larger rate constants than expected for the channels with one and two inactivation balls. Recovery from inactivation of Kv1.4 is much slower than that of the *Shaker* K⁺ channel. Kv1.4 has more positive charges than the *Shaker* K⁺ channel in its ball structure, which could result in a higher affinity of the inactivation ball for its receptor.

There is also another possibility that might arise from the experimental strategy. Structural changes induced by linkage of the subunits in a single polypeptide chain might alter accessibility of inactivation balls to their receptor sites.

The channels that have two tethered balls and two in the connecting loop showed an inactivation rate constant that was intermediate between channels with four tethered balls and those with two balls. This indicates that the ball structure positioned in the loop also functions as an inactivation gate, but

![Fig. 4. Time courses of recovery from inactivation.](http://www.jbc.org/)

Recovery from inactivation was determined by recording currents using the two-microelectrode voltage-clamp method. A control 400-ms depolarization from holding potential of −80 mV to +20 mV was given. Each control pulse was followed by a second identical depolarization after an interval of increasing duration from 0.3 to 30 s at −80 mV. The peak amplitude of the current evoked by the second depolarization was expressed as a fraction of the values obtained by the control depolarization. Fractional recovery was plotted as a function of the interpulse interval. Data points are means ± S.E. and were fitted by a single exponential. Time constants of recovery from inactivation for each channel are presented in Table I. The symbols used are as follows: ○, Kv1.4; □, Kv1.4-Kv1.4; △, Kv1.4-Kv1.4Δ1–145; ◇, Kv1.4-[Kv1.4Δ1–145]₂; and ●, Kv1.4-Kv1.2.

**Table II**

| Rate constants of inactivation and recovery from inactivation of the expressed channels |
|------------------------------------------|
| h<sub>inact</sub> Ratio to Kv1.4 | h<sub>rec</sub> Ratio to Kv1.4 |
| s<sup>–1</sup> | s<sup>–1</sup> |
|------------------|------------------|
| Kv1.4             | 16.8 ± 0.9 (24)  | 0.12 ± 0.01 (24) |
| Kv1.4-Kv1.4       | 13.2 ± 1.2<sup>a</sup> (13) | 0.78 ± 0.01 (13) | 1.00 |
| Kv1.4-Kv1.4Δ1–145 | 11.9 ± 0.5<sup>a</sup> (18) | 0.65 ± 0.01 (18) | 1.17 |
| Kv1.4-[Kv1.4Δ1–145]₂ | 6.7 ± 0.2<sup>a</sup> (20) | 0.40 ± 0.01 (20) | 1.10 |
| Kv1.4-Kv1.2       | 12.8 ± 1.4<sup>a</sup> (7) | 0.75 ± 0.005 (7) | 4.68 |

<sup>a</sup> p < 0.01.

<sup>b</sup> p < 0.001 versus Kv1.4.
its access to the receptor was decreased due to restricted movement.

In this study we have isolated the current of Kv1.4 channel with a single or two inactivation balls for the first time by expressing homogeneous populations of channels in *Xenopus* oocytes. It was demonstrated that rat Kv1.4 channel could inactivate even with only a single inactivation ball, although inactivation was not complete. The calculated microscopic rate constants for inactivation of the channels with a single and two inactivation balls were larger than would be expected if inactivation balls were independent, suggesting the possibility of some interaction between inactivation balls. Single channel analysis will provide unequivocal estimation of differences in microscopic rate constants of inactivation and the mechanism underlying the noninactivating fraction of the currents.

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**TABLE III**

Parameters of steady-state activation and inactivation for the expressed channels

| Channel | Activity | Inactivation |
|---------|----------|-------------|
|         | $V_a$    | $a_a$       | $V_h$    | $a_h$ |
| Kv1.4   | –12.2 ± 1.6 | 13.9 ± 0.5 (15) | –36.2 ± 1.1 | 5.9 ± 0.5 (15) |
| Kv1.4-Kv1.4 | –10.2 ± 2.4 | 13.9 ± 0.4 (17) | –34.6 ± 1.5 | 5.2 ± 0.2 (17) |
| Kv1.4-[Kv1.4](1–145) | –8.4 ± 1.3 | 13.9 ± 0.5 (14) | –32.9 ± 1.5 | 5.7 ± 0.3 (14) |
| Kv1.4-[Kv1.4](1–145) | –12.7 ± 1.5 | 14.3 ± 0.3 (18) | –38.5 ± 0.9 | 6.2 ± 0.2 (18) |
| Kv1.4-Kv1.4 | –8.9 ± 1.2 | 13.3 ± 0.3 (8) | –26.2 ± 1.7$^a$ | 6.9 ± 0.6 (8) |

$^a p < 0.001$ versus Kv1.4.

$^b p < 0.01$. 

$V_a$ and $V_h$ are half-activation and -inactivation voltages, respectively; $a_a$ and $a_h$ are slope factors for activation and inactivation, respectively; $\alpha$ is the noninactivating component. The values given are mean ± S.E. The number of oocytes tested is given in the parentheses. Differences of the values between channels were analyzed as in Table I.
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