A Mammalian Transient Type K⁺ Channel, Rat Kv1.4, Has Two Potential Domains That Could Produce Rapid Inactivation*  

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The “ball and chain” model has been shown to be suitable for explaining the rapid inactivation of voltage-dependent K⁺ channels. For the Drosophila Shaker K⁺ channel (ShB), the first 20 residues of the amino terminus have been identified as the inactivation ball that binds to the open channel pore and blocks ion flow (Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1990) Science 250, 533–538; Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990) Science 250, 568–571). We studied the structural elements responsible for rapid inactivation of a mammalian transient type K⁺ channel (rat Kv1.4) by constructing various mutants in the amino terminus and expressing them in Xenopus oocytes. Although it has been reported that the initial 37 residues might form the inactivation ball for rat Kv1.4 (Tseng-Crank, J., Yao, J.-A., Berman M. F., and Tseng, G.-N. (1993) J. Gen. Physiol. 102, 1057–1083), we found that not only the initial 37 residues, but also the following region, residues 40–68, could function independently as an inactivation gate. Like the Shaker inactivation ball, both potential inactivation domains have a hydrophobic amino-terminal region and a hydrophilic carboxyl-terminal region having net positive charge, which is essential for the domains to function as an inactivation gate.

Aldrich and co-workers have shown that a “ball and chain” model, originally proposed for Na⁺ channel inactivation (4), can also explain the rapid inactivation of a Drosophila Shaker K⁺ channel (1, 2). The amino-terminal domain (ball) tethered by the adjacent region (chain) to the channel protein binds to the channel pore after channel activation and blocks ion flow. In the Shaker K⁺ channel (ShB), the initial 20 amino acids have been identified as the inactivation ball. The following region preceding the assembly domain (5) has been identified as the chain tethering the ball to the channel (1). The 20-amino acid inactivation ball is composed of the 11 amino-terminal hydrophobic residues and the following 9 hydrophilic residues containing net positive charge. Both the hydrophobic stretch and the charged region are thought to be involved in the binding of the ball to its receptor via hydrophobic and electrostatic interactions. In contrast, in mammalian transient type K⁺ channels, the ball and chain structure had not been well defined, although it had been shown that deletion of various lengths from the amino-terminal region of Kv1.4 disrupted rapid inactivation suggesting the presence of a “ball” structure (6, 7). Tseng and co-workers (3) have studied this issue in more detail by deleting different domains in the amino-terminal region of rat Kv1.4. They have not identified “chain” structure but have shown that deletion of the amino-terminal hydrophobic domain, residues 3–25, resulted in loss of rapid inactivation. Deletion of the following hydrophilic region containing five positive and two negative charges, residues 26–37, greatly attenuated inactivation. Based on these and other findings, they suggested that the amino terminus of rat Kv1.4 might be similar to that of ShB in having one inactivation ball, which is composed of the initial 37 residues. In the present study, we investigated the structural elements responsible for rapid inactivation of rat Kv1.4 and have identified another domain that can produce rapid inactivation independently of the proposed inactivation ball.

EXPERIMENTAL PROCEDURES

In Vitro Mutagenesis—Fig. 1 shows the amino-terminal sequences of Kv1.4 and the mutants investigated in this study. Eleven deletion mutants and one addition mutant were made in the amino-terminal region of Kv1.4. In addition, one mutant in which amino acid residues 40–68 of Kv1.4 were inverted in Δ2–38 & Δ29–162 was constructed. Fragments for all the mutants except the one with inverted residues were generated by polymerase chain reaction (PCR). The 20–22-base pair sense primers used for generating Δ2–25, Δ2–26, Δ2–28, Δ2–30, Δ2–32, Δ2–39, and Δ2–61 corresponded to the appropriate region in Kv1.4 and contained an Apal site, unique within the multiple cloning site of the vector pBluescript II, and an ATG at the 5′-end. The antisense primer (AS1) complementary to nucleotides (nt) 532–551 of Kv1.4 was used for the above seven mutants. The sense primer used for generating Δ29–162 corresponded to nucleotides 487–506 of Kv1.4 and contained a Xhol site (which is unique in Kv1.4 at nt 80) at the 5′-end; the antisense primer was AS1. For constructing Δ2–39 & Δ69–162, the sense primer (S1), with an Apal site at the 5′-terminus and corresponding to nucleotides −35 to −16 of Kv1.4, was used with an antisense primer complementary to nucleotides 180–199 with a Xhol site at its 5′-end. To generate Δ38–162 and Δ2–39 & Δ61–162, two fragments, amplified by PCR, were ligated into the mutants. The upstream fragment for each mutant was designated fragment I; the downstream fragment was fragment II. Fragment II for both mutants was the same and corresponded to amino acid residues 163–185 of Kv1.4. The sense primer for fragment I of Δ38–162 was S1, and that of Δ2–39 & Δ61–162 was the same one used for Δ2–39. The antisense primer for the fragment I contained a Stvu site at the 5′-end and corresponded to nucleotides 96–114 for Δ38–162 and to nucleotides 161–181 for Δ2–39 & Δ61–162. The sense primer for fragment II corresponded to nucleotides 490–508 and contained a Smal site at the 5′-end; the antisense primer was AS1. Amino acid residues 26–39 of Kv1.4 were added to the amino terminus of Kv1.4 in the addition mutant. To make the addition mu-

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1 The abbreviations used are: PCR, polymerase chain reaction; nt, nucleotide(s).
Fig. 1. Diagram of the amino-terminal sequences of the mutants and the time constants for inactivation and recovery from inactivation of their currents. The first 190 amino acid residues of wild type Kv1.4 are shown at the top. Open circle shows positively charged amino acid residues, and closed circle shows negatively charged amino acid residues. Deleted regions in the mutants are shown by solid bars. Time constants for inactivation ($\tau_{\text{inact}}$) and recovery from inactivation ($\tau_{\text{rec}}$) of the currents are shown on the right. The mutants whose currents did not rapidly inactivate during a 400-ms pulse are shown by (−) in the column of $\tau_{\text{inact}}$ and $\tau_{\text{rec}}$. The values are expressed as the mean ± S.E. Differences between values were analyzed using Student’s unpaired t test.

Oocytes expressing Kv1.4 and all the mutant channels showed voltage-dependent outward currents upon depolarization (data not shown). They were held at −80 mV and depolarized to test potentials.

**RESULTS**

Oocytes expressing Kv1.4 and all the mutant channels showed voltage-dependent outward currents upon depolarization (data not shown). They were held at −80 mV and depolarized to test potentials.

**Presence of Two Potential Inactivation Balls**—Fig. 2A (upper panel) shows normalized currents of Δ2–28 and Kv1.4 recorded using a depolarizing pulse to +20 mV for 400 ms. The traces are superimposed to illustrate the differences in their wave forms. The peak current of Kv1.4 and Δ2–28 at +20 mV was 2.05 ± 0.35 μA (n = 7) and 4.96 ± 0.54 μA (n = 5), respectively. The Δ2–28 current showed little decline during the 400-ms test pulse, while the Kv1.4 current inactivated almost completely. $\tau_{\text{inact}}$ of Δ2–28 current was measured using a prolonged depolarization pulse (5000 ms). The $\tau_{\text{inact}}$ was 2090.9 ± 647.9 ms (n = 5), which was about 90 times larger than that of Kv1.4. The inactivation of Δ2–28 seems to be qualitatively different from that of Kv1.4. Since it has been shown for Shaker K⁺ channel and RHK1 (rat Kv1.4) that elevating [K⁺]o can accelerate the recovery rate (11, 12), we investigated the effects of

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Effects of Deleting Positive Charges—Since both potential inactivation balls contain net positive charge in the carboxyl termini, we constructed mutants in which some positive charges were removed to examine the contribution of charge to rapid inactivation. Δ2–69 was constructed to delete one net positive charge (3 arginine and 2 glutamic acid residues) from the first potential inactivation ball. Δ2–39 & Δ61–162 was constructed to delete one positive charge (arginine) from the second potential inactivation ball. Fig. 4A (upper panel) shows the normalized Δ29–162 current superimposed on the Δ38–162 current recorded at +20 mV. The peak current of Δ29–162 at +20 mV was 1.80 ± 0.54 μA (n = 4). Inactivation of the Δ29–162 current was much slower than that of Δ38–162. The τ_inact of Δ29–162 was 361.09 ± 37.69 ms (n = 4), which is about 9 times larger than that of Δ38–162 (Fig. 1). The τ_rec of Δ29–162 (1.55 ± 0.16 s at 2 mM [K+]o and 0.63 ± 0.10 s at 20 mM [K+]o) was significantly smaller than that of Δ38–162 (3.94 ± 0.32 s at 2 mM [K+]o and 1.53 ± 0.08 s at 20 mM [K+]o) (p < 0.01). Fig. 4A (lower panel) shows the effects of elevating [K+]o on the recovery time course of Δ29–162. Currents of Δ2–39 & Δ61–162 and Δ2–39 & Δ69–162 recorded at +20 mV are normalized and superimposed in Fig. 4B (upper panel). The peak current of Δ2–39 & Δ61–162 at +20 mV was 1.41 ± 0.20 μA (n = 7). The τ_inact of Δ2–39 & Δ61–162 was 59.22 ± 1.77 ms (n = 7), which is 5 times larger than that of Δ2–39 & Δ69–162 (Fig. 1). Recovery from inactivation was much faster in Δ2–39 & Δ61–162 than in Δ2–39 & Δ69–162 (Fig. 1). The τ_rec of Δ2–39 & Δ61–162 and Δ2–39 & Δ69–162 at 2 mM [K+]o were 2.85 ± 0.31 s and 20.83 ± 2.46 s, respectively, and those at 20 mM [K+]o were 1.74 ± 0.12 s and 15.13 ± 1.07 s, respectively. Fig. 4B (lower panel) shows the effects of elevating [K+]o on the τ_rec of Δ2–39 & Δ61–162.

Effects of Adding Positive Charges—When the structures of Δ2–28 and Δ2–39 are compared, they differ by a single net positive charge (3 arginine and 2 glutamic acid residues) at the amino terminus of the second potential inactivation ball. However, the currents of the two channels were completely different.
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t as Kv1.4 (400 ms) (Fig. 5A). These mutants showed little inactivation on the same time scale as Kv1.4 (400 ms) (Fig. 5A, lower panel) and no significant differences in \( \tau_{\text{inact}} \) measured during a test pulse of 5000 ms (data not shown).

We also constructed a mutant ((+)Kv1.4) in which residues 26–39 of Kv1.4 containing three net positive charges were added to the amino-terminal end of Kv1.4. Adding positive charges greatly reduced the rate of inactivation (Fig. 5B, upper panel). The \( \tau_{\text{inact}} \) of (+)Kv1.4 was 155.9 ± 15.83 ms \((n = 6)\), which was significantly larger than that of Kv1.4 (23.55 ± 3.48 ms; \(n = 7; p < 0.01\)) (Fig. 1). The \( \tau_{\text{rec}} \) of (+)Kv1.4 at 2 mM [K\(^+\)]\(_k\) (7.10 ± 1.57 s) was significantly larger than that at 20 mM [K\(^+\)]\(_k\) (3.41 ± 0.45 s; \(p < 0.05; n = 6\)) (Fig. 5B, lower panel).

Effects of Inverting the Amino Acid Sequence of the Second Potential Ball—We constructed a mutant in which the second potential inactivation ball was inverted (Inv(40–68)). The inverted ball has positive charge at the amino terminus and a hydrophobic region at the carboxyl terminus. This mutant showed little inactivation during a 400-ms test pulse to +20 mV, whereas the parent mutant showed rapid inactivation (Fig. 5C, upper panel). The \( \tau_{\text{inact}} \) of Inv(40–68) recorded during a 5000-ms pulse was 2616.3 ± 252.8 ms \((n = 5)\). Inverting the second potential ball resulted in the loss of rapid inactivation. There were no differences in \( \tau_{\text{rec}} \) of the mutant between 2 mM [K\(^+\)]\(_k\) (4.74 ± 0.16 s) and 20 mM [K\(^+\)]\(_k\) (4.88 ± 0.48 s; \(n = 5\)) (Fig. 5C, lower panel).

DISCUSSION

We found that there are two potential inactivation balls in the amino-terminal region of rat Kv1.4. Deletion of amino acids 2–28 resulted in loss of rapid inactivation. Surprisingly, deletion of 11 more residues resulted in reappearance of rapid inactivation even though the Δ2–39 mutant did not have the core hydrophobic region of the inactivation ball. With further deletion of residues 40–61, rapid inactivation disappeared again. It seems probable that besides the inactivation ball proposed by Tseng and co-workers (the initial 37 residues), there exists a second potential inactivation ball having residues 40–61 as an essential domain. To confirm the presence of two potential balls, we made deletion mutants that had only one potential ball and lacked most of the amino-terminal region preceding the assembly domain (Δ38–162 and Δ2–39 & Δ69–162). As expected, the currents of both the mutants showed rapid inactivation, which indicated that the two potential ball, residues 2–37 and residues 40–68, respectively, could produce rapid inactivation independently. Comparison of Δ38–162 and Δ2–39 & Δ69–162, both of which lack most of the possible chain region, gave some information about the characteristic differences between the first and the second ball. In the case of the second ball (Δ2–39 & Δ69–162), inactivation was more rapid and the recovery from inactivation was slower than in the case of the first ball (Δ38–162) (Figs. 1 and 3). This suggests that the second ball may have a higher affinity for the receptor than the first ball. Compared with Kv1.4, binding between the ball and the receptor seems to be much stronger for the second ball than for the ball in wild type Kv1.4, as Δ2–39 currents recover significantly more slowly than Kv1.4 currents (Fig. 1). Among the mutants investigated, the ones that have the second ball recovered from inactivation most slowly (Δ2–39 and Δ2–39 & Δ69–162). The recovery rates of their currents were significantly slower than those of the other mutants. The Δ2–39 & Δ69–162 currents recovered faster than Δ2–39 currents, probably reflecting the influence of the chain region on binding of the ball to the receptor. The presence of residues 69–162 caused slowing of the recovery from inactivation of the Δ2–39 current.

Similar to the structure of ShB inactivation ball, the two potential balls in Kv1.4 have an amino-terminal hydrophobic deletion of residues 3–25 disrupted rapid inactivation. Surprisingly, deletion of 11 more residues resulted in reappearance of rapid inactivation even though the Δ2–39 mutant did not have the core hydrophobic region of the inactivation ball. With further deletion of residues 40–61, rapid inactivation disappeared again. It seems probable that besides the inactivation ball proposed by Tseng and co-workers (the initial 37 residues), there exists a second potential inactivation ball having residues 40–61 as an essential domain. To confirm the presence of two potential balls, we made deletion mutants that had only one potential ball and lacked most of the amino-terminal region preceding the assembly domain (Δ38–162 and Δ2–39 & Δ69–162). As expected, the currents of both the mutants showed rapid inactivation, which indicated that the two potential ball, residues 2–37 and residues 40–68, respectively, could produce rapid inactivation independently. Comparison of Δ38–162 and Δ2–39 & Δ69–162, both of which lack most of the possible chain region, gave some information about the characteristic differences between the first and the second ball. In the case of the second ball (Δ2–39 & Δ69–162), inactivation was more rapid and the recovery from inactivation was slower than in the case of the first ball (Δ38–162) (Figs. 1 and 3). This suggests that the second ball may have a higher affinity for the receptor than the first ball. Compared with Kv1.4, binding between the ball and the receptor seems to be much stronger for the second ball than for the ball in wild type Kv1.4, as Δ2–39 currents recover significantly more slowly than Kv1.4 currents (Fig. 1). Among the mutants investigated, the ones that have the second ball recovered from inactivation most slowly (Δ2–39 and Δ2–39 & Δ69–162). The recovery rates of their currents were significantly slower than those of the other mutants. The Δ2–39 & Δ69–162 currents recovered faster than Δ2–39 currents, probably reflecting the influence of the chain region on binding of the ball to the receptor. The presence of residues 69–162 caused slowing of the recovery from inactivation of the Δ2–39 current.

Similar to the structure of ShB inactivation ball, the two potential balls in Kv1.4 have an amino-terminal hydrophobic
region and a carboxyl-terminal hydrophilic region containing net positive charge, which is thought to be involved in the binding of the inactivation particle to its receptor via electrostatic interactions (2, 13, 14). Therefore we investigated the contribution of positive charge. Deletion of positive charge from either ball greatly attenuated the inactivation rates and accelerated the recovery rates, which probably reflects the higher affinity of the ball to the receptor site with the carboxyl-terminal positive charge. This result clearly indicates that the positive charge at carboxyl-terminal region of the ball plays an important role. The structural requirements for the inactivation ball were further studied by deleting or adding positive charges in the amino-terminal region of Kv1.4 and by inverting the amino acid sequence of the potential inactivation ball. Since the structure of non-inactivating \(\Delta 2–28\) was just like having net positive charge (3 arginine and 2 glutamic acid residues) at the amino terminus of the second potential ball of rapidly inactivating \(\Delta 2–39\), the mutants with different numbers of charges were constructed \((\Delta 2–32, \Delta 2–30, \Delta 2–26\) and \(\Delta 2–25\)). The currents through these mutants hardly inactivated (Fig. 5A, lower panel). These results suggest that one extra positive charge at the amino terminus of the second inactivation ball is enough to disrupt its function. Therefore, the influences of net positive charges at the amino terminus of

**Fig. 4. Influences of deleting net positive charge from the carboxyl-terminal region of the potential ball domains.** Upper panel, currents of \(\Delta 29–162\) and its parent mutant \(\Delta 38–162\) (A) and \(\Delta 2–39 & \Delta 61–162\) and its parent mutant \(\Delta 2–39 & \Delta 69–162\) (B) are normalized and superimposed. Lower panel, influences of changing \([K^+]_o\), 2 mM \([K^+]_o\) ○, 20 mM \([K^+]_o\) on the recovery from inactivation are shown. A, \(\Delta 29–162, B, \Delta 2–39 & \Delta 61–162\). Pulse protocols are same as described in the legend of Fig. 2.

**Fig. 5. Influences of net positive charge at the amino-terminal region of the potential inactivation ball domains and by inverting the second potential ball domain.** Upper panel, currents of \(\Delta 2–28\) and \(\Delta 2–39\) (A), (+)Kv1.4 and Kv1.4 (B), and Inv(40–68) and its parent mutant \(\Delta 2–39 & \Delta 69–162\) (C) are normalized and superimposed. Lower panel, currents of \(\Delta 2–25, \Delta 2–26, \Delta 2–30,\) and \(\Delta 2–32\) are normalized and superimposed (A). Influences of changing \([K^+]_o\), 2 mM \([K^+]_o\) ○, 20 mM \([K^+]_o\) on the recovery from inactivation are shown. B, (+)Kv1.4; C, Inv(40–68). Pulse protocols are same as described in the legend of Fig. 2.
the inactivation ball of Kv1.4 were studied by adding residues 26–39 (three net positive charges) at the amino-terminal end of Kv1.4 (+Kv1.4). The currents through (+Kv1.4) inactivated but the rate of inactivation was significantly slower than for wild type Kv1.4 (Fig. 5B). These results indicate that net positive charge at the amino-terminal end of the inactivation ball of wild type Kv1.4 has a profound effect on function. Together the results indicate the structural requirements of the inactivation ball(s) are an amino-terminal hydrophobic region and a carboxyl-terminal hydrophilic region containing net positive charge.

In agreement with the results of Tseng and co-workers, changing [K+]o had no effects on the recovery rate for our mutants, which did not show rapid inactivation. Elevating [K+]o accelerated the recovery rate in mutants with rapid inactivation, which might reflect repulsion of the inactivation ball by K+ ions (11).

The most striking finding in the present study is that there exist two potential inactivation balls in the amino terminus of rat Kv1.4. It is not known how two inactivation balls could work in wild type Kv1.4. One of the two potential domains might function as the inactivation gate, or one inactivation gate might be composed of both domains. Alternatively, the redundancy of inactivation balls might be a safety device to ensure inactivation. The synthetic ShB inactivation ball peptide has been reported to block several types of K+ channels and also cyclic nucleotide gated channels (15–19). It will be of interest to synthesize the peptides corresponding to the first domain, the second domain and both the domains of Kv1.4, and to compare their effects on currents of the non-inactivating mutant of Kv1.4 and the other channels. Synthetic peptides could give useful information about how the two domains contribute to form the inactivation ball in wild type Kv1.4. Recently, NMR structures of the inactivation peptides of Kv3.4 (the initial 30 residues) and Kv1.4 (the initial 37 residues) have been reported. The inactivation peptides have a similar characteristic surface charge pattern with a positively charged, a hydrophobic, and a negatively charged region (20). The inactivation peptide of Kv1.4 whose NMR structure was determined corresponds to our first inactivation ball. It will be of interest to determine the NMR structure of the second domain and both the domains in the amino-terminal region of Kv1.4.

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