N-terminal inactivation in voltage-gated K\(^+\) (Kv) channels is a widespread means to modulate neuronal excitability and signaling. Here we have shown a novel mechanism of N-type inactivation in a Caenorhabditis elegans Kv channel. The N-terminal sequence of KVS-1 contains a domain of 22 amino acids that resembles the inactivation ball in A-type channels, which is preceded by a domain of eighteen amino acids. Wild type KVS-1 currents can be described as A-type; however, their kinetics are significantly (~5-fold) slower. When the putative inactivation ball is deleted, the current becomes non-inactivating. Inactivation is restored in non-inactivating channels by diffusion of the missing inactivation domain in the cytoplasm. Deletion of the domain in front of the ball speeds inactivation kinetics ~5-fold. We conclude that KVS-1 is the first example of a novel type of Kv channel simultaneously possessing an N-inactivating ball preceded by an N inactivation regulatory domain (NIRD) that acts to slow down inactivation through steric mechanisms.

A-type channels constitute an important group of voltage-gated K\(^+\) (Kv) subunits that play a prominent role in the control of neuronal and muscular excitability, synaptic input, and neurotransmitter release (1–4). The name “A-type” stems from the typical profile of these currents, rapid activation at sub-threshold voltages followed by fast inactivation (4). In many neurons, A-type channels are usually silent at resting membrane potentials. They, however, transiently activate during the decay of the after-hyperpolarization phase of the action potential, delaying depolarization (5). Thus, A-type inactivation is a key physiological feature that allows control of the neuronal firing frequency by regulating the interval between consecutive action potentials (4). In the late 70s, Armstrong and Bezanilla (6), in an effort to explain the mechanism of A-type inactivation, proposed the “ball-and-chain” model, which postulates that a positively charged inactivation particle (the α-ball) on a tether prevents the movement of ions by physically occluding the pore. Using the Drosophila channel Shaker, Hoshi, Zagotta, and Aldrich (7, 8) identified the location and molecular composition of the ball. This is composed of the first ±20 amino acids in the N terminus (thus the name “N-type” inactivation) followed by 40 or more residues constituting the chain. The inactivation ball possesses two essential chemical characteristics (Fig. 1): the first half is composed of hydrophobic residues, and the rest has a net positive charge that pushes the ball toward its channel receptor site upon depolarization (9, 10). Because of the dynamic nature of N-type inactivation, the details of the mechanisms have been unknown for a long time. Recently, using crystallography, MacKinnon and colleagues (11, 12) have proposed that N inactivation occurs as a sequential reaction; the ball initially binds to the T1 domain surface by electrostatic interactions, and then it enters through the lateral portals that connect the cytoplasm to the inner pore and reaches the inner cavity to eventually impair ion movement.

Accessory subunits of the Kvβ family can convert non-inactivating Kvα subunits into A-type channels (13). These may also possess an inactivation ball, or β-ball, that functions identically to an α-ball. Thus, Kvβ1 and Kvβ3 interact with the Kv1 family of α-subunits and induce rapid inactivation in these channels (13–16); however, there is one exception. Kvβ1.6 possesses an N-terminal domain termed the N inactivation prevention (NIP) domain, which neutralizes the β-ball of the Kvβ subunit through electrostatic forces (17). A KChIP isoform (KChIP4a) also exhibits a K-channel suppressor (KIS) domain, which abolishes fast inactivation in the Kv4.3 channel (18). The KIS domain, however, profoundly differs from the NIP domain. Whereas NIP antagonizes the inactivation ball, KIS acts on residues around the inner mouth of the pore to alter the gating of Kv4.3. To date, no other cases of channels and/or accessory subunits possessing regulatory domains of N-type inactivation have been reported in the literature.

Here we have shown the first example of an α-subunit that simultaneously possesses an inactivation ball and a domain that regulates its function. KVS-1 is a voltage-gated K\(^+\) channel that operates in the nervous system of the nematode Caenorhabditis elegans to maintain and modulate cell sensitivity (19). Even though the KVS-1 current can be described as A-type, its inactivation is considerably slower than normal. We discovered that, in fact, KVS-1 possesses both a functional inactivation ball and a receptor. In addition, KVS-1 exhibits a domain in front of the ball (here named N inactivation regulatory domain (NIRD)) that modulates the kinetics of inactivation. NIRD is different from NIP and KIS in several respects. It co-exists with the ball in the same subunit and slows down inactivation by a mechanism that is best described as steric.
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Thus, KVS-1 provides the first example of a novel mechanism of regulation of N-type inactivation.

EXPERIMENTAL PROCEDURES

Molecular Biology—KVS-1 mutants were constructed by PCR. For biochemistry, KVS-1 was epitope-tagged by replacing the terminal stop codon with nucleotides encoding HA residues (YPYDVPDYA-STOP). The constructs were subcloned into plc-neo vector (Promega) for expression in Chinese hamster ovary (CHO) cells. All sequences were confirmed by automated DNA sequencing. Transcripts were quantified with spectroscopy and compared with control samples separated by agarose gel electrophoresis stained with ethidium bromide.

Electrophysiology—CHO cells were transiently transfected with cDNA using the Superfect kit (Qiagen) and studied 24–36 h post-transfection. Data were recorded with an Axopatch 200B amplifier (Axon), a Dell personal computer, and Clampex software (Axon) after cells were filtered at a = 1 kHz and sampled at 2.5 kHz. Bath solution was (in mM) KCl, 100 NaCl, 10 Heps (pH = 7.5 with NaOH), 1.8 CaCl2 and 1.0 MgCl2. The pipette solution included 100 KCl, 10 Heps (pH = 7.5 with KOH), 1.0 MgCl2, 1.0 CaCl2, and 10 EGTA (pH = 7.5 with KOH). Whole-cell currents were evoked by 200-ms voltage sweeps from a holding potential of −80 to +120 mV in 20-mV increments. The time course of activation and inactivation was fitted to a single exponential function as follows,

\[ I = I_0 e^{-\frac{V}{\tau}} \]  

(Eq. 1)

where \( I_0 \) is a constant and \( \tau \) is the value at which Equation 1 = 0.37.

Macroscopic conductance (G) curves were calculated as,

\[ G = \frac{1}{V - V_{rev}} \]  

(Eq. 2)

where \( I \) is the macroscopic current and \( V_{rev} \) is the resting potential. \( G/G_{max} \) curves were fitted to the Boltzmann function,

\[ \frac{G}{G_{max}} = \frac{1}{1 + \exp \left( \frac{V_{1/2} - V}{\Delta V} \right)} \]  

(Eq. 3)

where \( V \) is the membrane voltage, \( V_{1/2} \) is the value of the voltage at which Equation 2 = 0.5 and \( \Delta V \) is the slope coefficient (in mV).

Data are presented as means ± S.E. The number of determinations is indicated by \( n \). Statistical significance of unpaired groups was estimated with the Student’s \( t \) test.

Synthetic Peptide—Peptides encoded by the sequence MVIPSTRKGAHASRDNFANGSNH (Δ19–40) and MSTERLMYDLNKNQNIAVMVIPSTRKGAHASRDNFANGSNH (Δ1–40) were synthesized dry at the University of Medicine and Dentistry of New Jersey peptide synthesis core facility. Prior to the experiment, the peptides were freshly diluted in the pipette solution to a final concentration of 10 nM.

Immunoprecipitations—CHO cells were washed with 10 ml of ice-cold phosphate-buffered saline and lysed with ~2 ml of ice-cold radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAHL CA-630, 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS) and freshly added 10 mM iodoacetamide, phosphatase, and protease inhibitors for 30 min at 4 °C. Cell lysates were centrifuged for 60 min at 4 °C, and the supernatant was mixed with HA-conjugated beads (Roche Applied Science) and rocked at 4 °C for 3 h. The beads were washed three times with ice-cold TBST (8.8 g of NaCl, 0.2 g of KCl, 3 g of Tris base, 500 μl of Tween 20, pH to 7.4, H2O to 1 liter) and incubated in SDS sample buffer at ~90–95 °C for 15 min.

Membrane Biotinylation—30 h after transfection, the CHO cells were washed three times with phosphate-buffered saline at room temperature (22–25 °C), and cell surface proteins were biotinylated by 1.0 mg/ml impermeant biotin analog EZ-link sulfo-NHS-LcBiotin (Pierce) in phosphate-buffered saline. After incubation at 4 °C for 1 h, the cells were washed five times with ice-cold phosphate-buffered saline to remove any remaining biotinylation reagent. Cells were then harvested in radioimmune precipitation assay buffer. Lysate proteins were precipitated by streptavidin-agarose-linked beads. The precipitated KVS-1 was detected by monoclonal anti-HA antibodies (Roche Applied Science).

RESULTS

A 22-amino-acid domain (Δ19–40) (Fig. 1) in the N terminus of KVS-1 (dubbed K1 in this “Result” section) exhibited homology to the N-inactivating domains in several A-type α- and β-subunits (7, 9, 35–38). This suggests that inactivation in K1 might proceed through a N-type mechanism. To determine whether Δ19–40 is an inactivation ball, we constructed a panel of K1 truncation mutants and used the whole-cell configuration of the patch clamp to characterize currents.

KVS-1 Possesses an N Inactivation Ball—In CHO cells, wild type (wt) K1 channels express robust voltage-dependent K+ currents exhibiting fast activation and inactivation (Fig. 2A). The voltage dependence of the extent of inactivation, expressed as the ratio between the residual and the peak current, is sharp (~12.7 ± 0.7 mV) with a midpoint at 45.5 ± 7 mV (Fig. 2B). The time course of inactivation, which is best fit with a single exponential function (Equation 1), weakly depends on the membrane voltage (Fig. 2C). Thus, the characteristic of the K1 current is A-type overall. However, compared with normal fast inactivating channels, the kinetics of K1 inactivation are considerably slower (~5-fold). Therefore, the time constant of inactivation represents the best parameter to describe changes in inactivation in wt or mutant K1 channels. Complete or par-
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FIGURE 2. KVS-1 possesses an N inactivation ball. A, representative whole-cell macroscopic currents elicited by voltage jumps from −80 mV to +120 mV in 20-mV increments (inset) from CHO cells expressing KVS-1 (K1) alone or the K1Δ40 mutant. B, voltage dependence of the extent of inactivation defined as the ratio between the residual and peak current in wt and K1Δ40. Fit of wt (squares) and K1Δ40 (circles) data to Equation 3 gave, respectively, \( V_{1/2} = 47.3 \pm 4 \) mV, \( n = 11.1 \pm 3 \) (n = 13), \( V_{1/2} = 36.7 \pm 4 \) mV, and \( n = 18.5 \pm 5 \) (n = 11). C, voltage dependence of the time course of inactivation in wt and K1Δ40. Currents were fitted to a single exponential function (Equation 1). Symbols are the same as described for A. D, steady-state dependence of activation in wt and K1Δ40. Macroscopic conducance curves (G) were calculated according to Equation 2, normalized to the maximum value, and fitted to the Boltzmann function with \( V_{1/2} = 57.1 \pm 6.0 \) mV, \( V_c = 25.0 \pm 2.0 \) mV (n = 11) for wt channels, \( V_{1/2} = 47.9 \pm 3.3 \) mV, \( V_c = 22.6 \pm 2.4 \) mV (n = 13) for K1Δ40 channels. E, instantaneous activation in wt and K1Δ40. The time course of activation was fitted to a single exponential function. Symbols are the same as described for A. F, total protein expression and surface expression of wt and K1Δ40 epitope tagged to the HA tag in the C terminus. Western blots were stained with an anti-HA primary antibody or with anti-tubulin for control and then visualized with a horseradish peroxidase chemiluminescence-coupled secondary antibody.

Tidinal deletion of the putative inactivation domain (Fig. 2A, K1Δ40) (K1Δ30 not shown) suppressed most of the inactivation. The current exhibited a residual time-dependent decline, suggesting that multiple inactivation mechanisms may co-exist in K1. The magnitude of the current in K1Δ40 and K1Δ30 (not shown) was roughly 2-fold larger in wt channels. The level of K1Δ40 protein as well as its expression in the plasma membrane were not significantly different (Fig. 2F). This ruled out effects due to altered channel biosynthesis, trafficking, or abundance in the membrane. The midpoint for voltage activation (\( V_{1/2} \)) was shifted ~8 mV to the left in the K1Δ40 mutant compared with wt channels (Fig. 2D). In contrast, the kinetics of activation did not significantly differ in the two channel types (Fig. 2E). These observations are consistent with the notion that the current increase in K1Δ40 and K1Δ30 was because of the removal of N-type inactivation. Under normal conditions, N inactivation does not allow the current to reach saturation. Thus, an increase in the current upon removal of the ball was expected, and it was, in fact, observed in several A-type channels (e.g. see Ref. 20). Alternative mechanisms such as increased unitary current are also possible, but they seem unlikely considering that the N terminus is not expected to contribute to the ion conduction pathway.

Synthetic Δ19–40 Peptide Restores N-type Inactivation in K1Δ40 Mutants—The ball-and-chain model makes predictions that can be tested: if deletion of the inactivating ball eliminates inactivation, then diffusion of the missing piece into the cytoplasm should restore inactivation. Therefore, to corroborate the notion that Δ19–40 is an inactivation ball and to ascertain whether K1 possesses a functional ball receptor, we synthesized a synthetic Δ19–40 peptide and assessed its ability to inactivate K1Δ40 mutant channels. Addition of the inactivation peptide to the pipette solution restored inactivation in K1Δ40 channels in a time-dependent fashion, consistent with progressive diffusion of the peptide into the cytoplasm (Fig. 3). As expected, we did not observe any change in the current in the absence of the peptide (Fig. 3B). Moreover, diffusion of a synthetic peptide formed by the first 40 amino acids of KVS-1 (Δ1–40) restored wild type inactivation in K1Δ40 currents (\( \tau_{120} = 51 \pm 7 \) ms) (Fig. 3B). We conclude that K1 possesses an α-inactivation ball and a functional receptor. Therefore, N-type inactivation is a primary (although not the only) mechanism contributing to the transient behavior of the K1 current.

NIRD Hinders N-type Inactivation—The kinetics of inactivation induced by the Δ19–40 peptide were considerably faster than in wt channels (compare them in Fig. 4B). This suggests that the domain in front of the ball might slow down N inactivation in wt channels. As expected, deletion of this domain (Fig. 4, K1Δ18) conferred full A-type attributes to the current. The inactivation in K1Δ18 currents did not significantly differ from that in K1Δ40 currents modified by the inactivating peptide (\( \tau_{120} = 11.2 \pm 3 \) versus 10.0 ± 4 ms) (Fig. 4B). We conclude that Δ18 specifically acts to modulate N-type inactivation without apparently affecting other gating parameters.
Steric Mechanisms Underlie NIRD-mediated Inhibition of Inactivation—To gain insight into the mechanisms underlying NIRD function, we probed the role of electrostatic interactions, which have been shown to play a key role for NIP function (13). Surprisingly, mutation of charged residues to neutral residues or to residues of opposite charge did not induce any apparent modification in the current (Table 1). Post-translational modifications such as phosphorylation and oxidation of methionine have also been shown to modulate N-type inactivation (14–16). Notably, neutralization of a protein kinase C consensus site in NIRD (T3V) or mutation of its methionines (M7L, M19L) gave rise to mutant channels that did not significantly differ from wt (Table 1). Conversely, progressive deletion of NIRD gradually accelerated inactivation in a fashion that did not depend on the overall electrical charge of NIRD (Fig. 5). We conclude that the NIRD acts primarily to slow down N-type inactivation through steric mechanisms.

**DISCUSSION**

Electrophysiological and biochemical analyses support the following conclusions. First, KVS-1 is an A-type channel. It has both a functional N-inactivating ball and a ball receptor. Second, KVS-1 possesses a domain in front of the ball (NIRD) that modulates the kinetics of N inactivation. The primary sequence of NIRD as well as its function lead us to conclude that NIRD represents a new mode of A-type current modulation.

To date, only two examples of N-terminal domains that can act to regulate N inactivation (NIP and KIS) have been reported. KIS affects multiple gating parameters in Kv4.3, including activation (18). In contrast, NIP specifically antagonizes N-type inactivation in heteromeric Kv1.6-Kvβ channels (17). NIRD differs from NIP and KIS in several respects. First, the ball and NIRD co-exist in the same subunit. Second, NIRD does not abolish N inactivation nor does it affect other gating parameters. Its mode of action is also different from those of the other domains. Although NIP exploits electrostatic forces and KIS binds to the inner mouth of the pore, the mechanism by which NIRD hinders inactivation is best described as steric. The details of the mechanism are not completely elucidated. NIRD might either decrease the affinity of the ball for its receptor, or alternatively, it might hamper its progression into the pore of the channel. Nonetheless, in the light of the known three-dimensional structures of Kv channels, these findings unveil unexpected insights into the molecular mechanisms of N-type inactivation.

Suppression of N inactivation in KVS-1 revealed the existence of secondary inactivation mechanisms. This is not unexpected, as many Kv channels exhibit multiple forms of inactivation that involve slow and fast processes. For example, two forms of inactivation (N-type and C-type) co-exist in Shaker (25). C-type inactivation is produced by rearrangements of the external mouth of the pore that involve movements of S6 and the S5-S6 linker (26, 27). The C terminus can also be a source of inactivation in Kv channels (28). The attributes of non N-type inactivation in KVS-1 are more consistent with a C-type mechanism than a C terminus mechanism, because this domain is remarkably small. Furthermore, accessory subunits might also contribute to secondary mechanisms of inactivation. In native

### Table 1

<table>
<thead>
<tr>
<th>Variant</th>
<th>Current density</th>
<th>(\tau_{120})</th>
<th>(V_{1/2})</th>
</tr>
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<tbody>
<tr>
<td>Wild type 202 ± 23</td>
<td>38.9 ± 4.5</td>
<td>47.3 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>T3A 200 ± 32</td>
<td>38.7 ± 5.4</td>
<td>45.4 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>M7L 187 ± 27</td>
<td>42.3 ± 6.2</td>
<td>49.4 ± 4.6</td>
<td></td>
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<tr>
<td>D10N 197 ± 39</td>
<td>49.3 ± 10.2</td>
<td>50.3 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>D10K 216 ± 25</td>
<td>37.5 ± 4.7</td>
<td>48.5 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>K13Q 205 ± 16</td>
<td>38.5 ± 5.7</td>
<td>45.6 ± 4.2</td>
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<tr>
<td>K13E 185 ± 23</td>
<td>39.0 ± 4.2</td>
<td>43.9 ± 6.0</td>
<td></td>
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<tr>
<td>M20L 198 ± 35</td>
<td>43.3 ± 6.7</td>
<td>47.5 ± 3.8</td>
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</table>

In the figure, it is plotted the \(\tau\) at +120 mV as a function of the indicated deletions (arrows) in the NIRD. A methionine was inserted before the indicated residues (for example, M-DLN). The number of determinations are \(n \pm \tau\) cells in each case.
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tissue, KVS-1 forms a complex with MPS-1, a bifunctional β-subunit that possesses kinase activity (29). When MPS-1 is co-expressed with KVS-1 in CHO cells, it accelerates the rate of inactivation through mechanisms that are independent of its enzymatic activity. This implies that MPS-1 might either interfere with N-type inactivation, with secondary mechanisms of inactivation, or induce de novo inactivation. Even though a complete elucidation of these issues is beyond the scope of this study, we can rule out that MPS-1 interferes with N-type inactivation. When MPS-1 was co-expressed with K1Δ40, the channel inactivated (data not shown). The most likely explanation for these effects is that they result from structural rearrangements in the channel protein induced by the incorporation of MPS-1.

A-type inactivation is a key attribute of neuronal K⁺ channels. This explains why this gating characteristic can be controlled by multiple, often redundant mechanisms. It can be regulated through the formation of heteromeric complexes of distinct A-type α-subunits and/or α and β-subunits by signaling molecules, including protein kinases and post-translational modifications (13–16, 21–24, 30–33). An example has been an experiment in which the channel can bind to phospholipids, thereby converting the channel from non-inactivating to inactivating and vice versa (34). We are currently testing whether in vivo deletion of NIRD is a physiological mechanism of regulation of neuronal excitability. Hence, exciting questions lay ahead that we expect will provide new insights into the molecular mechanisms determining K⁺ channel function.

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REFERENCES