C-type inactivation controls recovery in a fast inactivating cardiac K^+ channel (Kv1.4) expressed in *Xenopus* oocytes

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1. A fast inactivating transient K^+ current (FK1) cloned from ferret ventricle and expressed in *Xenopus* oocytes was studied using the two-electrode voltage clamp technique. Removal of the NH_2-terminal domain of FK1 (FK1Δ2-146) removed fast inactivation consistent with previous findings in Kv1.4 channels. The NH_2-terminal deletion mutation revealed a slow inactivation process, which matches the criteria for C-type inactivation described for *Shaker* B channels.

2. Inactivation of FK1Δ2-146 at depolarized potentials was well described by a single exponential process with a voltage-insensitive time constant. In the range −90 to +20 mV, steady-state C-type inactivation was well described by a Boltzmann relationship that compares closely with inactivation measured in the presence of the NH_2-terminus. These results suggest that C-type inactivation is coupled to activation.

3. The coupling of C-type inactivation to activation was assessed by mutation of the fourth positively charged residue (arginine 454) in the S4 voltage sensor to glutamine (R454Q). This mutation produced a hyperpolarizing shift in the inactivation relationship of both FK1 and FK1Δ2-146 without altering the rate of inactivation of either clone.

4. The rates of recovery from inactivation are nearly identical in FK1 and FK1Δ2-146.

5. To assess the mechanisms underlying recovery from inactivation the effects of elevated $[K^+]_o$ and selective mutations in the extracellular pore and the S4 voltage sensor were compared in FK1 and FK1Δ2-146. The similarity in recovery rates in response to these perturbations suggests that recovery from C-type inactivation governs the overall rate of recovery of inactivated channels for both FK1 and FK1Δ2-146.

6. Analysis of the rate of recovery of FK1 channels for inactivating pulses of different durations (70–2000 ms) indicates that recovery rate is insensitive to the duration of the inactivating pulse.

Voltage-gated potassium channels frequently inactivate following activation. The process of inactivation, however, is extremely variable in time course and can occur through various mechanisms. The best understood of these mechanisms is N-type inactivation which is well described by a 'ball and chain' model (Hoshi, Zagotta & Aldrich, 1990; Zagotta, Hoshi & Aldrich, 1990) similar to one originally proposed for the squid axon sodium channel (Armstrong, 1971). The molecular basis for this model was demonstrated in *Shaker* B channels to be dependent upon a small group of amino acids in the NH_2-terminus which bind to the activated channel and occlude the intracellular mouth of the channel (Hoshi *et al.* 1990; Zagotta *et al.* 1990). Although no sequence similarity has been found among the NH_2-termini of the various N-type inactivating *Shaker* K^+ channels and mammalian Kv1.4 channels, Kv1.4 channels have also been demonstrated to have an N-type inactivation mechanism (Ruppersberg, Frank, Pongs & Stocker, 1991; Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991; Tseng-Crank, Yao, Berman & Tseng, 1993; Comer *et al.* 1994).

In addition to the N-type mechanism, another type of inactivation, termed C-type, has been identified in *Shaker* K^+ channels. This mechanism is apparent in some *Shaker* splice variants which lack N-type inactivation and can be revealed in others when the NH_2-terminus is deleted from those channels which exhibit N-type inactivation (Hoshi, Zagotta & Aldrich, 1991). Thus, the two types of inactivation can exist simultaneously in *Shaker* K^+ channels. It has been proposed that C-type inactivation occurs by a mechanism in which the external mouth of the channel becomes occluded during sustained depolarization (Busch,

Hurst, North, Adelman & Kavanaugh, 1991). Despite being potentially as widespread a mechanism of K^+ channel inactivation as N-type, C-type inactivation has been less frequently studied and is less well understood. However, a set of criteria which serve to define the C-type mechanism has emerged: (1) insensitivity to removal or mutation of the NH_2-terminus (e.g. Hoshi *et al.* 1991), (2) sensitivity to the level of extracellular K^+, with a slowing of the C-type mechanism by elevated K^+ (Lopez-Barneo, Hoshi, Heinemann & Aldrich, 1993), (3) sensitivity to mutation of a particular amino acid near the extracellular mouth of the pore (position 449 in *Shaker* B; Lopez-Barneo *et al.* 1993) and (4) competition with external TEA^+ block in channels which possess an extracellular binding site for TEA^+ (Choi, Aldrich & Yellen, 1991).

Our knowledge of the molecular basis of the C-type inactivation mechanism in voltage-gated K^+ channels is derived almost exclusively from *Drosophila Shaker* K^+ channels; few studies have addressed the role or mechanism of slow inactivation in mammalian channels. A recent study of the effects of elevated $[K^+]_o$ on an NH_2-terminal deletion mutant of a rapidly inactivating rat heart channel (RHK1) has called into question the existence of the C-type mechanism in Kv1.4 channels (Tseng & Tseng-Crank, 1992). In addition, extrapolation of mutational studies in *Shaker* K^+ channels raise questions concerning the mechanism of slow inactivation in FK1. Mutation of threonine to lysine at position 449 in an NH_2-terminal deleted mutant of *Shaker* B produces a fast C-type inactivation time constant of less than 100 ms (Lopez-Barneo *et al.* 1993). However, Kv1.4 has a lysine at the analogous position (532) but has a slow inactivation time constant (in the order of seconds) in its NH_2-terminal deletion construct. Measurement of competition with external block by TEA^+ in Kv1.4 was not possible since this channel is insensitive to external TEA^+ (Comer *et al.* 1994). Thus, although a slow inactivation mechanism is present in NH_2-terminal deletion mutants of Kv1.4 channels, it is unclear that slow inactivation is mediated by a C-type mechanism (Tseng-Crank *et al.* 1993).

In this study we measured the properties of slow inactivation in a Kv1.4 channel (FK1) isolated from ferret ventricle. We demonstrated that slow inactivation has the appropriate properties previously described for C-type inactivation in *Shaker* K^+ channels. We further characterized the relationship between N-type and C-type inactivation by demonstrating that the kinetics of recovery from inactivation in the presence of the NH_2-terminus were closely correlated with the kinetics of recovery from C-type inactivation in the absence of the NH_2-terminus as modified by several perturbations that alter C-type inactivation. In addition, the recovery rate of FK1 was insensitive to inactivating pulse duration. This strongly suggests that the rate of recovery from inactivation in Kv1.4 is governed by reversal of C-type inactivation.

A preliminary account of this work has appeared in abstract form (Rasmusson, Zhang, Campbell, Comer & Strauss, 1994).

METHODS

Preparation of cRNA

Construction of the cDNA clone, pBSMC1-12, and its NH_2-terminal deletion mutant, pFK1Δ2-146, has been previously described (Comer *et al.* 1994). FK1Δ2-146 is a deletion mutant in which amino acids 2–146 have been deleted. This deletion removes the amino acid sequence required for fast inactivation without significantly altering activation characteristics (Comer *et al.* 1994). Plasmids pBSMC1-12 and pFK1Δ2-146 were linearized using *Asp*718 I. Two micrograms of linearized plasmid were transcribed *in vitro* and the RNA purified and quantified exactly as described (Comer *et al.* 1994). cRNA was stored in RNase-free water at −80 °C until use. Injection of 2–5 ng of our cRNA preparation in *Xenopus* oocytes typically gave 1–10 μA magnitude peak currents (at +50 mV) after 48 h incubation.

Site-directed mutagenesis

The FK1-R454Q mutation was constructed by a modified 'megaprimer' method as described (Steinberg & Gorman, 1994). The first polymerase chain reaction reaction (PCR) contained 20 mM Tris-Cl (pH 8·2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0·1% Triton X-100, 10 μg ml^{-1} BSA, 0·2 mM each of dATP, dCTP, dGTP, dTTP, 100 pg μl^{-1} pBSMC1-12, 1·0 μM FK1:S6 (GGC ACT GGC AAA GCA AT), 1·0 μM FK1:R454Q (CGA GTA TTC CAA ATA TTC AAA CTC TCC), and 0·5 units *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). After denaturation at 94 °C for 5 min, the reactions were cycled 30 times for 30 s at 94 °C, then 1 min at 53 °C, and 1 min at 72 °C. The reactions products were separated on a 3% Nusieve agarose gel (FMC, Rockland, ME, USA) and the PCR product gel-purified using Qiaex resin (Qiagen, Chatsworth, CA, USA). This PCR product (1·5 pmol) was mixed with 250 ng pBSMC1-12 (digested with *Sph* I and treated with ddCTP and terminal transferase), and the reaction mixture described above (without the oligonucleotides). This mixture was treated for 2 min at 100 °C, after which the temperature was lowered to 50 °C over 5 min, then increased to 72 °C for 2 min. Oligonucleotides FK1:S6 and FK1:58 (GTA TAA ACC TGC CTG TGC) were added and the reactions were cycled 15 times as above. The PCR product was then digested with *Sph* I and *Tth*111 I and cloned into those sites in the FK1 or FK1Δ2-146 cDNA

The FK1-K532Y mutation was constructed as described (Higuchi, 1990). The mutagenic oligonucleotides were FK1:K532Y-U (TGG ATA CAT GTC CCC GTA GCC TAC AGT TGT CAT GGT CAC CAC AGC) and FK1:K532Y-L (ACT GTA GGC TAC GGG GAC ATG TAT CCA ATC ACT GTG). The flanking oligonucleotides and temperature cycling conditions were identical to those above except that a 10:1 mixture of *Taq* DNA polymerase (Life Technologies, Inc., Grand Island, NY, USA) and *Pfu* DNA polymerase was used. The final PCR product containing the K532Y mutant was digested with *Sph* I and *Hpa* I and cloned into those sites in the FK1 or FK1Δ2-146 cDNA. The fidelity of all

mutants was verified by sequencing mutant cDNAs between the cloning sites.

Preparation of oocytes

Mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI, USA) were anaesthetized by immersion in tricaine solution (1·5 g l^{-1} in 25 mM NaH_2PO_4, pH 6·8). Ovarian lobes were removed through a small incision in the abdominal wall. The follicular layer was removed enzymatically by placing the lobes in a collagenase containing Ca^{2+}-free OR2 solution containing 82·5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes (pH 7·4), 1–2 mg ml^{-1} collagenase (Type I, Sigma). After removal of the ovarian lobe, the frogs were sutured (twice in the abdominal wall and twice in the external skin). The frogs were then allowed to recover in a small water-filled container, with their heads elevated above water level. Once the animal had recovered from anaesthesia, it was placed in a separate aquarium by itself and monitored until healed. Typically, lobes are obtained three times from a single frog. When individual frogs no longer yielded acceptable oocytes, anaesthetized frogs were killed by an overdose of tricaine (20 g l^{-1}). The oocytes were gently shaken for 3 h, with an enzyme solution replacement at 1·5 h, and collagenase activity was then halted as previously described (Comer *et al.* 1994). Stage V–VI oocytes were injected with 50 nl of cRNA using a 'Nanoject' microinjection system (Drummond Scientific Co., Broomall, PA, USA) and incubated at 18 °C for 24–72 h in an antibiotic containing Barth's solution (mM): 88 NaCl, 1 KCl, 2·4 $NaHCO_3$, 0·82 $MgSO_4$, 0·33 $Ca(NO_3)_2$, 0·41 $CaCl_2$, 10 Hepes (pH 7·4), 2% (v/v of 100 × stock) Antibiotic–Antimycotic (Gibco catalogue No. 600-5240PG, Life Technologies, Inc.).

Electrophysiological techniques

Oocytes were voltage clamped using a two-microelectrode 'bath clamp' amplifier (OC-725A; Warner Instruments Corp., Hamden, CT, USA) as has been described in detail elsewhere (Comer *et al.* 1994). Microelectrodes were fabricated from 1·5 mm o.d. borosilicate glass tubing (TW150F-4; WPI) using a two-stage puller (L/M-3P-A; Adams & List Associates, Ltd, Great Neck, NY, USA), filled with 3 M KCl, and had resistances of 0·6–1·5 MΩ. During recording, oocytes were continuously perfused with control solution (mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 10 Hepes, pH adjusted to 7·4, with NaOH). High K^+ solution contained (mM): 98 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 10 Hepes, pH adjusted to 7·4, with NaOH. Currents were recorded at room temperature (21–23 °C) and were filtered at 2·5 kHz for two-electrode clamp. Data were recorded on videotape using an A/D VCR adaptor (model PCM 4/8, Medical Systems Corporation, Greenvale, NY, USA) and digitized using pCLAMP software (Axon Instruments). Raw data traces shown were not leakage or capacitance subtracted. Analysis of steady-state inactivation used linear leak subtraction. No subtraction was employed in the analysis of recovery rates. Data are presented as means ± S.E.M.

RESULTS

FK1, a Kv1.4 channel, has been previously reported to display bi-exponential inactivation, with a large fast N-type component and a smaller slow component of unidentified origin. Removal of the NH_2-terminus from FK1 (FK1Δ2-146) greatly reduces inactivation rate (Fig. 1*A*) as described previously (Campbell, Rasmusson, Comer & Strauss, 1994). However, a slow component of inactivation remains, as can be seen on a reduced time scale for the NH_2-terminal deletion mutant (Fig. 1*B*). At +50 mV the time constant of inactivation in the absence of N-type inactivation is on average 2·7 ± 0·3 s.

FK1Δ2-146 current decline represents channel inactivation

The slow decay of FK1Δ2-146 current requires lengthy sustained depolarizations for analysis. Under such conditions the possibility that the sustained current is contaminated by depletion of intracellular K^+ ions must be considered. The envelope of tails test is frequently utilized to identify such depletion artifacts. Figure 1*C* and *D* shows the results of an envelope of tails test. FK1Δ2-146 was activated by stepping to +50 mV from a holding potential of −90 mV and allowed to inactivate for 0·1, 2 and 6 s and was then stepped to −40 mV as shown in the raw current traces of Fig. 1*C*. Figure 1*D* shows the inverse ratio of the activated current measured at the end of the +50 mV pulse to the peak current measured after changing membrane potential to −40 mV. This correspondence between time-dependent current reduction at two potentials indicates that the reduction in current observed during sustained depolarization to +50 mV was mediated through a reduction in a single conductance component. This was further confirmed by observing the change in the current–voltage relationship and the insensitivity of the *I–V* relation and reversal potential of the activated current to pulses of 0·1, 2 and 6 s at +50 mV (Fig. 1*E* and *F*). In conclusion, slow inactivation of FK1Δ2-146 reflects genuine inactivation of the channel without activation of non-K^+ selective channels or significant contamination by ion depletion artifacts.

Slow inactivation is coupled to activation

The time course of development of slow inactivation in FK1Δ2-146 was well described as a single exponential process, as shown in Fig. 2*A*. The time constant (τ, note: subscripts denote membrane potential in mV) of slow inactivation was voltage insensitive (Fig. 2*B*) over a wide range of potentials ($\tau_{-20} = 3{\cdot}1 \pm 1{\cdot}3$ s, $\tau_{+50} = 2{\cdot}7 \pm 0{\cdot}3$ s, $n = 6$) and became voltage sensitive only in the range of potentials for which the channel was only partially activated (< −20 mV; see Comer *et al.* 1994). A pseudo-steady-state inactivation relation was constructed using a standard two-pulse protocol with P1 inactivation pulses of 5 s duration over the range −90 to +50 mV followed by a P2 pulse to +50 as shown in Fig. 2*C*. Peak currents during P2 were normalized and averaged to give the average pseudo-steady-state inactivation relation shown in Fig. 2*D*. In the range −90 to +20 mV, slow inactivation was well described by a Boltzmann relationship with a $V_{½}$ (membrane

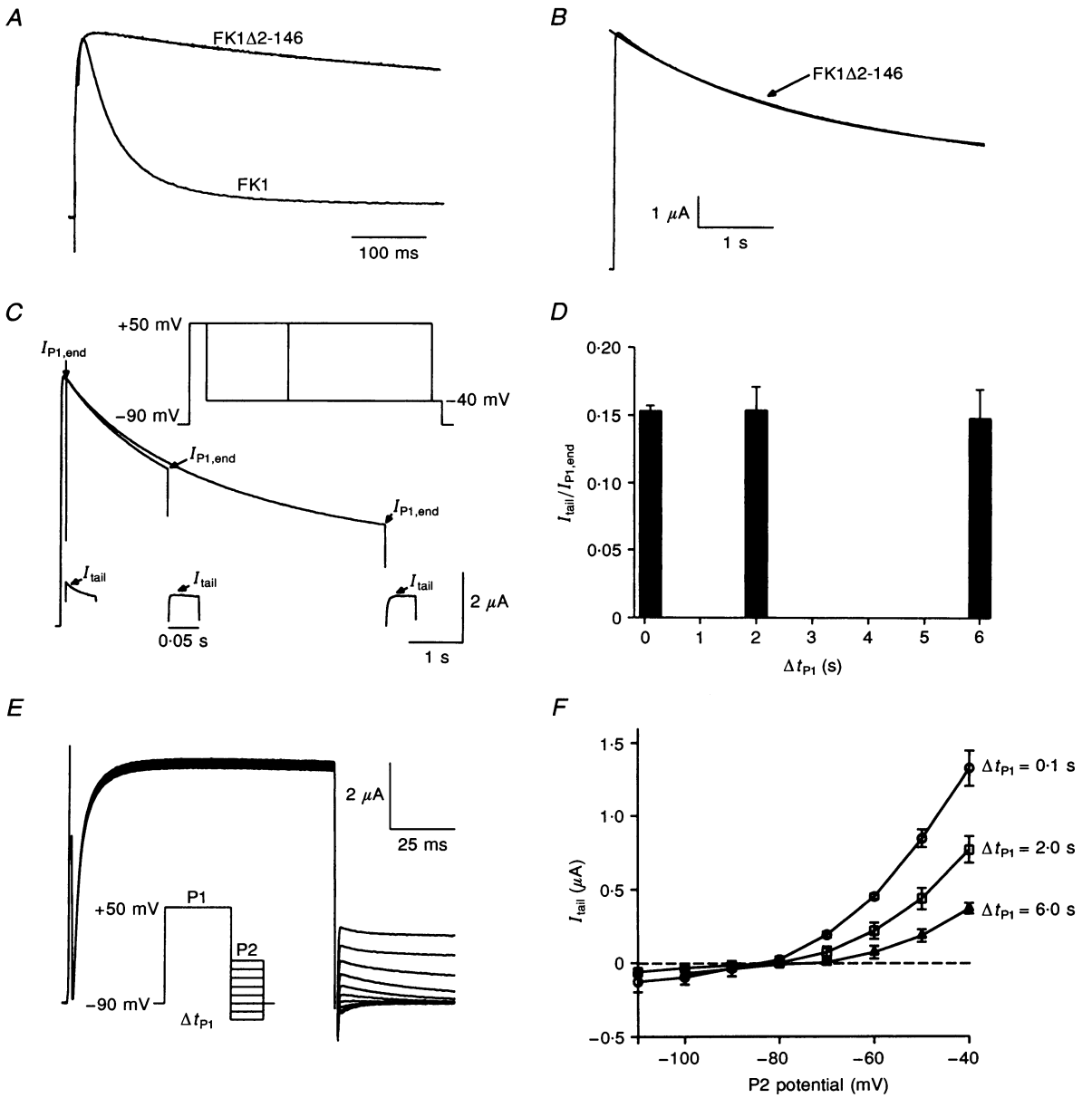


Figure 1. NH_2-terminal deletion unmasks a slow inactivation process in FK1

A, as described previously (Comer *et al.* 1994) removal of the NH_2-terminus removes fast inactivation. *B*, example of the slow component of inactivation remaining after deletion of the NH_2-terminus shown on a reduced time scale. *C*, envelope of tails test for slow inactivation of FK1Δ2-146. P1 pulses of 0·1, 2 and 6 s duration were applied to FK1Δ2-146 and were followed by a brief (50 ms) pulse to −40 mV (near the threshold for activation). The deactivation tail currents are displayed on an expanded time scale (total length of tail, 50 ms). The raw data traces were neither leakage nor capacitance subtracted, because the net linear leak current during these pulses was less than 5% of the smallest current measured. Note that deactivation at −40 mV is neither single exponential nor complete. Thus, a small component of the capacitance transient obscures a portion of the rapid component of tail current deactivation following the 6 s pulse. *D*, ratio of peak tail current to final current at the end of the P1 pulse. The ratio remained constant for pulse durations 0·1, 2 and 6 s (data presented as means ± S.E.M., $n = 3$), indicating the records did not contain artifacts due to spatial inhomogeneity, series resistance errors, or accumulation/depletion artifacts. *E*, the deactivation tail currents were measured after a P1 pulse to +50 mV at various P2 potentials varying between −20 and −110 mV. *F*, peak currents during P2 were measured and plotted as a function of P2 potential. The shape and reversal potential of the *I–V* relations were unaffected by the duration of the P1 pulse, providing further evidence against artifacts due to spatial inhomogeneity, series resistance errors, or accumulation/depletion. Zero current levels are indicated by a dashed line.

potential at half-maximal inactivation) of approximately −50 mV and a slope factor of 5·0. This compares well with the N-type inactivation relationship of FK1 shown in Fig. 2*D*. It should be noted that at all potentials slow inactivation was incomplete. When slow inactivation was extrapolated to infinite time using the measured inactivation time constants, inactivation was maximal near the threshold for channel activation but left a 23 ± 2·2% ($n = 6$) non-inactivated component. Steady-state slow inactivation also did not display monotonic Boltzmann-like behaviour but instead became significantly less inactivated at very depolarized potentials. This voltage-dependent 'tip up' in steady-state inactivation resembles the non-monotonic voltage-dependent inactivation of calcium channels (e.g. Campbell, Giles, Hume & Shibata, 1988), which have been previously noted to share other properties with C-type inactivation (Zhang, Ellinor, Aldrich & Tsien, 1994).

The insensitivity of the time constant of slow inactivation to membrane potential indicated that slow inactivation may not function as an independent, intrinsically voltage-sensitive process. Instead slow inactivation may depend upon movement of the channel activation voltage sensor to drive the channel into a suitable conformation for slow inactivation to proceed. But, once enabled, inactivation may have no intrinsic voltage dependence of its own (see Hille, 1992, for review). One way to assess the coupling of slow inactivation to activation is through mutation of the

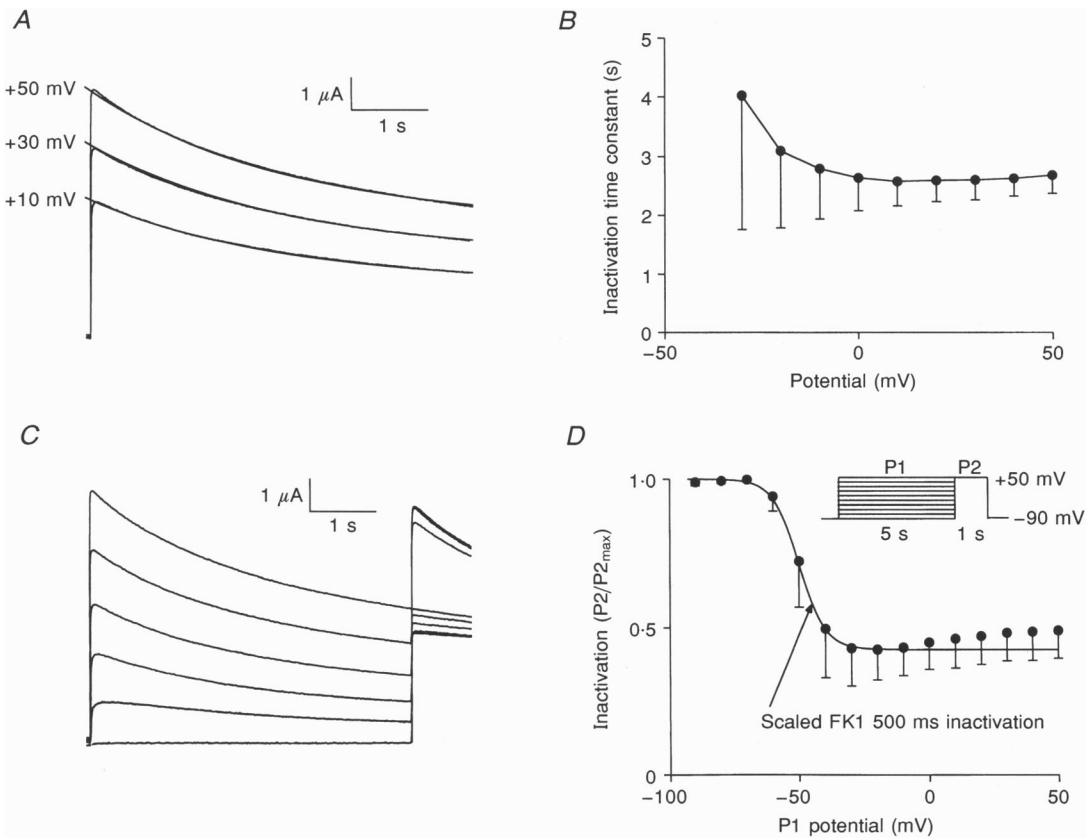


Figure 2. Properties of C-type inactivation in FK1Δ2-146

A, raw data records showing the time-dependent inactivation of FK1Δ2-146 during a 5 s pulse to 3 potentials (+10, +30 and +50 mV; holding potential (V_h) = −90 mV; stimulation rate = 0·017 Hz). Representative single exponential fits to inactivation are shown superimposed on the data. *B*, mean time constants of inactivation measured for FK1Δ2-146 as a function of membrane potential. Inactivation rate was voltage insensitive over the range −10 to +50 mV. *C*, current records used for determining steady-state inactivation relation for FK1Δ2-146. P1 was to varying potentials (−90 to +50 mV in 10 mV increments in ascending order; raw data traces are shown in 20 mV increments for clarity) for 5 s. P2 was to +50 mV for 1 s (V_h = −90 mV; stimulation rate = 0·017 Hz). *D*, voltage dependence of inactivation. Inactivation was measured as the percentage reduction in P2 peak current and plotted as a function of P1 potential. The continuous line is a Boltzmann relationship describing the steady-state N-type inactivation of FK1 by Comer *et al.* (1994) scaled to match the incomplete degree of inactivation of FK1Δ2-146 achieved during a 5 s pulse. The exact formula and parameters used were $0{\cdot}573/(1 + \exp((V_m + 50)/5{\cdot}0)) + 0{\cdot}427$. Data presented as means ± S.E.M. ($n = 4$).

S4 voltage sensor region. We tested the coupling of slow inactivation to movement of the S4 activation voltage sensor by a point mutation at position 454, the fourth positively charged residue in the S4 segment of FK1. Neutralization of this arginine by a point mutation to a polar glutamine (R454Q) in FK1 shifted the inactivation relationship in the hyperpolarized direction (Fig. 3*A*) similar to that reported for the analogous mutation in *Shaker* B (Papazian, Timpe, Jan & Jan, 1991). This shift demonstrated the coupling of the voltage sensor to N-type inactivation. When this same arginine was mutated to a glutamine in FK1Δ2-146, inactivation was also shifted in the hyperpolarizing direction (Fig. 3*B*). Neither FK1 nor FK1Δ2-146 showed any alteration in the rate of inactivation at +50 mV in response to this S4 mutation, consistent with the mutation simply altering the threshold for inactivation without altering other parameters of inactivation. These results indicate that slow inactivation is also coupled to movement of the activation voltage sensor, and may require a similar degree of channel activation to enable slow inactivation to proceed. This raises the question of whether slow inactivation proceeds independently or in parallel with N-type inactivation in the NH_2-terminal intact Kv1.4 channel or whether there is interaction between slow inactivation and N-type inactivation similar to the interaction suggested for *Shaker* K^+ channels (Hoshi *et al.* 1991).

Recovery from inactivation is governed by the C-type mechanism

Recovery from inactivation is the process by which inactivation is reversed following a return to hyperpolarized potentials and is an important determinant of K^+ channel function. Therefore, we examined the relative kinetics and roles of slow and N-type inactivation in determining the rate of recovery from inactivation. Figure 4*A* shows an example of raw data traces obtained from FK1 using a standard two-pulse recovery protocol. Figure 4*B* shows the results of applying the same protocol to FK1Δ2-146. Despite very different degrees of total inactivation produced during the first 800 ms P1 pulse to +50 mV, the peak current measured for both channel types recovered very slowly (Fig. 4*C*). When the peak current data from FK1 and FK1Δ2-146 was normalized to the same starting and ending values it became clear that the rate of recovery was unchanged by NH_2-terminal deletion (Fig. 4*D*). This surprising similarity suggested that either recovery from both C-type or N-type inactivation proceeded independently with nearly identical rates or that recovery in both cases proceeded by a common mechanism or state. If recovery was governed by a slow, or C-type, mechanism, this further suggested that in the presence of the NH_2-terminus nearly 100% of the current was driven into the slow or C-type inactivated state within the 800 ms pulse. This is consistent with the observed effect of the NH_2-terminus on C-type inactivation reported for *Shaker* B channels (Hoshi *et al.* 1991).

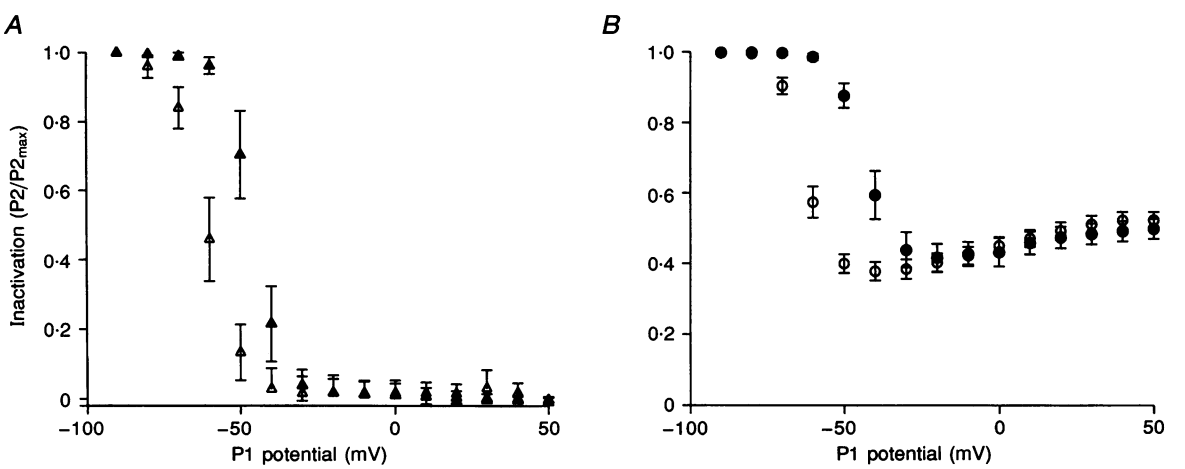


Figure 3. Charge neutralization at position 454 in the S4 voltage sensor produces similar shifts in both N-type and C-type inactivation

A, mutation of arginine to glutamine at position 454 in FK1 (△, denoted FK1-R454Q) shifts inactivation relative to FK1 wild type (▲). The voltage clamp protocol was as follows. A P1 pulse to varying potentials was applied (−90 to +50 mV in 10 mV increments in ascending order) for 500 ms. P2 was to +50 mV for 500 ms (V_h = −90 mV). Inactivation was measured as the percentage reduction in P2 peak current and plotted as a function of P1 potential. Data presented as means ± S.E.M. (n = 12 for FK1 and n = 11 for FK1-R454Q). *B*, application of a similar protocol as in *A* to FK1Δ2-146 wild type (●) and with the analogous arginine to glutamine substitution (○) resulted in a similar hyperpolarizing shift in inactivation (P1 duration = 6 s, P2 duration = 500 ms, n = 6).

In order to verify the hypothesis that both FK1 and FK1Δ2-146 recovered through reversal of a C-type mechanism we examined the effects of high extracellular potassium on the rate of recovery of both FK1 and FK1Δ2-146. In other cloned K^+ channel types, elevation of extracellular K^+ has been shown to compete with C-type inactivation (Lopez-Barneo *et al.* 1993) while development of N-type inactivation is thought to be relatively insensitive to extracellular K^+ concentration (Tseng & Tseng-Crank, 1992). It is generally thought that extracellular K^+ acts on C-type inactivation via a 'foot in the door' mechanism which serves to prevent C-type inactivation from closing the extracellular mouth of the pore through direct steric interference (Tseng & Tseng-Crank, 1992; Lopez-Barneo *et al.* 1993). The mechanism by which elevated extracellular K^+ accelerates recovery from inactivation has remained somewhat controversial, although direct electrostatic effects have been suggested as a factor in Kv1.4 channels (Tseng & Tseng-Crank, 1992). We investigated the possibility that the effects of elevated extracellular K^+ on recovery from inactivation were mediated through alteration of the slow inactivation mechanism. As shown in Fig. 5*A*, the rate of the fast inactivation component of FK1 was relatively insensitive to elevation of extracellular K^+ to 98 mM, yet it greatly reduced the degree and rate of inactivation of FK1Δ2-146 (Fig. 5*B*). However, the rates of recovery from inactivation of FK1 and FK1Δ2-146 were both greatly accelerated by elevation of extracellular K^+ (Fig. 5*C* and *D*). This further confirms the C-type nature of the slow inactivation component of FK1Δ2-146 and again implicates the C-type mechanism in governing the recovery from inactivation of

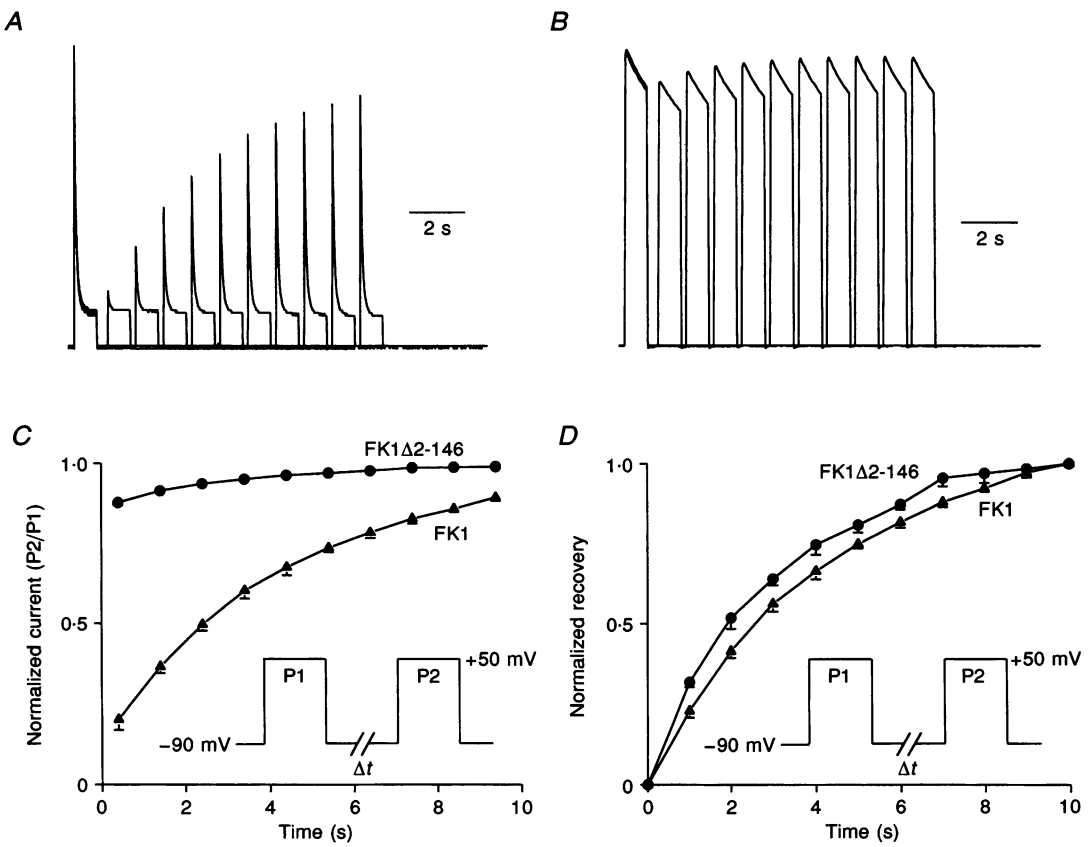


Figure 4. The rate of recovery from inactivation is insensitive to NH_2-terminal deletion

A, recovery of FK1 measured using a two-pulse protocol. A P1 pulse to +50 mV was applied for 800 ms and was repolarized to a recovery potential of −90 mV for a variable duration Δt followed by a second P2 pulse to +50 mV for 800 ms (stimulation rate = 0·033 Hz). As Δt increased to several seconds the peak current slowly returned to control values. *B*, recovery of FK1Δ2-146 using a voltage clamp protocol which was identical to the protocol in *A*. *C*, mean fractional recovery of peak FK1 and FK1Δ2-146 current as a function of time. Peak current during P2 obtained from FK1 ($n = 4$) and FK1Δ2-146 ($n = 4$) was obtained using the protocol described above, normalized to peak P1 current, and plotted as a function of Δt. *D*, the relative degree of inactivation at the end of the P1 pulse differed significantly between FK1 and FK1Δ2-146. For the purpose of comparison of the rate of recovery, recovery data in *A* were renormalized with respect to initial value ($\Delta t = 400$ ms) and final value ($\Delta t = 10{\cdot}4$ s). Despite the large difference in the magnitude of inactivation, the rate of recovery was virtually identical.

FK1 and conferring the sensitivity of recovery rate to extracellular $[K^+]$.

C-type inactivation can also be distinguished by its sensitivity to mutations near the mouth of the extracellular pore. Mutation of threonine at position 449 to tyrosine in *Shaker* B channels has been shown to remove C-type inactivation (Hoshi *et al.* 1991). We introduced the analogous point mutation lysine to tyrosine at position 532 (K532Y) in both FK1 and FK1Δ2-146 to further verify that slow inactivation was mediated by a C-type mechanism in these channels and that recovery rate was governed by a C-type inactivation mechanism. As can be seen in Fig. 6*B*, inactivation of FK1Δ2-146 was greatly slowed by the K532Y mutation while the fast inactivation time constant of FK1 (Fig. 6*A*) was insensitive to the mutation. However, the rate of recovery from inactivation of both FK1 and FK1Δ2-146 was greatly increased by the K532Y mutation (Fig. 6*C* and *D*), confirming that FK1Δ2-146 inactivation occurs via a C-type mechanism and that recovery of both FK1 and FK1Δ2-146 occurs through a C-type mechanism.

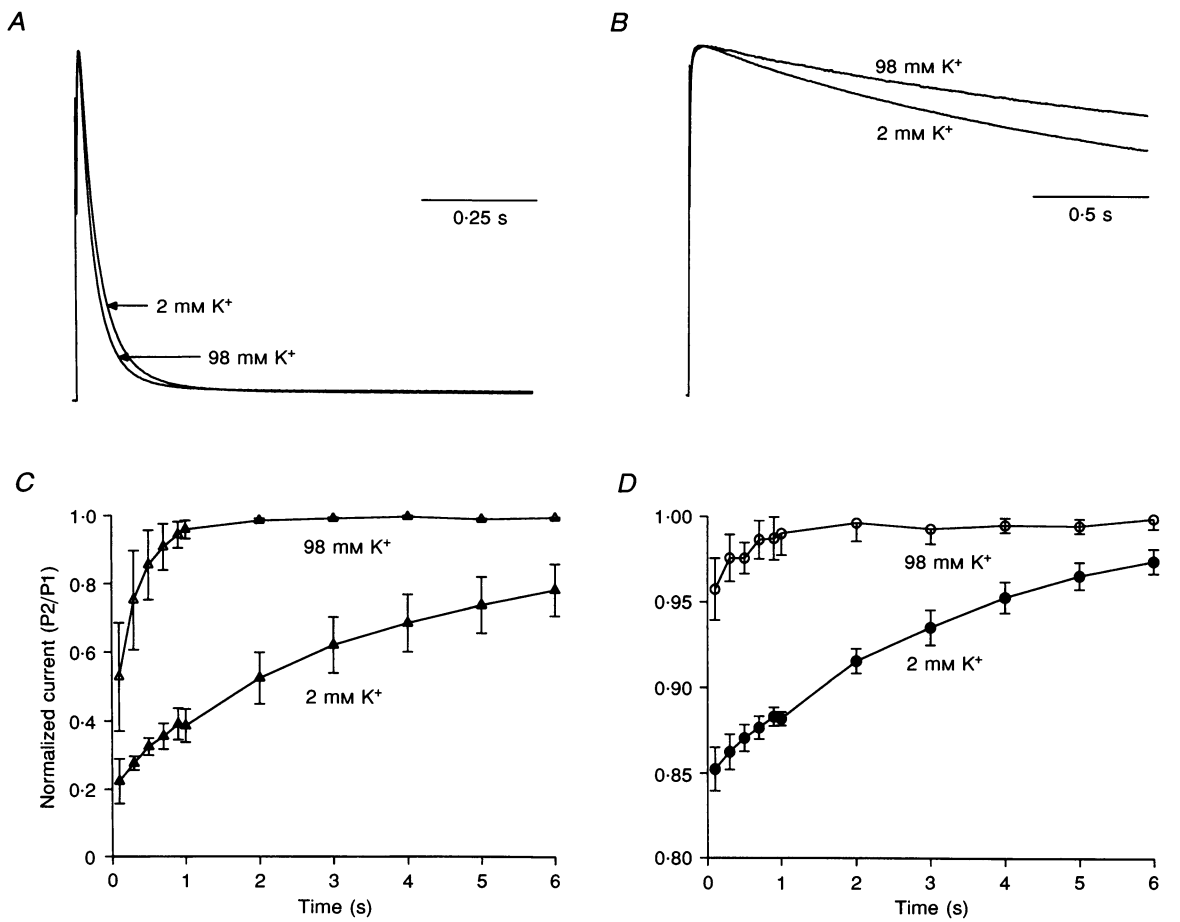


Figure 5. Recovery from inactivation of FK1 and FK1Δ2-146 is accelerated by high extracellular K^+

A, FK1 current traces recorded at +50 mV in the presence of control (2 mM) $[K^+]_o$ and high (98 mM) $[K^+]_o$ show no change in the rate of fast inactivation. Currents recorded during a pulse from −90 to +50 mV (stimulation rate = 0·03 Hz) and normalized for comparison (peak conductance and E_{rev} changed with elevated $[K^+]_o$). *B*, FK1Δ2-146 current traces recorded at +50 mV in the presence of 2 mM $[K^+]_o$ and 98 mM $[K^+]_o$ show a marked decrease in the rate of development of inactivation. Currents recorded during a pulse from −90 to +50 mV (rate = 0·03 Hz) and normalized for comparison. *C*, the rate of recovery of FK1 was accelerated by elevation of $[K^+]_o$. Mean fractional recovery of peak FK1 currents ($n = 3$) was plotted as a function of time in 2 mM and 98 mM K_o^+. Peak P2 current was obtained using the two-pulse protocol described in Fig. 4 with a 2 s P1 pulse and normalized to peak P1 current and plotted as a function of Δt. *D*, although application of 98 mM K^+ decreased the rate and degree of inactivation of FK1Δ2-146, some inactivation did occur. The rate of recovery from inactivation of FK1Δ2-146 was accelerated by elevation of $[K^+]_o$. Mean fractional recovery of peak FK1Δ2-146 currents ($n = 3$) was plotted as a function of time in 2 mM and 98 mM $[K^+]_o$. Peak P2 was measured using the two-pulse protocol described as in Fig. 4 and normalized to peak P1 current and plotted as a function of Δt.

Recovery from inactivation has also been suggested to be energetically coupled to movement of a portion of the voltage sensor in other K^+ channel types (e.g. Hille 1992). We examined the effects of the S4 R454Q mutation on the rate of recovery from inactivation. As can be seen in Fig. 7 the FK1Δ2-146 recovery rate at −90 mV was strongly slowed by the mutation. This is despite the fact that the inactivation rates of FK1Δ2-146 at +50 mV were unaffected by the S4 mutation. Thus, activation may be permissive with respect to development of both N-type and C-type inactivation, but during recovery, C-type inactivation may have kinetic requirements which depend upon backwards movement of the S4 voltage sensor. We speculate that activation merely brings regions involved in forming the C-type inactivated conformation into physical proximity to each other. These regions then form bonds which stabilize a conformation in which the extracellular mouth of the pore is occluded. Such a sequence of events would make the rate of C-type inactivation insensitive to S4 activation charge, provided the channel was fully activated. However, during deactivation movement or force transduced from the charges on S4 physically contributes to breaking or 'pulling apart' these bonds, thus making recovery from C-type inactivation sensitive to the charge mutations on S4.

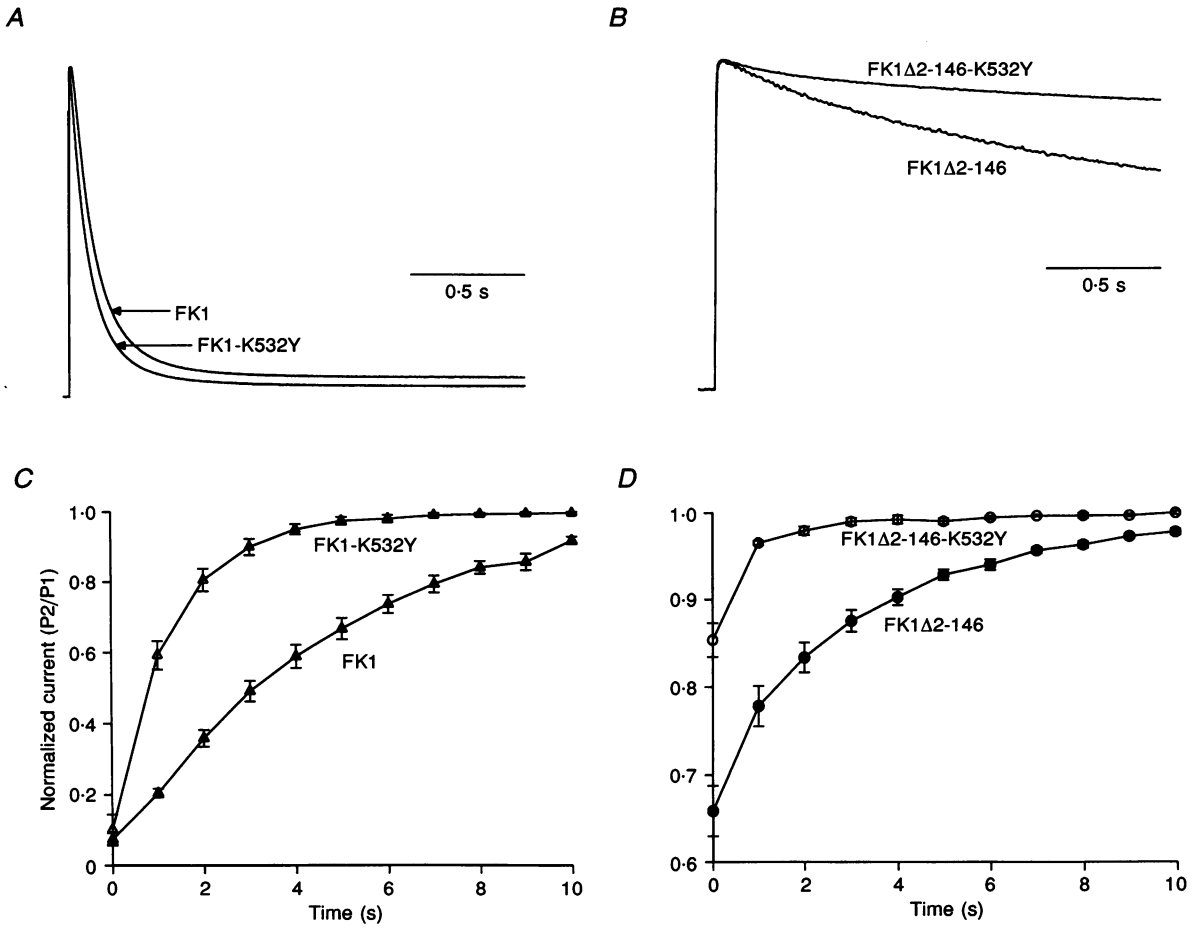


Figure 6. Recovery from inactivation of both FK1 and FK1Δ2-146 is accelerated by mutations at position 532 near the extracellular mouth of the pore

A, raw current traces of FK1 and FK1-K532Y in response to a depolarizing step from −90 to +50 mV. Note that inactivation rate is insensitive to the mutation near the extracellular mouth of the pore. Currents were normalized to peak for purposes of comparison. No leakage correction was applied. *B*, raw current traces of FK1Δ2-146 and FK1Δ2-146-K532Y in response to a depolarizing step from −90 to +50 mV. Currents were normalized to peak for purposes of comparison. Note that the degree of inactivation developed during the 2 s pulse is much less for the K523Y pore mutation, consistent with the C-type nature of this inactivation process. *C*, recovery from inactivation of FK1 ($n = 3$) and FK1-K532Y ($n = 4$). Note that recovery from inactivation is greatly accelerated by the mutation. No leakage correction was applied. *D*, recovery from inactivation of FK1Δ2-146 ($n = 4$) and FK1Δ2-146-K532Y ($n = 4$). Note that recovery from inactivation is greatly accelerated by the mutation. Recovery from a 2 s inactivating pulse to +50 mV was measured as described previously.

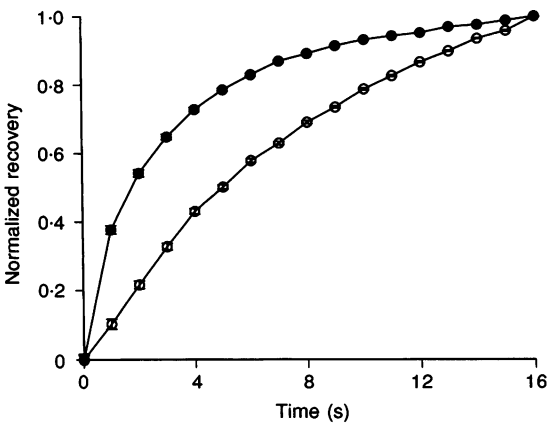
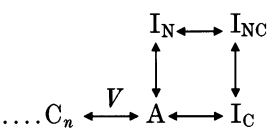


Figure 7. Normalized average recovery from inactivation of FK1Δ2-146 and FK1Δ2-146-R454Q

●, FK1Δ2-146; $n = 7$. ○, FK1Δ2-146-R454Q; $n = 7$. Recovery was measured as in previous figures except that the rate of protocol application for the charge neutralization mutants was once every 30 s. The rate of FK1Δ2-146 inactivation was slowed by S4 mutation.

C-type inactivation governs recovery for brief depolarizations

The preceding data indicate that recovery from inactivation of FK1 is governed by reversal of the C-type mechanism. However, it is clear that C-type inactivation proceeded much more quickly and to nearly 100% completion in the presence of N-type inactivation. Thus, N-type and C-type inactivation of FK1 might be described as coupled processes similar to those proposed for *Shaker* B channels.



Model A

where C_n is the nth closed state, V denotes the final voltage-sensitive transition in the activation pathway, A is the open or activated states, I_N is the state of the FK1 (Kv1.4) channel which has been inactivated by binding of the NH_2-terminal ball, I_C is the C-type inactivated state of FK1 alone and I_{NC} is the state in which FK1 is both C-type and N-type inactivated.

The degree to which C-type inactivation dominates recovery for such a model is dependent upon the duration of depolarization and the relative rates of the $I_N \rightarrow I_{NC}$, $A \rightarrow I_N$ and $I_N \rightarrow A$ transitions. If the $I_N \rightarrow I_{NC}$ transition rate is sufficiently slower than the $A \rightarrow I_N$ and $I_N \rightarrow A$ transition rates, Model A predicts that for brief pulses, recovery from inactivation will be governed by fast recovery from N-type inactivation and will slowly change to a slower C-type as the inactivating pulse increases in duration. Figure 8*A* shows the recovery from inactivation of FK1 from inactivation P1 pulses ranging in duration from 70 ms to 2 s. When these data were re-normalized to account for the different degrees of inactivation at the end of the P1 pulse (Fig. 8*B*) it became clear that the rate of recovery from

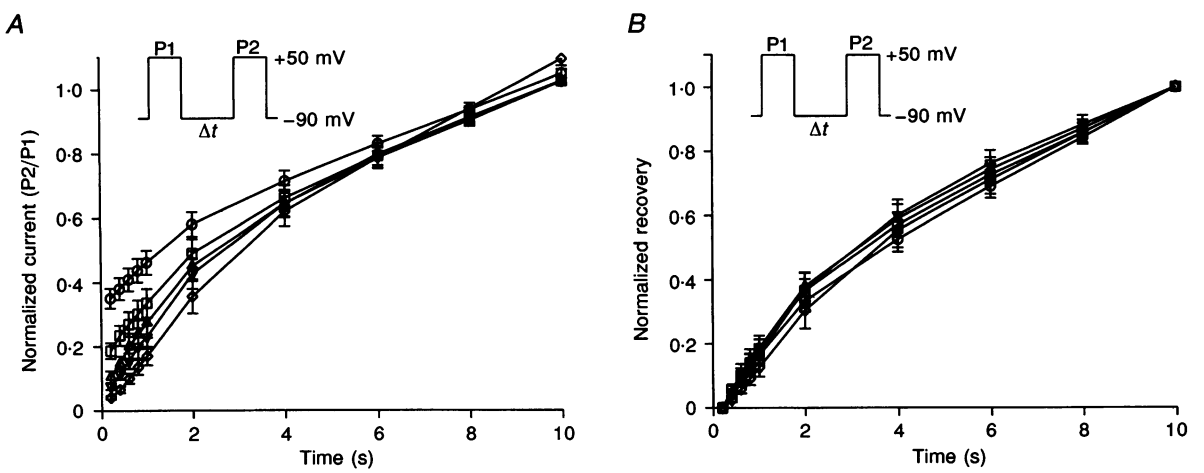


Figure 8. Development of C-type following N-type inactivation

A, average time course of recovery from inactivation of FK1 for P1 durations of 70 (○), 150 (□), 300 (△), 500 (▽) and 2000 ms (◇) (recovery was plotted as the ratio of peak P2 current to peak P1 current. P1 = +50 mV, $V_h = -90$ mV, P2 = +50 mV; data plotted as means ± S.E.M., $n = 4$). *B*, the data from *A* were normalized to the same relative amplitude (peak P2 current for $\Delta t = 200$ ms was defined equal to 0 and peak P2 current for $\Delta t = 10$ s was defined equal to 1) for comparison of the time course of recovery from inactivation for different P1 pulses. Despite the different levels of inactivation, the time course of recovery was unaltered by varying P1 duration.

inactivation was insensitive to the P1 pulse duration. Thus the $I_N \rightarrow I_{NC}$ transition rate is sufficiently fast relative to either $A \rightarrow I_N$ or $I_N \rightarrow A$ that, regardless of the duration of depolarization tested, recovery is governed by reversal of the C-type inactivation mechanism.

DISCUSSION

The two most important findings of this study are: (i) the identification of slow inactivation of Kv1.4 channels which is due to a C-type mechanism analogous to that previously described in *Shaker* K^+ channels (Hoshi *et al.* 1991), and (ii) the demonstration that slow recovery from inactivation observed for this channel type (i.e. in both FK1 and FK1Δ2-146) occurs at the same rate and is influenced by the same factors (e.g. extracellular K^+, S4 mutation at position 454 or mutation at position 532 in the mouth of the pore) regardless of the presence or absence of the NH_2-terminus. This indicates that recovery from inactivation is most probably rate limited by reversal of the C-type inactivation mechanism.

C-type inactivation in Kv1.4 channels

The existence of C-type inactivation in Kv1.4 channels was called into question experimentally by Tseng & Tseng-Crank (1992) on the basis of the insensitivity to K^+ elevation of an N-terminal deletion mutant of RHK1 (also a Kv1.4 channel). In addition, the amino acid residue in the wild-type channel at position 532 is a lysine. If we extrapolate directly from the mutational analysis of *Shaker* channels (Lopez-Barneo *et al.* 1993), a lysine at this position should produce an inactivation rate of approximately 25 ms for an NH_2-terminal deletion mutant. As shown in Fig. 1 the observed inactivation rate in FK1Δ2-146 is close to 3 s, implying that the C-type inactivation rate of FK1 (Kv1.4) cannot easily be implied or predicted from analysis of *Shaker* mutant channels on the basis of the identity of a single amino acid. However, it should be noted that the sensitivity of recovery from inactivation and the rate of slow inactivation in the NH_2-terminal deletion mutant to mutation at position 532 in Kv1.4 confirm the C-type nature of slow inactivation. We are uncertain as to the basis for the differential sensitivity to external K^+ between our NH_2-terminal deletion of FK1 and that of RHK1 by Tseng & Tseng-Crank (1992). Furthermore, it is not clear why recovery of RHK1 was accelerated by elevated K^+ but the NH_2-terminal deletion of RHK1 was insensitive. The differences between RHK1 and FK1 may reflect a genuine biophysical difference due to one of the seventeen non-identical amino acid residues scattered over the entire clone. Other possible explanations for the discrepancy may be due to the existence of additional inactivation mechanisms as suggested by Tseng & Tseng-Crank (1992) which may have been sensitive to the exact length of NH_2-terminal deletion. The existence of additional inactivation mechanisms is quite possible given the recent identification of another inactivation mechanism (P-type inactivation; DeBiasi, Hartmann, Drewe, Taglialatela, Brown & Kirsch, 1993). Indeed, the mutation of residue 532 to tyrosine in FK1 which abolishes C-type inactivation in *Shaker* K^+ channels does not completely abolish slow inactivation in FK1.

Recovery mechanisms in Kv1.4 and *Shaker* channels

In *Shaker* K^+ channels, where both N-type and C-type inactivation co-exist, the relative roles of N-type and C-type inactivation in determining the properties of recovery are significantly different from those described for Kv1.4 in this study. Hoshi *et al.* (1991) demonstrated that mutations in the S6 region which altered the stability and rate of inactivation of an NH_2-terminal deletion mutant of *Shaker* also altered the rate of recovery of the channel in the NH_2-terminal intact *Shaker* channel. On this basis, Hoshi *et al.* (1991) proposed that *Shaker* A channels which support C-type inactivation with time constants of the order of magnitude of 10–20 ms inactivate by both mechanisms, but that recovery was governed by C-type inactivation. For *Shaker* B channels, which have a much slower C-type inactivation, Hoshi *et al.* (1991) proposed that for pulses of up to 100 ms in duration inactivation was governed by an N-type mechanism. Hoshi *et al.* further proposed that the multi-exponential recovery observed in previous studies of *Shaker* B represented recovery from both the N- and C-type as well as potentially other inactivated states. The mechanism underlying recovery in *Shaker* B is further complicated by the recent report of multiple N-type inactivated states (I_{N1} and I_{N2}; Gomez-Lagunas & Armstrong, 1994). Recovery of *Shaker* B for 40 ms pulses was reported by Gomez-Lagunas & Armstrong (1994) to be governed by the I_{N2} state and C-type inactivation was reported to proceed with time constants of the order of seconds and not to be a factor in their analysis of recovery. In contrast, our studies suggest that recovery of FK1 is governed by reversal of C-type inactivation, regardless of pulse duration for a wide range of depolarization times.

The differences between *Shaker* and FK1 with respect to their N-type inactivation, C-type inactivation and the coupling between N-type and C-type inactivation are undoubtedly due to the differences in amino acid sequence. Between *Shaker* B and FK1 there is extensive amino acid identity only in the S1–S6 transmembrane spanning segments (ranging between 100% identity in S6 to approximately 50% identity in S2), the H5 loop, the intracellular S4–S5 linker and the putative subunit assembly domain (Li, Jan & Jan, 1992). The majority of the N- and C-termini and the linkers between the membrane spanning domains show little or no identity. Thus, although it is possible that some of the molecular determinants of N-type to C-type coupling reside in the transmembrane segment differences between these channels it seems equally likely that differences in non-transmembrane cytoplasmic and extracellular domains confer the difference in phenotype.

Functional coupling between N- and C-type inactivation

In the absence of the NH_2-terminal domain, C-type inactivation has an intrinsic coupling to the voltage sensor. The similarity in the parameters of the Boltzmann distribution describing C-type inactivation and the Boltzmann distribution describing N-type inactivation suggested that both N-type and C-type activation might be coupled by a similar degree to channel activation. This hypothesis was further supported by the observation that neutralization of S4 arginine 454 to glutamine resulted in hyperpolarizing shifts in inactivation of both FK1 and FK1Δ2-146. This is not to suggest that the two types of inactivation are mediated by similar mechanisms, but rather that a given degree of movement of the voltage sensor may result in conformational changes which permit both types of inactivation to proceed.

C-type inactivation can proceed in the absence of an intact NH_2-terminus but has a slow time constant of approximately 3 s. However, FK1 (in the presence of N-type inactivation) recovery rate is governed by reversal of the C-type mechanism after relatively brief depolarizing pulses, indicating that N-type inactivation facilitates C-type inactivation in a partially coupled fashion similar to that suggested by Hoshi *et al.* (1991) for the *Shaker* K^+ channel. The mechanism by which binding of the NH_2-terminus at an intracellular site can facilitate development of C-type inactivation near the extracellular face of the pore remains unclear. However, one known important consequence of N-type inactivation is immobilization of S4 gating charge movement. Given the shared dependence of C-type and N-type inactivation on the S4 sensor (Fig. 3), it seems likely that N-type inactivation immobilizes the S4 voltage sensor and perhaps shifts other regions of the channel into a conformation which is conducive to C-type inactivation.

Possible physiological consequences of C-type inactivation in Kv1.4

In the absence of the NH_2-terminus, C-type inactivation of Kv1.4 occurs very slowly and is incomplete at all potentials. As a consequence, the C-type mechanism is frequently regarded as a secondary mechanism compared with N-type inactivation. Indeed, in response to a single depolarizing pulse, the inactivation time course of FK1 is dominated by a first-order NH_2-terminal driven mechanism with only a very small second exponential component to suggest that inactivation may involve multiple mechanisms. However, we have shown that C-type inactivation is critically important in determining the rate of recovery and therefore is likely to be of primary physiological importance in determining the refractory period of Kv1.4 channels. In the case of cardiac muscle, the C-type mechanism could potentially govern the sensitivity of the recovery rate to extracellular K^+, which has been shown to accumulate in the extracellular spaces during ischaemia, fibrillation and tachycardia. In addition, C-type inactivation may play a role in determining the binding site access and use dependence of pharmacological agents (Russell *et al.* 1995).

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