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Slowed Inactivation at Positive Potentials in a Rat Axonal K^+ Channel is not Due to Preferential Closed-State Inactivation

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Summary

We have investigated slow inactivation in a rat axonal K^+ channel, the I channel. Using voltage steps to potentials between –70 mV and +80 mV, from a holding potential of –100 mV, we observed a marked slowing of inactivation at positive potentials: the time constant was 4.5±0.4 s at –40 mV (mean ± S.E.M.), increasing to 14.7±2.0 s at +40 mV. Slowed inactivation at positive potentials is not consistent with published descriptions of C-type inactivation, but can be explained by models in which inactivation is preferentially from closed states (which have been developed for Kv2.1 and some Ca^{2+} channels). We tested two predictions of preferential closed-state models: inactivation should be more rapid during a train of brief pulses than during a long pulse to the same potential, and the cumulative inactivation measured with paired pulses should be greater than the inactivation at the same time during a continuous pulse. The I channel does not behave according to these predictions, indicating that preferential closed-state inactivation does not explain the slowing of inactivation we observe at positive potentials. Inactivation of the I channel therefore differs both from C-type inactivation, as presently understood, and from the inactivation of Kv2.1.

Key words
Potassium channel • Inactivation • Axon • Rat

Introduction

The objective of this study was to establish whether slowing of inactivation of an axonal K^+ channel at positive potentials could be accounted for by a kinetic model of preferential closed-state inactivation. Two mechanisms of K^+ channel inactivation have been described (for review see Yellen 1998). The faster process, N-type inactivation, involves binding of an inactivation particle at the N-terminal end of the channel protein to a site within the pore (described by the "ball and chain" model, Hoshi *et al.* 1990). The slower process (with time constants between 200 ms and 10 s), generally known as C-type inactivation in Kv1 K^+ channels, is less clearly understood. However, there is accumulating evidence that it involves conformational changes at the outer mouth of the channel pore by which it becomes occluded during long depolarizations (Lopez-Barneo *et al.* 1993, Yellen *et al.* 1994). The important effects of permeant ions on the time course of slow inactivation (Fedida *et al.* 1999) and the changes in selectivity associated with the process (Starkus *et al.* 1997, Kiss and

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Korn 1998) indicate that the selectivity filter participates in C-type inactivation. It has been shown that many mutations in the vicinity of the selectivity filter, as well as within the filter itself, affect the rate of C-type inactivation (Perozo *et al.* 1993, Yang *et al.* 1997). Studies of inactivation in a Kv1.4 mutant with N-type inactivation removed indicate that the process is coupled to activation and that the voltage-dependence of C-type inactivation can be entirely accounted for by this coupling; inactivation becomes more rapid with more positive potentials in the voltage range where the activation of the channel is incomplete, and the rate is independent of voltage in the range where the channel is fully activated (Rasmusson *et al.* 1995).

The starting point of this study was the observation that inactivation of the major axonal delayed rectifier K^+ channel, the I channel, becomes slower at more positive potentials (Reid *et al.* 1999). Similar behavior was noted in an earlier study on K^+ currents in *Xenopus* nodes of Ranvier, in a current component later identified as being carried by the I channel (Schwarz and Vogel 1971). The I channel belongs to the delayed rectifier family and carries most of the potassium current in vertebrate axons (Jonas *et al.* 1989, Safronov *et al.* 1993, Scholz *et al.* 1993, Koh and Vogel 1996, Reid *et al.* 1999). The human I channel appears to lack N-type inactivation, but it is not yet clear whether its slow inactivation is C-type (Reid *et al.* 1999).

In this study, we have shown that the inactivation of the rat I channel current, like that in human and *Xenopus* axons, becomes slower at positive potentials, in the voltage range where the channel is already fully activated. This slowing of inactivation at positive potentials is not consistent with published descriptions of C-type inactivation (Rasmusson *et al.* 1995), but a similar phenomenon has been reported for the Kv2.1 K^+ channel and neuronal N, P/Q and R Ca^{2+} channels, and can be explained using models in which inactivation occurs preferentially from partially activated closed states (Klemic *et al.* 1998, Patil *et al.* 1998).

Our aim in this study was to test whether preferential closed-state inactivation can explain the slower inactivation of the I channel at positive potentials. In addition, as studies of slow inactivation have until now been performed almost exclusively on a small set of cloned K^+ channel types, we wanted to clarify to what extent these studies describe the behavior of a native K^+ channel.

We therefore tested some predictions of models incorporating preferential closed-state inactivation. The axonal I channel failed to confirm these predictions. Inactivation of the rat I channel is therefore not adequately explained either by C-type inactivation, as presently understood from studies in Kv1 K^+ channels, or by the preferential closed-state inactivation models developed to describe the slow inactivation in the Kv2.1 channel and neuronal Ca^{2+} channels.

Methods

Preparation

The preparation was similar to that previously described for human axons (Reid *et al.* 1999). Briefly, ventral and dorsal roots were dissected from male Wistar rats (300-450 g) which had been killed by asphyxiation with CO_2 followed by exsanguination. The roots were split longitudinally to expose the endoneurium to the enzyme solution, tied at the ends and pinned to the base of a chamber moulded from silicone resin (Wackersilicone 601-A, Wacker Chemie GmbH, München, Germany). They were then incubated at 37 ºC in the presence of the following enzymes: first stage – 1 mg ml^{-1} collagenase (C-7657; Sigma, Deisenhofen, Germany) for 60 min; second stage – 0.5 mg ml^{-1} protease (P-8038; Sigma) for 30 min. Enzymes were dissolved in the standard extracellular solution described below. After enzyme treatment the roots were gently shaken to promote the separation of individual axons, which were then allowed to adhere to the base of 35 mm plastic tissue culture dishes (29000, Corning Glass Works, Corning, NY, USA) which had been coated with uncured silicone (applied onto a thin layer of cured silicone) to improve adhesion. Recordings were made from 2 to 7 h after preparation.

Recording system

Outside-out patches (Hamill *et al.* 1981) were obtained from internodal and paranodal regions of demyelinated axons, and recordings were performed in symmetrical 155 mM KCl (see below for solutions). Recordings were made using an EPC-7 amplifier (HEKA-Elektronik, Lambrecht, Germany). Temperature during recordings was kept at 25 °C using a TC-202A temperature controller and PDMI-2 microincubator (Medical Systems Corp., Greenvale, NY, USA). Pipettes were pulled from borosilicate glass (GC150F, Clark Electromedical Instruments, Pangbourne, England) and

heat-polished to give resistances between 5 and 20 MΩ (bubble numbers around 5.5-6.5). The current was filtered at 10 kHz (–3 dB; 3-pole Bessel filter) and recorded on a video cassette recorder using digital pulse code modulation (modified Sony PCM-701ES digital audio processor). The current was also filtered at 3 kHz (–3 dB; 3-pole Bessel filter) and the output of this filter was led to the input of an A/D converter (MC-DAS 1612, Scientific Solutions, Mentor, OH, USA), mounted in an IBM PS/2 computer. Data acquisition and control of the pulse protocols was performed using pCLAMP software, version 5.6 (Axon Instruments, Foster City, CA, USA). Data analysis was done with pCLAMP and with software written by ourselves. The data are presented as mean ± S.E.M.

Solutions

The extracellular solution used for enzyme treatment and storage of the dissociated axons contained (in mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, HEPES 10, NaOH 4.54, D-glucose 20 (pH 7.4 at 25 °C). The intracellular recording solution contained (in mM): KCl 149, NaCl 5, EGTA 3, KOH 6, HEPES 10, $MgCl_2$ 1.35 (free $[Mg^{2+}]$ 1.1 mM). The pH was adjusted with NaOH to 7.25 at room temperature. In the initial experiments, we used a solution which also contained $CaCl_2$ 1.2 mM and $MgCl_2$ 1.2 mM (free $[Ca^{2+}]$ 10^{-7} M and $[Mg^{2+}]$ 1.1 mM). Free Ca^{2+} and Mg^{2+} concentrations were calculated using React II software (Prof. G.L. Smith, University of Glasgow, Scotland). The high-$[K^+]$ extracellular solution used for all recordings contained (in mM): KCl 155, NaCl 6.5, $CaCl_2$ 2.2, $MgCl_2$ 1.2, HEPES 10, NaOH 4.54 (pH 7.4 at 25 °C). All materials were reagent grade, from Sigma or Fisher Scientific (Loughborough, UK).

Simulations

Simulations were done using the model of Klemic *et al.* (1998) implemented in a program written by G.R. in Microsoft Professional Basic version 7.1. The accuracy of the implementation was verified by repeating the simulations presented by the original authors. The only modification made to the model was that the gating charge on the $O_4 \rightarrow C_4$ transition was set to zero in Figure 4B.

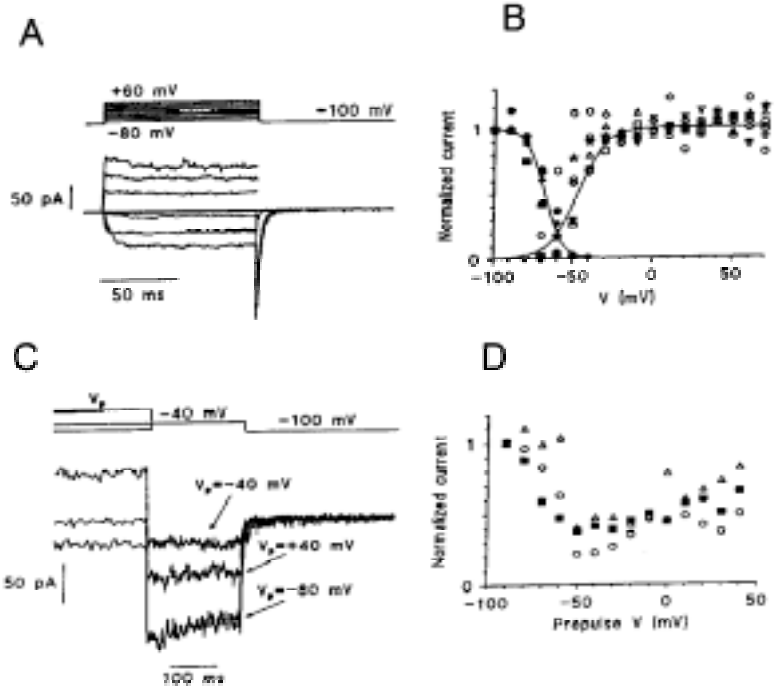

Fig. 1. *Activation and inactivation of the I channel current in rat axons.* ***A****. Currents in response to voltage steps between –80 and +60 mV at 20 mV intervals. All recordings in this and other figures are from outside-out patches from paranodes and internodes of rat axons, and all are in symmetrical 155 mM* K^+ *(see Solutions). Holding potential was –100 mV. Correction of leak currents and capacitive transients was performed offline by digital subtraction using the current during the pulse to 0 mV.* ***B.*** *Voltage dependence of activation of the I channel current in 7 patches (open symbols) and of inactivation in 4 different patches (filled symbols), measured as described in the text. Smooth curves show fits of the Boltzmann equation* $f(V) = 1/(1 + \exp((V_{0.5} - V)/k))$ *with the following values: activation,* $V_{0.5}$ *= –47.3 mV, k = 9.0 mV; inactivation,* $V_{0.5}$ *= –67.9 mV, k = –5.7 mV (see text).* ***C****. Voltage dependence of inactivation following brief prepulses. The voltage protocol consisted of a 5 s prepulse to voltages between –90 and +40 mV, followed by a 200 ms test pulse to –40 mV. Recordings using three different prepulse voltages (–80, –40 and +40 mV) are shown. The different noise level is due to the change in sampling frequency from 200 Hz to 1 kHz at the beginning of the test pulse. No leak or capacitance correction.* ***D****. Current amplitude during the test pulse to –40 mV (normalized to that after a –90 mV prepulse) in the recordings shown in C (open circles) and two other patches (other symbols), plotted against the prepulse potential.*

Results

Properties of the I channel current

The current component contributed by the I channel was identified by comparison with the known activation, deactivation and inactivation properties of single I channels in amphibian, rat and human axons (Jonas *et al.* 1989, Safranov *et al.* 1993, Scholz *et al.* 1993, Koh and Vogel 1996, Reid *et al.* 1999). Figure 1A shows the K^+ current in a large outside-out patch in which the current was dominated by this component. The greater part of the current in all patches (mean 91.4 %, range 62.2-100 %) deactivated with a time constant of 1.5-3.9 ms at –100 mV (n = 24), and the voltage dependence of activation of this component in seven different patches is shown in Figure 1B. The voltage dependence is derived from the amplitude of the current component deactivating with a time constant of 1.5-3.9 ms in tail currents after depolarizing pulses of 100 ms duration. The current activates at around –70 mV, and the activation reaches a plateau at around –40 mV. The voltage dependence of activation for each patch (normalized as described in Reid *et al.* 1999) was fitted separately with a Boltzmann function (see legend to Fig. 1B) which yielded a half-maximal activation voltage ($V_{0.5}$) of –47.3±1.0 mV and a slope factor (k) of 9.0±1.2 mV (n = 24).

Inactivation of the I channel current is slowed at positive potentials

Steady-state inactivation of this current was measured by applying long (1 min) prepulses to potentials between –100 and –40 mV, each followed by four 50 ms pulses to –40 mV. The current in response to these pulses was averaged over the last 20 ms of each pulse and normalized to that from a holding potential of –100 mV. The normalized current amplitudes are shown in Figure 1B. Half of the current was inactivated at –67.9±1.7 mV and the slope factor k was –5.7±0.3 mV (n=4); inactivation was complete at –40 mV. This voltage dependence is similar to that described in human axons (Reid *et al.* 1999) and for the slowly inactivating K^+ current in *Xenopus* nodes (Schwarz and Vogel 1971). In all patches, the slow component of inactivation had a time constant consistent with that published for the I channel (Safronov *et al.* 1993, Reid *et al.* 1999). We conclude that the slowly inactivating K^+ current component with the activation and deactivation properties mentioned above was carried predominantly by the I channel, and we will therefore refer to it as the I channel current in the reminder of this article.

When depolarizing prepulses of 5 s duration were applied to partially inactivate the I channel current (Fig. 1C), the resulting voltage dependence of inactivation was U-shaped (Fig. 1D). Inactivation was greatest around –40 mV, and less at more negative or more positive potentials. At negative voltages, the less complete inactivation is explained by the steady-state voltage dependence of inactivation in Figure 1B; at positive potentials, inactivation during these relatively short prepulses is incomplete not because steady-state inactivation is incomplete, but because the time course of inactivation is slower at more positive potentials.

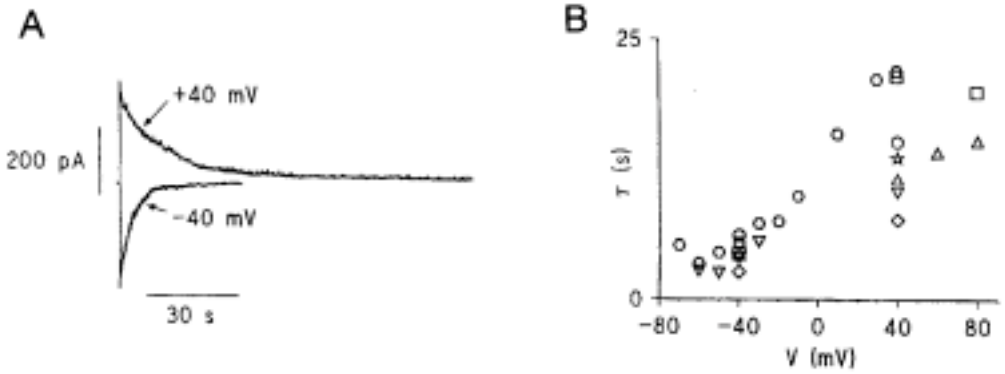

Fig. 2. *Slowing of inactivation of the I channel current at positive potentials* ***A.*** *Current in response to pulses to –40 mV (pulse duration was 40 s) and +40 mV (2 min). Holding potential was –100 mV. No leak or capacitance correction.* ***B.*** *Voltage dependence of the inactivation time constant measured from the currents in panel A (circles) and in 6 other patches (other symbols). These are the same seven patches as shown in Fig. 1B.*

This can be seen in Figure 2A, which shows currents in response to pulses to –40 mV and +40 mV from a holding potential of –100 mV. The duration of the depolarizing pulse at each potential (40 s at –40 mV and 2 min at +40 mV) was chosen in order to allow inactivation to reach a steady state. Inactivation was

complete except for isolated channel openings at potentials positive to +60 mV. The slow phase of K^+ current inactivation is well fitted with a single exponential, and the voltage dependence of the fitted inactivation time constant in seven patches is shown in Fig. 2B. It can be seen that inactivation becomes substantially slower with more positive potentials: the time constant (τ) was 4.5±0.4 s at –40 mV (n=7), increasing linearly to 14.7±2.0 s at +40 mV (n=7). This slowing of inactivation occurs at potentials where the channel is fully activated (positive to –40 mV). Although the variability between patches is large, especially at voltages positive to 0 mV, inactivation in every patch became monotonically slower with more positive potentials, with a single exception (squares in Fig. 2B at +40 mV and +80 mV).

Preferential closed state inactivation does not account for the slowing of inactivation at positive potentials

As mentioned in the Introduction, this behavior is inconsistent with published descriptions of C-type inactivation. However, a similar slowing has been observed in the Kv2.1 delayed rectifier K^+ channel and in some neuronal Ca^{2+} channels, and can be accounted for by a kinetic model of preferential closed-state inactivation (Klemic *et al.* 1998, Patil *et al.* 1998). We therefore tested two predictions of preferential closed-state inactivation models: firstly, that inactivation should be more rapid during a train of brief pulses than during a long pulse to the same potential (Klemic *et al.* 1998), and secondly, that more inactivation should take place in the interval between a pair of depolarizing pulses than during a continuous pulse to the same potential, leading to a greater extent of inactivation during the second pulse of a pair than at the same time during a long pulse ("excess cumulative inactivation", Klemic *et al.* 1998, Patil *et al.* 1998).

In order to test the first prediction, we compared the time course of inactivation during a long depolarizing pulse with that during a train of pulses to the same potential. Figure 3A shows the current during a 40 s pulse from a holding potential of –100 mV to –40 mV (only the beginning is shown; trace a), along with that during a train of pulses to the same potential, each with a duration of 80 ms and a 20 ms interpulse interval (trace b, superimposed). The potentials were chosen as just enough to allow full activation at –40 mV and deactivation at –100 mV, while the interpulse interval of 20 ms during the pulse train was chosen to allow just enough time for deactivation while minimizing the time allowed for recovery from inactivation. The pulse potential of –40 mV was also chosen in order to minimize any contribution from the F channel, which activates at potentials positive to –40 mV (Safronov *et al.* 1993, Reid *et al.* 1999). In both cases, the current decayed with a double-exponential time course. The small fast phase, possibly resulting from a different channel type (see Discussion), did not differ between the long pulse and a train of interrupted pulses; however, the remaining slow phase of inactivation was more rapid during the long pulse (time constant 4.3 s in the patch in Fig. 3A) than during the train of brief pulses (time constant 9.3 s), in contrast to the prediction of models involving preferential closed-state inactivation.

The relationship between rates of macroscopic inactivation in the preceding experiment and the transition rates of the channel molecule between its putative states is not easy to interpret. A simpler and more direct test of preferential closed-state inactivation is provided by a second prediction, excess cumulative inactivation. Preferential closed-state inactivation models predict that more inactivation should occur during the interval between two depolarizing pulses (when the channel would travel along the deactivation and activation pathways and hence would enter the closed state(s) which favor inactivation) than during a continuous pulse which would instead maximize the time in the open state. In order to test this prediction, we applied paired pulses with various interpulse intervals. We used a constant pulse duration of 80 ms and interpulse intervals between 2 and 256 ms. Again, the holding potential was –100 mV and all pulses were to –40 mV. Each pair of 80 ms pulses was bracketed between two long pulses (500 ms). The current amplitude was measured during the last 55 ms of the second short pulse, after activation was complete (I_{S2}), and at the same time during the first short pulse (I_{S1}). The amplitudes at the corresponding times during the long pulses were also measured (I_{L2} and I_{L1}, respectively); the long pulses before and after the pair of short pulses were averaged before measuring the current amplitudes. The corrected amplitude ratio was then calculated as $(I_{S2} * I_{L1}) / (I_{S1} * I_{L2})$.

Figure 3B shows currents during three pairs of pulses, with interpulse intervals of 16, 128 and 256 ms, superimposed on the current during a continuous pulse. The amplitude of the current during the second pulse is the same as or larger than at the corresponding time during the continuous pulse; this observation is valid for all interpulse intervals used (Fig. 3C). Inactivation during a continuous depolarizing pulse is therefore the same or greater than during the interval between two brief depolarizing pulses, and the I channel current does not show excess cumulative inactivation.

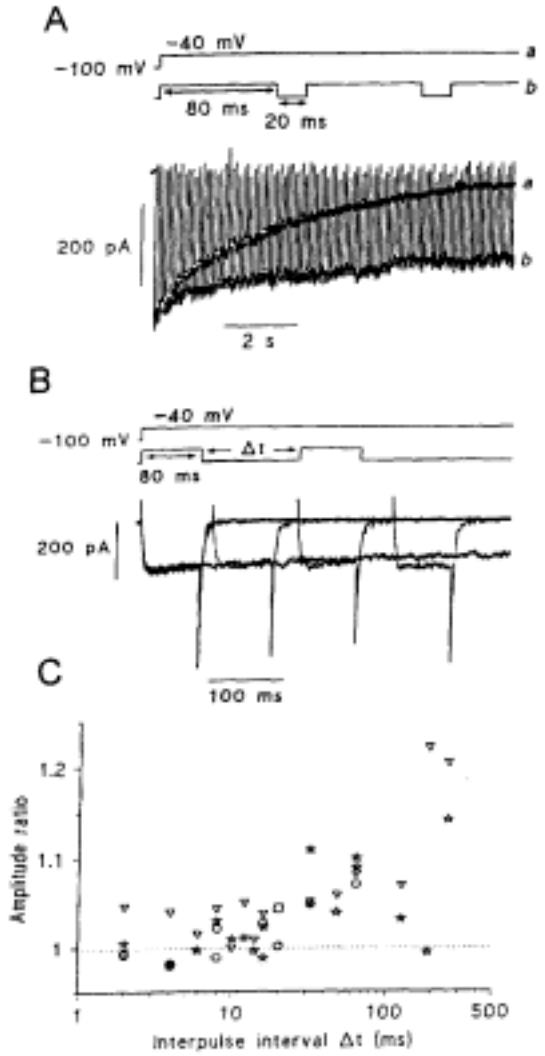

Fig. 3. *Tests of preferential closed-state inactivation.* ***A.*** *The I channel current inactivates faster during a continuous pulse (a) than during a train of brief pulses to the same potential (b). The holding potential was -100 mV and the pulse potential -40 mV. Pulse duration and interval between pulses were 80 ms and 20 ms, respectively. No leak or capacitance correction. Tail currents during the pulse train were deleted for clarity.* ***B.*** *The I channel does not show excess cumulative inactivation during paired-pulse experiments. Currents during three sets of paired 80 ms pulses to -40 mV (interpulse intervals Δt were 16, 128 and 256 ms) are shown superimposed, along with the current during a continuous pulse to the same potential. No leak or capacitance correction.* ***C.*** *The ratio between the current amplitude during the second of the paired pulses and the current amplitude at the same time during the long pulse, plotted against the interpulse interval Δt. Measurements are from the recordings shown in panel B (open circles) and from three other patches (other symbols) The current amplitude ratio was calculated as described in the text.*

Discussion

Inactivation of the I channel current in rat ventral and dorsal root axons becomes slower with more positive potential in the range where the channel is fully activated, in contrast to the behavior reported in K^+ channels which inactivate by a C-type mechanism (Rasmusson *et al.* 1995). This slowing cannot be reproduced by a coupled model where inactivation is voltage independent and occurs primarily from the open state. We have tested the hypothesis that it could be accounted for by preferential closed-state inactivation; our observations lead us to reject this hypothesis, because the I channel current does not behave as predicted by preferential closed-state inactivation models. Inactivation during a train of pulses is slower than during a long pulse to the same potential (Fig. 3A; cf. Klemic *et al.* 1998); and during paired-pulse experiments, no "dip" in the amplitude ratio is seen at short interpulse intervals (Figs 3B and 3C; cf. Patil *et al.* 1998 where a dip of up to 30 % was reported). A comparison of the points in Figure 3C with the time course of deactivation suggests that at an early stage during deactivation, the channel enters a state where inactivation is either slower than from the open state, or is dominated by recovery from inactivation. It should be noted that the predictions we have tested are not only made by the two specific models we have selected (Klemic *et al.* 1998, Patil *et al.* 1998), but are general to this class of model. We can therefore conclude that the I channel does not display preferential closed-state inactivation.

Published reports on rat axonal K^+ channels indicate that the current we have studied here is homogeneous and due to the I channel (Safronov *et al.* 1993, Schneider *et al.* 1993). However, a note of caution is raised by recent work on human axons (Reid *et al.* 1999), where we showed that the axonal α-dendrotoxin-sensitive current, usually attributed to the I channel alone, includes currents through at least two additional channels. One of these, with a 40 pS conductance, appears to inactivate much more rapidly than the I channel: its possible contribution in Figure 3A has already been noted, but the possible effects of its presence on the measurements shown in Figures 3B and 3C need to be considered. If channels of this type were to be present in a patch, to inactivate substantially during the long pulse, and to recover substantially in the interval between the two short pulses, the amplitude ratio could be overestimated. We consider any distortion due to this

cause to be small, for two reasons: firstly, in the patches shown in Figures 3B and 3C, there was no detectable current component inactivating with kinetic properties which could be attributed to the 40 pS channel, and secondly, the human 40 pS channel recovers from inactivation with a time constant of several tens of milliseconds (Reid *et al.* 1999, Reid, unpublished data). The other channel described by Reid *et al.* (1999), with about 25 pS conductance, could not easily be separated from the I channel and could therefore not be fully characterized; it is therefore possible that the current we have studied includes a contribution from this channel type, if it also exists in rat axons. However, our own single-channel recordings (Babes *et al*., unpublished data) as well as published studies in the rat (Safronov *et al.* 1993) and human (Reid *et al.* 1999) suggest that its density is likely to be substantially less than that of the I channel, making it unlikely that it would distort the conclusions drawn from our recordings.

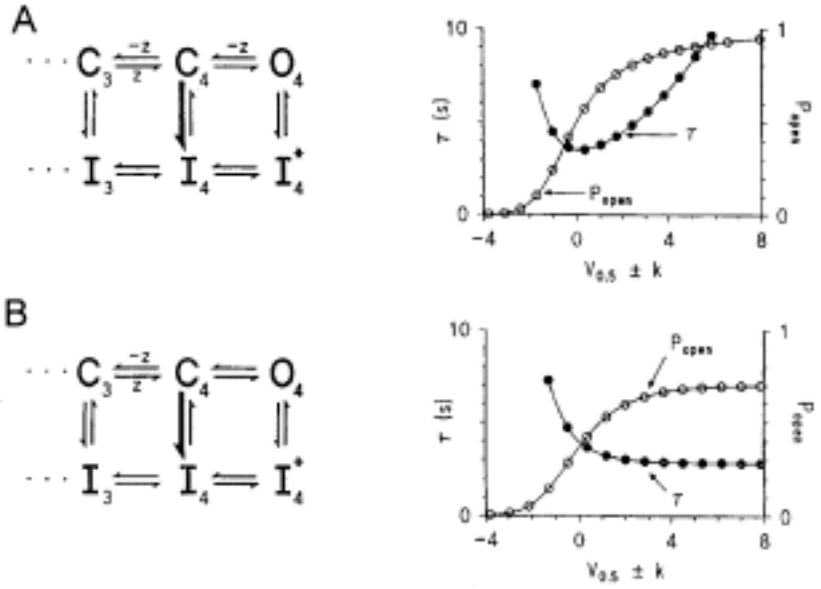

Fig. 4. *Preferential closed-state inactivation models do not predict slowing of inactivation in the voltage range where the channel is fully activated.* ***A.*** *Model from Klemic et al. (1998): voltage dependence of peak open probability (P_{open}) does not have a plateau and the inactivation time constant (τ) is slower with more positive potentials. The x axis is defined in terms of the half-maximal activation voltage ($V_{0.5}$) and slope factor (k) in the Boltzmann equation (see Fig. 1). The bold arrow in the left panel indicates the transition for which the inactivation rate is most rapid.* ***B.*** *The same model with the gating charge on the $O_4 \rightarrow C_4$ transition removed: inactivation rate is now independent of voltage in the range where the channel is fully activated.*

A closer look at the behavior of preferential closed-state inactivation models reveals that they are not able to explain the slowing of inactivation at potentials where a channel is already fully activated, as we have observed here. In the model developed to explain the behavior of Kv2.1 (Klemic *et al.* 1998), the transition between the open state and the nearest closed state has an asymmetrical voltage dependence: charge moves during the open-to-closed transition, but the reverse transition is voltage independent (see also Fig. 4A). This explains why activation in the model does not reach a plateau even at large positive potentials. In contrast, I channel activation reaches a plateau at about –40 mV (Fig. 1B; cf. Jonas *et al.* 1989, Safronov *et al.* 1993, Scholz *et al.* 1993, Koh and Vogel 1996, Reid *et al.* 1999). This plateau can be modeled by removing the asymmetric voltage dependence mentioned above from the model; but interestingly, this modification also removes the slowing of inactivation at positive potentials (Fig. 4B). Inactivation now becomes monotonically more rapid as the potential becomes more positive, and its rate is nearly independent of voltage at potentials where the channel is fully activated; this behavior is indistinguishable from that of a coupled model with voltage-independent inactivation from the open state, and is similar to that actually recorded in N-terminal deleted Kv1.4 channels and attributed to C-type inactivation (Rasmusson *et al.* 1995). A slightly different preferential closed-state inactivation model proposed for neuronal N-, P/Q-, and R-type Ca^{2+} channels (Patil *et al.* 1998) also predicts a slowing of inactivation at positive potentials only in the voltage range where the channel is not fully activated. We conclude that it is a general property of models of this type that they predict slowing of inactivation at positive potentials only at potentials where the channel is not fully activated, and only when there is a voltage- dependent

transition between the open state and the state from which inactivation is most rapid.

The voltage dependence of inactivation of Kv2.1 and neuronal Ca^{2+} channels after relatively short prepulses is U-shaped – less inactivation at extreme negative and positive potentials than at intermediate potentials (Klemic *et al.* 1998, Patil *et al.* 1998). This behavior is predicted by models of preferential closed-state inactivation. When we measured I channel inactivation using brief (5 s) prepulses, we found that the I channel current also has a U-shaped voltage dependence of inactivation (Fig. 1D); but this is accounted for simply by the slowing of inactivation of the I channel in this potential range, as true steady-state inactivation does not have a U-shaped voltage dependence (Fig. 1B). A U-shaped voltage dependence of inactivation is therefore not an appropriate test of preferential closed-state inactivation: it is predicted by preferential closed-state inactivation models simply because they predict slowed inactivation at positive potentials.

It is possible that the slowing of inactivation we have observed is due to the high external potassium we used. This would increase the occupancy by K^+ ions of a site near the outer mouth of the channel which has been shown to modulate C-type inactivation (Lopez-Barneo *et al.* 1993). However, we consider this to be unlikely because the same degree of slowed inactivation of the macroscopic potassium current was observed by Schwarz and Vogel (1971) in Ringer solution and in a solution with high external potassium. It is more likely that the direction of the current itself influences the occupancy of that site: an outward K^+ current may cause a local increase in potassium concentration at the outer mouth and a decrease at the inner mouth of the channel.

On the other hand, the slowing of inactivation at positive potentials could be due to a modulating effect of permeant ions within the pore (Fedida *et al.* 1999) or to an intrinsic voltage dependence of slow inactivation gating. The latter hypothesis is not easy to test: the slope of the relation between the inactivation time constant and voltage (Fig. 2B) is consistent with an effective gating charge of about 0.3, and such a small and slow charge movement would not lead to a measurable gating current.

To summarize, we have shown in this study that inactivation of the rat I channel current becomes slower with more positive potentials, in a voltage range where the channel is already fully activated. This behavior differs from that observed in Kv1 K^+ channels whose slow inactivation is classified as C-type, and is not consistent with voltage-independent inactivation from the open state. Slowing of inactivation at positive potentials can be explained in some circumstances by models in which inactivation occurs preferentially from closed states, but preferential closed-state inactivation does not account for the slowing of inactivation at large positive voltages which we have observed in the rat I channel current, for two reasons. Firstly, preferential closed-state inactivation is not a property of the rat I channel, and secondly, preferential closed-state inactivation models do not predict a slowing of inactivation at potentials positive to those where the channel is fully activated, which is the voltage range where we have observed this effect. We conclude that inactivation of the rat I channel is not adequately explained by existing C-type inactivation models, and that it also differs from the inactivation of the Kv2.1 K^+ channel.

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References

FEDIDA D, MARUOKA ND, LIN S: Modulation of slow inactivation in human cardiac Kv1.5 channels by extra- and intracellular permeant cations. *J Physiol Lond* **515**: 315-329, 1999.

HAMILL, OP, MARTY A, NEHER E, SAKMANN B, SIGWORTH FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* **391**: 85-100, 1981.

HOSHI T, ZAGOTTA, WN, ALDRICH, RW: Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science* **250**: 533-538, 1990.

JONAS P, BRÄU, ME, HERMSTEINER M, VOGEL W: Single-channel recording in myelinated nerve fibers reveals one type of Na channel but different K channels. *Proc Natl Acad Sci USA* **86**: 7238-7242, 1989.

KISS L, KORN SJ: Modulation of C-type inactivation by K^+ at the potassium channel selectivity filter. *Biophys J* **74**: 1840-1849, 1998.

KLEMIC KG, SHIEH CC, KIRSCH GE, JONES SW: Inactivation of Kv2.1 potassium channels. *Biophys J* **74**: 1779-1789, 1998.

KOH DS, VOGEL W: Single-channel analysis of a delayed rectifier K^+ channel in *Xenopus* myelinated nerve. *J Membr Biol* **149**: 221-232, 1996.

LOPEZ-BARNEO J, HOSHI T, HEINEMANN S, ALDRICH R: Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. *Receptors Channels* **1**: 61-71, 1993.

MAROM S, LEVITAN IB: State-dependent inactivation of the Kv3 potassium channel. *Biophys J* **67**: 579-589, 1994.

PATIL PG, BRODY DL, YUE DT: Preferential closed-state inactivation of neuronal calcium channels. *Neuron* **20**: 1027-1038, 1998.

PEROZO E, MACKINNON R, BEZANILLA F, STEFANI E: Gating currents from a nonconducting mutant reveal open-closed conformations in *Shaker* K^+ channels. *Neuron* **11**: 353-358, 1993.

RASMUSSON RL, MORALES MJ, CASTELLINO RC, ZHANG Y, CAMPBELL DL, STRAUSS HC: C-type inactivation controls recovery in a fast inactivating cardiac K^+ channel (Kv1.4) expressed in *Xenopus* oocytes. *J Physiol Lond* **489**: 709-721, 1995.

REID G, SCHOLZ A, BOSTOCK H, VOGEL W: Human axons contain at least five types of voltage-dependent potassium channel. *J Physiol Lond* **518**: 681-696, 1999.

SAFRONOV BV, KAMPE K, VOGEL W: Single voltage-dependent potassium channels in rat peripheral nerve membrane. *J Physiol Lond* **460**: 675-691, 1993.

SCHNEIDER U, QUASTHOFF S, MITROVIĆ N, GRAFE P: Hyperglycaemic hypoxia alters after-potential and fast K^+ conductance of rat axons by cytoplasmic acidification. *J Physiol Lond* **465**: 679-697, 1993.

SCHOLZ A, REID G, VOGEL W, BOSTOCK H: Ion channels in human axons. *J Neurophysiol* **70**: 1274-1279, 1993.

SCHWARZ JR, VOGEL W: Potassium inactivation in single myelinated nerve fibres of Xenopus laevis. *Pflügers Arch* **330**: 61-73, 1971.

STARKUS JG, KUSCHEL L, RAYNER MD, HEINEMANN SH: Ion conduction through C-type inactivated *Shaker* channels. *J Gen Physiol* **110**: 539-550, 1997.

YANG Y, YAN Y, SIGWORTH FJ: How does the W434F mutation block current in *Shaker* potassium channels. *J Gen Physiol* **109**: 779-789, 1997.

YELLEN G: The moving parts of voltage-gated ion channels. *Q Rev Biophys* **31**: 239-295, 1998.

YELLEN G, SODICKSON D, CHEN T, JURMAN M: An engineered cysteine in the external mouth of a K^+ channel allows inactivation to be modulated by metal binding. *Biophys J* **66**:1068-1075, 1994.

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