Modulation of Jellyfish Potassium Channels by External Potassium Ions

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INTRODUCTION

The large diversity of potassium channels provides richness to the functional repertoire of excitable cells. Their associated currents shape action potentials and regulate firing frequency in addition to maintaining the membrane resting potential (Connor and Stevens 1971; Hille 1992). The amplitude of the ionic current passing through the selective pore of these channels is determined by channel conductance, the transmembrane electrical field, and the potassium gradient. When the extracellular potassium concentration ([K+]out) is increased, the chemical driving force is reduced, which results in reduced currents. In addition to this intrinsic property of all ionic channel membranes, it has been shown that altering [K+]out produces a broad spectrum of modulatory effects on the delayed rectifier current in Xenopus axonal membrane (Safronov and Vogel 1996), on the fast inactivating K-current in rat hippocampal neurons (Pardo et al. 1992), and on heterologously expressed cloned representatives of the Shaker subfamily of rapidly inactivating channels (Baukrowitz and Yellen 1995; Lopez-Barneo et al. 1993; Tseng and Tseng-Crank 1992). These modulatory effects include the following: regulation of the number of channels available for activation, alterations in the rate of C-type inactivation, and changes in frequency-dependent cumulative inactivation resulting from an interaction between N- and C-type inactivation. It has been suggested that these modulatory effects might allow excitable cells to compensate for increases in [K+]out in intracellular space as a result of repetitive firing. Hounsgaard and Nicholson (1983) clearly demonstrated that an elevation of [K+]out by as little as 1 mM may alter the pattern of spontaneous activity in guinea-pig Purkinje neurons. Synchronous activation of a large population of these cells in brain slices can raise the level of [K+]out from 6 to 10 mM.

In higher vertebrates, extracellular potassium concentrations are regulated as a result of homeostatic mechanisms at the organ, tissue, and cellular levels. In contrast, there are no known organs or tissues maintaining relatively constant extracellular potassium levels in lower metazoans such as the cnidarians (hydroids, jellyfish, corals, etc). It is likely that neurons and other excitable cells in jellyfish have to be able to adapt to unstable extracellular potassium concentrations as a result of phasic accumulation of potassium during firing, or tonic changes due to naturally occurring salinity differences.

Despite their phylogenetic position at the base of the metazoans, hydrozoan jellyfish display a variety of potassium channels and currents (Meech and Mackie 1993; Przysiezniak and Spencer 1994). Several members of the Shaker and Shal subfamilies of genes encoding α-subunits of voltage-gated potassium channels were cloned from the jellyfish Polyorchis penicillatus. Their products, when expressed in the Xenopus oocyte expression system, demonstrate biophysical properties similar to other known A-like currents (Jegla et al. 1995; Jegla and Stevens 1991). These modulatory effects include the following: regulation of the number of channels available for activation, alterations in the rate of C-type inactivation, and changes in frequency-dependent cumulative inactivation resulting from an interaction between N- and C-type inactivation. It has been suggested that these modulatory effects might allow excitable cells to compensate for increases in [K+]out in intracellular space as a result of repetitive firing. Hounsgaard and Nicholson (1983) clearly demonstrated that an elevation of [K+]out by as little as 1 mM may alter the pattern of spontaneous activity in guinea-pig Purkinje neurons. Synchronous activation of a large population of these cells in brain slices can raise the level of [K+]out from 6 to 10 mM.

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while recovery from inactivation was also affected by variations in $[K^+]_{\text{out}}$. Shaker potassium channels have at least two inactivation mechanisms: N- and C-type. N-type inactivation is associated with blockage of ionic current by the cytoplasmic N-terminus of the α-subunit of the channel protein, often called the “ball and chain” mechanism of inactivation (Hoshi et al. 1990; Zagotta et al. 1990). The molecular mechanism of C-type inactivation is less clear, but it appears to be associated with conformational changes in the external mouth of the channel (Baukrowitz and Yellen 1995; Liu et al. 1996; Molina et al. 1997).

Mutational analysis indicated that $j$Shak2 channels show a strong, potassium-dependent C-type inactivation mechanism and that N-type inactivation is weak and “implicit.” Enhancement of N-type inactivation of $j$Shak2 by transplantation of a charged amino acid cluster from the N-terminus of the $j$Shak1 channel sequence ($j$Shak1 channels possess strong “explicit” N-type inactivation) rendered the inactivation rate less susceptible to variation of $[K^+]_{\text{out}}$. We suggest that an interplay between weak, “implicit,” N-type inactivation and strong, and fast, C-type inactivation of $j$Shak2 is responsible for the potassium-dependent cumulative inactivation observed in this channel.

**METHODS**

**Molecular biology**

All $j$Shak2 mutants were constructed using cassette, PCR-based, site-directed mutagenesis as described previously (Grigoriev et al. 1997). Mutants were verified by sequencing in both directions using a Perkin-Elmer ABI 373A sequencer and an ABI Prism Dye-Terminator cycle sequencing kit. Construction of the mutant $j$Shak1Δ2–24 ($j$Shak1T) was described by Jegla et al. (1995). Capped mRNAs were prepared by run-off transcription using mMessage mMachine kits (Ambion) for T3 ($j$Shak1 and $j$Shak1Δ2–24) or T7 ($j$Shak2 and $j$Shak2 mutants). The plasmid containing a rat skeletal muscle sodium channel, the α-subunit gene, rSkM1, was linearized with SalI and transcribed using the Ambion kit for T7.

**Electrophysiological recording from swimming motor neurons**

Primary cultures of swimming motor neurons of the jellyfish Polyorchis penicillatus were prepared as described previously (Przysiezniak and Spencer 1994) with the modification that cells were exposed, with agitation, to collagenase for only 1 h. Swimming motor neurons were identified by their large size, clear cytoplasm, and a nucleus surrounded by membranous structures. Whole cell patch recordings were made using 1–2 MΩ borosilicate glass pipettes filled with a solution that contained (in mM) 500 KCl, 2 MgCl$_2$, 10 HEPES, 1 CaCl$_2$, and 11 EGTA at pH 7.5 adjusted with N-methylglucamine (NMG). The extracellular bathing solution contained (in mM) 450 NMG-Cl, 50 MgCl$_2$, and 10 HEPES, at pH 7.5 adjusted with HCl. Potassium was introduced at the indicated concentrations by equimolar NMG substitution. Cells were microperfused using a manifold with a dead volume of $<1\mu$L. Solutions were completely exchanged within 2 s. All recordings were carried out at room temperature (20–22°C).

**Whole cell, two-electrode recording from Xenopus oocytes**

Xenopus oocytes were prepared and injected with mRNA as previously described (Grigoriev et al. 1997). mRNA (1–5 ng) was injected in each oocyte using a volume of 50 nl. The amount of injected RNA was adjusted for each expressed channel type to minimize the effects introduced by high levels of channel expression (Grigoriev et al. 1999). Whole cell currents were recorded between 2 and 3 days after injection using a two-microelectrode voltage clamp (CA-1, Dagan, Minneapolis, MN). Cells were constantly microperfused with a gravity fed system.

**Outside-out macropatch recording from Xenopus oocytes**

Outside-out macropatches were obtained and recordings made as described by Stühmer et al. (1992). Patch recordings, as well as whole cell patch recordings from swimming motor neurons, were made using an Axopatch 1D amplifier (Axon Instruments). Fast perfusion was via two PE tubes (7405, Intramedic) glued to the bottom of a 35 mm plastic Petri dish with their orifices perpendicular and in contact with one another. The two resulting perfusion streams were deflected where they met and then ran parallel to one another. Because of the small dimensions and relatively low flow rates, the perfusion system was operating at a low enough Reynolds’s number as to produce laminar flow. Because NMG substituted for potassium, it was possible to visualize an optical density difference between the K mM and the K mM = 100 mM streams under phase contrast optics, and mixing was not seen at the boundary between the two solutions. Each of these tubes was connected to a reservoir filled with the relevant solution. These reservoirs were separately connected to a pair of Picospitzers (General Valve Corporation, Fairfield, NJ) that were controlled by a computer. At the beginning of each recording session, control and test solutions were perfused at 0.5 cm s$^{-1}$ by gravity. Pipettes containing the macropatch were positioned closer to the orifice of the tube containing the control solution than the test solution. Pressure pulses (1 bar) applied to the reservoir containing the test solution by a Picospitzer increased the velocity of the test solution to 5 cm s$^{-1}$, thereby deflecting the control stream and exposing the macropatch to the test solution. Solutions could be changed in this way within 1 ms. The extracellular solution without potassium, $[K^+]_{\text{out}} = 0$, contained 100 mM NMG-Cl, 3 mM MgCl$_2$, 10 mM HEPES-acid adjusted to pH 7.5; whereas the solution, $[K^+]_{\text{out}} = 100$ mM contained KCl 95 mM, MgCl$_2$ 3 mM, 10 mM HEPES$^2$/K adjusted to pH 7.5. Intermediate concentrations of K$^+$ were made by mixing these two solutions in the required proportion. The intracellular solution contained (in mM) 100 KCl 100, 3 MgCl$_2$, 10 EGTA, and 10 HEPES adjusted to pH 7.5. Experiments were carried out at 20°C using a temperature controller TC-10 (Dagan, Minneapolis, MN). Conductance calculations for whole oocyte recordings were made using intracellular potassium activity of 147 mM as reported by Kusano et al. (1982).

**Data acquisition and experimental control**

All data acquisition and experimental control was achieved with a Digidata 1200 acquisition system (Axon Instruments, Foster City, CA) running pClamp 6.1 software (Axon Instruments). Analysis and fitting of experimental data were done using the Clampfit program of the pClamp 6.1 suite and SigmaPlot 4.00 (SPSS, Chicago, IL). All results are expressed as means ± SE.

**RESULTS**

External potassium modulates A-like currents in identified neurons and heterologously expressed jellyfish Shaker channels

Recordings from cultured, identified, motor neurons that control swimming demonstrate the presence of two potassium currents; a fast inactivating A-like current, $I_{K_{fast}}$, and a delayed rectifier, $I_{K_{slow}}$ (Przysiezniak and Spencer 1994). In this study we were able to show that $I_{K_{fast}}$ current was strongly modu-
lated by \([K^+]_{\text{out}}\) (Fig. 1). Eliminating potassium ions from the external solution completely inhibited this current (Fig. 1, inset), leaving a "potassium insensitive," slowly activating current, \(I_{K\text{-slow}}\). Increasing \([K^+]_{\text{out}}\) from 1 to 100 mM amplified the peak, whole cell conductance of channels passing \(I_{K\text{-fast}}\) more than threefold (Fig. 1). We were able to demonstrate the potassium insensitivity of \(I_{K\text{-slow}}\) by using a holding potential of \(-240\) mV, which completely inactivated \(I_{K\text{-fast}}\) (Przysiezniak and Spencer 1994), and then altering \([K^+]_{\text{out}}\) from 1 to 100 mM (data not shown).

Jellyfish \(jShak1\) and \(jShak2\) genes encode high-threshold, A-like currents (Jegla et al. 1995). When expressed in Xenopus oocytes, \(jShak1\) and \(jShak2\) currents showed a strong dependence on the concentration of extracellular potassium ions (Fig. 2, A and B). When \([K^+]_{\text{out}}\) was increased from 1 to 100 mM, the conductance for both channels increased by approximately fourfold and the current by 33%; we call this the amplitude effect. The effect of increasing \([K^+]_{\text{out}}\) on the conductances of \(jShak1\) and \(jShak2\) could be fitted by a combination of an equation similar to a Michaelis-Menten relationship, having an apparent \(K_m\) of \(\approx 1.5\) mM, and a linear relationship with a slope of 0.005 mM\(^{-2}\) (Fig. 2C). We suggest that this nonhyperbolic component represents a low-affinity mechanism that appears as a linear slope within the range of concentrations used.

With increasing concentrations of external \(K^+\), \(jShak2\) inactivated more slowly (Fig. 2D, \(\tau = 15.67 \pm 0.27\) ms and 71.81 \(\pm 7.75\) ms at \([K^+]_{\text{out}}\) = 1 mM and 100 mM, respectively, \(n = 9\)). This change in \(\tau\) with alterations in \([K^+]_{\text{out}}\) was not seen in either \(jShak1\) (Fig. 2D) or \(I_{K\text{-fast}}\) (Fig. 1, inset). The time constant of \(I_{K\text{-fast}}\) inactivation was not significantly different (\(P = 0.45\), \(n = 5\)) between 1 and 100 mM \([K^+]_{\text{out}}\).

Several monovalent cations, other than \(K^+\), produced qualitatively similar effects on \(jShak2\) current, but with substantially lower efficacy than potassium ions. Their modulatory effectiveness for both current amplitude and inactivation kinetics could be ranked as follows: \(K^+ > Rb^+ > Cs^+ > Na^+\) (data not shown). A similar sequence of sensitivity of current peak amplitude to extracellular monovalent cations was dem-
onstrated for the rat neuronal channel, RCK4 or Kv1.4 (Pardo et al. 1992).

Modulatory effects of [K⁺]out involve N-type inactivation

It is well established that N-type inactivation in Shaker potassium channels involves a “ball and chain” mechanism (Hoshi et al. 1990; Zagotta et al. 1990). Deletion of the cytoplasmic N-terminus of the Shaker channel protein eliminates fast (N-type) inactivation revealing a slow (C-type) inactivation that has been associated with conformational changes in the external mouth of the channel (Baukrowitz and Yellen 1995; Liu et al. 1996; Molina et al. 1997).

Extracellular potassium strongly affects the inactivation rate of jShak2 but not of jShak1 currents, which may reflect differences in the molecular mechanism of inactivation. It is known that partial blockade of Shaker channels by extracellular application of TEA is accompanied by a concomitant slowing down of C-type inactivation. Conversely, rapid N-type inactivation is TEA-insensitive (Choi et al. 1991; Grissmer and Cahalan 1989). Extracellular administration of TEA slowed down the rate of inactivation of jShak2 but not fast inactivation of jShak1 (Fig. 3), indicating that the mechanisms of inactivation of jShak1 and jShak2 differ.

To examine N- and C-type inactivation mechanisms more closely, we constructed mutants with modified N-termini (Fig. 4). A previous study (Jegla et al. 1995) showed that deletion of the first 23 residues of jShak1 yielded channels lacking fast inactivation (Fig. 5A). This mutant, jShak1Δ2–24, showed noticeable conductance even in the absence of external potassium. In the absence of [K⁺]out there were two distinct components of activation of jShak1Δ2–24, slow and fast. Presum-
ably the slow component resulted from additional activation of the channels by potassium effluxing through the already open channels and accumulating in the intermicrovillar space of the oocyte (Grigoriev et al. 1999). Dose-response curves suggest an increase in the apparent affinity of this mutant channel to external potassium: the K value for \( g_{app} \) of \( jShak1\Delta2–24 \) was 0.2 mM compared with 1.5 mM for Wild-type \( jShak1 \) (Fig. 5, A and C). A truncated \( jShak2 \) mutant, \( jShak2\Delta2–38 \) was not as sensitive to \([K^+]_{out}\) having a \( K_{app} \) of \( \sim 2 \) mM (Fig. 5, B and C). The effect of N-terminal deletion on the inactivation rate of \( jShak2 \) was not pronounced (Fig. 5, D). Comparisons of the sequences of \( jShak1 \), \( jShak2 \), and fly \( Shaker \) N-termini indicate some structural conservation (Jegla et al. 1995); however, there are differences in the number and distribution of charged amino acids in the first 25 amino acids (Fig. 4). \( jShak1 \Delta2–24 \) and \( jShak2 \Delta2–38 \) are more difficult to associate with such a mechanism and suggest that inactivation is due to rapid, C-type inactivation. To determine whether any N-type inactivation is present in \( jShak2 \), we compared the rates of recovery from inactivation in both \( jShak1 \) and \( jShak2 \) and their N-terminal deleted mutants using a two pulse protocol (Fig. 6). Recovery from inactivation of Wild \( jShak2 \) could be fitted with the sum of slow (\( \tau = 1 \) s) and fast (\( \tau = 0.1 \) s) exponent, suggesting that there are two distinct processes involved in inactivation. The relative contribution of the slow and fast components depended on \([K^+]_{out}\) such that increasing the external potassium concentration increased the proportion of the fast process. Although the \( jShak2\Delta2–38 \) mutant also demonstrated potassium dependence, recovery from inactivation became monoeponential, indicating the presence of only one mechanism. Disappearance of the slow-recovering component of the \( jShak2 \) current after N-terminal truncation shows that this part of the protein is involved in the inactivation process. Thus the presence of the second component of recovery in Wild-type \( jShak2 \) and its absence in \( jShak2\Delta2–38 \) together with the absence of a pronounced change in the inactivation rate at low \([K^+]_{out}\) leads us to the assumption that \( jShak2 \) experiences both weak, N-type inactivation and strong, fast, C-type inactivation. It is important to note that, under similar ionic conditions, C-type inactivation in \( Drosophila Shaker \) channels was two orders of magnitude slower than in \( jShak2 \) (Baukrowitz and Yellen 1995).

**Figure 5.** Effect of external potassium ion concentration on conductance and the time constant of inactivation in the N-terminal deleted mutants \( jShak1\Delta2–24 \) and \( jShak2\Delta2–38 \). A and B are current traces obtained under different \([K^+]_{out}\) conditions from oocytes expressing \( jShak1\Delta2–24 \) and \( jShak2\Delta2–38 \), respectively. The stimulus protocol used was 100 ms depolarizing pulses of +65 mV from a holding potential of \( \sim 80 \) mV. C: plot of the peak conductance (\( g \)) vs. the concentration of extracellular potassium for the 2 mutants, \( jShak1\Delta2–24 \) (●) and \( jShak2\Delta2–38 \) (○). Conductance for \( jShak1\Delta2–24 \) at 0 mM of \([K^+]_{out}\) was calculated for the fast-activating part of the current. D: plot of the time constant of inactivation for \( jShak2\Delta2–38 \) (●) vs. the concentration of extracellular potassium. Solid line results from fitting with the equation shown in Fig. 2. Dashed line is the result of fitting experimental data for the Wild-type, which still has the N-terminal region present.
tural analysis of the N-termini of RCK4 and Raw3 showed that negatively and positively charged residues may be distributed in such a way as to confer dipole properties on the ball (Antz et al. 1997). Therefore it is possible that the transmembrane electrical field orients the ball in the cytoplasmic mouth of the channel during the inactivation process. It is also possible that the presence of the charged residues in the N-terminus sequence might be required for its effective interaction with the ball receptor located in the internal channel mouth. The presence of electrostatic and steric interactions has been suggested for the ball peptide and the ball receptor (Holmgren et al. 1996).

To examine the influence of these clusters of charged residues on the sensitivity of inactivation kinetics to extracellular potassium, we constructed mutants of jShak2 (Fig. 4) in which positively (RRKKE) and negatively charged (KDDE) clusters were transplanted from jShak1 to the jShak2 N-terminal region, both separately (jShak2N1 and jShak2N2) and together (jShak2N1/2). Introduction of a positively charged cluster reduced the influence of [K+]out on the rate of inactivation, whereas inclusion of a negatively charged cluster of amino acids enhanced the potassium dependency of this phenomenon (Fig. 7A). The decrease in potassium sensitivity of inactivation shown by jShak2N+ was accompanied by a far slower (almost 100-fold) rate of recovery from inactivation (Fig. 7B). Conversely, addition of a negative cluster slightly increased the rate of recovery (Fig. 7B). Inclusion of both charge clusters (jShak2N1/2) had intermediate effects on both the potassium sensitivity of the inactivation rate and the time for recovery from inactivation (Fig. 7B).

The effectiveness of the N-terminal region as an inactivation particle can be judged from the rate of recovery from inactivation. Charge-dependent changes in the recovery rates seen in N-terminal mutants probably reflect differences in the strength of interaction between the inactivation particle and presumed receptor sites in the internal channel mouth. Figure 7C shows the effect of these mutations on the effectiveness of [K+]out modulation of both peak current and the inactivation time constant. Altering the effectiveness of the “ball and chain” mechanism did not significantly

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**FIG. 6.** N-terminal deletion affects recovery from inactivation in jShak2. Recovery from inactivation was tested using a 2-pulse protocol. A conditioning pulse to +65 mV (300 ms duration) from a holding potential of −100 mV was followed by a test pulse of the same amplitude (150–200 ms duration), after a variable time T. The ratio of the peak current amplitude during the second pulse (P2) to the peak current amplitude during the 1st pulse (P1) was plotted against time T. ■ and ●, data obtained for Wild-type jShak2 at [K+]out = 1 and 100 mM, respectively; ▼ and ○, data obtained for the N-terminal deleted mutant jShak2Δ2–38 at [K+]out = 1 and 100 mM, respectively. Dotted lines result from fitting the means with a single exponent for jShak2 Δ2–38 and the solid lines from fitting with 2 exponents for Wild-type jShak2. Fitting yielded the following time constants: for jShak2 Wild-type at [K+]out = 1 mM, τslow = 944 ± 57.4 ms, τfast = 85.6 ± 18.5 ms, Aslow/Atotal = 5.4 (Aslow/Atotal is the ratio of the amplitudes of slow and fast exponents); for Wild-type jShak2 at [K+]out = 100 mM, τslow = 1,017 ± 59.1 ms, τfast = 70.3 ± 2.2 ms, Aslow/Atotal = 0.82; for jShak2Δ2–38 at [K+]out = 1 mM, τ = 415 ± 22.6 ms; for jShak2 Δ2–38 at [K+]out = 100 mM, τ = 100 ± 8.5 ms. Data were obtained from 8 to 10 independent measurements from different oocytes.
change the amplitude effect of $[K^+]_{out}$, but these mutations did noticeably alter the sensitivity of the rate of inactivation to the extracellular potassium concentration. For example, when N-type inactivation was more effective ($jShak2^N$), the external potassium concentration no longer had a strong influence on the inactivation rate. Decreasing the effectiveness of N-terminal inactivation ($jShak2^N$) had the opposite effect. Compared with the Wild-type, there was an additional increase in the sensitivity of the inactivation mechanism to $[K^+]_{out}$. The mutant $jShak2^{N+/-}$ demonstrated an intermediate sensitivity of the inactivation rate to $[K^+]_{out}$.

**Fast perfusion experiments reveal differences between amplitude and inactivation effects of $[K^+]_{out}$**

When patch recordings of $jShak2$ current were made in the outside-out configuration at a high external $[K^+]$ (100 mM), there was a noticeable increase in the inactivation rate ($\tau = 12.8 \pm 1.3$ ms, $n = 6$) compared with whole cell recordings ($\tau = 71.8 \pm 7.7$ ms, $n = 6$; Fig. 8A). We suggest that this phenomenon is associated with the accumulation of potassium ions in intermicrovillar space close to the oocyte membrane during channel activation when making whole cell recordings. The original microvillar structure is not preserved in outside-
out patch recordings, thus eliminating many of the barriers to diffusion of potassium ions as they efflux through the pore (Grigoriev et al. 1999).

Using a fast perfusion system (solution exchange in <1 ms), we were able to detect differences in the number of channels available on depolarization and in their rates of inactivation when \([K^+]_{\text{out}}\) was rapidly altered (Fig. 8A). Channels that had been activated instantly changed their time constants of inactivation from 12.8±1.3 ms (n=6) to 3.5±0.3 ms (n=6) following a rapid switch from 100 to 0 mM \([K^+]_{\text{out}}\). In Fig. 8A the amplitude effect is seen as a decrease in the peak amplitude of currents recorded at increasing intervals after switching from 100 to 0 mM. The rate of reduction in the peak current reflects the rate of elimination of potassium from the channel mouth in the resting state. This removal of \([K^+]\) from the channel mouth by diffusion (dekalification) occurred more slowly in the resting state (τ = 13 ms) than in the open state when the rate of dekalification is comparable with the speed of switching between solution (i.e., <1 ms). These results indicate that both the amplitude and inactivation effects of \([K^+]_{\text{out}}\) are due to processes occurring at different sites with different retention times for potassium ions or at the same site where retention time is determined by conformation of
the channel protein. Experiments in which dekalification of channels occurred after opening indicate that, as a result of losing potassium, they were converted to a state from which recovery was slow. This happened in spite of a high \([K^+]_{\text{out}}\) being restored rapidly after inactivation (Fig. 8B).

**Physiological implications of channel sensitivity to \([K^+]_{\text{out}}\)**

Although the data presented above showed that \([K^+]_{\text{out}}\) can regulate both the conductance and inactivation rate of \(jShak2\), we wanted to determine whether such modulation of channel properties could modify the excitability properties so as to be physiologically significant. We were able to produce synthetic action potentials by co-expressing \(jShak2\) and the rat skeletal muscle sodium channel \(a\)-subunit, \(rSkM1\) (Fig. 9A). These action potentials repolarized with two distinct phases: a rapid, early phase provided by the \(A\)-like properties of \(jShak2\), followed by a slow phase presumably associated with an inward rectifier current endogenous to \(Xenopus\) oocytes (Bauer et al. 1996). Increasing \([K^+]_{\text{out}}\) from 1 to 40 mM in the presence of constant \([Na^+]_{\text{out}}\) caused an increase in the rate of early repolarization from 73.3 ± 2 s\(^{-1}\) \((n = 4)\) to \(68.7 ± 4.5\) s\(^{-1}\) \((n = 4)\) at \([K^+]_{\text{out}} = 40\) mM \((P = 0.034)\) and an associated exaggeration of the plateau phase. Increasing \([K^+]_{\text{out}}\) caused late repolarization to become much slower and the spike broader as a result of the decreased electrochemical

**FIG. 9.** Physiological effects of \([K^+]_{\text{out}}\) and 4-aminopyridine (4-AP) on the shape of molecularly synthesized action potentials and the effect of \([K^+]_{\text{out}}\) on the cumulative inactivation of Wild \(jShak2\) and \(jShak2\) \(\Delta2\sim38\) during repetitive stimulation. A: synthetic action potentials generated by oocytes injected with a mixture of RNAs encoding channels for inward and outward currents (the \(K^+\) channel, \(jShak2\) plus sodium channel, \(rSkM1\)). Action potentials were evoked by 3-ms depolarizing current pulses (5 \(\mu\)A) using an extracellular solution containing 1 mM \(K^+\) (black trace), 40 mM of \(K^+\) (gray trace), and 40 mM of \(K^+\) with 2 mM of 4-AP (gray dashed trace). In all cases the extracellular solution contained 60 mM \(Na^+\). The membrane potential was continuously adjusted to \(-80\) mV by injection of constant hyperpolarizing current. B: cumulative inactivation of Wild \(jShak2\) (circles) and \(jShak2\) \(\Delta2\sim38\) (squares) as peak current (normalized to the amplitude at a stimulation rate 0.05 Hz) at different stimulation frequencies and different \([K^+]_{\text{out}}\). Stimulus pulses were of 60 ms duration applied from \(-100\) to +65 mV at frequencies of 0.1, 1, and 2 Hz at \([K^+]_{\text{out}} = 1\) mM (black symbols) and 100 mM (gray symbols).
driving force on K$^+$ through endogenous inward rectifier channels. In these experiments the decreased resting potential resulting from an increase in $[K^+]_{\text{out}}$ was compensated by adjusting the holding current in current clamp mode. Inhibition of $jShak2$ current by application of 2 mM 4-aminopyridine (4-AP) decreased the rate of early repolarization and positioned the plateau closer to the level of the sodium reversal potential. It should be noted that the endogenous inward rectifier current in oocytes is not sensitive to 4-AP (Bauer et al. 1996). We suggest that this modulation by $[K^+]_{\text{out}}$ could stabilize the plateau phase of action potentials when potassium accumulates in restricted extracellular spaces during repetitive firing.

Another mechanism that could counteract the effect of potassium accumulation on the driving force and hence action potential shape is the inhibitory effect of $[K^+]_{\text{out}}$ on the cumulative inactivation of $jShak2$ in the course of repetitive stimulation (Fig. 9B). Elevation of extracellular potassium concentration makes cumulative inactivation of channels less pronounced. The severe reduction of cumulative inactivation observed for the $jShak2$ Δ2–38 mutant suggests that the N-terminal “ball” might play a pivotal role in this process.

**DISCUSSION**

Figure 10 is a kinetic model for both $jShak1$ and $jShak2$ and provides a background for the discussion.

Jellyfish A-like current ($I_{K,\text{Fast}}$) recorded from swimming motor neurons and currents in oocytes expressing $jShak1$ and $jShak2$ showed modulation of the peak current amplitude by altering the external potassium concentration. Only $jShak2$ experienced modulation of its inactivation rate by changes in $[K^+]_{\text{out}}$. An effect of external $[K^+]_{\text{out}}$ on current amplitude has been reported for other potassium channels, such as the delayed rectifier in *Xenopus* axonal membrane (Safronov and Vogel 1996), heterologously expressed mammalian neuronal RCK4 channels (Pardo et al. 1992), and the T449K mutant of fly *Shaker* (Lopez-Barneo et al. 1993). RCK4 channels in the absence of external potassium became nonconducting, although gating currents could still be recorded, indicating that the voltage-sensing mechanism remains operational. Mutation of the pore region of the fly *Shaker* channel protein by substitution of tyrosine residue 449 with lysine made peak current amplitude of this channel strongly dependent on $[K^+]_{\text{out}}$ (Lopez-Barneo et al. 1993). It was suggested that potassium ions, as well as other monovalent cations, can occupy site(s) in the external channel mouth preventing development of C-type inactivation by a “foot in the door” mechanism (Labarca and MacKinnon 1992; Lopez-Barneo et al. 1993). If depolarization occurs in the absence of $[K^+]_{\text{out}}$, channels proceed rapidly to the C-type inactivated state after opening (Fig. 10). C-type inactivation occurs sufficiently rapidly so as to convert many K$^+$ channels to a nonconducting state before any significant current can be recorded and before sites in the mouth become occupied by potassium effluxing from the cytoplasm. Current can only be detected when C-type inactivation is slowed sufficiently by extracellular potassium ions. An efficient ball and
chain mechanism, as was seen for jShak1 in this study, prevents occupation of the proposed potassium binding site(s) by effluxing potassium ions, which can explain the increased apparent affinity to $[K^+]_{\text{out}}$ observed for the N-terminus truncated mutant. The “foot in the door” hypothesis for modulation by $[K^+]_{\text{out}}$ also provides an explanation for the effect of $[K^+]_{\text{out}}$ and other monovalent cations, on inactivation of jShak2. Inactivation of this channel occurred predominantly by a C-type mechanism, with occupation of the potassium binding site(s) slowing inactivation. The differences in retention times of $K^+$ for closed and open channels observed in experiments involving fast dekallification probably reflects state-dependent conformational changes, or accessibility, of potassium binding site(s) located in the external channel vestibule. N-type inactivation of jShak2 channels is “implicit,” but experiments examining recovery from inactivation of Wild-type and jShak2Δ2–38 unmasks the presence of an N-type inactivation mechanism. Its presence can also be detected at $[K^+]_{\text{out}}$ of $>30$ mM when C-type inactivation becomes very slow (Fig. 5D). Conversely, inactivation of jShak1 is mostly by an efficient ball and chain mechanism (Fig. 10). In the case of jShak2, enhancement of N-type inactivation by transplantation of the jShak1 RRKKE cluster (jShak2N+) makes the potassium dependence of inactivation less pronounced because the potassium-independent ball and chain mechanism becomes more explicit.

According to the kinetic model suggested by Baukrowitz and Yellen (1995) for fly Shaker channels, recovery from N-type inactivation is fast, whereas recovery from C-type inactivation is much slower. Interaction between slowly recovering, potassium-dependent, C-type inactivation and rapidly recovering, potassium-independent N-type inactivation explains the potassium dependency of cumulative inactivation observed for fly Shaker channels. In our experiments with jShak2 channels, the slower component of recovery appears to be tightly associated with N-type inactivation. Truncation of jShak2 channels was accompanied by disappearance of the slower component of recovery, and introduction of the RRKKE cluster in the N-terminal sequence made recovery dramatically slower. It is reasonable to suggest that the increase in effectiveness of interaction between the inactivation particle and the receptor in the internal channel mouth simultaneously slows down the process of unbinding of this particle from the receptor during recovery from inactivation. Our data indicate that N-type inactivation in jShak2 channels is the major mechanism involved in potassium-dependent cumulative inactivation.

At the end of a depolarizing pulse, jShak2 channels can be in N, C, and C + N inactivated states (Fig. 10). jShak2 channels with the N-terminus present show potassium-sensitive recovery from inactivation indicating that channels in the C + N inactivated state recover more slowly than those in the N state. Why do channels in the C + N state recover slowly and N-type inactivated channels recover rapidly? Displacement of the N-particle from the internal channel mouth will allow the channel to proceed to the resting state and determine the rate of recovery from inactivation. Considering that impermeable Cs+ ions (data not shown) can imitate the effect of $K^+$ on the process of recovery from inactivation in jShak2 then occupancy of the site(s) at the external channel vestibule, rather than ion flow through the reopened channels, can explain this effect. Similarly, Gomez-Lagunas and Armstrong (1994) reported that Cs+ could substitute for $K^+$ in the process of recovery of Shaker B channel from inactivation. There are at least two possible mechanisms by which occupation of this site(s) can promote displacement of the inactivation ball. One explanation involves electrostatic interaction between ion(s) occupying site(s) in the external channel mouth and the inactivation ball (Gomez-Lagunas and Armstrong 1994). Another suggested mechanism is that occupation of the $K^+$ binding site prevents the conformational changes associated with C-type inactivation. The latter mechanism assumes that such conformational changes can have a remote influence on more internalized parts of the protein participating in ball-receptor interaction. The ultimate effect of the conformational change is to hold the inactivation particle in place for a longer time. This mechanism can also explain the absence of visible jShak2 tail currents at low concentrations of $[K^+]_{\text{out}}$ as seen in Fig. 2B, because most of the inactivated channels are in a C + N type inactivated state and channel reopening after repolarization (Ruppersberg et al. 1991) cannot be observed.

Both jShak2 and fly Shaker exhibit potassium-dependent cumulative inactivation. This dependence was explained by Baukrowitz and Yellen (1995) by assuming that there is an interplay between an “explicit” N-type mechanism and slow C-type inactivation. However, for jShak2 channels, we suggest that the potassium dependence of cumulative inactivation is caused by a combination of “implicit” N-type inactivation and fast C-type inactivation.

All Polyorchis potassium-dependent currents, such as jShak1, jShak2 and $I_{K_{\text{fast}}}$, show a high activation threshold that is associated with their roles in shaping the early repolarization of the action potential. We also observed that when jShak2 contributes outward current in synthetic action potentials expressed in oocytes, this current is capable of both repolarizing the action potential and forming a plateau. The endogenous repolarizing outward current in swimming motor neurons of Polyorchis, $I_{K_{\text{fast}}}$, also truncates the plateau of action potentials, which has been shown to modulate neuromuscular transmission (Przysiezniak and Spencer 1994; Spencer 1984; Spencer et al. 1989). Jellyfish have no known tissues or cells, such as glia, that are specialized for $K^+$ homeostasis in the immediate extracellular space surrounding neurons. It is also important to note that nearly all cell types in hydromedusae are electrically excitable, including epithelial cells (Satterlie and Spencer 1987), which would drastically increase the number of potential sources for potassium accumulation in extracellular space. Therefore it is likely that potassium that accumulates during repetitive firing could reduce outward current amplitude and duration, thereby altering action potential shape. Because $I_{K_{\text{fast}}}$ can be modulated by $[K^+]_{\text{out}}$, one can imagine that these negative influences by accumulating $K^+$ can be compensated by increased current amplitude. Although the kinetics and electrical properties of $I_{K_{\text{fast}}}$ are markedly different from those of heterologously expressed jShak1 and jShak2 currents, we cannot rule out the possibility that $I_{K_{\text{fast}}}$ is conducted by channels composed of jShak1 or jShak2 a-subunits because their properties may be altered by differences in lipid environments (Schetz and Anderson 1993) and/or by the presence of auxiliary subunits when these channels are expressed in vivo.

In the mammalian CNS, extracellular potassium concentrations can increase by several millimoles as a result of high-frequency firing (Hounsgaard and Nicholson 1983; Sykova 1983). It is
probably significant that the potassium sensitivity shown by various vertebrate (Pardo et al. 1992; Safronov and Vogel 1996) and fly (Baukrowitz and Yellen 1995) K$^+$ channels can be satisfactorily described by curves with a $K_{\text{app}}$ close to 2 mM. By comparison, there are additional low-affinity sites that are modulating potassium currents in Polyorchis. These sites are presumably an adaptation for the lack of glia cells and the consequently greater potassium accumulation in extracellular space.

We thank Bamfield Marine Station for providing excellent facilities. We are especially grateful to W. Gallin for advice on molecular techniques and P. Ruben for providing the plasmid containing the rSkM1 channel gene. We are also grateful to T. Baukrowitz, P. Ruben, and S. Buckingham for stimulating discussion and valuable advice. We especially thank Dr. W. Gallin for providing technical guidance for the molecular biological aspects of this study, which were carried out in his laboratory.

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