Atypical Phenotypes From Flatworm Kv3 Channels

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1Department of Biological Sciences, University of Alberta, Edmonton, Alberta; and Bamfield Marine Sciences Centre, Bamfield, British Columbia, Canada; 2Medical Research Council Functional Genetics Unit, Department of Human Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; and 3Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Submitted 16 August 2005; accepted in final form 27 January 2006

Klassen, Tara L., Steven D. Buckingham, Donna M. Atherton, Joel B. Dacks, Warren J. Gallin, and Andrew N. Spencer. Atypical phenotypes from flatworm Kv3 channels. J Neurophysiol 95: 3035–3046, 2006. First published February 1, 2006; doi:10.1152/jn.00858.2005. Divergence of the Shaker superfamily of voltage-gated (Kv) ion channels early in metazoan evolution created numerous electrical phenotypes that were presumably selected to produce a wide range of excitability characteristics in neurons, myocytes, and other cells. A comparative approach that emphasizes this early radiation provides a comprehensive sampling of sequence space that is necessary to develop generally applicable models of the structure–function relationship in the Kv potassium channel family. We have cloned and characterized two Shaw-type potassium channels from a flatworm (Notoplana atomata) that is arguably a representative of early diverging bilaterians. When expressed in Xenopus oocytes, one of these cloned channels, Nat-Kv3.1, exhibits a noninactivating, outward current with slow opening kinetics that are dependent on both the holding potential and the activating potential. A second Shaw-type channel, Nat-Kv3.2, has very different properties, showing weak inward rectification. These results demonstrate that broad phylogenetic sampling of proteins of a single family will reveal unexpected properties that lead to new interpretations of structure–function relationships.

INTRODUCTION

Members of the Shaker superfamily of voltage-gated (Vg) ion channels (consisting of the Shak [Kv1], Shah [Kv2], Shaw [Kv3], and Shal [Kv4] families of channels) are major determinants of the electrical phenotype of excitable cells such as neurons, glia, myocytes, and some epithelial cells. This superfamily has its origins in the early metazoans (Jeglaj et al. 1995) and their consequent functional radiation was presumably in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. For models to have more general validity, and for structure–function relationships to be set in context, it is important to examine potassium channels from more diverse taxa, especially more basal metazoan phyla.

Indeed, the few potassium channels from basal phyla that have been described provide a rich source of structural and functional novelty. Potassium currents from jellyfish and flatworms have been characterized (Blair and Anderson 1993; Buckingham and Spencer 2000; Day et al. 1995; Holman and Anderson 1991; Keenan and Koopowitz 1984; Meech and Mackie 1993; Przysiezniak and Spencer 1994), and three functional potassium channel proteins have been cloned from a jellyfish (Jeglaj and Salkoff 1997; Jegla et al. 1995) and one from Schistosoma mansoni (Kim et al. 1995), a parasitic trematode. What little is known of the K+ channels from these two phyla indicates that they differ in several respects from many of their homologues in vertebrates, insects, and molluscs. The potassium channels of flatworms and jellyfish tend to be shifted in their conductance–voltage (I–V) curves in a more detailed fashion than that seen in vertebrates. Presumably, the selection pressure to produce increasingly more complex membrane potential changes intensified as metazoans radiated. Thus studying the range of electrophysiological properties that can be found in extant examples of the major, early diverging, metazoan taxa such as the Porifera (sponges), Cnidaria (jellyfish, hydroids, anemones, and corals), and Platyhelminthes (flatworms) will likely reveal the range of basic phenotypes that are possible within this family of proteins and may, with appropriate phylogenetic analysis, reveal ancestral properties.

Rather than using a comparative approach to understand structure–function relationships in Vg potassium channels, most studies intended to understand structure and function of Vg channels formulate hypotheses for a specific property of this channel family, based on a current structural model, and then test these hypotheses through site-directed mutagenesis experiments. One drawback to this approach is that it is based on information for a relatively small number of potassium channels, isolated from few taxa. Consequently, only a relatively small region of total sequence space for Vg potassium channels has been sampled to date, and only a limited part of the total possible range of electrophysiological activities for these channels has been characterized. For models to have more general validity, and for structure–function relationships to be set in context, it is important to examine potassium channels from more diverse taxa, especially more basal metazoan phyla.

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depolared direction (Blair and Anderson 1993; Buckingham and Spencer 2000; Day et al. 1995; Grigoriev et al. 1997; Holman and Anderson 1991; Jegla and Salkoff 1997; Jegla et al. 1995; Keenan and Koopowitz 1984; Kim et al. 1995; Meech and Mackie 1993; Przysiecki and Spencer 1994) and one flatworm potassium current in central neurons activates very rapidly, allowing for high-frequency firing (Buckingham and Spencer 2002). The potassium channels of “basal” phyla, those that diverged earliest within the metazoan clade, are more likely to exhibit novel properties because of their long divergence times from commonly studied channels in phyla such as the nematodes, arthropods, and vertebrates.

To date, no potassium channels have been cloned from nonderived, free-living flatworms. We therefore applied a degenerate PCR approach to characterize voltage-gated potassium channels from Notoplana atomata, a marine, polyclad flatworm, that is representative of early bilaterians. We cloned and characterized two Shaw-type voltage-gated channels with disparate and unusual properties for this channel family. When expressed in oocytes, N.at-Kv3.1 exhibited a nonactivating, outward current with slow opening kinetics arising from a number of voltage-dependent, preopening intermediate events. In contrast, a second channel, N.at-Kv3.2, was constitutively open in the range of normal resting potentials but showed weak inward rectification at potentials more positive than −40 mV. Both of these flatworm Shaw-type channels express currents with properties that are not normally seen in other members of the Shaw subfamily (Rudy and McBain 2001). Evolutionarily early natural selection in a basal metazoan has produced two Shaw channels with diverse electrical properties that are convergent with phenotypes produced by distantly related families of K⁺ channels in “higher” metazoa.

**METHOFS**

**Collection and isolation of DNA and RNA**

Specimens of Notoplana atomata were collected from the mid-intertidal zone on cobble beaches at Bamfield Inlet, BC, Canada. Animals were held in running seawater and starved for 4 days before isolation of DNA. Genomic DNA was prepared either by SDS–proteinase K lysis followed by phenol extraction (Strauss 1998) or by the Carlson lysis method (Carlson et al. 1991) followed by methylene chloride extraction (M. Krause, Department of Biology, Hofstra University, Hempstead NY, personal communication).

Total RNA was isolated from whole organisms using the Totally RNA Kit (Ambion).

**Cloning and sequencing**

Initial PCR fragments of voltage-gated potassium channels were obtained using degenerate primers as previously described (Jegla et al. 1995) and cloned into pGEM-T (Promega). Individual plasmids were then sequenced and the translated sequence was used to search the National Center for Biotechnology Information (NCBI) nonredundant protein sequence database using BLASTP (Altschul et al. 1997) to identify potassium channel fragments. Complete nucleotide sequences for the two channel mRNAs were determined using a combination of inverse PCR on genomic DNA (Ochman et al. 1988) and RACE-PCR on cDNA (Invitrogen GeneRacer kit). Sequences were determined from both strands of uncloned PCR product from at least two independent reactions to avoid unintended PCR errors. Open reading frames for each channel were amplified using appropriately designed primers, cloned into pXT7 expression plasmid (Dominguez et al. 1995), and fully sequenced to confirm that no errors had been introduced.

**Phylogenetic analysis**

Amino acid sequences were aligned using the XALIGN algorithm (Wishart et al. 1994), as implemented in Peptool v1.0 (www.biotool.com), and hand adjusted. Four distinct phylogenetic analyses were performed to address four different questions. First, to test whether the Notoplana sequences were more related to ERG or Shaker superfamily proteins, an alignment of 43 taxa and 327 positions was assembled. Second, resolution within the Shaker superfamily was sought from a data set of 38 taxa and 292 positions. Third, a data set of 31 taxa and 324 positions was used to resolve the position of the Notoplana sequences in the Shaw/Shab clade. Fourth, a data set, using N.at-Kv3.1 as a surrogate for both N. atomata clades, was assembled with 46 taxa and 324 positions. All data sets are available on request.

Optimal tree topologies of the 46, 43, and 38 taxon data sets were determined from distance matrices calculated by Tree-Puzzle 4.0.2 (Schmidt et al. 2002) with 8 plus 1 rate categories estimated from a starting Neighbor Joining tree (Saitou and Nei 1987). Trees were constructed from these matrices using the Fitch program, which implements the Fitch–Margoliash algorithm (Fitch and Margoliash 1967) with global rearrangements and jumbling implemented. This analysis also yielded Quartet Puzzling support values. Additional confidence estimates for the 38 taxon data set were obtained using ProtML 2.2 (Adachi and Hasegawa 1996) with 10,000 replicates. For the 31 taxon data set, the optimal tree topology and Bayesian posterior values were obtained using MrBayes v3.0b4 (Ronquist and Huelsenbeck 2003) with the default parameters for amino acid sequences.

For maximum likelihood (ML) distance bootstrapping, Seqboot (from the PHYLIP 3.6 package) was used to produce 100 pseudoreplicate data sets. These were analyzed using Tree-Puzzle to produce distance matrices with parameters estimated from the original data set in coordination with Puzzleboot (www.tree-puzzle.de). These distance matrices were then analyzed using Fitch (for the smaller data sets) or Neighbor (for the larger data sets). Analyses with Neighbor incorporated the Jumbling option, whereas Fitch analyses were done with 10 × jumbling and global rearrangements.

Seqboot, Fitch, and Neighbor are all programs from the PHYLIP v3.6 (Felsenstein 1989) suite of phylogenetic analysis programs.

**Expression of channels**

Expression plasmids were linearized and mRNA was produced by in vitro transcription from the T7 promoter (mMessage mMachine kit, Ambion). Ovarian lobes were surgically removed from adult Xenopus laevis and separated into clumps of about 50 oocytes. These were then washed in calcium-free oocyte saline [ND96, consisting of (in mM): NaCl, 96; KCl, 2; CaCl₂, 1; MgCl₂, 1; HEPES, 5; pH 7.6] and incubated in 2 mg/ml collagenase (Sigma type IV) in calcium-free ND96 for about 1 h. Selected stages V to VI oocytes were then manually defolliculated. Oocytes were injected with 50 nl of mRNA (1 ng/μl) and held at room temperature with daily changes of incubation medium (ND96 supplemented with sodium pyruvate at a final concentration of 2.5 mM).

**Electrophysiological methods**

Electrophysiological experiments were performed 1 to 2 days after injection. Recordings were made in an RZ-17 perfusion chamber (Warner Instruments) and oocytes were perfused with ND96. Oocytes were impaled with glass microelectrodes fabricated from borosilicate glass, filled with 3 M KCl and having a resistance of 0.5 to 1 MΩ. Experiments were driven by a GeneClamp 500B amplifier (Axon Instruments) controlled by pClamp 9.0 software (Axon Instruments). Data were acquired through an Axon Instruments 1322A analogue/
digital converter and analyzed using Clampfit 9.0 (Axon Instruments). Measurements were performed with leak subtraction (P/N = 4) for N.at-Kv3.1, but not for N.at-Kv3.2 because these channels conducted over the range of command membrane potentials used.

For N.at-Kv3.1 currents, the I–V relationship was determined by applying 100-ms step depolarizations from a holding potential of −90 mV to a range of potentials from −140 to +70 mV in 10-mV increments followed by a return to −90 mV. Maximal current was measured 95 ms after the initial stimulus artifact. The steady-state activation properties were determined from the amplitude of tail currents measured immediately after the stimulus artifact and plotted against the voltage used to elicit the current. The amplitudes of the tail currents were normalized and fitted to a standard, first-order Boltzmann function using Clampfit 9.2. Clamp currents were measured while holding the oocyte at the holding potential (−60 mV) for 15 ms followed by a 50-ms step to −90 mV then applying a range of 800-ms depolarization steps from −90 to +50 mV in 10-mV increments immediately followed by a 200-ms step to +50 mV to fully open all channels. The kinetics of channel opening was analyzed by fitting the current traces to formulas representing different models using the model comparison function in Clampfit 9.2 (Axon Instruments).

For N.at-Kv3.2 currents, the I–V relationship was determined from oocytes held at −60 mV by applying a 50-ms step to −90 mV before a 500-ms step to a range of potentials from −140 to +80 mV in 10-mV steps followed by a 50-ms step back to −90 mV. Current amplitudes were measured after inactivation had occurred and constant current was obtained. The resulting currents were normalized and plotted against the voltage used to elicit the current.

Perfusion experiments in which [K]out was varied from 2, 10, 50, and 98 mM were performed in ND96 and ND96 with choline replacing 100 ms step depolarizations from a holding potential of 70 mM BaCl2, and (in mM) HEPES, 10; EDTA, 1; pH 7.6. Pharmacological blockers were dissolved in ND96 at the concentrations specified.

RESULTS

N.at-Kv3.1 and N.at-Kv3.2 are Shaw-type channels

PCR with degenerate primers applied to genomic DNA of Notoplana atomata yielded several small fragments whose sequences were characteristic of the pore and S6 regions of potassium channels. New primers, whose design was based on the unique sequences obtained from these clones, were used for inverse PCR of genomic DNA and 5′–RACE (Rapid Amplification of cDNA Ends) and 3′–RACE reactions using cDNA to obtain full-length cDNA sequences, N.at-Kv3.1 and N.at-Kv3.2 (GenBank Accession numbers AY186793.1 and AY186794.1, respectively), that encode proteins with strong similarities to characterized potassium channels in the Shaker superfamily.

BLASTP searches of the nonredundant NCBI protein database using N.at-Kv3.1 and N.at-Kv3.2 sequences retrieved members of the Shaw subfamily of six transmembrane domain K+ channels as the best scoring match (e-values of 6e-75 and 8e-70, respectively). However, K+ channels from other Shaker subfamilies were also retrieved at nearly equivalent scores (e-values of 8e-55 and 8e-69, respectively) for the next most significant, non-Shaw, match.

To confirm that these channel proteins are members of the Shaker superfamily, and not of the ERG-type with which N.at-Kv3.2 shares inward-rectifying properties (see following text), N.at-Kv3.1 and N.at-Kv3.2 were aligned with 36 taxonomically diverse sequences from the four Shaker subfamilies, and with five ERG-type channels (a total of 43 channel sequences). Analysis of this data set clearly separated the ERG channels from the clade of Shaker family channels, in which the N. atomata sequences were embedded by 100% resampling of estimated log likelihood (RELL) and bootstrap support in maximum likelihood (ML) and ML distance (MDL) analyses (not shown). In analyses of the Shaker superfamily proteins, both N.at-Kv3.1 and N.at-Kv3.2 were clearly positioned within the Shaw/Shab clade, with 97% RELL and 85% bootstrap support (Fig. 1, node D). Strongly supported nodes excluded them from the Shaw (100/100%), Shab (100/100%), and Shak (100/95%) groups (Fig. 1, nodes E, F, and A, respectively). To further clarify this placement, a data set of 38 sequences containing diverse Shaw and Shab homologues, rooted by Shal sequences, was investigated by Bayesian and MLD methods (Fig. 2). Below we show that N.at-Kv3.2 shares a distinct electrophysiological trait with the nematode C. elegans Shab 3 channel (EXP-2), that is, inward rectification. However, three strongly supported nodes separate these two sequences (Fig. 2). Therefore the inward-rectifying properties of N.at-Kv3.2 and C. elegans Shab 3 must have evolved separately.

In all analyses, the two N. atomata sequences strongly group together in ML and Bayesian analyses despite lower support from MLD methods. It is therefore likely that they represent the product of lineage-specific gene duplication. When N.at-Kv3.1 is used as a surrogate for the two sequences, it strongly groups with Shaw-type channels (99/77%) by ML and MLD methods. This grouping along with the exclusion of the two Notoplana channels from the Shal clade (Figs. 1 and 2) further confirms the Shaw affiliation of both sequences.

The clade consisting of the two channels from N. atomata branches basal to the Kv3-type channel from the cnidarian, Polyorchis penicillatus, in Fig. 2. Thus there is an alternative interpretation, that the two N. atomata channels form a clade representing a novel Kv channel family. Given the fact that all the channels within the Kv group evolved in common ancestor of metazoans, this would mean that another channel family was lost in all other phyla for which the Kv channels have been studied. However, if only N.at-Kv3.1 is included in the phylogenetic analysis it groups robustly within the Kv3 clade and weakly as a sister group to the P. penicillatus Kv3 channel. Given the long branch lengths of the N. atomata channel clade and the relatively weak support for placing this clade basal to the P. penicillatus Kv3 channel, we think that this formal possibility is unlikely to be true.

Both N.at-Kv3.1 and N.at-Kv3.2 sequences exhibit relatively long branches when compared with other Shaw-type proteins (Fig. 1), consistent with the differences in electrophysiological properties exhibited by these flatworm channels.
N.at-Kv3.1 expresses a noninactivating \(K^+\) current with slow, early opening transitions.

*X. laevis* oocytes injected with *N.at-Kv3.1* mRNA expressed currents with delayed rectifier properties. Voltage-clamped oocytes expressing this channel responded to step depolarizations to potentials greater than about 30 mV with a slowly activating outward current (Fig. 3, A and B). Steady-state activation was characterized by a \(V_{50}\) of \(+9.3 \pm 1.2\) mV, with a Boltzmann slope parameter of \(+14.5 \pm 1.01\) mV/e (\(n = 9\)) (Fig. 3C).

Unlike currents characteristic of the *Shaw* subfamily of potassium channels, the *N.at-Kv3.1*–mediated currents have a...
slow rate of activation, with a 10–90% rise time of 150 ms, compared with 3 to 7 ms for mammalian Kv3.1 and Kv3.2 (Rudy and McBain 2001). The opening kinetics include an unusually long, late-rising phase after the initial opening phases (Figs. 4A and 5A). Single exponential functions and single sigmoidal functions failed to fit the opening kinetics observed in N.at-Kv3.1 because of the combination of the sigmoidal shape early in the time course of the opening transitions and the long, late phase of activation. Increasing the number of terms yielded an improved fit with the sum of two sigmoidal terms (Fig. 4, Eq. 2) or the sum of three exponential terms (Fig. 4, Eq. 3) or the sum of two sigmoidal functions and a noncooperative exponential term all with different time constants (Figs. 4C and 5A)

$$I(t) = [A(1 - \exp(-u_1))] + [B(1 - \exp(-u_2))] + C$$

$$I(t) = [A(1 - \exp(-u_1))] + [B(1 - \exp(-u_2))] + [C(1 - \exp(-u_1))] + D$$

However, neither of these forms fitted the early phase of the kinetics well (Fig. 4B). A better fit was obtained with Eq. 3 (i.e., the sum of two terms), each the product of a cooperative exponential term (n = 4, Fig. 4C) and a noncooperative exponential term all with different time constants (Figs. 4C and 5A)

$$I(t) = [A(1 - \exp(-u_{fast1}))][1 - \exp(-u_{slow1})] + [B(1 - \exp(-u_{fast2}))[1 - \exp(-u_{slow2})] + [C(1 - \exp(-u_{fast3}))[1 - \exp(-u_{slow3})]]$$

The steepness of the activation curve increased as the holding potential became more positive (Fig. 5B), suggesting that the kinetic parameters vary not only as a function of the depolarizing potential, but also as a function of holding potential. However, the form of the curves did not vary with the holding potential (i.e., Eq. 3), providing a good fit for activation curves elicited from different holding potentials.

In currents elicited from a holding potential of −90 mV to a range of potentials (−20 to +50 mV) the contributions of the fast (A) and slow (B) components in Eq. 3 were invariant (A = 0.315 ± 0.017, B = 0.685 ± 0.017) (n = 6). For these currents the time constants of activation τ_{fast1} and τ_{slow1} showed linear dependency on stimulation voltage (Fig. 5C). Similarly, τ_{slow2} showed linear voltage dependency, decreasing substantially with membrane depolarization. However, τ_{fast2} was independent of depolarization voltage over the range tested (Fig. 5D).

The shape of the opening curve is a function of the holding potential, manifesting much faster opening with less delay when the holding potential is more positive (Fig. 5B). Little or no inactivation was observed, even when depolarization was extended to 1 s.

When currents were elicited from a range of holding potentials (−20 to +40 mV) to a final potential of +50 mV, the ratio of A to B was independent of the holding potential (A = 0.425 ± 0.039, B = 0.575 ± 0.039) (n = 6). Both τ_{fast1} and τ_{slow1} were sensitive to the holding potential: τ_{fast1} showed a sigmoidal dependency on holding potential (inflection point at −22 mV), whereas τ_{slow1} showed an exponential dependency (Fig. 5E). Both τ_{fast2} and τ_{slow2} showed an exponential dependency on holding potential (Fig. 5F).
N. at-Kv3.2 expresses an inward-rectifying K⁺ current

Voltage-clamped oocytes injected with N. at-Kv3.2 mRNA responded to step depolarizations from a holding potential of −90 mV with currents that show inward rectification (Fig. 6A). At membrane potentials more negative than −40 mV the channel exhibited inward currents that were approximately proportional to the test potential. At extremely hyperpolarized potentials (−140 to −110 mV) the current was slow to reach peak steady-state current. This delay might arise from poor

FIG. 4. Curve-fit analysis of the opening kinetics of N. at-Kv3.1 channel. A: curve-fit analysis of the first 400 ms of channel opening in response to a step to +50 mV from a holding potential of −90 mV. Combination of a sigmoidal shape early in the time course of the opening transitions and the long, late phase of activation prevented fitting of a single exponent or single sigmoid curve. Double sigmoidal curve of Eq. 1 (orange circles) and the triple exponential curve of Eq. 2 (blue circles) both fit well to the later portion of the curve. B: curve-fit analysis from A showing the first 70 ms of channel opening. Neither the double sigmoidal curve (Eq. 1, orange circles) or the triple exponential curve (Eq. 2, blue circles) fit the slow early transitions well. C: kinetic analysis of activation demonstrated that the opening transitions require 4 subunits. Current traces for the initial 80 ms of current development is shown for depolarizations to either +50 or +20 mV from a holding potential of −90 mV (as in Fig. 3A). Curves were fit to Eq. 3 with different values of n (where n = 1, green; 2, pink; 3, blue; 4, red; 5, aqua; 6, gray). Best fit was obtained for n = 4 (heavy red line).
voltage clamp at hyperpolarized potentials. When the membrane was depolarized beyond $-40$ mV, the incremental increase in peak steady-state current became less with each successive voltage step. At the more depolarized potentials (greater than $+10$ mV) peak current was observed immediately after the voltage step but within 20 ms a reduction to the steady-state current level was observed (Fig. 6A). The current–voltage relationships shown in Figs. 6B are based on measurements taken after steady-state current levels had been reached.

A “relaxation” of the peak outward current in response to test pulses more positive than $-40$ mV was observed (Fig. 7A). This relaxation to the steady-state current level was best fitted by the sum of three exponential functions providing three separate time constants of inactivation (Figs. 7, B, C, and D).

The relative contribution of the exponential term A (slow term) remains constant over the voltage range, whereas the contribution of exponential term B (intermediate term) increases with increasing voltage. Exponential term C (fast term) decreases with increasingly depolarized potentials. Thus the increased steepness of the relaxation at more positive voltages is attributed to the decrease of tau1 and tau3 (Fig. 7B) and not a shift in relative contributions of the faster terms.

Oocytes expressing N.at-Kv3.2 had markedly hyperpolarized resting potentials ($-68 \pm 12.7$ mV, $n = 11$) when compared with both control oocytes injected with nuclease-free water ($-39 \pm 3.8$ mV, $n = 15$) and with oocytes injected with the delayed rectifier channel N.at-Kv3.1 ($-38 \pm 4.1$ mV, $n = 8$) described above. Current–voltage curves (Fig. 6B) suggested that N.at-Kv3.2 channels were constitutively open over the voltage range of test protocols, accounting for the relatively hyperpolarized resting potentials. The Goldman equation was used to calculate the relative permeabilities for Na$^+$ and K$^+$, assuming that X. laevis oocytes have [K$^+$]$_{\text{internal}} = 150$ mM.

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**FIG. 5.** Activation kinetics of the N.at-Kv3.2 channel. A: current traces for the N.at-Kv3.2 channel showing the first 400 ms of channel opening from a holding potential of $-90$ mV to a range of potentials from $-140$ to $+50$ mV in 10-mV steps. Best fit for kinetic analysis was obtained by fitting with Eq. 3 where $n = 4$ (red lines). B: scaled, smoothed superimposed current traces showing that predepolarization of the membrane reduced the initial delay in channel opening and increased the rate at which equilibrium was achieved. An 800-ms predepolarization step was followed by a 100-ms step to $+50$ mV to fully open the channels. Sigmoidal shape of the current trace varied in response to the predepolarization voltage. As the predepolarizations became more positive the time constants of activation decreased, reflecting the relatively steeper activation curve. Predepolarization voltages (holding potentials) were $a = +30$ mV, $b = 0$ mV, $c = -40$ mV, and $d = -90$ mV. C and D: kinetic analysis of activation of currents from a constant holding potential to a range of suprathreshold potentials. All parameters were generated using Eq. 3 where $n = 4$. Total contributions of the fast components (A for $\tau_{\text{fast1}}$ and $\tau_{\text{fast2}}$) and slow components (B for $\tau_{\text{slow1}}$ and $\tau_{\text{slow2}}$) remained constant for the potentials tested (0.315 ± 0.017 and 0.685 ± 0.017, respectively). C: both $\tau_{\text{fast1}}$ (filled circles) and $\tau_{\text{slow1}}$ (open circles) of activation decreased in a linear, voltage-dependent manner. D: $\tau_{\text{fast2}}$ (filled circles) did not show any voltage dependency, whereas $\tau_{\text{slow2}}$ (open circle) decreased linearly in a voltage-dependent manner. E and F: kinetic analysis of activation of currents after prolonged submaximal depolarization. Relative contributions of the fast components (A for $\tau_{\text{fast1}}$ and $\tau_{\text{fast2}}$) and slow components (B for $\tau_{\text{slow1}}$ and $\tau_{\text{slow2}}$) remained constant over the range of predepolarization voltages tested (0.425 ± 0.039 and 0.575 ± 0.039 respectively). Time constants are plotted against the voltage deviation from the expected relationship for potassium ions suggests that

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**FIG. 6.** Currents mediated by the N.at-Kv3.2 channel expressed in X. laevis oocytes. A: currents evoked by 500-ms step depolarizations from a 50-ms prepulse at $-90$ mV to a range of potentials from $-140$ to $+80$ mV in 10-mV increments followed by a 50-ms step back to $-90$ mV. Holding potential was $-60$ mV. Currents were activated very rapidly throughout this range, although currents evoked by hyperpolarizing steps also expressed a slowly activating component and currents produced by strong depolarizations showed evidence of rapid and slow partial inactivation. B: current–voltage relationships for the N.at-Kv3.2 channel at different $[K^+]_{\text{out}}$. Reversal potential of N.at-Kv3.2-mediated currents depended on the external concentration of potassium ions. Reversal potentials were measured by the same protocol as illustrated in A: first in normal ND96 (2 mM K$^+$, filled circles) then in saline in which a proportion of the sodium ions had been replaced with potassium ions (10 mM K$^+$, open circles; 50 mM K$^+$, filled triangles; 98 mM K$^+$, filled squares). Note the extent of rectification was variable, as evidenced by the large error bars at positive voltages. C: reversal potentials measured for different values of $[K^+]_{\text{out}}$ deviate from the theoretical Nernst potential for potassium ions ($[K^+]_{\text{internal}} = 150$ mM as reported in Kusano et al. 1982). Reversal potentials were extrapolated from the experiment in B in which a proportion of the sodium ions in the saline were replaced with potassium ions (filled circles), as well as the same experiment in which choline chloride was used instead of sodium (open triangles). This deviation from the expected relationship for potassium ions suggests that N.at-Kv3.2 is permeable to ions other than potassium.
and \( [\text{Na}^+]_{\text{internal}} = 20 \text{ mM} \) (Kusano et al. 1982). For uninjected and \( \text{N.at-Kv3.1} \) injected eggs, the ratio of \( \text{Na}^+ \) permeability to \( \text{K}^+ \) permeability for the leak current is 0.34 and 0.33, respectively. Expression of constitutively open \( \text{N.at-Kv3.2} \) channels hyperpolarizes the membrane, as would be expected for a potassium-selective channel, but the resting potential (~69 mV) is substantially more positive than the equilibrium potential for \( \text{K}^+ \) (~110 mV) as shown in Fig. 6C. The calculated ratio of \( \text{Na}^+ \) permeability to \( \text{K}^+ \) permeability for cells expressing the \( \text{N.at-Kv3.2} \) channel is 0.08.

Perfusion experiments in which \( [\text{K}^+]_{\text{out}} \) was varied from 2, 10, 50, and 98 mM also showed that the major permeant ion was \( \text{K}^+ \) (Fig. 6B). However, the \( E_{\text{rev}} \) values measured when \( \text{K}^+ \) replaced \( \text{Na}^+ \) in solution are significantly different from the theoretical calculation for \( E_{\text{rev}} \) if \( \text{K}^+ \) was the sole permeant ion (Fig. 6C). When choline was substituted for \( \text{Na}^+ \) in the bath solution, the extrapolated \( E_{\text{rev}} \) was even more positive than predicted if it is assumed that the channels are permeant only to potassium. Thus there must be other significantly permeant ions (Fig. 6C). Ion substitution experiments were performed in which a single cation was present in the bath solution. \( \text{N.at-Kv3.2} \) is shown to preferentially pass potassium ions but this channel also passed other cations (Fig. 8B). Potassium inward currents were greater than those for any other cations tested, although a significant inward current was observed for all ions tested (Fig. 8B). Although \( \text{N.at-Kv3.2} \) is permeable to two cationic K-channel blockers, CsCl and BaCl2, the channel’s permeability to potassium ions was insensitive to 10 mM extracellular tetraethylammonium (TEA).

**DISCUSSION**

Although these two \( \text{N. atomata} \) channel sequences are members of the Shaw family of potassium channels their long branch lengths on the phylogenetic tree suggest that their electrophysiological characteristics should differ substantially from the other Shaw-type channels. In the case of \( \text{N.at-Kv3.1} \), the large number of differences in sequence were reflected in a phenotype with far slower opening kinetics compared with the other Shaw-type channels. The inward currents of \( \text{N.at-Kv3.2} \) are shown to be affected most by changes in potassium and sodium and this channel has limited permeability to calcium (Fig. 8A). This limited ionic selectivity accounts for the reversal potentials not following the expected Nernst relationship for potassium ions. A second set of ion substitution experiments were performed in which a single cation was present in the bath solution. \( \text{N.at-Kv3.2} \) is shown to preferentially pass potassium ions but this channel also passed other cations (Fig. 8B). Potassium inward currents were greater than those for any other cations tested, although a significant inward current was observed for all ions tested (Fig. 8B). Although \( \text{N.at-Kv3.2} \) is permeable to two cationic K-channel blockers, CsCl and BaCl2, the channel’s permeability to potassium ions was insensitive to 10 mM extracellular tetraethylammonium (TEA).
consistent with the kinetics of potential. curve, but not its position on the time axis, varies with holding al. 2004). Although a number of different activation schemes would be consistent with the kinetics of N.at-Kv3.1–mediated currents, it appears that there are two independent activation mechanisms and that both include slow transitions that are activated at relatively negative voltages and fast transitions that are activated at more depolarizing voltages. The fact that this channel has such a complex kinetic behavior that is affected by the predepolarization holding potential suggests that the voltage-dependent transitions in the channel-opening process have different voltage sensitivities. It is important to note that this behavior is different from the Cole–Moore effect (Cole and Moore 1960), in which the onset of channel opening varies with the holding potential but the shapes of the activation curve are superimposable by simple translation along the time axis. The shape of the N.at-Kv3.1 channel activation curve, but not its position on the time axis, varies with holding potential.

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Currents mediated by N.at-Kv3.2 were evident at all physiologically significant potentials. The characteristic decrease in conductance in the top right quadrant of current–voltage curves is a weak inward rectification that is incomplete. Inward rectification is found in a number of channels from different families, such as ERG, Kir, C. elegans Shab3 (EXP-2), and KAT1. However, a number of different mechanisms appear to be at play in producing inward rectification.

One possible mechanism of inward rectification in N.at-Kv3.2 might be that channels were constitutively open (at potentials between −140 and −40 mV), but are inactivated with membrane depolarization above −40 mV. Miller and Aldrich (1996) were able to convert Shaker B channels to inward rectifiers by a triple mutation of the S4 voltage sensor. Unlike N.at-Kv3.2, this Shaker B mutant is not constitutively open at rest because channels are mostly inactivated at rest. Hyperpolarization-induced “activation” of the Shaker B mutant is by recovery from N-type inactivation occurring through the open state. This is the mechanism suggested for intrinsic, voltage-dependent activation of other six transmembrane (TM) inward rectifiers such as KAT1 and HERG (Muller-Rober et al. 1995; Sanguinetti et al. 1995).

Second, inward rectification might result from relatively slow activation and ultrafast, N-type inactivation as was seen in another 6TM channel, the EXP-2 channel (Shab3) of Caenorhabditis elegans (Fleischhauer et al. 2000). In N.at-Kv3.2, at the most positive test potentials (above +30 mV), the rectification may also be a result of inactivation having both an initial fast phase and a slower phase (Fig. 7). The slow development of inward current that was apparent only at the
most hyperpolarized command potentials used might be caused by a relatively slow removal of inactivation at very hyperpolarized potentials. However, inactivation cannot account for all of the rectification observed in N.at-Kv3.2. A steady-state reduction in current was observed at potentials (−40 mV) before any inactivation was apparent. Rectification without inactivation first appeared at potentials more positive than about −10 mV (Fig. 6A).

It is also possible that the voltage sensor of N.at-Kv3.2 is modified such that the mechanics of activation is altered significantly. In Shaker channels the transmembrane electric field is focused across aqueous crevices in the membrane (Starace and Bezanilla 2004), implying that the S4 segment moves a short distance (Blunck et al. 2004; Cha et al. 1999) between crevices and specific acidic residues in S2 and S3 segments (Laine et al. 2004; Silverman et al. 2003; Swartz 2004). Substitution of a proline with hydrophilic residues at a specific site in the S6 activation gate of some Shaker channels can destabilize the closed state of the channel, making it constitutively open and uncoupling the gate from the voltage sensor (Sukhareva et al. 2003). In this case, inward rectification may be caused by intracellular block by other cations. Partial block by intracellular Na⁺ and Mg²⁺ occurs at very positive potentials in H. sapiens Kv 2.1, causing weak inward rectification of what is in other respects a typical delayed rectifier (Lopatin and Nichols 1994). Polyamine or Mg²⁺ block is seen in the 2TM, strongly inward-rectifying potassium channels such as IRK1 (Lopatin et al. 1994; Stanfield et al. 1994).

Finally, mutation of the S4 transmembrane domain can allow an alternative permeation pathway through the channel (Starace and Bezanilla 2004). For example, mutation of the first arginine in Shaker B removes a side chain that blocks an alternate, parallel ionic pathway to the pore. This Omega current has been shown to be a cation-selective permeation pathway along the length of S4, which is present only when S4 is in the resting state (Tombola et al. 2005). As shown in Fig. 8, the N.at-Kv3.2 channel is permeable to many cations, although K⁺ is the most permeant ion. The canonical pore sequence in N.at-Kv3.2 shows no sequence differences that would indicate that this channel should show such a low specificity (see Fig. 9). Thus the unusual permeability properties of this channel would be consistent with its manifesting a naturally occurring Omega current. It is also worth noting that N.at-Kv3.2 has a tyrosine residue at a position three residues C-terminal to the GYG selectivity sequence in Shaker, the mutation T449Y, at the homologous position, has been strongly correlated with the sensitivity of that channel to block by TEA (Taglialetela et al. 1994). N.at-Kv3.2 current is not affected by TEA up to a concentration of 10 mM, indicating that the ion conductance path may not involve the canonical pore region, but rather the conductance pathway characteristic of the Omega current.

![FIG. 9. A comparative alignment of the S4 voltage sensor and pore regions of N.at-Kv3.2 with other Kv channels. A: alignment of the highly conserved pore region. Characteristic GYG selectivity sequence is highlighted in gray. Location of the nonconducting Shaker mutation W434F is indicated in black. Sequence analysis of the pore suggests N.at-Kv3.2 should be tetraethylammonium (TEA) sensitive as the result of a tyrosine position after the pore (circled) as the homologous site T449 in Shaker when mutated to tyrosine gave increased TEA sensitivity in that channel. B: alignment of the S4 voltage sensor in selected channels. Characteristic positive arginine lysine repeats (R1–R7) are shaded in gray. Omega current is caused by mutating R1 in Shaker to R362A, R362C, R362S, or R362V. N.at-Kv3.2 has a histidine (H325) at position R1 and a glycine (G331) at position R3. Combination of the hinging action of the glycine in the middle of the helix coupled with the presence of the planar side chain at R1 may create a native Omega current.](http://jn.physiology.org/)

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The relatively nonselective cationic Omega current in the *D. melanogaster* Shaker B channel is caused by mutation of the first Arg residue in the S4 domain to one of Ala, Cys, Ser, or Val (Tombola et al. 2005). The S4 helix of the *N. at-Kv3.2* channel differs from the normal S4 sequence in that the first residue is His, not Arg, and the third Arg residue is replaced with Gly (see Fig. 9). Thus the S4 region of *N. at-Kv3.2* contains amino acid substitutions that might be expected to create an Omega current conductance pathway and uncouple S4 from the canonical channel gate.

If this is an example of a naturally occurring Omega current, then the canonical ion conductance pathway must be uncoupled from the normal activation mechanism because depolarization is not associated with the appearance of a more K\(^{+}\)-specific current, as is seen in the case of the synthetic Omega family channels (Hasegawa M. and Adachi J.).

**ACKNOWLEDGMENTS**

We thank Bamfield Marine Sciences Centre for providing collection and holding facilities.

**GRANTS**

This research was supported by Operating Grant MOP-62685 from the Canadian Institutes of Health Research (CIHR) to A. N. Spencer and W. J. Gallin. T. L. Klassen was supported by a Natural Sciences and Engineering Research Council of Canada Post Graduate Scholarship. J. B. Dacks was supported by a joint CIHR/Wellcome Trust Travelling Research Fellowship. Research Council of Canada Post Graduate Scholarship. J. B. Dacks was supported by a Natural Sciences and Engineering Research Council of Canada Post Graduate Scholarship. J. B. Dacks was supported by a joint CIHR/Wellcome Trust Travelling Research Fellowship.

**REFERENCES**

- Kusano K, Miledi R, and Stinnakre J. Comparative data can be complemented by mutagenesis studies focused on the S2, S3, S4, and pore regions that will determine the structural basis for these unusual activation properties. Although macroscopic current recordings demonstrate that the two N. atomata channels have unusual kinetic properties, a detailed structure–function analysis will require detailed single-channel recordings. As the scope of genetic and genomic studies expands to encompass an evolutionary diversity of organisms wider than that of the intensively studied model organisms, this general approach to sampling functional sequence space should prove fruitful in the analysis of other examples of protein machinery.


