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Primary Sensory Neurons Express a *Shaker*-like Potassium Channel Gene

Angeles B. Ribera and Duy-Ai Nguyen

Department of Physiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Developmentally regulated action potentials are a hallmark of Rohon-Beard cells, a class of sensory neurons. In these neurons as well as other primary spinal neurons of *Xenopus laevis*, the functional differentiation of delayed-rectifier potassium current regulates the waveform of the action potential during the initial day of its appearance. Later, the acquisition of another voltage-dependent potassium current—the A current—plays a major role in regulating excitability. In order to understand the molecular basis of this functional differentiation, genes encoding voltage-dependent potassium currents expressed in the embryonic amphibian nervous system are being cloned. Here, we report the functional properties and developmental localization of a second *Xenopus Shaker*-like gene (*Xenopus* $K_v1.1$; XSha1; GenBank accession number M94258) encoding a potassium current. Homology screening with the mouse gene MBK1 led to its isolation. Functional expression in oocytes identifies it as a delayed-rectifier current when assembled as a homooligomeric structure. Specific transcripts corresponding to XSha1 and to the previously cloned gene XSha2 are both detectable by RNase protection in RNA isolated from the embryonic nervous system. However, whole-mount *in situ* hybridization reveals the temporal pattern and cellular localization of XSha1 but not XSha2 mRNA, suggesting that the concentration of XSha2 transcripts in individual cells is lower than the threshold for detection by this method. Of particular interest, Rohon-Beard cells express XSha1 mRNA. In addition, XSha1 mRNA is detected in several structures containing neural crest derivatives including spinal ganglia, the trigeminal ganglion, and branchial arches; its presence in motor nerves and lateral spinal tracts suggests that both CNS and PNS glia express the mRNA. The results are consistent with a role for XSha1 in regulating electrical excitability in Rohon-Beard cells, neural crest derivatives, and glia.

[Key words: delayed rectifier potassium current,* Xenopus *embryo,* Shaker *gene,* in situ *hybridization, Rohon-Beard cells, neural crest]

The acquisition and further development of electrical excitability have been extensively studied in amphibian spinal neurons. The results indicate that electrical excitability is one of the first functions acquired by postmitotic neurons (Warner, 1973; Lamborghini, 1980). Action potentials are initially detected at the neural tube stage (22 hr after fertilization) and are calcium dependent and of long duration (Baccaglini and Spitzer, 1977). These action potentials precede neurite extension by 2–4 hr and the appearance of chemosensitivity by 4–6 hr (Bixby and Spitzer, 1982, 1984; Taylor and Roberts, 1983).

During the following day *in vivo* and *in vitro,* the impulse matures to a brief sodium dependent spike (Spitzer and Lamborghini, 1976; Baccaglini and Spitzer, 1977). This program is expressed in a cell autonomous manner, since a neuron developing in the absence of other cells also exhibits a transient period of long duration impulses (Henderson and Spitzer, 1986). Thus, electrical excitability is not only an early acquired phenotype but one that continues to be modified as development proceeds.

Biophysical analysis of the voltage-dependent currents that underlie the action potential indicates that the transition in ionic dependence of the impulse is due primarily to the maturation of a delayed-rectifier potassium current (I_{Kv}; Barish, 1986; O'Dowd et al., 1988; Lockery and Spitzer, 1992). During this time the calcium current shows very little change, while the sodium current doubles in density. The delayed-rectifier potassium current, however, triples in density and its kinetics are accelerated (O'Dowd et al., 1988). Later, another voltage-dependent potassium current, the A current (I_{KA}), appears. Its contribution results in further shortening of the duration of the action potential leading to additional changes in excitability (Ribera and Spitzer, 1990).

What are the molecular events involved in encoding the early appearance of electrical excitability? What is the basis of its continued regulation during early neuronal development? Investigation of these questions requires probes for genes encoding the ion channels that underlie action potentials. Given that potassium channels are pivotal in regulating action potential wave form, our efforts have focused on these proteins. Here we report the cloning of a second *Xenopus Shaker*-like potassium channel gene, XSha1, that is expressed in the embryonic nervous system. Its expression pattern is similar in some respects to that of XSha2: it is detectable in RNA extracted from the embryonic brain, and expression in oocytes produces a delayed-rectifier potassium current. However, important differences are apparent. Notably, *in situ* hybridization reveals the tissue specific expression of XSha1 and shows that it is localized to a subset of primary spinal neurons (Rohon-Beard cells), whose action potentials and currents have been extensively studied both in culture and *in situ.* In addition, XSha1 mRNA is found in neural

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Correspondence should be addressed to Angeles B. Ribera, Department of Physiology, C-240, University of Colorado Health Sciences Center, Denver, CO 80262.

crest derivatives and nerve tracts. These characteristics of XSha1 make it an ideal candidate for molecular analyses of the regulation of electrical excitability in developing spinal sensory and neural crest cells.

Materials and Methods

Animals. Embryos were produced by breeding pairs of adult *Xenopus* primed with human chorionic gonadotropin (U.S. Biochemicals) and staged according to Nieuwkoop and Faber (1967). In addition some embryos were obtained by *in vitro* fertilization using standard methods (Moon and Christian, 1989).

Isolation of Xenopus Shaker *homolog, DNA sequencing, and analysis.* *Xenopus* cDNA libraries were screened as previously described (Ribera, 1990). A partial clone was first isolated from a *Xenopus* tadpole brain nervous system λUNIZAPII cDNA library using the mouse MBK1 sequence as a probe (generously provided by Dr. Bruce Tempel, Columbia Univ.) for reduced stringency screening. This clone was then used to screen a *Xenopus* larynx λZAPII cDNA library (generously provided by Dr. Darcy Kelley and Leslie Fisher, Univ. of WA) at high stringency. A clone containing the entire coding sequence within a ~1.6 kilobase (kb) insert was identified (Fig. 1).

The two *Xenopus* clones were identical in their region of overlap (~0.6 kb). Double-stranded DNA was subjected to alkaline denaturation. Primers designed to give overlapping sequences were synthesized on an Applied Biosystems PCR-Mate. The majority of the sequence was clearly read from both strands. Standard chain termination reactions with dideoxy nucleotides were carried out (Sanger et al., 1977) and DNA was synthesized with Sequenase 2.0 (U.S. Biochemicals) in the presence of ^{35}S labeled dATP (New England Nuclear). DNA sequences were read and entered into a computer using a GEL READER interface and software (CBS Scientific) and analyzed using DNASTAR software (Madison, WI).

Oocyte recording. The entire coding region of the XSha1 potassium channel gene was cloned into the pSP64T expression vector (Melton et al., 1984). It was excised from the larynx cDNA phagemid by cutting the 5′ and 3′ flanking regions of the polylinker with Xba1 and EcoRV. The fragment was blunt ended with Klenow, and cloned into pSP64T that had been cut with BglII and blunt ended to yield compatible ends. The resulting recombinant pSP64T was linearized with Xba1. Capped sense RNA was generated by *in vitro* transcription with SP6 RNA polymerase in the presence of rNTPs and cap analog (Boehringer-Mannheim). Oocytes were removed, defolliculated, and injected with 10–50 ng of RNA as described previously (Ribera, 1990). Currents were detectable 1.5 d after injection of XSha1 RNA, and recordings were generally carried out between 3 and 5 d postinjection.

Comparison of the current induced by XSha1 with the current recorded from *Xenopus* spinal neurons was of particular interest and thus the *standard solution* was that used to study the endogenous neuronal current (O'Dowd et al., 1988; Ribera and Spitzer, 1989, 1990): 80 mM NaCl, 3 mM KCl, 5 mM $MgCl_2$, 10 mM $CoCl_2$, 5 mM HEPES, pH 7.4. As previously noted, the high concentrations of divalent cations in this solution interfered with determination of the potassium selectivity (O'Dowd et al., 1988; Ribera, 1990). In experiments designed to examine the potassium dependence of the XSha1 current the bath solution was more similar to a typical oocyte recording solution (*oocyte solution*): 88 mM NaCl; 1, 3, or 10 mM KCl, 0.33 mM $Ca(NO_3)_2$; 2.4 mM $NaHCO_3$; 0.82 mM $MgSO_4$; and 5 mM Na-HEPES, pH 7.4. In some cases, potassium selectivity was also examined in the standard solution to permit comparisons with the endogenous current.

Tetraethylammonium (TEA) sensitivity was examined by addition of either 1, 1.5, 3, 15, or 40 mM TEA to the recording solutions. Electrodes were filled with 3 M KCl and had resistances ranging between 0.1 and 3 MΩ. Voltage protocols and data analysis were accomplished with the pCLAMP suite of programs (Axon Instruments). Comparisons for endogenous current are from data presented in Ribera and Spitzer (1990) or S. M. Jones and A. B. Ribera (unpublished observations).

RNA isolation and RNase protection. The guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) was used to isolate RNA from isolated brains. Homogenization in 10 ml of guanidinium buffer (4 M guanidinium thiocyanate, 25 mM Na-citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) per gram of tissue was followed by phenol extraction, and precipitation in isopropanol.

The regions of XSha1 or XSha2 chosen as templates for probe synthesis were specific to each and contained 3′ untranslated sequences. In order to maximize the amount of 3′ untranslated region in the probes and thus their specificity, the brain library XSha1 clone was linearized with SalI. The transcribed RNA was 493 base pairs (bp) containing ~30 nucleotides of vector sequences. The XSha2 probe was that described previously (Ribera, 1990), 396 nucleotides in length of which ~70 represent vector sequences. The different sizes of the two probes permitted simultaneous use in each RNA sample. Short complementary ("antisense") radioactive riboprobes were generated and used as described previously (Ribera, 1990). EF-1α (a constitutively ubiquitously expressed gene; Krieg et al., 1989) and N-CAM (neural-specific long cytoplasmic domain form; Kintner and Melton, 1987; kindly provided by Dr. Chris Kintner, Salk Inst.) probes were used to control for the amount of cellular RNA and neural tissue, respectively, in samples.

In situ *hybridization.* For whole-mount preparations, the nonradioactive detection method (Harland, 1991) was followed with minor modifications (Ferreiro et al., 1993). In order to generate a short probe that contained 3′ untranslated sequences, the clone from the brain library was linearized at a unique Hpa1 site. A 362-nucleotide-long RNA probe that consisted essentially of untranslated sequences was synthesized in the presence of digoxigenin-labeled UTP (Boehringer-Mannheim). Both pigmented and albino embryos were fixed in MEMPFA (4% paraformaldehyde, 0.1 M MOPS, pH 7.4, 1 mM $MgSO_4$, 2 mM EGTA) for 12–15 hr at 4°C. Hybridization was carried out overnight. After incubation with antibody, washes were carried out over ≥24 hr with a minimum of seven changes. The alkaline phosphatase reaction product was developed in the presence of chromogenic substrate for 8–30 hr.

Whole-mount embryos were either cleared in Murray's solution (2:1 benzyl benzoate : benzyl alcohol) or embedded in Eponate 12 (Pella), sectioned at 10–20 μm, and stained with Eosin (2–5 sec). Photography of whole-mounts and sections was done with Kodak Ektachrome 160T film using appropriate color filters.

Results

Identification of a second Xenopus Shaker *homolog*

A cDNA clone containing a portion of the XSha1 coding sequence was isolated from a *Xenopus* tadpole nervous system cDNA library (Stratagene) on the basis of its homology to MBK1. This clone contained a 0.9 kb insert that had its 5′ end immediately preceding the S4 region and extended in the 3′ direction 290 bp past the stop codon. This DNA was then used to screen a postmetamorphic *Xenopus* larynx cDNA library at high stringency. A single hybridizing clone was identified. DNA sequencing indicated that the entire coding region was contained within its ~1.6 kb insert.

The predicted amino acid sequence has 489 residues and a molecular weight of ~56 kD (Fig. 1). It is identical to the clone isolated from the nervous system library over the region of overlap. Overall, this amphibian clone is most conserved with the mammalian sequences MBK1 and RCK1 (88%; Fig. 2; Baumann et al., 1988; Tempel et al., 1988). The last 13 residues of XSha1, MBK1, and RCK1 are the same and distinguish them from other *Shaker*-like gene family members. The next highest identities, 75–77%, are found between XSha1 and either XSha2 or the mammalian MK2 and RBK2 peptides (McKinnon, 1989; Chandy et al., 1990; Ribera, 1990). Other regions of high homology include the domain thought to contribute to specificity of subunit assembly (residues 22–133; Li et al., 1992); SS1-2, which is thought to constitute the pore (amino acids 354–374; MacKinnon and Yellen, 1990; Hartmann et al., 1991; Yellen et al., 1991; Yool and Schwartz, 1991); and the six putative transmembrane domains (S1–S6). In view of these similarities, this *Xenopus Shaker*-like gene has been named XSha1 and $K_v1.1$ of *Xenopus* according to the standard nomenclature (Chandy et al., 1991). Similar conclusions are drawn from parsimony analysis (G. Gutman, personal communication).

A region rich in basic and serine residues (amino acids 437–445), containing consensus sites for cAMP as well as protein

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-2 +1
 AA ATG ACC GTA ATC GCA GGG GAG AAT ATG GAC GAG ACC TCA GTA TTG CCC GGT CAC CCT CAG GAC AGC TAC CAT CCA GAC CAA GAT GAC CAC   90
    Met Thr Val Ile Ala Gly Glu Asn Met Asp Glu Thr Ser Val Leu Pro Gly His Pro Gln Asp Ser Tyr His Pro Asp Gln Asp Asp His   30

    GAA TGC TGT GAG AGG GTG GTC ATC AAT GTG TCC GGC CTA CGC TTC GAG ACC CAG CTT AAG ACT CTC GCT CAG TTC CCC AGC ACT CTG CTA  180
    Glu Cys Cys Glu Arg Val Val Ile Asn Val Ser Gly Leu Arg Phe Glu Thr Gln Leu Lys Thr Leu Ala Gln Phe Pro Ser Thr Leu Leu   60

    GGG AAC CCC AAA AAA CGG ATG CGT TAC TTT GAC CCC CTG AGG AAC GAG TAC TTC TTT GAC CGA AAC CGT CCA AGT TTC GAT GCC ATA TTA  270
    Gly Asn Pro Lys Lys Arg Met Arg Tyr Phe Asp Pro Leu Arg Asn Glu Tyr Phe Phe Asp Arg Asn Arg Pro Ser Phe Asp Ala Ile Leu   90

    TAT TAC TAC CAG TCT GGG GGT CGA CTC CGG AGA CCT GTT AAT GTT CCA CTG GAC ATG TTC TCT GAG GAA ATC AAG TTT TAT GAG TTA GGG  360
    Tyr Tyr Tyr Gln Ser Gly Gly Arg Leu Arg Arg Pro Val Asn Val Pro Leu Asp Met Phe Ser Glu Glu Ile Lys Phe Tyr Glu Leu Gly  120

    GAA GAG GCC ATG GAG AAG TTT AGG GAG GAT GAG GGT TTC GTA AAG GAA GAG GAA CGC CCT CTG CCA GAT AAA GAG TTC CAA CGC CAG GTG  450
    Glu Glu Ala Met Glu Lys Phe Arg Glu Asp Glu Gly Phe Val Lys Glu Glu Glu Arg Pro Leu Pro Asp Lys Glu Phe Gln Arg Gln Val  150

    TGG CTC TTG TTT GAG TTC CCC GAA AGC TCC GGC CCG GCC AGG ATC ATT GCC ATA ATA TCT GTG ATG GTC ATC CTT ATA TCA ATT GTT ATC  540
    Trp Leu Leu Phe Glu Phe Pro Glu Ser Ser Gly Pro Ala Arg Ile Ile Ala Ile Ile Ser Val Met Val Ile Leu Ile Ser Ile Val Ile  180

    TTC TGC TTG GAG ACT TTG CCA GAA TTA AAA GAT GAG CGG ATC TTC AGT CGA CGG GTG AAC AAC AGC ACA GTT TTC TAC AAA TCC AAC ATC  630
    Phe Cys Leu Glu Thr Leu Pro Glu Leu Lys Asp Glu Arg Ile Phe Ser Arg Arg Val Asn Asn Ser Thr Val Phe Tyr Lys Ser Asn Ile  210

    TTC ACG GAT CCA TTC TTT GTG GTG GAG ACC CTC TGC ATT ATC TGG TTT TCC TTT GAA TTG GTG GTG AGG TTC TTT GCA TGT CCC AGC AAA  720
    Phe Thr Asp Pro Phe Phe Val Val Glu Thr Leu Cys Ile Ile Trp Phe Ser Phe Glu Leu Val Val Arg Phe Phe Ala Cys Pro Ser Lys  240

    CCG GAA TTC TTT AAG AAC ATC ATG AAC TTC ATT GAC ATT GTG GCC ATC ATC CCT TAC TTT ATC ACC TTG GGG ACT GAA ATG GCA GAG CAA  810
    Pro Glu Phe Phe Lys Asn Ile Met Asn Phe Ile Asp Ile Val Ala Ile Ile Pro Tyr Phe Ile Thr Leu Gly Thr Glu Met Ala Glu Gln  270

    GAA GGT CCC CAA AAA GGA GAA CAG GCA ACA TCT TTG GCA ATC CTG AGG GTC ATC AGA CTG GTA AGA GTG TTT AGA ATC TTC AAA CTC TCC  900
    Glu Gly Pro Gln Lys Gly Glu Gln Ala Thr Ser Leu Ala Ile Leu Arg Val Ile Arg Leu Val Arg Val Phe Arg Ile Phe Lys Leu Ser  300

    AGG CAT TCT AAG GGA CTC CAG ATT TTG GGA CAG ACC TTG AAA GCT AGC ATG AGA GAA TTA GGG TTG CTA ATT TTT TTT CTA TTC ATT GGG  990
    Arg His Ser Lys Gly Leu Gln Ile Leu Gly Gln Thr Leu Lys Ala Ser Met Arg Glu Leu Gly Leu Leu Ile Phe Phe Leu Phe Ile Gly  330

    GTC ATC TTG TTC TCC AGT GCA GTG TAC TTT GCT GAA GCT GAA GAG GAT GAA TCT CAT TTT ACA AGT ATC CCT GAT GCT TTC TGG TGG GCG 1080
    Val Ile Leu Phe Ser Ser Ala Val Tyr Phe Ala Glu Ala Glu Glu Asp Glu Ser His Phe Thr Ser Ile Pro Asp Ala Phe Trp Trp Ala  360

    GTG GTA TCC ATG ACC ACT GTG GGC TAT GGT GAC ATG TAC CCT GTG ACA ATT GGA GGC AAA ATC GTG GGC TCC TTG TGT GCC ATC GCT GGT 1170
    Val Val Ser Met Thr Thr Val Gly Tyr Gly Asp Met Tyr Pro Val Thr Ile Gly Gly Lys Ile Val Gly Ser Leu Cys Ala Ile Ala Gly  360

    GTG CTG ACA ATT GCC CTG CCT GTA CCT GTC ATC GTG TCC AAC TTC AAC TAC TTC TAC CAC CGA GAA ACT GAA GGG GAG GAA CAG GCT CAG 1260
    Val Leu Thr Ile Ala Leu Pro Val Pro Val Ile Val Ser Asn Phe Asn Tyr Phe Tyr His Arg Glu Thr Glu Gly Glu Glu Gln Ala Gln  420

    TTA CTC CAT GTT AGC TCC CCC AAT TTA GCC TCT AAC AGT GAT CTG AGT CGA CGA AGT TCC TCC GCA ATG AGC AAA TCT GAG TAC ATG GAG 1350
    Leu Leu His Val Ser Ser Pro Asn Leu Ala Ser Asn Ser Asp Leu Ser Arg Arg Ser Ser Ser Ala Met Ser Lys Ser Glu Tyr Met Glu  450

    ATT GAA GAG GAT CTG AAT AAT AGC ATA GAT AAC TTT AGA GAG GCA AAT ATC AGA ACT GGC AAT TGC ACC ATA GCC AAT CAG AAC TGT GTT 1440
    Ile Glu Glu Asp Leu Asn Asn Ser Ile Asp Asn Phe Arg Glu Ala Asn Ile Arg Thr Gly Asn Cys Thr Ile Ala Asn Gln Asn Cys Val  480

    AAC AAA AGC AAG CTC CTG ACA GAT GTG TAG ACA CGA CAG TCC CAT TAC AGA ATT ATC ATG ACC ACT TCC CTC TTT GCA GGG CCA CCA TGA 1530
    Asn Lys Ser Lys Leu Leu Thr Asp Val STOP                                                                              489

    ACT TAA CCT TCA AAA ATA CAG AGG CCT AGT ACA AAT TAG GGG AGC AGC CCC                                                   1581
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Figure 1. Sequence of a *Shaker* homolog in *Xenopus:* nucleotide and predicted amino acid sequence of XSha1. The three-letter code for amino acids is used.

kinase C–dependent phosphorylation, is found near the carboxy terminus in a putative intracellular domain; this position is analogous to the location of a consensus sequence for cAMP-dependent phosphorylation in mouse and fly *Shaker*-like sequences (e.g., Tempel et al., 1988). The possibility of protein kinase C–dependent phosphorylation is particularly intriguing given the known developmental regulation of the kinetics of the endogenous delayed-rectifier current by a mechanism involving this enzyme (Desarmenien and Spitzer, 1991).

XSha1 encodes a delayed-rectifier-type current

Based upon the amino acid sequence, XSha1 is predicted to be a delayed-rectifier-type current since it does not contain an inactivating ball in the amino terminus region (Hoshi et al., 1990). Functional expression of XSha1 verified this prediction. Capped sense transcripts were generated (Melton et al., 1984) and injected into *Xenopus* oocytes. The oocyte membrane was clamped at −80 mV, and then stepped for 60 msec intervals to depolarized potentials expected to activate potassium currents. This protocol demonstrated a voltage-activated current in 24 of 27 XSha1-injected oocytes (three batches of oocytes) that was not found in uninjected oocytes. The current was sustained during the 60 msec voltage pulse (Fig. 3*A*), indicating that it is of the delayed-rectifier type. The average current amplitude induced by XSha1 was 6.8 ± 1.1 μA (+30 mV). Currents greater than 1.8 μA (+30 mV) were never observed after expression of XSha2, even when fourfold greater concentrations of RNA were injected into oocytes.

Under recording conditions similar to those used to study the endogenous delayed rectifier, activation of the XSha1 current is observed at potentials positive to −40 mV, which is more hyperpolarized than that observed for the endogenous current (O'Dowd et al., 1988; Ribera and Spitzer, 1989, 1990) or for XSha2 (Fig. 3*B,C*; Ribera, 1990). In this regard, XSha2 is more similar to the endogenous current.

The ionic dependence of the XSha1 RNA induced current is strongly potassium selective. Elevating the external potassium concentration from 1 to 10 mM leads to a 60 ± 2 mV shift in the reversal potential of tail currents ($n = 10$). The Nernst equation predicts a 58 mV shift for a purely potassium-dependent current. The data confirm the prediction and are consistent with results for mammalian homologs as well as the endogenous

voltage-dependent potassium currents (O'Dowd et al., 1988; Christie et al., 1989; Stuhmer et al., 1989; Ribera and Spitzer, 1990).

Inspection of the putative pore sequence (amino acids 354–374; Figs. 1, 2) suggests that the potassium channel blocker TEA will strongly inhibit the current, given the tyrosine residue at position 373 (Stuhmer et al., 1989; MacKinnon and Yellen, 1990). In fact, at concentrations of 1.5 mM and 15 mM TEA, the current was reduced by 64 ± 6% (n = 8) and 88 ± 2% (n = 13) in oocyte solution (Fig. 4). However, in the presence of the standard solution, low concentrations of TEA were less effective. For example, 1.5 mM TEA in standard solution resulted in only an 18 ± 6% (n = 4) reduction of the current; at higher concentrations of TEA, the apparent competition between it and divalent cations was less pronounced. XSha2, with a valine at the analogous position, is expected to show low sensitivity to TEA on the basis of structure (Stuhmer et al., 1989; MacKinnon and Yellen, 1990). Extension of previous work showed that 40 mM TEA produced only 20% reduction of the XSha2-induced current expressed in oocytes (Fig. 4; Ribera, 1990).

Pharmacological agents distinguish among the various potassium currents that function within a single cell (e.g., Thompson, 1977; Wu and Barish, 1992). The endogenous delayed-rectifier potassium current (I_{Kv}) is sensitive to external TEA, being reduced by 80% at a concentration of 40 mM; at a similar concentration the endogenous A current (I_{KA}) is inhibited only by 20% (Fig. 4; Ribera and Spitzer, 1990). Thus, with respect to TEA sensitivity, the endogenous delayed-rectifier potassium current is more similar to XSha1 than it is to XSha2.

XSha1 is expressed in the nervous system

Potassium channels are expressed in a wide variety of tissues (see Ribera and Spitzer, 1992, for review). In order to determine whether XSha1 might contribute to the formation of neuronal potassium channels, its presence in neural tissue was first determined by the sensitive method of RNase protection, as done previously (Ribera, 1990). Further, to compare XSha1 levels to those of XSha2, probes for both were simultaneously included in the hybridization reaction. In RNA extracted from tadpole brains, both XSha1 and XSha2 mRNA are detectable (Fig. 5). The autoradiographic signals are similar, suggesting that the levels of the two mRNAs in whole-brain extracts are comparable.

In situ *hybridization indicates that XSha1 is expressed in both the CNS and PNS*

The cellular distribution of XSha1 expression was addressed using whole-mount *in situ* hybridization. For 1–3 d embryos, a whole-mount consists of the entire embryo. Visualization quickly locates the hybridization signal to specific regions or tissues of the embryo. Subsequently, the embryo can be sectioned to examine more precisely the distribution of the mRNA.

XSha1 mRNA has an unusual pattern of expression. In 3-d-old embryos, XSha1 mRNA is specifically detected in the head and throughout the length of the embryo in the dorsal side (Fig. 6*a*). Thus, XSha1 expression predominates in the nervous system. However, the gills (Fig. 6*b*) also show an intense signal. Of particular interest is the signal that is seen throughout the dorsal aspect of the spinal cord (Fig. 6*c*). As shown in more detail in Figures 7 and 8, this signal is due to expression in Rohon-Beard neurons.

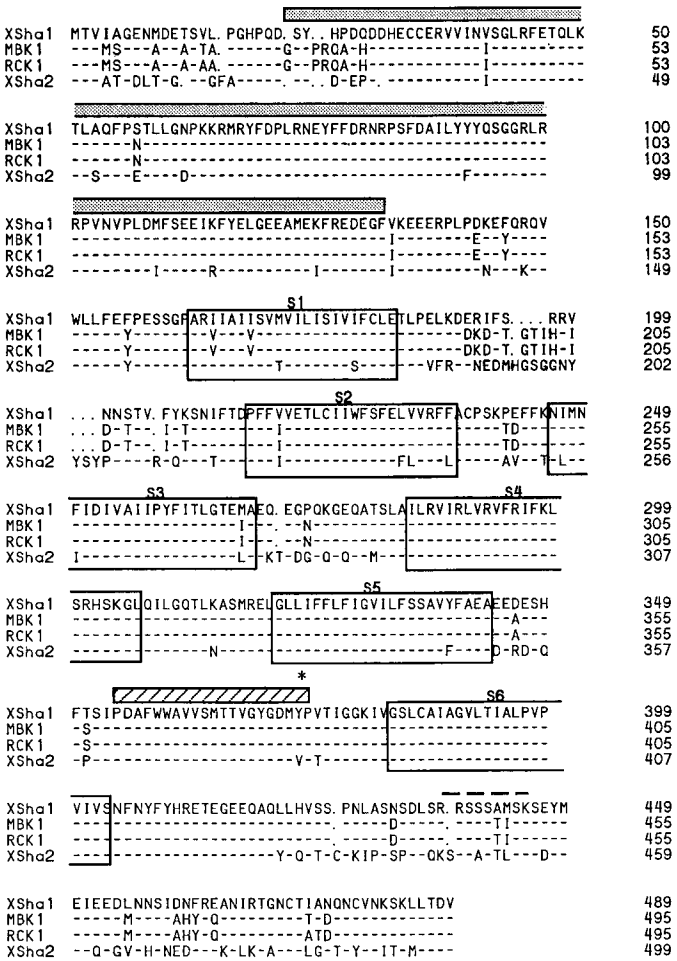


Figure 2. Alignment of the XSha1, XSha2, MBK1, and RCK1 peptides. XSha1 shares 89% identity with MBK1 and RCK1 (Tempel et al., 1988; Stuhmer et al., 1989) but only 77% identity with XSha2, a previously cloned *Xenopus Shaker* gene (Ribera, 1990). Identity with XSha1 is indicated by *dashes*; gaps that were introduced to enhance the alignment are indicated by *dots*. The one-letter amino acid code is used. Unique features of the $K_v1.1$ *Shaker* genes include the carboxy-terminal residues and the tyrosine at position 373. The region thought to specify subunit assembly specificity (*shaded bar*) extends from amino acids 22 to 133 and 94% and 89% identical to the similar region in MBK1/RCK1 and XSha2, respectively. The putative transmembrane domains *S1–S6* are drawn into *boxes*. The putative pore (*hatched bar*, residues 354–374) contains the tyrosine residue (373, *asterisk*) implicated in determination of TEA sensitivity. Consensus sites for cAMP-dependent protein kinase C phosphorylation are found near the carboxy terminus in the region indicated by a *dashed line* (residues 437–445). Alignment was achieved using the MEGALIGN program (DNASTAR) followed by adjustments done by eye. MBK1 and RCK1 sequences were downloaded from the GenBank/EMBL database.

The neural structures exhibiting XSha1 are better defined in sections. At the level of the eye (Fig. 7*a,d*), the trigeminal ganglion presents a robust reaction product. The signal in the eye was also found in control embryos, and thus not specific. At the levels of the hindbrain and spinal cord (Fig. 7*b,c*) Rohon-Beard cells and lateral tracts present the signal. In the trunk region, at repeated intervals, sections show an XSha1 signal in the location of condensing spinal ganglia (Fig. 7*c*). In a longitudinal view, at the level of the notochord, the reaction product is observed in the intermyotomal junction region (Fig. 7*e*). The motor nerves run into the junction at this level (Chu and Klymkowsky, 1989; Hemmati-Brivanlou et al., 1992); the staining may reflect the

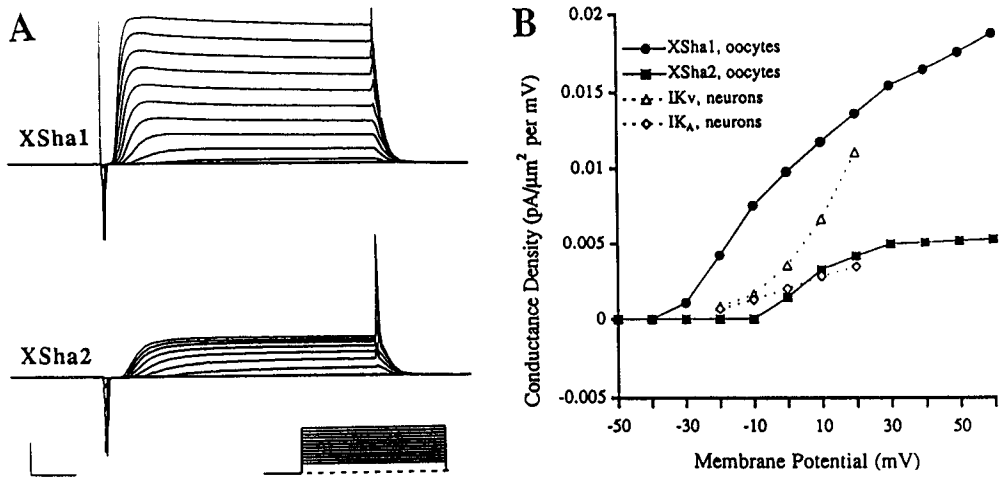


Figure 3. Characterization of the voltage-dependent potassium current induced by XSha1 transcripts. *A*, Currents induced by expression of XSha1 (*top*) or XSha2 (*bottom*) RNA in oocytes were elicited by depolarizing steps to voltage levels ranging between −50 to +60 mV from a holding potential of −80. The XSha1-induced current begins to activate at −40 mV and is sustained for the duration of the 60 msec pulse, indicating that is a delayed-rectifier-like current. XSha2 RNA induces a delayed-rectifier potassium current, which has a voltage dependence of activation that is depolarized with respect to XSha1. Calibration: 2 μA, 10 msec. *B*, Conductance density–voltage plots compare the voltage dependence of activation of XSha1- and XSha2-induced currents in oocytes to that of the endogenous delayed rectifier (I_{K_v}) and A potassium currents (I_{K_A}) recorded from primary spinal neurons (after Ribera and Spitzer 1989, 1990). Oocyte current densities were calculated assuming a cell diameter of 1 mm. The endogenous delayed-rectifier current has activation properties that are more similar to that of XSha2. Recordings were done in standard solution.

presence of XSha1 in the Schwann cells that ensheath the motor nerve. These data indicate that XSha1 expression is found in a subset of CNS and PNS structures.

In addition, XSha1 is found in a non-neural tissue, the gill arches (Figs. 6*b*, 7*f*). The gills arise from the branchial arches, a neural crest derivative. In fact, the majority of the neural presence of XSha1 is localized to neural crest derivatives.

Two aspects of XSha1 expression differed in 2 versus 3 d embryos. The first reflected the more medial position of the Rohon-Beard cells in 3 versus 2 d embryos. For example, these cells are found in a dorsal lateral position in a section from a 2 d embryo at the level of the spinal cord (Fig. 7*c*), whereas in a section from a 3 d embryo, the cells have a more medial location (Fig. 8*a*). This difference is also detectable at the level of the whole-mount by optically sectioning the specimen (Fig. 8*c,e*). Second, the presence of superficial tracts that run dorsoventrally in repeated fashion is apparent only in 3 d embryos. The tracts follow the pattern expected for motor nerves (Fig. 8*c–h*; see also Fig. 7*e*; Chu and Klymkowsky, 1989; Hemmati-Brivanlou et al., 1992), and may reflect the presence of XSha1 in the neural crest derivatives forming peripheral glia, Schwann cells (Fig. 8*e–h*). In a 2 d embryo, neural crest cells are migrating (Sadaghiani and Thiebaud, 1987; Krotoski et al., 1988) and thus may have a more internal location (Fig. 8*c,d*).

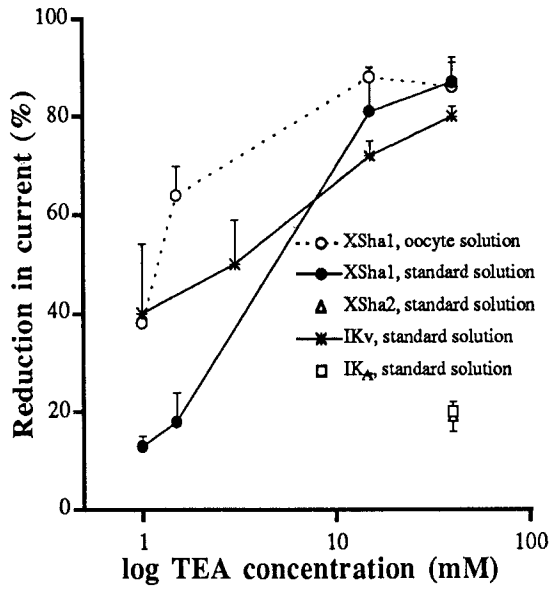


Figure 4. TEA sensitivity of XSha1-induced current in oocytes. The current is efficiently blocked by TEA, in particular in the presence of oocyte buffer. In the presence of the standard solution, which contains 10 mM cobalt and 5 mM magnesium, TEA sensitivity is reduced at low concentrations (1 and 1.5 mM) but not at higher concentrations (15 and 40 mM). The sensitivities of the XSha2-induced current in oocytes as well as the endogenous delayed-rectifier (I_{K_v}) and A currents (I_{K_A}) to 40 mM TEA in standard solution are also shown. Data for the endogenous currents are obtained either from Ribera and Spitzer (1990) or from S. M. Jones and A. B. Ribera (unpublished observations). Data are presented as mean ± SEM for three to eight oocytes or neurons.

Discussion

We find that a variety of structures containing neural crest derivatives express a *Xenopus Shaker*-like gene, XSha1. The pattern includes such diverse tissues as the trigeminal and spinal ganglia and the branchial arches; several nerve tracts also show XSha1 mRNA, suggesting that it may be in glia. The restriction of XSha1 mRNA to numerous structures containing neural crest derivatives supports the idea that it is the crest derivatives rather than other cells (e.g., placodal cells in the trigeminal ganglion) that express the transcript.

Of particular interest is the detection of the mRNA in Rohon-Beard cells, for which the development of electrical excitability has been extensively studied. However, these cells are not widely considered to be neural crest derivatives (Jacobson, 1991), although they are found in the region of the neural tube most proximate to the neural crest (Sadaghiani and Thiebaud, 1987;

Hartenstein, 1989; Eagleson and Harris, 1990). The presence of XSha1 in Rohon-Beard cells may reflect a shared lineage with the neural crest.

XSha1 mRNA demonstrates novel distributions. First, its appearance in gill arches suggests that it may be in cartilage cells, the expected neural crest derivative in this location. Expression of potassium channels in these cells is hard to reconcile with an expected role in determining excitability, although the broad roles of ion channels are increasingly appreciated (Ribera and Spitzer, 1992, for review). Second, the clone was isolated from a larynx cDNA library. Its presence in muscle contrasts with the pattern observed here. The larynx was isolated from older animals and could reflect developmental changes in the distribution of the mRNA. Alternatively, since XSha1 mRNA was present in several nerve tracts and the larynx was not denervated, the presence of the XSha1 clone may reflect RNAs found in the innervating nerve fibers.

The majority of the *in situ* hybridization data were obtained from albino specimens to avoid the signal due to pigmentation. However, physiological analyses of the endogenous potassium currents expressed in *Xenopus* spinal neurons have been done using cultures prepared from pigmented embryos. We found that the distribution of XSha1 mRNA was the same in pigmented and albino embryos (Figs. 7, 8).

The development of potassium currents and electrical excitability in neural crest derivatives has been studied in avian and mammalian systems (Bader et al., 1985; Belluzzi et al., 1985; Nerbonne and Gurney, 1989; McFarlane and Cooper, 1992). Both delayed-rectifier and A potassium currents function in these cells. Although no single pattern emerges, the densities and other properties of these potassium currents are developmentally regulated (see Ribera and Spitzer, 1992, for review). Since the exact schedule followed requires specific examination in each system, neural crest–derived cells do not have a universal program for the functional expression of excitability.

Functional expression of XSha1 in oocytes induces a delayed-rectifier-type potassium current resembling that studied in *Xenopus* spinal neurons both in culture and *in vivo* with respect to properties of inactivation, TEA sensitivity, and potassium selectivity (Harris et al., 1988; O'Dowd et al., 1988; Ribera and Spitzer, 1990; Desarmenien et al., 1993). However, the voltage dependence of activation is considerably different and is more reminiscent of the endogenous A current, which has a less steep voltage dependence of activation with respect to the delayed rectifier (Fig. 3; Ribera and Spitzer, 1990). XSha1 mRNA was not apparent in Rohon-Beard cells until 2 d of development. This is later than would be expected if it participated in forming the endogenous delayed-rectifier current, but is consistent with a contribution to the endogenous A current. If so, this would require that XSha1 peptides form heteromeric structures with other *Shaker*-like peptides that have an inactivating ball (Christie et al., 1990; Hoshi et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990).

Several issues are important to consider when drawing comparisons between natively expressed currents and those induced in the oocyte by expression of injected RNA. First, XSha1 peptides expressed in oocytes and in neurons may undergo different extents of posttranslational processing. Second, endogenous subunits may combine with other *Shaker*-like subunits to form heteromultimeric channels with novel properties (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). Third, potassium channels may share common associated subunits as

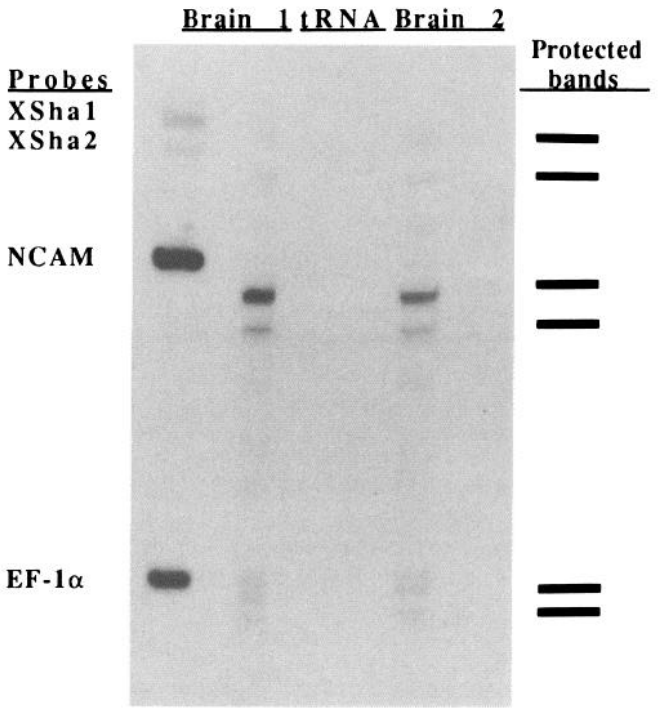
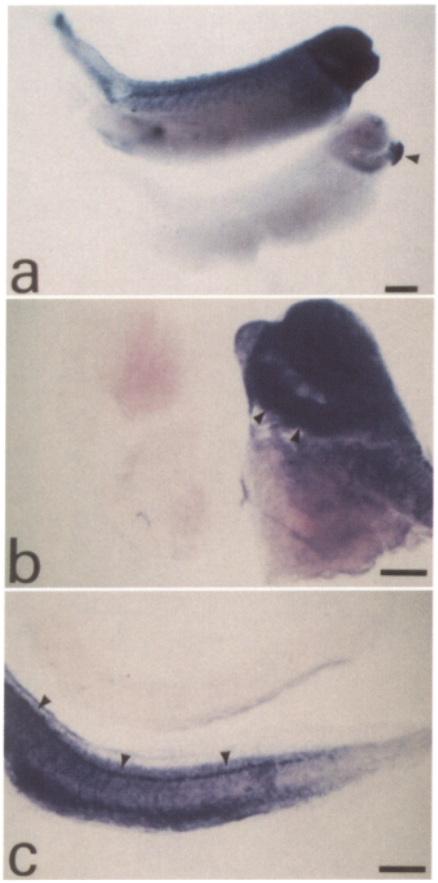


Figure 5. Relative levels of XSha1 and XSha2 mRNA in tadpole brain RNA. RNase protection assay indicates that XSha1 is expressed in the brain of 2–3-week-old embryos at levels comparable to that of XSha2. The results for two different brain RNA preparations are shown. A neural-specific N-CAM probe, an early marker of neural induction (Kintner and Melton, 1987), is used to assess the relative amounts of neural tissue in the samples. EF-1α protection is presented to indicate the relative amounts of total RNA that were incubated with the probes. The *tRNA lane* demonstrates that incubation with nonhybridizing tRNA does not protect the probes from degradation and that signals are due to true protection from digestion. Note that the protected bands run slightly faster than the full-length probe, since the probe contains vector sequences at its 3′ and 5′ ends that will not hybridize to the extracted RNA.

demonstrated by analysis of potassium currents in larval muscle of *ether a go-go* mutants (Zhong and Wu, 1991).

XSha1 is consistently detectable in 2 and 3 d embryos. XSha1 transcripts are not detected in younger embryos, although the method of *in situ* hybridization is sufficiently sensitive to detect low-abundance mRNAs, such as *En-2* (Harland, 1991; Hemmati-Brivanlou et al., 1991). At 2–3 d, several neural cells derived from the crest have reached their characteristic position and are beginning to undergo final differentiation (Sadaghiani and Thiebaud, 1987; Krotoski et al., 1988). For example, functional innervation of the head by trigeminal neurons is detectable in a 2 d embryo (stage 33; Davies et al., 1982); a potassium A current functions in Rohon-Beard neurons at this time (Ribera and Spitzer, 1990). Activation of ion channel genes may characterize terminal differentiation in these cells.

The previously cloned gene XSha2 showed highest identity to the mammalian $K_v1.2$ genes (MBK2, RBK2, RCK5; McKinnon, 1989; Stuhmer et al., 1989; Chandy et al., 1990). However, reconstruction of phylogenetic trees places it outside the mammalian group, and calls into question assignment of XSha2 as the *Xenopus* homolog of $K_v1.2$ (Strong et al., 1993). The finding that this second *Xenopus* gene, XSha1, is more similar to mammalian $K_v1.1$ genes rather than to XSha2 supports assignment of XSha1 and XSha2 as $K_v1.1$ and $K_v1.2$ of *Xenopus*,



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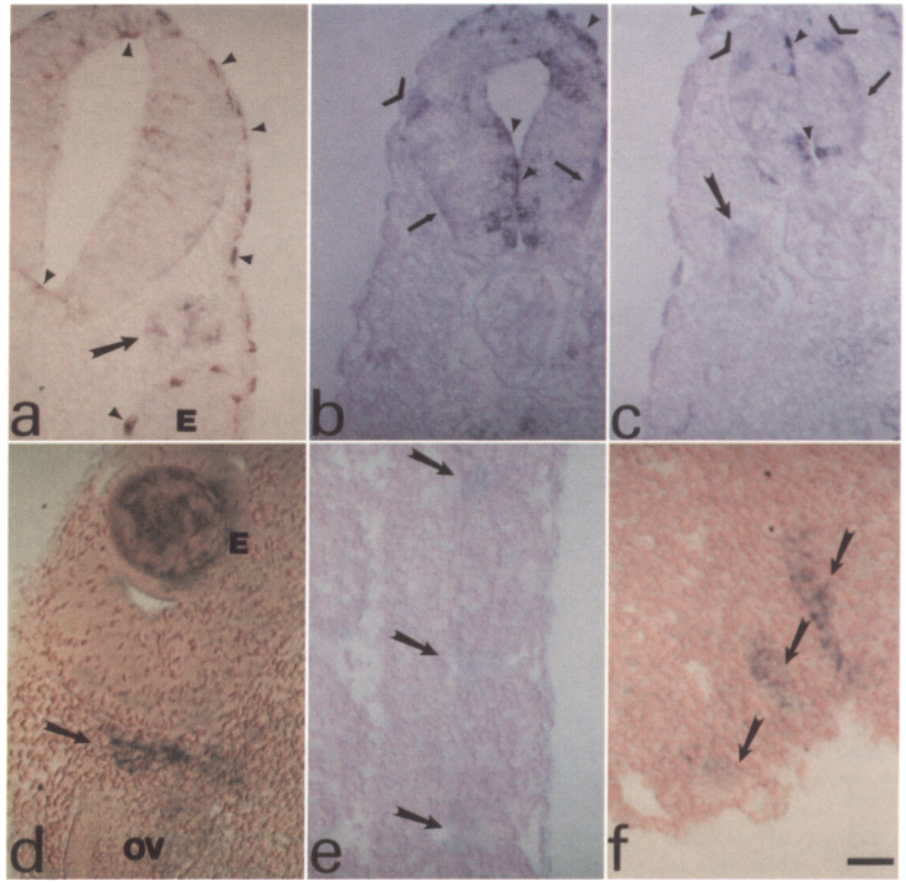

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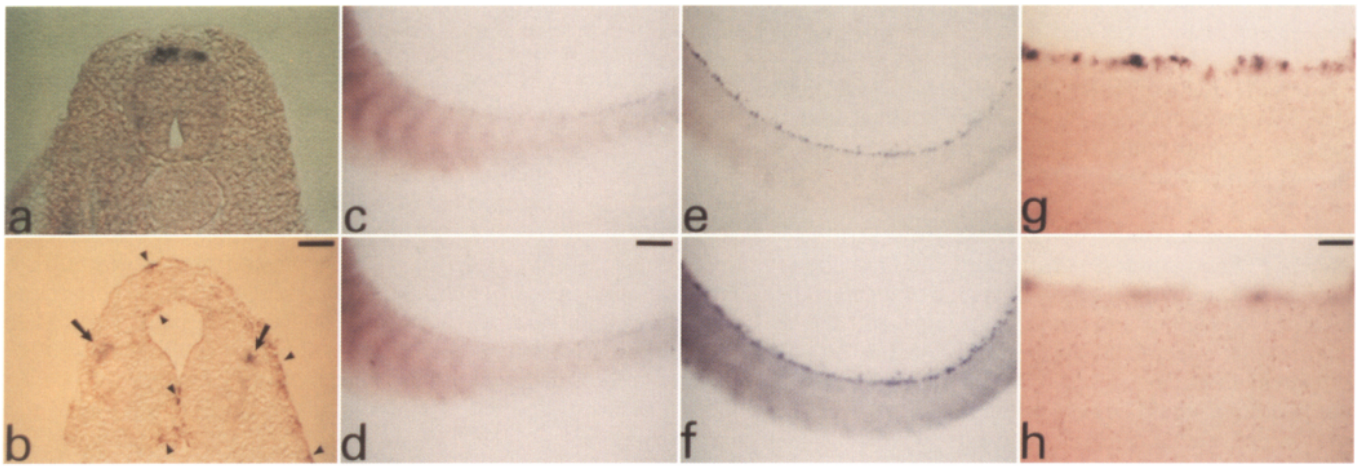

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Figure 6. XSha1 transcripts are detectable in young embryos by whole-mount *in situ* hybridization. Embryos were incubated as whole-mounts with sense control or antisense XSha1 probes. The RNA probes were labeled with digoxigenin (Boehringer-Mannheim), which permitted detection of hybridization with anti-digoxigenin antibodies coupled to alkaline phosphatase followed by reaction with the substrates NBT/BCIP to form the purple precipitate. *a,* XSha1 hybridization signal is localized to the nervous system. Three day embryos [Nieuwkoop and Faber (NF) stage 40] hybridized either to antisense XSha1 probe (*top*) or sense control probe (*bottom*) were processed similarly. Note the intense staining in the head, dorsal and ventrolateral aspects of the spinal cord. Occasionally in controls, the cement gland (*arrowhead*) showed background staining. However, in the control of *b,* the cement gland does not show background staining. This difference may be due to the time the embryos are in substrate solution (30 hr in *a* vs. 20 hr in *b* and *c*; see Materials and Methods). *b* and *c,* Slightly older embryos (NF stage 42) hybridized to antisense XSha1 probes (*b,* right, and *c,* bottom) or sense control probes (*b,* left, and *c,* top) and examined at higher power demonstrate further the specificity of the XSha1 signal. *b,* In the head, both the brain and the gill area (*arrowheads*) show intense staining. *c,* Along the length of the spinal cord, the dorsal aspect (*arrowheads*) contains XSha1 mRNA. Scale bars, *a,* 700 μm; *b* and *c,* 1 mm.

Figure 7. XSha1 is detected in several neural tissues, many of which contain neural crest derivatives. *a–c,* Transverse sections through pigmented embryos (NF stage 38/39) at the level of the forebrain (*a*), hindbrain (*b*), and spinal cord (*c*). The *brown-black staining in a–c* is due to pigment

respectively. However, the absence of a glycine, a proline, and an arginine residue at the beginning of the region associated with subunit assembly (Fig. 2) is a feature specific to the *Xenopus* peptides. Resolution of the evolutionary origins of these channels will be aided by a larger data set acquired as more *Xenopus* sequences are cloned. Preliminary examination of a more recent and larger data base by parsimony analysis does indeed indicate that XSha1 and XSha2 cluster with the mammalian $K_v1.1$ and $K_v1.2$ genes, respectively (G. Gutman, personal communication).

Analysis of several potassium channel mRNAs indicates that each shows a specific tissue distribution and developmental regulation (McKinnon, 1989; Beckh and Pongs, 1990; Swanson et al., 1990; Drewe et al., 1992; Hwang et al., 1992; Perney et al., 1992; Rudy et al., 1992; Tsaur et al., 1992). How does the pattern of mammalian $K_v1.1$ mRNA expression compare to that of XSha1? In rat, *in situ* hybridization studies demonstrate that $K_v1.1$ mRNA is limited to the nervous system, and that its levels are highest in the adult and not detected in the embryo, although $K_v1.3$ and $K_v1.4$ mRNAs are present (Beckh and Pongs, 1990). In the brain, $K_v1.1$ mRNA is found in several areas including the hippocampus, thalamus, cerebral cortex, and cerebellum (Tsaur et al., 1992). Its distribution overlaps with but is distinct from that of $K_v1.2$ mRNA. With respect to neural crest derivatives, all peripheral nervous tissues examined express $K_v1.1$ mRNA (Beckh and Pongs, 1990).

The roles of XSha1 remain to be further elucidated. The developmental program of excitability in Rohon-Beard cells is only partially accounted for by the properties of XSha1. The functional differentiation of potassium currents in trigeminal neurons, spinal ganglia, and Schwann cells has not yet been studied, and it is not possible to relate their expression of XSha1 mRNA to specific cellular properties. Several studies indicate that different voltage-dependent potassium channel gene subfamilies function independently within cells (Baker and Salkoff, 1990; Covarrubias et al., 1991; Furukawa et al., 1992). Further, within a cell transcripts have specific subcellular localizations (Sheng et al., 1992). Thus, molecular definition of the events directing functional differentiation of potassium currents requires the identification of other *Shaker*- as well as *Shab*-, *Shaw*-, and *Shal*-like genes (Butler et al., 1989) expressed in the embryonic *Xenopus* nervous system.

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(*arrowheads*), which is typically found in the skin, the lining of the spinal canal and brain ventricles, and the gut epithelia. *a*, The trigeminal ganglion (*arrow*) contains XSha1 mRNA. *b*, Rohon-Beard cognates (*carats*) and lateral tracts (*arrows*) contain XSha1 mRNA. *c*, In a 2 d embryo, the Rohon-Beard neurons (*carats*) have a slightly lateral position. Lateral tracts (*thin arrow*) in the spinal cord contain XSha1 RNA. A spinal ganglion (*thick arrow*) that is beginning to condense shows the XSha1 signal. *d–f*, Longitudinal sections at the level of the eye and otic vesicle (*d*), spinal cord and myotomes (*e*), and gill arches (*f*) of albino embryos (NF stage 40). *d*, The trigeminal ganglion (*arrow*) is positive for XSha1 as seen in a transverse section (*a*). *e*, The region surrounding the myotomal junctions has XSha1 staining. Anterior is toward the bottom. *f*, The gill (branchial) arches present the XSha1 signal. Anterior is toward the left. *E*, eye; *OV*, otic vesicle. Scale bar, 200 μm.

Figure 8. XSha1 mRNA is localized to Rohon-Beard cells in the spinal cord and their cognates in the hindbrain. *a*, XSha1 is found in Rohon-Beard neuron cell bodies that line the dorsal midline of the spinal cord of a stage 40 albino embryo. *b*, At the level of the hindbrain, Rohon-Beard like cells (*arrows*) show XSha1 mRNA in a stage 38 pigmented embryo. As in Figure 7, the pigment (*arrowheads*) appears *brown*. *c* and *d*, A lateral view of a stage 33 whole-mount albino embryo that was optically sectioned either at a midlevel (*c*) to focus on the Rohon-Beard cells, or at a more superficial level. *d*, Note that the repeated banding appears equally well in either plane of focus. *e* and *f*, A lateral view of a stage 42 whole-mount albino embryo that was optically sectioned either at a midlevel (*e*) to focus on the Rohon-Beard cells, or at a more superficial level (*f*). Note that the repeated banding appears faint at a midlevel (*e*), but is more clear at a superficial level (*f*). *g* and *h*, Similar to *e* and *f*, viewed at higher power. In *g*, note the Rohon-Beard cell bodies, whereas in *h*, note the superficial labeling that appears as repeating dorsoventral tracts. Scale bars: *a*, *b*, *g*, and *h*, 200 μm; *c–f*, 500 μm.

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