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Xenopus Spinal Neurons Express Kv2 Potassium Channel Transcripts during Embryonic Development

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Developmentally regulated delayed rectifier potassium currents determine the waveform of the action potential in all *Xenopus* embryonic primary spinal neurons. To examine this developmental program at the molecular level, we have isolated *Xenopus* Kv2 potassium channel genes Kv2.1 and Kv2.2. Both genes induce functional heterologous expression of delayed rectifier potassium currents. Transcripts from both Kv2 genes are present in developing embryos; however, only Kv2.2 mRNA is detectable in embryonic spinal neurons. Notably, Kv2.2 transcripts localize to ventral spinal neurons, whereas previously described Kv1.1 transcripts are found in dorsal spinal neurons. Thus, spinal neuron subtypes express distinct potassium channel genes, yet they temporally coordinate functional expression of delayed rectifier potassium currents.

Key words: potassium channels; gene expression; Xenopus *embryo; electrical excitability; neuronal differentiation; spinal neurons*

In the developing *Xenopus* nervous system, ion channel gene expression is amenable to both molecular and functional analyses. Previous biophysical studies of *Xenopus* embryonic spinal neurons have demonstrated that developmental regulation of the delayed rectifier potassium current (I_{Kv}) causes modulation of the action potential duration. I_{Kv} is initially a small, slowly activating current that matures to a larger, more rapidly activating one (O'Dowd et al., 1988) (for review, see Ribera and Spitzer, 1992).

Although much is known about differentiation of I_{Kv} at the functional level, the molecular mechanisms that synchronize development of I_{Kv} in motor, inter-, and sensory spinal neurons are unknown. Two lines of evidence are consistent with the notion that regulation of functional expression of I_{Kv} occurs at the trancriptional level. First, maturation of I_{Kv} requires new transcription during a critical period (Ribera and Spitzer, 1989). Second, increasing the levels of a delayed rectifier potassium channel mRNA suffices to drive premature maturation of I_{Kv}. The latter study suggests that neither the addition of associated subunits nor post-translational modifications are rate-limiting (Jones and Ribera, 1994).

Identification of potassium channel genes that are expressed in embryonic *Xenopus* spinal neurons is necessary for analysis of the molecular basis of the development of electrical excitability. Four different subfamilies of voltage-dependent potassium channel genes, *Shaker, Shab, Shaw,* and *Shal* (Kv1, Kv2, Kv3, Kv4; standard nomenclature of Chandy, 1991), have been identified in other systems. Previous work has demonstrated that members of the *Xenopus* Kv1 subfamily, Kv1.1 and Kv1.2, are expressed in cells of the embryonic nervous system. In particular, Kv1.1 transcripts localize specifically to embryonic sensory neurons (Ribera and Nguyen, 1993). The restricted expression of Kv1.1 mRNA, however, suggests that additional genes account for the endogenous delayed rectifier potassium current of *Xenopus* embryonic spinal neurons. Furthermore, the type of current carried by endogenous Kv1 channels is difficult to predict, because Kv1 α subunits may combine *in situ* with β subunits to form inactivating channels (Rettig et al., 1994).

This search for additional relevant potassium channel genes targets the Kv2 subfamily, because both invertebrate (Butler et al., 1989; Quattrocki et al., 1994) and vertebrate Kv2 genes (Frech et al., 1989; Pak et al., 1991; Hwang et al., 1992; Albrecht et al., 1993; Benndorf et al., 1994) encode delayed rectifier-type potassium currents in heterologous expression systems. Here we report the full-length sequences of two *Xenopus* Kv2 homologs, Kv2.1 and Kv2.2, their functional properties in *Xenopus* oocytes, and their patterns of expression in the developing *Xenopus* embryo. *In situ* hybridization demonstrates that Kv2.2 transcripts are restricted to the ventral portion of the spinal cord, whereas the previously reported Kv1.1 transcripts localize to the dorsal spinal cord (Ribera and Nguyen, 1993). Accordingly, Kv2.2 and Kv1.1 mRNAs would contribute to I_{Kv} in distinct subsets of embryonic spinal neurons. These results suggest that diverse potassium channel genes temporally coordinate functional expression across different neuronal subtypes during embryonic development.

MATERIALS AND METHODS

Animals. Embryos were produced by adult *Xenopus* breeding pairs or *in vitro* fertilization (Moon and Christian, 1989) and staged according to Nieuwkoop and Faber (1967).

Isolation of Xenopus *Kv2 cDNAs, DNA sequencing, and analysis.* Tadpole brain mRNA was used for reverse transcription-PCR (RT-PCR). Degenerate oligonucleotides (designed by Tim Jegla and Dr. Larry Salkoff, Department of Neurobiology and Anatomy, Washington University, St. Louis, MO) were used to generate PCR products; sequencing confirmed their identity as *Shab* cDNAs. ^{32}P-labeled (DuPont NEN,

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Boston, MA) random-primed probes (Prime-It Kit, Stratagene, La Jolla, CA) were generated from the PCR clones (pXb3 and pXb4) for screening at high stringency of a *Xenopus* tadpole brain λUniZapII cDNA library, as described previously (Ribera, 1990). The isolated clones were sequenced over both strands using the Sanger dideoxy method (Sanger et al., 1977) in the presence of Sequenase 2.0 enzyme (United States Biochemical, Cleveland, OH), ^{35}S-labeled dATP (DuPont NEN), and internal primers that generated overlapping sequences. DNA sequences were read and analyzed using Gel Reader (CBS Scientific, Del Mar, CA) and DNASTAR software (Madison, WI).

Oocyte recording. The entire coding regions of *Xenopus* Kv2.1 and Kv2.2 were subcloned into pGEMHE (provided by Dr. E. Liman, Department of Neurobiology, Harvard University, Cambridge, MA), which flanks the polylinker with *Xenopus* β-globin 5′- and 3′-untranslated regions (UTRs) to provide high expression from *in vitro*-transcribed RNAs (Liman et al., 1992). Kv2.1 was digested with *Sma*I and *Eco*RV to remove 279 bases of 3′-UTR and ligated to *Sma*I-digested pGEMHE to generate the pGHEX9 subclone. Kv2.2 was digested with *Xho*I to remove 408 bp of 5′-UTR, blunt-ended with Klenow, digested with *Pst*I to remove ~2 kb of 3′-UTR, and then directionally cloned into pGEMHE that had been predigested with *Sma*I and *Pst*I to generate the pGHEX12-2 subclone. Although digestion with *Pst*I removed the 3′-UTR of β-globin in pGEMHE, high expression was obtained from pGHEX12-2. For RNA synthesis, pGHEX9 and pGHEX12-2 were linearized with *Sph*I and *Nhe*I, respectively. Capped sense RNA was generated by *in vitro* transcription with T7 RNA polymerase (Promega, Madison, WI) in the presence of rNTPs (Pharmacia, Piscataway, NJ) and Cap analog (Boehringer Mannheim, Indianapolis, IN). A negative control consisted of an N-terminal truncation mutant of Kv2.2 that lacked the first 140 amino acids. Injection of RNA generated from this construct did not lead to the induction of currents that differed from endogenous background ones. Oocytes were removed, defolliculated, and injected as described previously (Ribera and Nguyen, 1993). Standard two-electrode voltage-clamp techniques were used; voltage protocols and data analysis were accomplished with the pCLAMP suite of programs (Axon Instruments, Foster City, CA). Currents were sampled at 200 μsec, and the leak and capacitative transient currents were subtracted using the P/4 protocol of the Clampex program (pCLAMP). The electrode solution consisted of 3 M KCl and 10 mM HEPES, pH 7.4. Electrode resistances ranged between 0.1 and 0.5 MΩ. Oocytes were not used if the holding current exceeded −200 nA at a potential of −80 mV. Recordings were generally carried out 1–3 d after injection. To examine the potassium dependence of Kv2.1 or Kv2.2 currents, the bath consisted of Barth's solution [88 mM NaCl, 1 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] containing 1, 3, 5, 10, or 20 mM KCl. For calculation of the potassium equilibrium potential, internal potassium was assumed to be 100 mM. Tail currents were measured after the capacitative transients settled, as assessed from recordings that were not leak-subtracted. Tetraethylammonium (TEA; Aldrich, Milwaukee, WI) and 4-aminopyridine (4-AP; Research Biochemicals, Natick, MA) sensitivities were examined by addition of the drugs to the recording solution.

RNA isolation and RNase protection. Total cellular mRNA was isolated from whole embryos by homogenization in proteinase K buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 30 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K), as described previously (Ribera, 1990). Antisense cRNAs corresponding to the 3′-UTRs of *Xenopus* Kv2.1 or Kv2.2 were synthesized *in vitro* using [^{32}P]uridine triphosphate (UTP; DuPont NEN) in the presence of T7 RNA polymerase (Promega). Kv2.1 DNA was linearized with *Eco*RV to generate a 300 bp probe, and Kv2.2 DNA was linearized with *Sst*I to generate a 450 bp probe. The different sizes of the two probes permitted simultaneous use in RNase protection assays. Antisense [^{32}P]cRNA probes were hybridized to 10 μg of mRNA extracted from embryos of different developmental stages. Hybridization was carried out as described previously (Ribera, 1990). A 100 bp elongation factor 1-α (EF-1α) cRNA probe (a constitutively, ubiquitously expressed gene) (Krieg et al., 1989) was used to control for the amount of cellular RNA, and a 200 bp neural-specific neural-cell adhesion molecule (N-CAM) probe (long cytoplasmic domain form) (Kintner and Melton, 1987) (provided by Dr. Chris Kintner, Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, CA) was used to control for the amount of neural tissue in samples. Densitometry analysis was performed by exposure of the gel to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) and quantitated using Image Quant Software v.3.0 (Molecular Dynamics).

Whole-mount in situ *hybridization.* The nonradioactive detection method (Harland, 1991; Ferreiro et al., 1992) was followed, with minor modifications described below. cRNA probes were synthesized in the presence of digoxigenin-labeled UTP (Boehringer Mannheim). Antisense probes corresponding to the 3′-UTR, the entire sequence of *Xenopus* Kv2.2 with or without the 3′UTR, or the pXb3 PCR product were generated, and identical results were obtained. Albino embryos were fixed in 4% *p*-formaldehyde, 0.1 M 3-(*N*-morpholino)propanesulfonic acid, pH 7.4, 1 mM $MgSO_4$, and 2 mM EGTA at 4°C overnight. Hybridization was carried out for 16 hr at 60°C. Incubation with alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) was carried out in the presence of maleic acid buffer (MAB) [100 mM maleic acid (Sigma, St. Louis, MO) and 150 mM NaCl, pH 7.5], 2% Boehringer Mannheim blocking reagent, and 20% heat-inactivated lamb serum (Gibco, Gaithersburg, MD). Washes were carried out in MAB for 24 hr with a minimum of five changes. The final wash was carried out overnight at 4°C. The alkaline phosphatase reaction product was developed in the presence of alkaline phosphatase-precipitating substrate-purple (Boehringer Mannheim) for at least 12 hr. Whole-mount embryos were either cleared in Murray's solution (2:1 benzyl benzoate/benzyl alcohol) or embedded in plastic (JB-4 embedding kit; Polysciences, Warrington, PA). Thirty micrometer sections were counterstained with methyl green (Fisher Scientific, Pittsburgh, PA) and photographed with Kodak Ektachrome 160T film (Rochester, NY).

RESULTS

Identification of two *Xenopus* Kv2 homologs

RT-PCR analyses indicate that *Xenopus* embryos express Kv2 genes. Two different 306 bp PCR products (pXb3 and pXb4) are generated (Fig. 1, *underlined*) using degenerate Kv2-specific PCR primers (designed by Tim Jegla and Dr. Larry Salkoff, Washington University) that span the region between the third and fifth putative transmembrane domains. Screening of a *Xenopus* tadpole brain cDNA library with pXb3 and pXb4 probes identifies two clones, initially called XShab9 and XShab12, which contain full-length coding sequences. XShab9 has a 3.2 kb insert that includes 2628 bp of coding region (Fig. 1*A*), 153 bp of 5′-UTR, and 428 bp of 3′-UTR. XShab12 is a 5.4 kb clone that contains 2694 bp of coding region (Fig. 1*B*), 588 bp of 5′UTR, and ~2 kb of 3′-UTR. Both XShab9 and XShab12 contain polyA$^+$ tracts.

Comparison of the nucleotide sequences of XShab9, XShab12, pXb3, and pXb4 over the region of overlap indicates that XShab9 and XShab12 are 75% identical at the nucleotide level. The nucleotide sequence of pXb4 is 99 and 76% identical to that of XShab9 and XShab12, respectively, suggesting that pXb4 is related to XShab9. The two nucleotide differences between XShab9 and pXb4 occur at the third position of a codon and do not cause amino acid changes (Fig. 1*A*). pXb3 is 76 and 97% identical to XShab9 and XShab12, respectively, indicating that pXb3 is related to XShab12. Eight of the nine nucleotide differences between pXb3 and XShab12 occur at the third position of a codon, yielding only one amino acid change (Fig. 1*B*). Because most of the nucleotide differences between the PCR products and the library cDNAs conserve the amino acid sequence, it is unlikely that the PCR variants are attributable to random errors introduced by reverse transcriptase or *Taq* polymerase. It is more likely that the differences reflect allelic variants, alternative splice variants, or the presence of additional *Shab* genes. In summary, these data suggest that at least two different Kv2 isoforms, corresponding to XShab9 and XShab12, are present in *Xenopus* embryos.

Sequence analysis and structure of *Xenopus* Kv2 genes

Kv2 genes from several species share highest amino acid identity in the S1–S6 putative transmembrane domain, followed by the

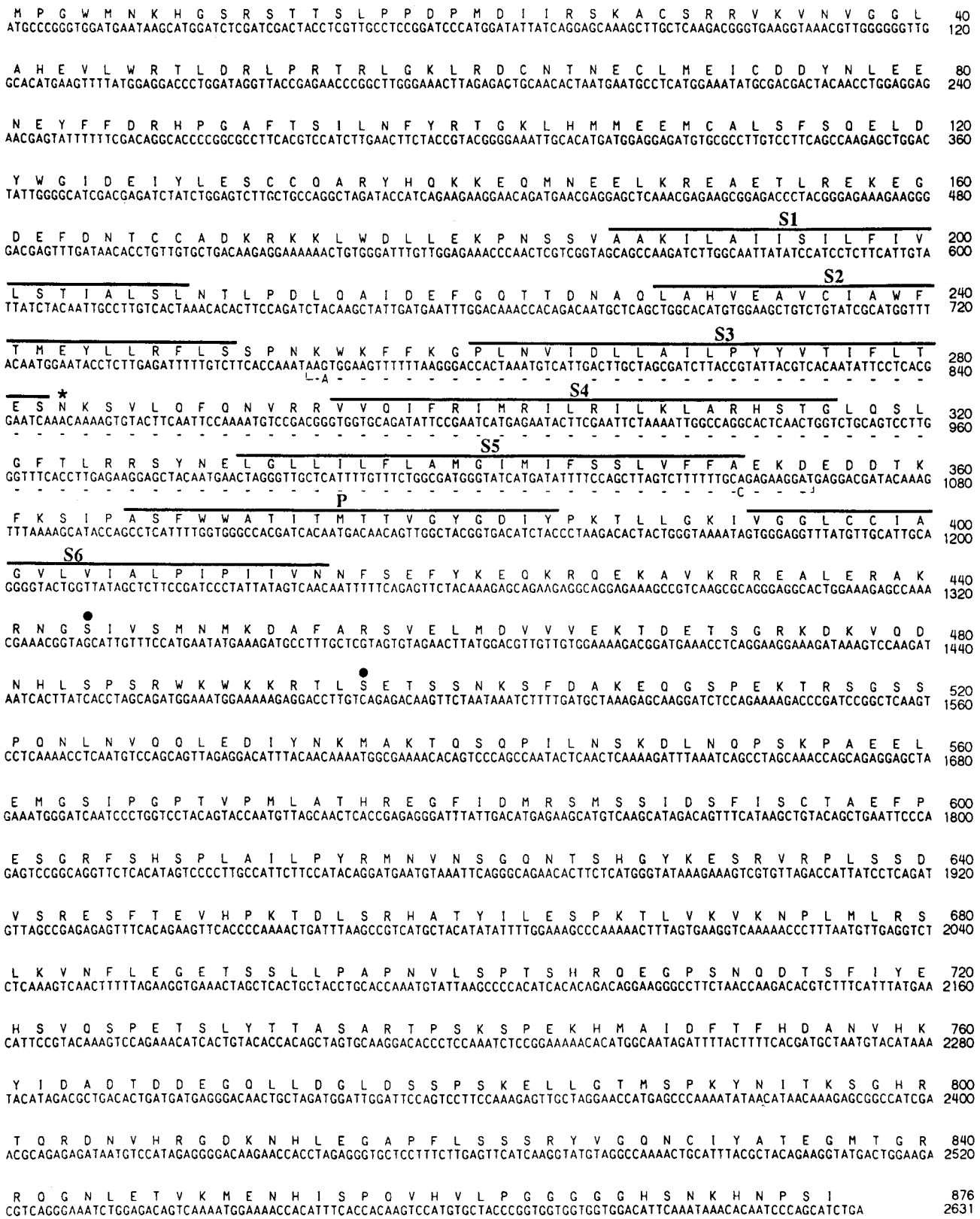
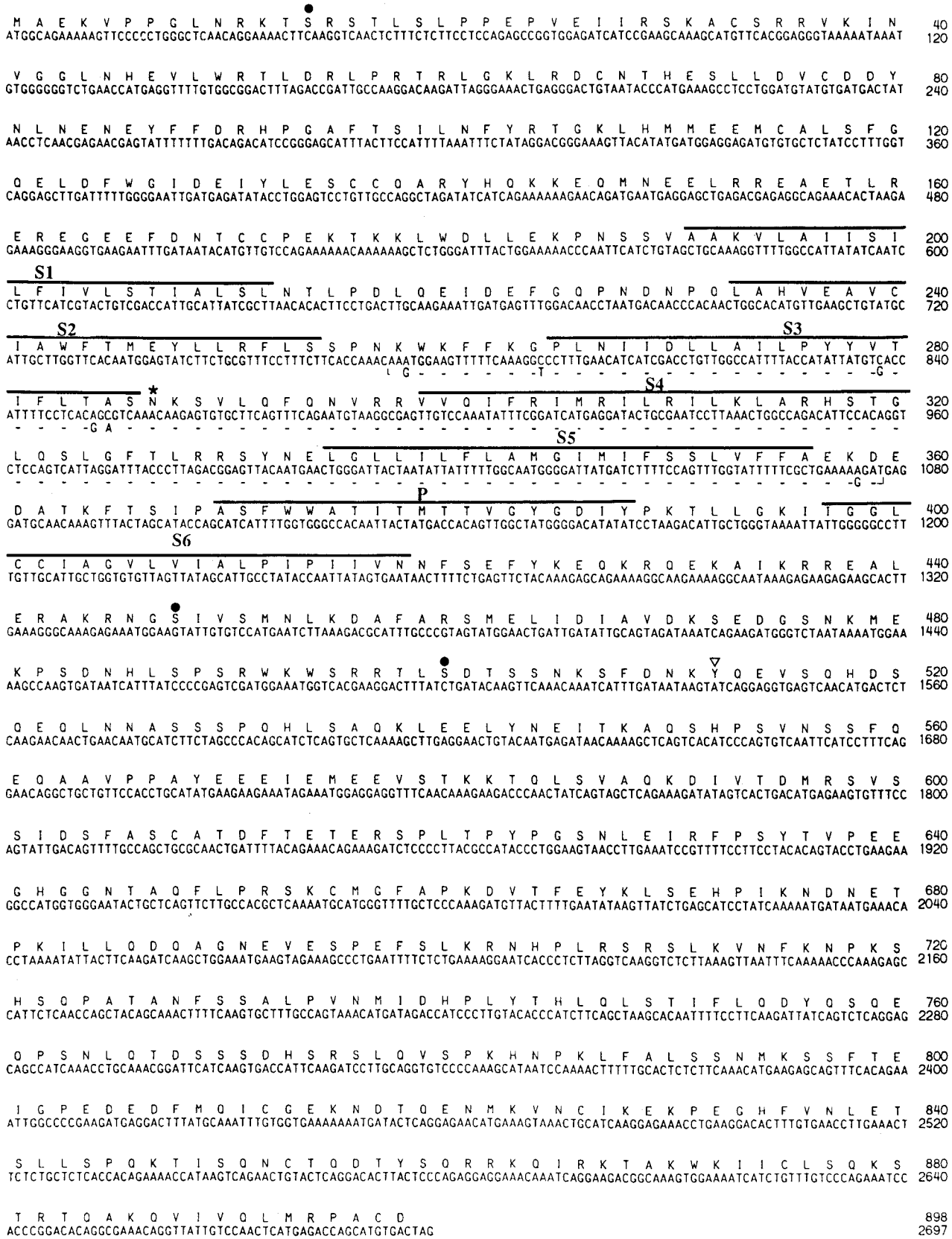
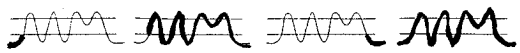


Figure 1. Nucleotide and deduced amino acid sequences of the coding regions of *Xenopus* Kv2.1 and Kv2.2 cDNAs. Amino acid and nucleotide positions are indicated in the *right-hand column.* The putative transmembrane domains (*S1–S6*) and the pore region (*P*) are *overlined.* A putative glycosylation site between *S3* and *S4* is indicated by a *star.* Consensus cAMP phosphorylation sites are indicated by *filled circles* above the amino acid position. The *Xenopus* Kv2.1 and Kv2.2 nucleotide sequences have been assigned Genbank accession numbers U20342 and U20343, respectively. *A*, Nucleotide and amino acid sequence of XShab9 (*Xenopus* Kv2.1). *Dashes* appear under the region spanned by the pXb4 PCR product. pXb4 nucleotide sequences that differ from Kv2.1 sites are shown below the corresponding Kv2.1 nucleotides (nucleotides 762 and 1056). *B*, Nucleotide and amino acid sequence of XShab12



(*Xenopus* Kv2.2). *Dashes* appear under the region spanned by the pXb3 PCR product. pXb3 nucleotide sequences that differ from Kv2.2 are shown underneath the corresponding Kv2.2 nucleotides. Except for one nucleotide change at position 854, all nucleotide changes occur in the third codon position. A tyrosine phosphorylation site is marked by a *triangle*. Although two of three cAMP phosphorylation sites are conserved between Kv2.1 and Kv2.2, the putative tyrosine phosphorylation site is unique to Kv2.2 (amino acid 512)

Table 1. Percent amino acid identities were calculated by alignment with known mammalian Kv2 genes.

A. Region of Kv2 gene under comparison: 

% IDENTITY	amino end	S1-S6 domain	carboxy end	overall
B. Compared to Xenopus Kv2.1				
mouse Kv2.1	89	97	54	73
rat Kv2.1	89	97	54	73
human Kv2.1	89	97	57	74
rat Kv2.2	86	96	33	63
Xenopus Kv2.2	86	96	29	58
C. Compared to Xenopus Kv2.2				
mouse Kv2.1	87	94	31	59
rat Kv2.1	87	94	32	60
human Kv2.1	87	94	32	61
rat Kv2.2	95	96	50	74
Xenopus Kv2.1	86	96	29	58

A, *Xenopus* Kv2.1 shows highest identity to previously cloned Kv2.1 genes in the transmembrane core and N-terminal domains. The greatest divergence is noted in the carboxy terminal domain. B, *Xenopus* Kv2.2 shows highest identity to previously cloned Kv2.2 genes in the transmembrane core and N-terminal domains. The greatest divergence is noted in the carboxy terminal domain.

N-terminal domain; the C-terminal domains display the highest degree of sequence divergence. At the amino acid level, XShab9 is 96% identical to XShab12 over the S1–S6 region, 86% identical over the N-terminal region, and only 29% identical over the C-terminal region (Table 1). Overall, XShab9 and XShab12 are 58% identical to each other, whereas they show only 30% identity with *Xenopus* Kv1 potassium channel genes (Ribera, 1990; Ribera and Nguyen, 1993).

Two Kv2 genes, Kv2.1 (Frech et al., 1989; Pak et al., 1991; Albrecht et al., 1993) and Kv2.2 (Hwang et al., 1992), have been identified thus far in mammals. Based on amino acid identity comparisons (Table 1*A,B*) and following standard nomenclature (Chandy, 1991), XShab9 and XShab12 are *Xenopus* Kv2.1 and Kv2.2, respectively.

Xenopus Kv2.1 encodes a protein with 876 amino acids, which has a predicted molecular weight of 96 kDa (Fig. 1*A*). *Xenopus* Kv2.2 encodes an 898-amino-acid protein with a predicted molecular weight of 99 kDa (Fig. 1*B*). A single putative N-glycosylation site (Asn-X-Thr/Ser; Kornfeld and Kornfeld, 1985), located in the S3–S4 putative extracellular loop, is present in *Xenopus* Kv2.1 and Kv2.2 as well as in all previously identified Kv2 genes (Fig. 1*A,B*) (Frech et al., 1989; Pak et al., 1991; Hwang et al., 1992; Albrecht et al., 1993; Quattrocki et al., 1994). Although Western analyses suggest that post-translational modifications increase the M_r of rat brain Kv2.1 polypeptides, similar analysis of rat brain Kv2.2 polypeptides revealed sharp bands of an M_r (97 kDa) that is close to the weight predicted by the primary sequence (95 kDa) (Trimmer, 1991; Hwang et al., 1992; Shi et al., 1994).

Given the known functional regulation of the endogenous delayed rectifier current achieved by stimulation of protein kinase C (Desarmenien and Spitzer, 1991), the presence of consensus phosphorylation motifs (Kemp and Pearson, 1990) is of interest. In the N- and C-terminal cytoplasmic domains, *Xenopus* Kv2.1 contains 17 putative protein kinase C phosphorylation sites, and Kv2.2 contains 20 sites. *Xenopus* Kv2.1 and Kv2.2 both have cAMP-dependent phosphorylation consensus sequences that are conserved in other vertebrate Kv2 genes (Frech et al., 1989; Hwang et al., 1992; Albrecht et al., 1993). *Xenopus* Kv2.1 has two conserved cAMP phosphorylation sites in the C-terminal domain (Fig. 1*A*). *Xenopus* Kv2.2 has these two cAMP consensus sites in the C-terminal domain, as well as a third site in the N-terminal domain (Fig. 1*B*). This additional N-terminal site is also present in rat Kv2.2 (Hwang et al., 1992). Tyrosine phosphorylation may also affect Kv2.2 function, given a consensus site that is conserved in *Xenopus* and rat Kv2.2 genes but absent in all Kv2.1 genes (Fig. 1*B*). Based on these observations, *Xenopus* Kv2.1 and Kv2.2 polypeptides are potential substrates for several different kinases known to affect potassium channel activity.

Functional expression of *Xenopus* Kv2.1 and Kv2.2 in oocytes

Based on sequence homology to Kv2 genes, *Xenopus* Kv2.1 and Kv2.2 are predicted to encode potassium channels with delayed rectifier properties. Injection of mRNAs corresponding to *Xenopus* Kv2.1 and Kv2.2 into oocytes leads to functional expression of sustained voltage-dependent potassium currents (Fig. 2*A,B*), which are similar to voltage-dependent currents induced in heterologous expression systems by other Kv2 genes (Frech et al., 1989; Pak et al., 1991; Hwang et al., 1992; Albrecht et al., 1993; Benndorf et al., 1994). Activation of the current occurs at relatively positive membrane potentials. The thresholds of activation for *Xenopus* Kv2.1 and Kv2.2 currents are −15 and 0 mV. Under conditions of two-electrode voltage clamp in the oocyte, *Xenopus* Kv2.1 currents activate more rapidly than do Kv2.2 currents (Fig. 2*C*).

Xenopus Kv2.1 channels are potassium-selective as determined by examination of the reversal potential of tail currents recorded in the presence of various external K^+ concentrations (Fig. 2*D*). For Kv2.1-induced currents recorded in the presence of 1 mM external K^+, the potassium tail current is outward at potentials more positive than −100 mV, as expected from the Nernst equilibrium potential for K^+ (−116 mV). The reversal potential with 10 mM external K^+ is −62.6 ± 2 mV (n = 3) and −38 ± 2 mV (n = 3) with 23 mM external K^+, which approximate the theoretical Nernst values of −58 and −37 mV. *Xenopus* Kv2.2 mRNA-induced currents show similar potassium selectivity (n = 2; data not shown).

A characteristic of mammalian Kv2 currents is their sensitivity to the potassium channel blocker TEA (Frech et al., 1989; Pak et al., 1991; Hwang et al., 1992; Albrecht et al., 1993). *Xenopus* Kv2.1 and Kv2.2 currents are less sensitive to TEA (Fig. 2*E*) than their mammalian homologs: 25 and 40 mM TEA are required to block 50% of *Xenopus* Kv2.2 and Kv2.1 currents, respectively, whereas 5–10 mM TEA inhibits half of mammalian Kv2 currents. Both *Xenopus* currents are relatively insensitive to 4-AP; <20% of either current is blocked by 50 mM 4-AP (data not shown).

The steady-state activation properties of *Xenopus* Kv2.1 and Kv2.2 currents resemble those reported for other Kv2.1 currents

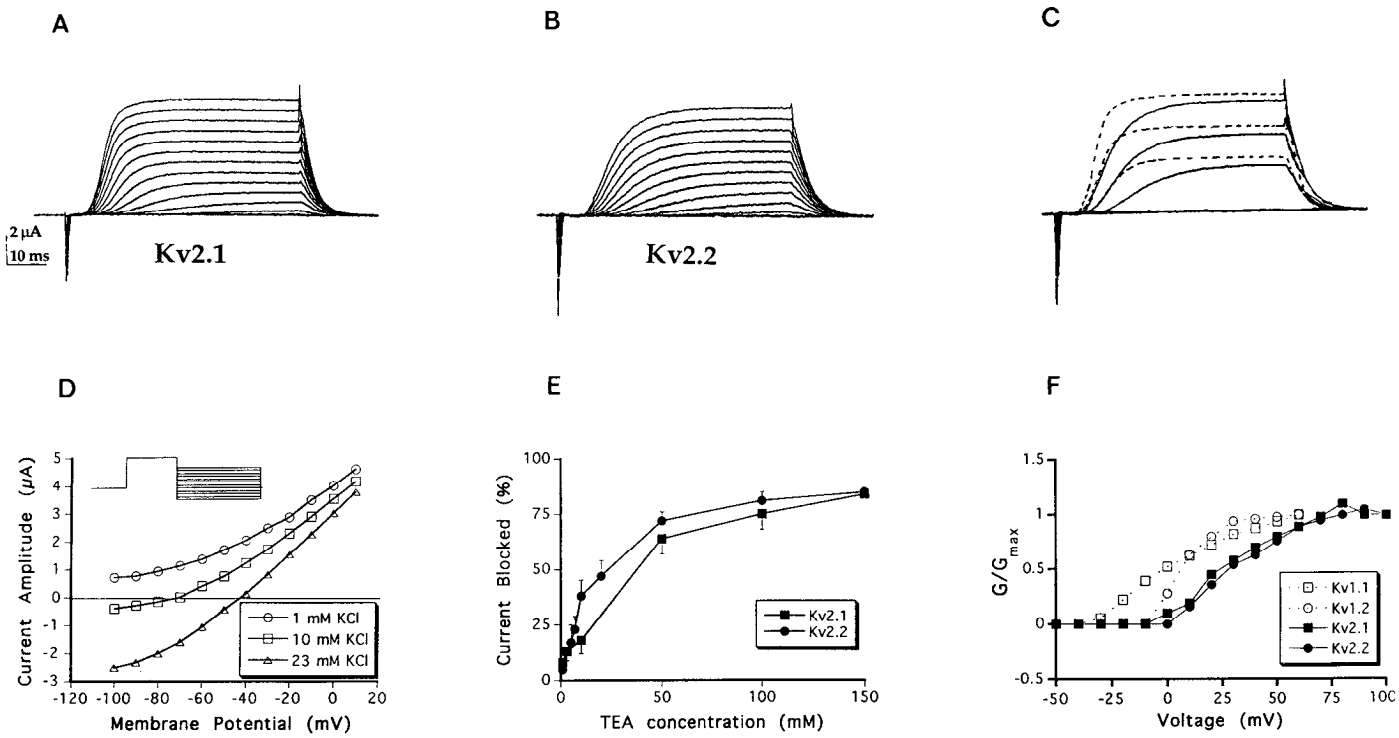


Figure 2. *Xenopus* Kv2.1 and Kv2.2 mRNAs induce expression of delayed rectifier-type potassium currents in *Xenopus* oocytes. For *A–C*, currents were generated in response to 60 msec voltage steps to potentials ranging from −50 to +100 mV from a holding potential of −80 mV; leak-subtracted currents are shown (see Materials and Methods). *A*, Kv2.1 mRNA induces expression of delayed rectifier-type potassium currents in *Xenopus* oocytes. The average current size at +20 mV is 1.1 ± 0.6 μA (mean ± SD; $n = 7$) for 1.7 ng of injected cRNA. *B*, Kv2.2 mRNA-induced current is sustained for the duration of the depolarizing pulse. The average current size at +20 mV is 1.3 ± 0.3 μA (mean ± SD; $n = 6$) for 1 ng of injected cRNA. *C*, Comparison of the kinetics of activation of Kv2.1 and Kv2.2 mRNA-induced currents. Current traces from *A* and *B* were superimposed for comparison. The Kv2.1 mRNA-induced current (*dotted line*) rises faster than the Kv2.2 mRNA-induced current (*solid line*). *D*, Tail current analysis indicates the Kv2.1 mRNA-induced channels are potassium-selective. Currents were activated with prepulses of 100 msec duration to +25 mV, then stepped to different hyperpolarizing pulses ranging from −100 to 10 mV (*inset*). Varying the external K^+ concentration shifts the reversal potential as predicted by the Nernst equation for a K^+-selective channel. Results from a representative experiment are shown, and average results are indicated in the text. *E*, Sensitivity of Kv2.1 and Kv2.2 currents to TEA. Current amplitude was measured in the presence of variable concentrations of TEA. Current amplitude was plotted against TEA concentration. Current values ± SEM were taken from voltage pulses to +20 mV. *F*, Normalized conductance versus voltage relationships for *Xenopus* Kv2.1, Kv2.2, Kv1.1, and Kv1.2 mRNA-induced currents. The conductance was calculated for a given voltage command (V) and its corresponding steady-state current response (I) from the formula $G = I/(V - E_K)$, where the equilibrium potential for K^+ (E_K) is −116 mV. Half-maximal activation ($V_{1/2}$) is achieved at values of +25.0 ± 0.1 mV (mean ± SD; $n = 7$) for Kv2.1 currents and at +30.0 ± 5.5 mV (mean ± SD; $n = 3$) for Kv2.2 currents. Kv1 currents activate and achieve G_{max} at less positive membrane potentials than do the Kv2 currents. Data from representative recordings were used to generate the graphs. Single Boltzmann distributions fit Kv1.1 and Kv1.2 data well (Jones and Ribera, 1994) (A. Ribera, unpublished observations), whereas Kv2.1 and Kv2.2 data deviate consistently from the Boltzmann isotherm (data not shown). This type of behavior has been observed previously in human Kv2.1 channels expressed in oocytes (Benndorf et al., 1994).

(Frech et al., 1989; Pak et al., 1991; Albrecht et al., 1993; Benndorf et al., 1994; Quattrocki et al., 1994) but contrast with those of two *Xenopus* Kv1 channels (Ribera, 1990; Ribera and Nguyen, 1993). Both the $V_{1/2}$ (25–30 mV) and G_{max} (90–100 mV) of *Xenopus* Kv2.1 and Kv2.2 currents (Fig. 2*F*) are more depolarized than those of *Xenopus* Kv1.1 and Kv1.2 currents. Previous studies have shown that endogenous potassium currents recorded from mature individual primary spinal neurons reveal subtle differences (Ribera and Spitzer, 1989; Jones and Ribera, 1994) (A. Ribera, unpublished observations). The properties of some mature neurons in culture resemble those of Kv1.1 homomultimers expressed in the oocyte; for example, the voltage threshold of activation has a hyperpolarized value of −35 mV and G_{max} plateaus at potentials of +30 to +40 mV. In some neurons, however, G_{max} is achieved more positive to +50 mV, as is found for Kv2 currents expressed in oocytes. Unfortunately, it is not presently possible to determine the identity (e.g., motor vs sensory) of a neuron in culture and, therefore, it is not known whether the minor differences observed are attributable to cell type.

Kv2.1 and Kv2.2 mRNAs are expressed during embryonic development

RNase protection assays reveal the temporal pattern of expression of *Xenopus* Kv2 transcripts during embryogenesis. mRNA isolated from embryos at various developmental stages was hybridized simultaneously to antisense cRNA probes for *Xenopus* Kv2.1, Kv2.2, EF-1α, and N-CAM. The latter two probes monitor relative amounts of RNA and neural tissue (Kintner and Melton, 1987; Krieg et al., 1989).

Kv2.1 and Kv2.2 transcript expression is differentially regulated during development (Fig. 3). Kv2.1 transcripts are not detectable until stage 22 (1 d). mRNA levels increase 2.5-fold between stages 22 and 35 (2 d). A threefold increase occurs between stages 41 (3 d) and 45 (4 d). In contrast, Kv2.2 mRNA is detected as early as the one-cell embryo (depending on the identity of the frog serving as a source of eggs). Zygotic transcription does not begin until the midblastula transition (stage 8, 6 hr after fertilization) (Newport

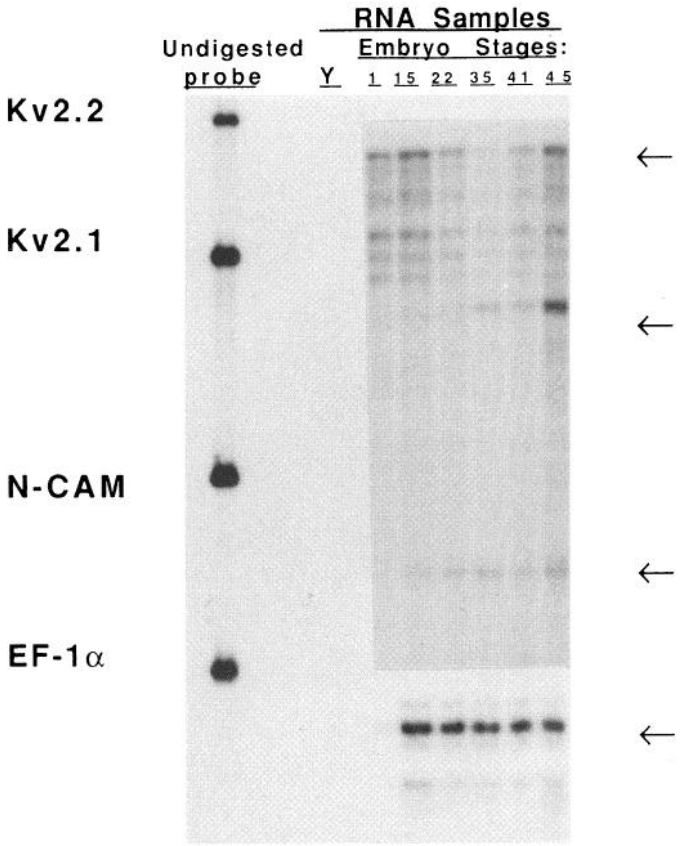


Figure 3. *Xenopus* Kv2.1 and Kv2.2 mRNA levels are differentially regulated during embryonic development. RNA extracted from embryos of the indicated stages was hybridized simultaneously to *Kv2.1*, *Kv2.2*, *N-CAM*, and *EF-1α* antisense probes. After RNase treatment, the hybridized products were run on an 8% acrylamide gel. Kv2.1 transcripts are first detected by stage 22 (1 d, early tail bud), and the levels gradually increase at subsequent times. Kv2.2 mRNA is present in the fertilized egg, and its levels change during development. N-CAM signal is initially detected in the stage 15 embryo (neural plate stage). EF-1α mRNA increases dramatically between stages 1 and 15, reflecting the fact that zygotic transcription initiates during this interval (Krieg et al., 1989). The relatively constant levels of EF-1α after stage 15 indicate that equal amounts of total mRNA were hybridized to the cRNA probes. Yeast RNA (*Y*, control lane) was used to control for nonspecific and self-hybridization of the cRNA probes. Undigested probes contain ~50 nucleotides that correspond to vector sequences and thus run slightly slower than the protected bands; the positions of protected bands are indicated by *arrows* at *right*. EF-1α-protected bands were visible after an overnight exposure to x-ray film. N-CAM-, Kv2.1-, and Kv2.2-protected bands were visible after a 15 d exposure. The probes for Kv2.1 and Kv2.2 had different specific activities than the EF-1α and N-CAM probes did. Thus, it was not possible to estimate the relative abundance of Kv2.1 or Kv2.2 transcripts with respect to N-CAM or EF-1α mRNA during development.

and Kirschner, 1982), indicating that only maternal transcripts are present at this early stage. Kv2.2 mRNA levels remain relatively constant during the first day of development (stages 1–22). A transient decrease in transcript expression is observed at stage 35, which coincides with the increase in expression of Kv2.1 mRNA. By stages 41–45, Kv2.2 transcript levels augment.

Kv2.2 transcript expression is restricted to the nervous system and somites

Whole-mount *in situ* hybridization demonstrates that *Xenopus* Kv2.2 transcripts are present in primary neurons and embryonic myocytes during differentiation of electrical excitability. Surprisingly, Kv2.1 transcripts are not observed with this method, although RNase protection indicates that they are present in embryos of similar developmental stage (Fig. 3). One explanation for this apparent discrepancy is that Kv2.1 transcripts are found throughout the embryo but at very low levels.

Kv2.2 mRNA is consistently detected during neural induction, which initiates in the dorsal ectoderm with the involution of the dorsal lip of the blastopore (stage 10, 9 hr after fertilization). At this time, Kv2.2 mRNA localizes to the dorsal ectoderm (Fig. 4*A*). During late gastrulation (stage 12, 13 hr), transcripts are present in presumptive neural tissue (Fig. 4*B*). After neural tube closure (stage 19, 21 hr), the hybridization signal appears throughout the neural tube (Fig. 4*C*). Therefore, during the first day of development, Kv2.2 transcripts localize to presumptive and differentiating neural tissue (Fig. 4*A–C*).

During the second day of embryonic life, Kv2.2 transcripts continue to reveal a developmentally regulated pattern of expression. In the early tail bud embryo (stage 23), transcripts are detected in the brain and spinal cord and around the nuclei of somites in the rostral to midtrunk portion of the embryo (Fig. 4*D*). Six hours later (stage 26), the signal in the somites is visible more caudally (Fig. 4*E*), which parallels the rostrocaudal gradient of somite segregation in the developing embryo (Nieuwkoop and Faber, 1967). In the 2 d embryo (stage 35), the signal is faintly visible in the brain and spinal cord but stronger in the midsomite regions (Fig. 4*F*).

Histological sections identify the specific cellular localizations of Kv2.2 mRNA. In cross-sections, Kv2.2 mRNA is observed in the ventrolateral region of the spinal cord where the cell bodies of primary motor neurons are located (Fig. 5*A,C,F*); sagittal sections confirm this ventral localization (Fig. 5*E*). Kv2.2 expression in the spinal cord maintains this characteristic ventral localization at the three stages examined (stages 23, 26, and 35; Fig. 5*A,C,E,F*). In sagittal sections, the staining along the ventral spinal cord occurs in a patchy pattern at stage 26 (Fig. 5*E*); by stage 35, the staining in the spinal cord is very faint (Fig. 5*F*), as observed at the whole-mount level (Fig. 4). In addition to its detection in the nervous system, Kv2.2 mRNA is also found in somites (Fig. 5*A,C,D*). Kv2.2 transcripts concentrate around nuclei in the somites (Fig. 5*D*). In summary, Kv2.2 transcripts are present in the developing spinal cord and in somites during the periods of maturation of electrical excitability.

DISCUSSION

Only two Kv2 genes Kv2.1 and Kv2.2 have been identified to date in a variety of organisms. Based on sequence identity, XShab9 and XShab12 are the *Xenopus* homologs of Kv2.1 and Kv2.2 genes. In addition, RT-PCR analyses of *Xenopus* brain mRNA and Southern analysis of rat genomic DNA suggest that the vertebrate Kv2 gene family has more than these two members (Fig. 1*A,B*; pXb3 and pXb4 data) (Hwang et al., 1992). The possibility of additional Kv2 isoforms is also consistent with sequence information from another *Xenopus* Kv2 cDNA that is not full length (XShab6; our unpublished observations).

Comparison of Kv2 proteins from many species indicates that the C-terminal domain presents the highest degree of sequence divergence (Table 1). As expected, the major differences between the *Xenopus* Kv2.1 and Kv2.2 gene products occur in this region. The C-terminal domain has numerous phosphorylation consensus motifs for several protein kinases, the activity of which modulates several ion channel properties (Levitan, 1988; Perozo and Bezanilla, 1990; Desarmenien and Spitzer, 1991; Drain et al., 1994). In *Xenopus* spinal neurons, protein kinase C affects the kinetics of

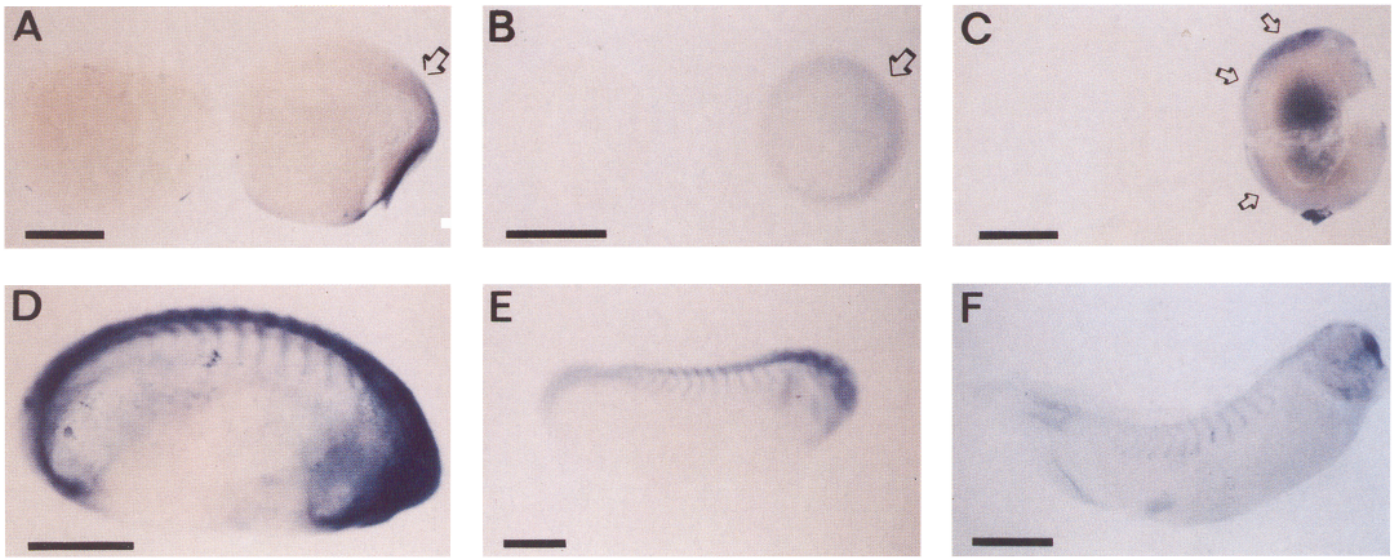


Figure 4. Kv2.2 transcripts are present in excitable tissues of developing *Xenopus* embryos. *A–C*, In each view, the embryo on the *left* was hybridized to a digoxigenin sense control cRNA probe, whereas the embryo on the *right* was hybridized to a Kv2.2 antisense cRNA probe (scale bars: *A*, *C*, 0.5 mm; *B*, 1 mm). *A*, Early gastrula stage embryo (stage 9; 7 hr after fertilization). Staining is apparent in the dorsal lip of the blastopore (*arrow*). *B*, Late gastrula stage (stage 12; 14 hr). Kv2.2 staining localizes to dorsal ectoderm and presumptive neural tissue (*arrow*). *C*, Neurula stage (stage 19; 20 hr). Kv2.2 mRNA is localized along the entire neural tube (*arrows*). Rostral is *up*; dorsal is to the *left*. The *dark shadow* in the gut of the embryo is attributable to incomplete clearing of the embryo. *D–F*, In these lateral views, rostral is to the *right*, and dorsal is *up* (scale bars: *D–F*, 0.5 mm). Sense controls for these older stages are not shown but are similar to those shown in *A–C*. *D*, Expression of Kv2.2 mRNA in the early tail bud embryo (stage 23; 1 d). Staining is present in the brain, spinal cord, and anterior midsomite regions. *E*, A similar pattern of Kv2.2 mRNA expression is observed 6 hr later in the stage 26 embryo, except that the signal in the midsomite regions has extended caudally. *F*, In the stage 35 embryo (2 d), Kv2.2 staining is faint in the majority of the brain and spinal cord but still visible in the midsomite regions. The *dark signal* at the tip of the embryo corresponds to the forebrain.

activation of the endogenous delayed rectifier current during development (Desarmenien and Spitzer, 1991). Investigation of the ability of protein kinases to modulate the kinetic properties of *Xenopus* Kv2.1 and Kv2.2 currents may provide insights into the mechanisms used by embryonic neurons to regulate ion channel function.

Xenopus Kv2 channels are functionally related to mammalian Kv2.1 channels (Frech et al., 1989; Pak et al., 1991; Albrecht et al., 1993; Benndorf et al., 1994); they activate at depolarized potentials and show sensitivity to the blocker TEA. Detailed comparison with mammalian Kv2.2 currents is not possible because it is known only that expressed rat Kv2.2 induces TEA-sensitive outward currents (Hwang et al., 1992). By examining the voltage dependence of activation and pharmacology of *Xenopus* Kv2.2 currents, the present work provides new information regarding the Kv2.2 isoform and a basis for comparison of the properties of Kv2.1 and Kv2.2 channels. Surprisingly, although *Xenopus* Kv2.1 and Kv2.2 sequences share only 58% amino acid identity, their expressed currents are not remarkably different. Perhaps the endogenous channel activities of the two isoforms are subject to differential functional modulation given that the major sequence divergences occur in the phosphorylation site-rich C-terminal domains.

Of potential interest to investigators using the oocyte expression system are the observations that Kv2.2 mRNA is present in eggs from some female frogs and that the properties of activation of Kv2.2 currents resemble the delayed rectifier current expressed endogenously in some oocyte clutches (Lu et al., 1990). Perhaps the occasional background current can be eliminated by treatment of oocytes with Kv2.2 antisense oligonucleotides (Melton, 1985).

Xenopus Kv2.1 and Kv2.2 demonstrate distinct developmental patterns of expression when examined by RNase protection. Contrasting patterns of expression are also observed for rat Kv2.1 and Kv2.2 genes in the adult brain (Drewe et al., 1992; Hwang et al., 1993). Rat Kv2.2 mRNA localizes specifically to the olfactory bulb, cerebellum, and hippocampus, in contrast to the homogeneous distribution of Kv2.1 transcripts in brain tissue. Information regarding the distribution of rat Kv2.1 and Kv2.2 mRNAs in the embryonic spinal cord is not available, and thus comparisons with the present work are limited.

In situ hybridization demonstrates the presence of *Xenopus* Kv2.2 transcripts in primary ventral spinal neurons and embryonic myocytes. In *Drosophila* embryos, the predominant delayed rectifier current in muscle and neurons is encoded by Kv2 (Tsunoda and Salkoff, 1995). Interestingly, the properties of muscle delayed rectifier currents resemble those of Kv2.2 currents expressed in oocytes. The development of electrical excitability in both of these tissues in *Xenopus* is well characterized (for review, see Ribera and Spitzer, 1991, 1992; Spruce and Moody, 1992). The temporal *in situ* pattern of expression of Kv2.2 in ventral neurons is expected if developmental regulation of function occurs at the level of transcription. The expression pattern in somites is also consistent with a role in developmental regulation of potassium current function.

At least two different potassium channel gene transcripts, Kv2.2 and Kv1.1, are present in primary spinal neurons. Do the properties of I_{Kv} in individual neurons reveal both the functional and temporal heterogeneity predicted by Kv1.1 and Kv2.2 transcript expression? Mature *Xenopus* spinal neurons possess delayed rectifier potassium currents (O'Dowd et al., 1988) that, based on voltage-dependent properties, are overall more similar to oocyte Kv1.1 than Kv2.2 currents. The currents recorded from individual mature neurons, however, demonstrate differences in voltage of activation and G_{max} (Ribera and Spitzer, 1989; Jones and Ribera,

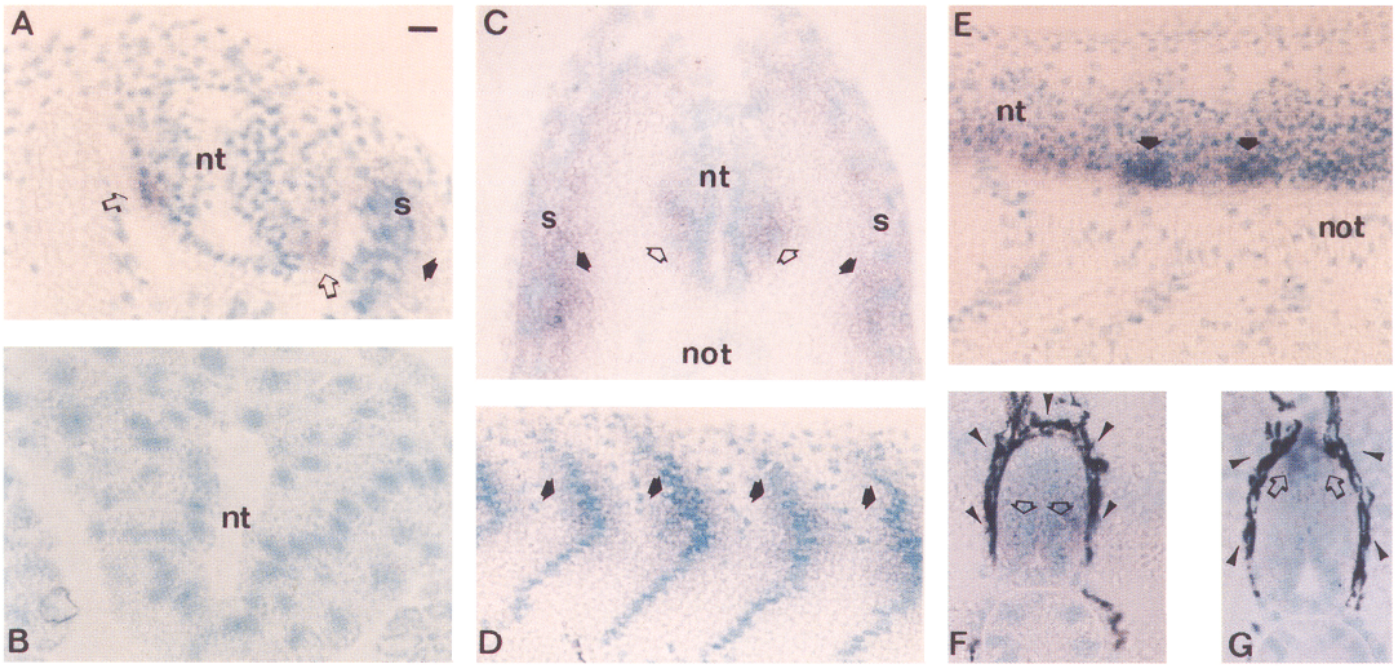


Figure 5. Kv2.2 mRNA localizes to the ventral spinal cord and perinuclear somite regions during development of excitability. These views are obtained from 30 μm sections of whole-mount embryos that were hybridized to a Kv2.2 antisense probe. Sections were counterstained with methyl green, which intensely colors nuclei. Dorsal is *up* in all panels. In the sagittal sections, rostral is to the *right*. *A,* In a cross-section from a stage 23 embryo, Kv2.2 mRNA is restricted to the ventrolateral portion of the neural tube (*open arrows*) and region around the somite nuclei (*filled arrowhead*). *B,* A sense control stage 23 embryo is observed in cross-section; the color is attributable solely to methyl green counterstaining. Sense controls for other stages and planes of section provide similar results. *C,* At stage 26, the signal in the ventral portion of the neural tube (*open arrowheads*) resembles the pattern observed at stage 23 (*A*). In the somites, the signal predominates around nuclei (*filled arrowheads*). *D,* In a parasagittal section of a stage 26 embryo, the Kv2.2 hybridization signal concentrates around the somite nuclei. *E,* In a sagittal section of a stage 26 embryo, the Kv2.2 hybridization signal concentrates in the ventral region of the neural tube in discrete patches. The Kv2.2 signal delimits the ventral extent of the neural tube, whereas the *nt* is positioned within the dorsal region. *F, G,* Sections obtained from stage 35 embryos probed with either antisense Kv2.2 (*F*) or Kv1.1 (*G*) probes. The black varicosities surrounding the spinal cord (*dark thin arrowheads*) are attributable to pigmented cells found in the embryos used for this experiment, which were caused by the mating of an albino female and a pigmented male. By stage 35, pigment begins to appear in albino/pigmented embryos. *F,* By stage 35, Kv2.2 expression in the neural tube (*open arrowheads*) appears fainter than at stage 23 (*C*), which is consistent with what is observed at the whole-mount level (Fig. 4*F*). *G,* Kv1.1 mRNA localizes to the dorsal region of the spinal cord indicated by the *open arrows.* Scale bar (shown in *A*): *A,* 50 μm; *B,* 75 μm; *C,* 100 μm; *D,* 100 μm; *E,* 200 μm; *F,* 100 μm; *G,* 100 μm. Abbreviations: *nt,* neural tube (spinal cord); *s,* somites; *not,* notochord.

1994) (A. Ribera, unpublished observations), confirming the functional differences predicted by the molecular heterogeneity. In addition, only a subset of primary neurons is susceptible to current reduction by overexpression of a Kv1-dominant negative subunit (Ribera, 1996). The delayed rectifier currents in all primary spinal neurons, however, undergo similar developmental regulation. Which other potassium channel genes contribute to functional expression in this functionally synchronized population of diverse spinal neurons remains to be determined.

The results raise questions regarding the mechanism that coordinates temporal expression of delayed rectifier potassium current function in *Xenopus* motor, inter-, and sensory neurons. It is possible that transcription of the relevant potassium channel genes is under a common genetic control. For example, in the olfactory system, the transcription factor OLF-1 coordinately activates expression of several important olfactory neuron-specific genes (Wang and Reed, 1993). More recently, the *Xenopus* basic helix-loop-helix factor NeuroD has been shown to lead to expression of several neural-specific genes during early neuronal differentiation (Lee et al., 1995). NeuroD is normally expressed in primary sensory and motor neurons during early stages of differentiation. These two cell types express either Kv1.1 or Kv2.2. Potassium channel gene expression, therefore, may serve as a sensitive functional and molecular reporter of the activity of neural master genes, because embryonic neurons exhibit potassium currents at early stages of differentiation. It is of particular interest that ectopic overexpression of NeuroD induces expression of Kv1.1 (Lee et al., 1995). Future work will determine whether Kv2.2 gene expression is similarly affected.

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