***Xenopus* Embryonic Spinal Neurons Express Potassium Channel Kv β Subunits**

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Developmental regulation of voltage-dependent delayed rectifier potassium current (I_{Kv}) of *Xenopus* primary spinal neurons regulates the waveform of the action potential. I_{Kv} undergoes a tripling in density and acceleration of its activation kinetics during the initial day of its appearance. Another voltage-dependent potassium current, the A current, is acquired during the subsequent day and contributes to further shortening of the impulse duration. To decipher the molecular mechanisms underlying this functional differentiation, we are identifying potassium channel genes expressed in the embryonic amphibian nervous system. Potassium channels consist of pore-forming (α) as well as auxiliary (β) subunits. Here, we report the primary sequence, developmental localization, and functional properties of two *Xenopus* Kv β genes. On the basis of primary sequence, one of these (xKv β 2) is highly conserved with Kv β 2 genes identified in

other species, whereas the other (xKv β 4) appears to identify a new member of the Kv β family. Both are expressed in developing spinal neurons during the period of impulse maturation but in different neuronal populations. In a heterologous system, coexpression of xKv β subunits modulates properties of potassium current that are developmentally regulated in the endogenous I_{Kv} . Consistent with xKv β 4's unique primary sequence, the repertoire of functional effects it has on coexpressed Kv1 α subunits is novel. Taken together, the results implicate auxiliary subunits in regulation of potassium current function and action potential waveforms in subpopulations of embryonic primary spinal neurons.

Key words: potassium channels; Kv1 α subunits; auxiliary Kv β subunits; *Xenopus* embryos; electrical excitability; spinal cord neurons

In several systems, voltage-dependent potassium current (I_{Kv}) undergoes developmental regulation and dynamically regulates emerging patterns of neuronal excitability (for review, see Spitzer and Ribera, 1998). In embryonic amphibian spinal neurons, maturation of I_{Kv} converts long-duration action potentials to brief sodium-dependent spikes (Barish, 1986; O'Dowd et al., 1988; Lockery and Spitzer, 1992). Maturation of I_{Kv} involves a tripling in its density and acceleration of activation and inactivation kinetics (Barish, 1986; O'Dowd et al., 1988; Ribera and Spitzer, 1990). Definition of the molecular mechanisms that underlie development of excitability requires identification of the potassium channels expressed in embryonic *Xenopus* spinal neurons.

Voltage-dependent potassium channels are oligomeric proteins composed of pore-forming (Kv α) and auxiliary subunits. Kv α subunit genes belong to one of several related subfamilies (Kv1–Kv9). Kv1 α subunits are expressed and contribute to potassium current maturation in the embryonic *Xenopus* spinal cord (Ribera and Nguyen, 1993; Jones and Ribera, 1994; Ribera, 1996). Auxiliary cytoplasmic Kv β subunits tightly associate with Kv1 α subunits (Rehm and Lazdunski, 1988; Parcej et al., 1992; Rettig et al., 1994; Scott et al., 1994; Rhodes et al., 1995, 1996; Nakahira et al., 1996; Sewing et al., 1996). The majority of studies of verte-

brate Kv β gene function have examined channels expressed in heterologous systems, and consequently their *in vivo* function is not yet defined. In heterologous systems, the effects of Kv β subunits are diverse and range from increasing surface expression/current density (Shi et al., 1996; Accili et al., 1997a) to accelerating activation and inactivation kinetics (Rettig et al., 1994; England et al., 1995a,b; Majumder et al., 1995; Heinemann et al., 1996; Morales et al., 1996). In addition, Kv β subunits share sequence and structural similarity with aldo-keto reductases, raising the possibility that they function as enzymes with as yet unspecified substrates (McCormack and McCormack, 1994; Gulbis et al., 1999).

Although little is known regarding the *in vivo* function of Kv β subunits, less is known about their contribution to emerging properties of neuronal excitability [but see, Butler et al. (1998)]. We sought to determine whether embryonic spinal neurons express Kv β subunits, and if so, whether their mRNAs are detectable in spinal neurons that express xKv1 α subunits (Ribera, 1990; Ribera and Nguyen, 1993). Here we report that *Xenopus* embryonic spinal neurons express Kv β subunit genes during the period of maturation of I_{Kv} . One of these (xKv β 2) appears to be the *Xenopus* ortholog of mammalian Kv β 2 on the basis of high amino acid identity (97%). The other gene shares less identity (<73%) with previously identified Kv β genes. We propose that it is a new member of the Kv β family, xKv β 4. Heterologous coexpression of xKv β s with xKv1 α subunits leads to modulation of properties of potassium current that are developmentally regulated in the endogenous I_{Kv} : current density, activation kinetics, and extent of inactivation. *In situ* hybridization indicates that xKv β 2 and xKv β 4 are expressed contemporaneously with previously studied Kv1 α genes in subpopulations of spinal neurons. Taken together, the

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The cDNA sequences reported have been assigned GenBank accession numbers AF172144 (xKv β 2) and AF172145 (xKv β 4).

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results implicate xKv β subunits in regulation of I_{Kv} and emerging excitability of embryonic spinal neurons.

MATERIALS AND METHODS

Animals. *Xenopus* embryos were produced by *in vitro* fertilization and staged according to Nieuwkoop and Faber (1967).

Isolation of *Xenopus* Kv β cDNAs, DNA sequencing, and analysis. ³²P-labeled probes directed against the entire coding regions of rat brain Kv β 1 and Kv β 2 genes (kindly provided by Drs. Ken Nakahira and James Trimmer, Department of Biochemistry, SUNY Stony Brook, Stony Brook, NY) were used to screen a *Xenopus* tadpole brain cDNA library (gift of Dr. Nicholas C. Spitzer, Department of Biology, University of California San Diego, La Jolla, CA) at reduced stringency. One full-length clone (xKv β -A) and two partial-length clones (xKv β -B1p, xKv β -B2p) were obtained. The missing 5' ends of the partial clones were obtained by further screening of cDNA libraries using either conventional methods or PCR. All isolated clones were sequenced over both strands. DNA sequences were read and entered into a computer using a GEL READER interface and software (CBS Scientific, Del Mar, CA) and analyzed using DNASTAR software (Madison, WI).

Whole-mount *in situ* hybridization. The nonradioactive whole-mount detection method (Harland, 1991) was used with minor modifications as described previously (Ferreiro et al., 1993; Burger and Ribera, 1996). cRNA sense and antisense probes corresponding to the 3' untranslated region (UTR) and 3' coding region of xKv β -A, xKv β -B1, and xKv β -B2 were used. For xKv β -A, the probe contained 700 bp of coding region and 150 bp of 3' UTR; for both xKv β -B1 and xKv β -B2, the probes contained 500 bp of coding region and 700 bp of 3' UTR. cRNA probes were synthesized in the presence of digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, IN) and hybridized to whole-mount albino embryos. After removal of probe, embryos were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). The alkaline phosphatase reaction product was developed in the presence of chromogenic substrate (Boehringer Mannheim). Whole-mount embryos were either cleared in Murray's solution (2:1 benzyl benzoate/benzyl alcohol) for photography or embedded in plastic (JB-4 embedding kit; Polysciences, Warrington, PA). Photography of whole mounts and sections was performed with Kodak Ektachrome 160T film using appropriate color filters. Photographic images were digitized (Nikon Cool Scanner), and composite figures were constructed using Adobe Photoshop Software.

Oocyte recording. The entire coding regions of xKv1.1 α and xKv1.2 α potassium channel genes were previously cloned into the pSP64T expression vector, and cRNA was synthesized as described (Ribera and Nguyen, 1993). An *EcoRI*-*XhoI* fragment containing the entire coding region of xKv β 4 along with 671 bp of 3' UTR was cloned into the pCS2+ expression vector (Rupp et al., 1994; Turner and Weintraub, 1994); the xKv β 2-pCS2+ expression construct was constructed by insertion of a PCR-generated cDNA encoding xKv β 2 into pCS2+. Synthesis of capped cRNA was achieved by linearization of a xKv β -pCS2+ expression vector with *NotI* followed by *in vitro* transcription with SP6 RNA polymerase (Promega, Madison, WI) in the presence of ribonucleotide triphosphates (Pharmacia Biotech, Piscataway, NJ) and cap analog (Boehringer Mannheim). RNA concentrations were determined spectrophotometrically and confirmed by electrophoresis in agarose-formaldehyde gels. Stage VI oocytes were removed and defolliculated as described previously (Ribera, 1990; Ribera and Nguyen, 1993; Burger and Ribera, 1996). xKv β and xKv1 α cRNAs were either injected alone or coinjected at ratios of 1–30:1 (xKv β /xKv1 α). For two-electrode voltage-clamp (TEVC) experiments, oocytes were injected with 50 nl of RNA solution (Kv1 RNA, 2.5–5 μ g/ml; Kv β RNA, 75–150 μ g/ml). Higher expression was required for macropatch experiments, and oocytes were injected with 100 nl of a more concentrated RNA solution (Kv1 RNA, 5–21 μ g/ml; Kv β RNA, 150–630 μ g/ml). Oocytes were incubated at 18°C in Barth's solution containing the following (in mM): 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 2.4 NaHCO₃, 0.82 MgSO₄ and 5 Na HEPES, pH 7.4.

TEVC methods were used [Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) or Oocyte Clamp OC-725C (Warner Instruments, Hamden, CT)] to record voltage-activated outward currents induced in oocytes 24–48 hr after injection of RNA. For detailed analysis of activation kinetics, data were obtained 48–72 hr after injection using the cell-attached macropatch configuration and a patch-clamp amplifier (Axopatch 200B amplifier, Axon Instruments) to avoid the constraints imposed by the large capacitive transients of the whole oocyte. Data acquisition and analysis were accomplished with the pCLAMP suite of

programs and AxoGraph software (Axon Instruments). For TEVC recordings, currents were sampled at 100–200 μ sec and filtered at 5–10 kHz. For macropatch recordings, currents were sampled at 30–100 μ sec and filtered at 5 kHz, and data from 10 runs were averaged to improve the signal-to-noise ratio. The leak and capacitive transient currents were subtracted using the P/4 protocol of the Clampex program (pCLAMP). For TEVC recordings, the bath consisted of Barth's solution and the electrode solution consisted of 3 M KCl and 10 mM HEPES, pH 7.4. Electrode resistances ranged between 0.1 and 0.7 M Ω . Oocytes were not used if the holding current exceeded –200 nA at a potential of –80 mV or if the resting membrane potential was positive to –40 mV. For macropatch recordings, the bath consisted of 115 mM KCl, 1.8 mM EGTA, and 10 mM HEPES, pH 7.4, and electrodes were filled with Barth's solution. Electrode resistances ranged between 1.5 and 2.5 M Ω .

Current amplitudes were measured 50–55 msec into a 60 msec pulse (steady-state level), except for data obtained on coexpression of xKv β 4. In the latter case, peak values were measured to avoid the effects produced by inactivation; induction of inactivation was analyzed separately (see Fig. 7). Conductance–voltage (G – V) relationships were determined for recordings from oocytes that did not saturate the amplifier at the most depolarized voltage step (+100 mV). Conductance was determined by dividing current amplitudes by the driving force assuming an internal K⁺ concentration of 100 mM and $E_K = -116$ mV; recordings that did not achieve a plateau value of conductance (G_{max} ; determined from Boltzmann fits) were excluded. Normalized G – V plots were obtained by dividing values of G by maximum G , G_{max} , as determined from a fit to the Boltzmann equation. The Boltzmann parameters, $V_{1/2}$ and k , were obtained from fits of the Boltzmann equation to G – V plots. Time to half-maximum ($t_{1/2}$) was determined as the time required to achieve half-maximum amplitude during a 60 msec voltage step. Current densities were determined by dividing current amplitudes by the oocyte surface area, assuming a 1 mm diameter ($\sim 10^6 \mu\text{m}^2$). Levels of statistical significance were assessed using the nonparametric Mann–Whitney test; p values <0.05 were indicative of statistical significance.

RESULTS

Identification of *Xenopus* Kv β Potassium Channel Subunits

Given the high identity among previously identified Kv β subunits, we began our search for *Xenopus* Kv β clones by screening cDNA libraries with probes derived from rat (kindly provided by Drs. K. Nakahira and J. S. Trimmer, Department of Biochemistry, SUNY, Stony Brook, NY). One full-length (xKv β -A) and two partial-length cDNA (xKv β -B1p and xKv β -B2p) clones were isolated. The missing 5' ends of the partial clones were obtained through a combination of further library screening and PCR-based approaches (see Materials and Methods) to yield cDNA clones containing complete open reading frames (xKv β -B1 and xKv β -B2, respectively).

The xKv β -A coding region contains 1101 base pairs (bp) and predicts a protein of 367 amino acids and a molecular weight (MW) of 40 kDa (Fig. 1). Alignment of the predicted amino acid sequence of *Xenopus* Kv β -A with mammalian Kv β sequences reveals >97% identity with Kv β 2 protein (Table 1). In contrast, only 76–77 and 62–67% exists with Kv β 1 and Kv β 3 subfamily members. On the basis of these comparisons, we consider Kv β -A to be the *Xenopus* ortholog of Kv β 2 and refer to it as xKv β 2.

xKv β -B1 and xKv β -B2 both contain open reading frames that are 1203 bp in length. They share 93 and 94% overall identity at both the nucleotide and predicted amino acid levels over the coding region. Kv β subunits belong to the superfamily of aldoketo reductases; for this superfamily, Jez et al. (1997) proposed that $\geq 97\%$ amino acid identity is indicative of allelic forms. Given their 94% amino acid identity and 94 and 89% nucleotide identity of coding regions and 3' UTR, respectively, xKv β -B1 and

xKv β 2	M.....YPESTTDSPARLSLRQTGS.PGMIYSARYG.....SPK	33
xKv β 4	-QVSFACTEQTLRSRT-ED--CPSRPSGGQNGVSM-QTKQRTTPP...MGAKNHNQVL-	55
hKv β 1	-QVSIACTEHNLSRNGED--LSK-SSTA-NVNNA--AKFRTVA...IIARSLGTFT-Q	56
hKv β 2	-----G-----T-----	33
hKv β 3	-QVSIACTEQNLSRS-ED--CGPRP-PGG-NGGP-GG-HGNPPGGGGSGPKARAAVV-G	60
xKv β 2	RQLQ.....FYRNLGKSGLRVSCIGLGTWVTFGGQITDEMAEQMLTLAYDN	79
xKv β 4	..-LSHILRESTVKSTGMK-----S--S--V--N--V--EH	113
hKv β 1	HHIS...LKESTAKQTGMK-----S--V--R--I--ES	113
hKv β 2	-----	79
hKv β 3	PPAPGGAVRESTGRGTGMK-----C-V-----S--S--T--DVL-V--EH	120
xKv β 2	GINLFDTAEVYAAGKAEVVLGNIKKKGWRRSSLVITTKIFWGGKAETERGLSRKHIEG	139
xKv β 4	-V-----R-RT-K-L-E---Y-V---Y-Q-----	173
hKv β 1	-V-----I-S-----LY-----	173
hKv β 2	-----	139
hKv β 3	-V-----RT---L-S-----Y-----Q-----	180
xKv β 2	LKASLERLQLDYVDVVFANRPDPNTPMEETVRAMTHVINQGMAMYWGTSRWSSMEIMEAY	199
xKv β 4	-RG-----I---M---S---I---F-----A-----	233
hKv β 1	-G-Q---E-----S---I-----A-----	233
hKv β 2	-----E-----	199
hKv β 3	-RG-----G---I---S---C---I---Y---L-L-----GAA-----	240
xKv β 2	SVARQFNLIPIICEQAEYHMFQREKVEVQLPELFHKIGVGAMTWSPLACGIVSGKYDGGI	259
xKv β 4	-----V-----L-----T---Y-----LIT---TDTV	293
hKv β 1	-----M--V-----L-----Y-----I---GN-V	293
hKv β 2	-----T-----	259
hKv β 3	-M-----V-----H-L-----M---Y-----V-Y-----LITS---RV	300
xKv β 2	PPYSRASLKGQWLKDKILSEEGRQQAKLKEFQAIAERLGCTLPQLATAWCLRNEGVSS	319
xKv β 4	-EK----F---H---E-AI-Q--KK-HS-V--LHP--D--N--VT-----S-----	353
hKv β 1	-ES-----C-----ER-V-----K--N--DLSP-----V-----	353
hKv β 2	-----	319
hKv β 3	-DTC---I-----VQ--D-KK---VMDLLPV-HQ---VA-----S-----	360
xKv β 2	VLLGASNADQLLENIGAIQVLPKLSSSIIEIDGILGNKPYSKKDYRS	367
xKv β 4	-----V--IE---L-----H-TPQLVT---Q-----NL---S-A	401
hKv β 1	-----S-TPE--I--L-----MT-HVVN---N--R-----	401
hKv β 2	-----M-----S-----	367
hKv β 3	-----V-S-E--I-HL--L---SQ-TPQTMV---L-----H---	404

Figure 1. *Xenopus* Kv β subunits. The predicted amino acid sequences of *Xenopus* xKv β 2 and xKv β 4 coding regions are aligned against mammalian Kv β 1, Kv β 2, and Kv β 3 sequences [hKv β 1.1 and hKv β 2.1 (McCormack et al., 1995); hKv β 3.1 (Leicher et al., 1998)]. Gaps (dots) have been introduced to improve the alignment. Residues that are identical to those of xKv β 2 are indicated by a hyphen. Overall, xKv β 2 and xKv β 4 share 71% identity at the amino acid level (Table 1). α -Helices and β -sheets are shaded light and dark gray, respectively; residues that contribute to the putative active site are indicated by an overlying asterisk (Gulbis et al., 1999).

xKv β -B2 sequences are likely to be allelic variants. Consistent with this, both genes have similar mRNA expression patterns (data not shown). Only results for the xKv β -B1 allelic form are presented below.

The coding sequence of xKv β -B1 predicts a protein containing 401 amino acids (44 kDa MW) (Fig. 1). Alignment of the predicted amino acid sequence of xKv β -B1 with mammalian Kv β subfamily members reveals 69–74, 70–73, and 70–74% identity with mammalian Kv β 1, Kv β 2, and Kv β 3 subfamily members (Table 1). In contrast, xKv β 2 is $\geq$ 97% identical to Kv β 2 genes previously cloned in other vertebrate species. Within a species (e.g., human), the different Kv β genes (i.e., Kv β 1, Kv β 2, Kv β 3) share $\sim$ 70–75% identity (Table 1), which is the degree of identity

found between xKv β -B1 and previously identified Kv β genes. On this basis, xKv β -B1 appears to identify a new Kv β subfamily, and we refer to xKv β -B1 as xKv β 4.

Kv β genes share sequence and structural similarities with the NAD(P)H-dependent oxidoreductase superfamily (McCormack and McCormack, 1994; Gulbis et al., 1999). The xKv β proteins show the same extent of sequence identity (13–49%) with these enzymes as do mammalian Kv β subunits. Although aspects of the xKv β 4 sequence are novel, motifs that define characteristic β - α - β barrel structures and critical active site residues are conserved (D119, Y124, and K154 of xKv β 4) (Fig. 1), indicating that xKv β 4 belongs to the β subunit cluster of aldo-keto reductases (Jez et al., 1997; Gulbis et al., 1999).

Table 1. Comparison of *Xenopus*, human, mouse and fly Kvβ amino acid sequences

Subfamily	hKvβ1			hKvβ2		h/mKvβ3		Hk	xKvβ	
Isotype	1.1	1.2	1.3	2.1	2.2	3.1	3.2		2	4
xKvβ2	77	76	76	98	97	67	62	42	100	71
xKvβ4	74	69	69	70	73	74	70	39	71	100

The percent identities between the amino acid sequences of Kvβ subunits are presented. All Kvβ1 and Kvβ2 sequences are the human version, as is Kvβ3.1. Kvβ3.2 is from mouse, and Hk is from *Drosophila*. References: Kvβ1.1, McCormack et al. (1995); Kvβ1.2, Majumder et al. (1995); Kvβ1.3, England et al. (1995a); Kvβ2.1, McCormack et al. (1995); Kvβ2.2, Rae et al. (1998); Kvβ3.1, Leicher et al. (1998); Kvβ3.2, Fink et al. (1996); Hk, Chouinard et al. (1995).

xKvβ2 and xKvβ4 transcripts are present in excitable tissues of the embryo

Whole-mount *in situ* hybridization was performed to determine the expression patterns of xKvβ2 and xKvβ4 genes in *Xenopus*

embryos. Embryos ranging in age between 1 and 2 d [Stage (St) 8–36] were examined, because maturation of *I*_{Kv} in spinal neurons occurs during this period. Action potentials are first detected in sensory spinal neurons at 20 hr (St 18) (Baccaglini and Spitzer,

Figure 2. xKvβ2 and xKvβ4 transcripts are present in excitable tissues of developing *Xenopus* embryos. Embryos were hybridized as whole mounts to antisense or sense cRNA probes corresponding to either xKvβ2 (A–F) or xKvβ4 (G–I). The *in situ* hybridization signal is recognizable as a deep blue-purple precipitate. The whole-mount embryos (A–C, G–I) are oriented with their dorsal sides up and anterior ends to the left. Embryos that were hybridized to sense control probes are identified by s; these demonstrate the background signal (caused by trapping of probe in internal cavities), which is higher in older embryos. The transverse sections (D–F) are oriented dorsal side up. A, At 26 hr (St 24), xKvβ2 mRNA is prominent in developing somites. B, Between 1 and 2 d (top, middle, and bottom embryos are St 28, 29, and 30, respectively), the pattern of expression of xKvβ2 mRNA undergoes a dramatic change. Initially the signal predominates in the somites but with time diminishes in this tissue and begins to appear in the spinal cord (arrowheads). At St 35–36 (50 hr; C), the signal is no longer observed in the somites but is present in the spinal cord and head. D, In a transverse section through a 1 d embryo hybridized to xKvβ2 antisense probe, the signal is present in the developing somites. E, In a transverse section of a 2 d embryo hybridized to xKvβ2 antisense probe, the signal is detectable in the dorsal spinal cord, where Rohon-Beard cells reside. F, In a transverse section of a 2 d embryo hybridized to a sense control probe, no signal is present. G–I, Whole-mount embryos hybridized to xKvβ4 antisense probe. At all stages, the signal predominates in the nervous system. In the older embryos (e.g., I), the signal is stronger. Comparison of H and I (xKvβ4) with B and C (xKvβ2) reveals that xKvβ4 mRNA localizes to a more ventral position in the spinal cord than does xKvβ2. Scale bar: A, G, 1 mm; B, C, 1.5 mm; H, 1.2 mm; I, 1.1 mm; D–F, 700 μm.

Figure 3. Coexpression of xKv β 2, but not xKv β 4, with xKv1.1 α or xKv1.2 α subunits leads to an increase in current amplitude. *A*, Representative whole-oocyte recordings of currents induced after injection of Kv1.1 α (top) and Kv1.2 α (bottom) RNAs in the absence (left) or presence of xKv β 2 (middle) or xKv β 4 RNA (right). Currents were elicited by depolarizing the membrane in 10 mV increments to potentials ranging between -60 and +130 mV from a holding potential of -80 mV; currents elicited by depolarizations to -60, -30, 0, +30, +60, +90, and +120 mV are shown. *B*, Normalized current-voltage (*I-V*) relationships for currents recorded from oocytes injected with xKv1.1 α (left) and Kv1.2 α (right) $\pm$ xKv β 2 or xKv β 4 RNAs. Current amplitudes were normalized to the mean current amplitude obtained at +50 mV for xKv1.1 α or Kv1.2 α alone; numbers in parentheses = *n*. Data obtained in the presence of Kv β 2 (■) are significantly different from those obtained in its absence (○); $p \leq 0.0002$.

1977). At this time, potassium currents are of small density (Barish, 1986; O'Dowd et al., 1988). During the subsequent day, there is a progressive increase in I_{Kv} density and consequent shortening of the action-potential duration. Appearance of I_{Kv} in myocytes (somitic tissue) is delayed with respect to that in neurons but also undergoes functional upregulation once it is present (25 hr, St 23) (Ribera and Spitzer, 1990).

In the developing embryo, excitable tissues express xKv β 2 in a spatially and temporally dynamic pattern (Fig. 2). xKv β 2 transcripts are first detected in somites (26 hr, St 24). Within 9 hr, (St 29, 35 hr), Kv β 2 is detected in the spinal cord as well. One hour later (St 30, 36 hr), xKv β 2 mRNA expression is downregulated in somites and upregulated in the spinal cord. By St 35–36 (50 hr), xKv β 2 expression is limited to the spinal cord and no longer present in the somites. In transverse sections of 2 d embryos (St

35), xKv β 2 mRNA expression is detected only in dorsal spinal neurons, including the mechanosensory Rohon-Beard cells.

xKv β 4 expression is limited to the nervous system in a spatially and temporally dynamic pattern. Kv β 4 transcripts are first detected in the brain and only the most anterior regions of the spinal cord (St 24, 26 hr). Nine hours later (St 29), Kv β 4 mRNA is detected in posterior regions of the spinal cord as well. During the subsequent day (Fig. 2*I*), the spatial pattern of expression remains constant, although the intensity of staining obtained is stronger, suggesting upregulation of gene expression during this period.

xKv β 2, but not xKv β 4, increases functional expression of xKv1 α channels

Coexpression of mammalian Kv β with Kv1 subunits leads to increases in current density and acceleration of activation and inactivation kinetics (Rettig et al., 1994; Majumder et al., 1995; McCormack et al., 1995; Heinemann et al., 1995, 1996; Morales et al., 1996; Shi et al., 1996; Uebele et al., 1996; Accili et al., 1997a,b). These properties of potassium current are of particular interest, because they are developmentally regulated in the endogenous I_{Kv} . We coexpressed xKv β with xKv1.1 α and Kv1.2 α subunits, because the developing spinal cord expresses the mRNAs for these α -subunits (Ribera, 1990; Ribera and Nguyen, 1993).

xKv1 α and xKv β mRNAs were coinjected into *Xenopus* oocytes at several different ratios ranging between 1 and 30:1 (xKv β /xKv1 α). For ratios of 1–4:1, no effects are observed. At ratios of 15–30:1, similar effects are observed, which are statistically significant at a ratio of 30:1 (xKv β /xKv1 α). Thus, only data obtained using the 30:1 ratio are presented.

Coexpression of xKv β 2 with either xKv1.1 α or xKv1.2 α subunits increases current amplitude 1.6- and 2.0-fold, respectively (+20 mV) (Fig. 3*A*). In contrast, coexpression of xKv β 4 with either xKv1 α subunit has no effect on current amplitude. One possible explanation for the lack of effect of xKv β 4 coexpression is that this auxiliary subunit was not expressed at a sufficiently high level. However, xKv β 4 coexpression does have functional effects on kinetic properties (see below). On this basis, lack of expression of xKv β 4 subunits at the 30:1 ratio (xKv β /xKv1 α) is unlikely to account for the absence of effect on current density.

Current densities for channels expressed in oocytes ranged between 1 and 10 pA/ μ m², which includes the range found in developing spinal neurons (0.5–1.5 pA/ μ m² at +30 mV) (O'Dowd et al., 1988; Jones and Ribera, 1994). On coexpression of xKv β 2, current densities increased two- to threefold, just as I_{Kv} density does in developing spinal neurons.

xKv β 1 and xKv β 2 modify functional properties of xKv1 α channels

The increase in current densities observed on coexpression of xKv β 2 subunits may reflect changes in the conductance-voltage relationship of the current, the number of functional channels present in the plasma membrane, or both. To distinguish between these possibilities, we examined conductance-voltage relationships of currents carried by the various subunit combinations. We determined the maximum value of the conductance (G_{max}) and voltage-dependent parameters derived from fits to the Boltzmann equation ($V_{1/2}$ and k).

For Kv1.1 α and Kv1.2 α subunit-containing channels, coexpression of xKv β 2 subunits leads to 1.6- and 1.8-fold increases in G_{max} (Fig. 4*A,B*; Table 2). These data indicate that an increase in the number of functional channels contributes to the increased cur-

Figure 4. Coexpression of xKv β 2 versus xKv β 4 subunits differentially modulates steady-state activation properties of xKv1 α channels. *A, B*, Conductance–voltage (G – V) relationships indicate that coexpression of either xKv1 α subunit (*A*, xKv1.1 α ; *B*, xKv1.2 α) with xKv β 2 subunits results in an increase in $G_{\max}$. In contrast, coexpression of xKv1 α subunits with xKv β 4 has no significant effect on $G_{\max}$. *C, D*, Normalized G – V curves for xKv1.1 α (*C*) or xKv1.2 α (*D*) currents obtained in the absence or presence of xKv β subunits. Coexpression of xKv1.2 α with either xKv β 2 or xKv β 4 subunits leads to a depolarizing shift in the G – V relationship (Table 2). In *C*, data obtained in the presence of xKv β 4 ($\blacktriangle$) are statistically different from those obtained in its absence ($\circ$) in the range of -20 to $+40$ mV; p values range between 0.0001 and 0.006. In *D*, data obtained in the presence of xKv β 2 ($\blacksquare$) or xKv β 4 ($\blacktriangle$) are statistically different from those obtained in its absence ($\circ$) in the range of $+10$ to $+50$ mV (xKv β 2) and 0 and 50 mV (xKv β 4); p values range between 0.0001 and 0.04.

rent amplitudes obtained on xKv β coexpression (Fig. 3). The increase in $G_{\max}$ found on xKv β 2 coexpression with xKv1.1 α subunits accounts for the increase in current density. For Kv1.2 α , xKv β 2 coexpression has a slightly smaller effect on $G_{\max}$ than it did on current amplitudes. If the single-channel conductance is unaltered, the result suggests that there are about twice as many functional channels in the plasma membrane on coexpression of xKv β 2 with either xKv1.1 α or xKv1.2 α subunits. In contrast, xKv β 4 coexpression does not alter either $G_{\max}$ values (Fig. 4*A, B*; Table 2) or current amplitudes (Fig. 3).

Next, we examined the possibility that coexpression of either xKv β 2 or xKv β 4 affects voltage-dependent properties of steady-state activation. In fact, coexpression of xKv β 4, but not xKv β 2, with xKv1.1 α shifts the activation curve to more positive voltages (Fig. 4*C*); $V_{1/2}$ is increased by ~ 5 mV (Table 2). Such a shift in $V_{1/2}$ on Kv β coexpression would lead to a reduction in current amplitude in the range of -20 to $+40$ mV. However, current amplitudes are unaffected by xKv β 4 coexpression (Fig. 3).

Table 2. Boltzmann parameters for xKv1 α currents in the absence or presence of xKv β subunits

	$G_{\max}$ (μ sec)	$V_{1/2}$ (mV)	k (mV)
Kv1.1 α alone	61 $\pm$ 6	-19 ± 1	11 $\pm$ 0.3
+ Kv β 2	98 $\pm$ 10*	-19 ± 1	11 $\pm$ 0.4
+ Kv β 4	60 $\pm$ 10	$-14 \pm 1^*$	13 $\pm$ 0.3*
Kv1.2 α alone	12 $\pm$ 1	-4 ± 1	11 $\pm$ 0.3
+ Kv β 2	21 $\pm$ 2*	-2 ± 1	13 $\pm$ 0.6*
+ Kv β 4	14 $\pm$ 1	$-1 \pm 1^*$	13 $\pm$ 0.3*

Asterisks indicate statistical significance; p values range between 0.005 and ≤ 0.0001 .

Similar analyses were performed for Kv1.2 α -containing channels. Coexpression of either xKv β 2 or xKv β 4 leads to small depolarizing shifts in the G – V relationship (Fig. 4*D*; Table 2). Such shifts would lead to a decrease in current amplitude in the range of $+10$ to $+50$ mV. However, current amplitude is unaffected by xKv β 4 coexpression and increased by xKv β 2 coexpression. On the basis of these data, the increased current amplitudes observed on xKv β 2 coexpression are most likely caused by an

Figure 5. Coexpression of xKv β with xKv1 α subunits accelerates kinetics of activation. Time to half-maximum activation ($t_{1/2}$) as a function of voltage is plotted for recordings obtained from whole oocytes injected with Kv1.1 α (left) or Kv1.2 α (right) cRNAs in the absence and presence of xKv β 2 or xKv β 4 cRNAs. Representative currents are presented in Figure 3. Data obtained in the presence of either xKv β are significantly different from those obtained in its absence; p values are all < 0.0001 . The number of oocytes recorded from are as follows: Kv1.1 α alone, 72; Kv1.1 α + xKv β 2, 70; Kv1.1 α + xKv β 4, 35; Kv1.2 α alone, 83; Kv1.2 α + xKv β 2, 82; Kv1.2 α + xKv β 4, 49.

Figure 6. Activation of xKv1 α currents recorded in oocyte macropatches is accelerated when xKv β subunits are coexpressed. Representative macropatch recordings (A) and time to half-activation ($t_{1/2}$)–voltage relations (B, C) are presented for currents recorded from oocytes injected with xKv1.1 α (A, left; B) or xKv1.2 α (A, right; C) coexpressed with xKv β subunits. Currents were elicited by depolarizing the membrane in 10 mV increments to potentials ranging between -60 and $+130$ mV from a holding potential of -80 mV; the currents elicited by depolarizations to -60 , -30 , 0 , $+30$, $+60$, $+90$, and $+120$ mV are shown. In B, data obtained in the presence of Kv β 2 are significantly different from those obtained in its absence in the range of $+10$ to $+60$ mV, except at $+40$ mV; p values range between 0.02 and 0.05. Data obtained in the presence of Kv β 4 are significantly different from those obtained in its absence in the range of -30 to $+70$ mV; p values range between 0.0005 and 0.04. In C, data obtained in the presence of Kv β 2 are significantly different from those obtained in its absence in the range of -30 to $+60$ mV; p values range between 0.0006 and 0.02. Data obtained in the presence of Kv β 4 are significantly different from those obtained in its absence in the range of $+10$ to $+60$ mV; p values range between 0.02 and 0.04.

increase in channel number rather than changes in steady-state voltage-dependent properties of the expressed channels.

xKv β subunits affect activation kinetics of Kv1 currents

The endogenous I_{Kv} undergoes a developmentally regulated acceleration of activation kinetics (O'Dowd et al., 1988). Mammalian Kv β subunits accelerate activation kinetics of Kv1 currents (Heinemann et al., 1995, 1996; Uebele et al., 1996). In whole-oocyte recordings, xKv1 currents activate more rapidly on coexpression of either xKv β 2 or xKv β 4 (Fig. 5). The time to half-maximum current ($t_{1/2}$) decreases, indicating that activation of the current is accelerated. Larger effects are observed when xKv β subunits are coexpressed with xKv1.2 α versus xKv1.1 α . The effects of xKv β coexpression are voltage dependent and less pronounced with increasing depolarization. Overall, coexpression of an xKv β subunit can lead to a 25–50% decrease in $t_{1/2}$, which is reminiscent of the 50% decrease in $t_{1/2}$ observed for the endogenous I_{Kv} during maturation of the action potential.

To avoid the large, long-lasting capacitive transients obtained in whole-oocyte recordings that compromise analysis of kinetics of current activation, we also examined kinetics of activation in recordings from oocyte macropatches (Fig. 6). The values of $t_{1/2}$ obtained from the macropatch data are smaller than those obtained for whole-oocyte recordings (Fig. 5 vs Fig. 6), which most likely reflects the better temporal resolution of the macropatch configuration. Nonetheless, as found for whole-oocyte recordings,

coexpression of xKv β 2 or xKv β 4 with xKv1.1 α leads to a decrease in the activation $t_{1/2}$. xKv β coexpression effects are voltage-dependent and greater at less depolarized potentials. At voltages positive to $+60$ and $+70$ mV (xKv β 2 and xKv β 4, respectively), the effects are no longer significant.

Coexpression of xKv β subunits with xKv1.2 α subunits also accelerates current activation (Fig. 6A,C). $t_{1/2}$ values are reduced by as much as a factor of 2. Effects are voltage dependent, being more pronounced at less depolarized membrane potentials. In sum, Kv β coexpression accelerates the kinetics of activation, and the specific effects depend on the particular combination of α - and β -subunits.

xKv β 4 subunits increase and accelerate inactivation of xKv1 α currents

The endogenous I_{Kv} undergoes a developmentally regulated change in inactivation properties (Ribera and Spitzer, 1990). Because mammalian Kv β 1 and Kv β 3 subunits enhance inactivation of Kv1 currents (Rettig et al., 1994; Heinemann et al., 1995, 1996; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1996; Accili et al., 1997a; Leicher et al., 1998), we also determined whether xKv β subunits are capable of modulating inactivation of channels containing either xKv1.1 α or xKv1.2 α subunits. Inactivation was evaluated by calculating the ratio of the steady-state versus peak-current amplitudes achieved during a pulse (I_{ss}/I_{pk}). This ratio has a value close to 1 for sustained currents and decreases when inactivation is apparent. Typically,

Figure 7. Coexpression of xKv β 4 induces inactivation of both xKv1.1 α and xKv1.2 α currents. *A*, Coexpression of xKv β 4, but not xKv β 2, induces inactivation of xKv1 α channels ($*p \leq 0.0001$ vs Kv1 α alone). *B*, For both xKv1.1 α and xKv1.2 α subunits, coexpression of xKv β 4 induces a voltage-dependent inactivation; more inactivation is observed at stronger depolarizations.

xKv1.1 α and xKv1.2 α currents have I_{ss}/I_{pk} values of ≥ 0.98 ($n = 74$ and 29, respectively; +100 mV), as expected for sustained currents.

Consistent with results found for Kv β 2 of other species, coexpression of xKv β 2 has no effect on inactivation of either Kv1.1 α or Kv1.2 α currents (Fig. 7*A*). However, coexpression of xKv β 4 with either xKv1.1 α or xKv1.2 α subunits induces inactivation (i.e., $I_{ss}/I_{pk} \leq 0.9$) in 79 and 100%, respectively, of oocytes. For those oocytes having I_{ss}/I_{pk} values ≤ 0.90 (+100 mV), inactivation was examined over a more extended range of voltages (Fig. 7*B*). xKv β 4 increased the extent of inactivation of both xKv1.1 α and xKv1.2 α currents in a voltage-dependent manner, with more inactivation observed at more depolarized voltages. These effects of xKv β 4 coexpression resemble those reported for mammalian Kv β 1 and Kv β 3 subunits (Rettig et al., 1994; England et al.,

1995a,b; Heinemann et al., 1995, 1996; Majumder et al., 1995; Morales et al., 1996; Accili et al., 1997a).

DISCUSSION

Our interest in Kv β subunits derives from the fact that they modulate properties of voltage-dependent potassium current that are developmentally regulated in the I_{Kv} of *Xenopus* spinal neurons. Although it is well known that I_{Kv} undergoes changes in density and kinetics of activation and inactivation, the underlying molecular mechanisms have yet to be defined. We find that xKv β genes are expressed in spinal neurons of *Xenopus* embryos during the period of action-potential maturation. Although one xKv β gene (xKv β 2) is highly conserved with mammalian forms, the other (xKv β 4) is unique with respect to both its primary sequence and functional effects. Coexpression of xKv β with xKv1 α subunits modifies those properties of potassium current that are required for maturation of the impulse and excitability.

xKv β 2 shares >97% identity with mammalian Kv β 2. In contrast, xKv β 4 shares only 69–74% identity with mammalian Kv β 1, Kv β 2, and Kv β 3. The level of identity found between the different Kv β families within a species (e.g., human Kv β 1 vs Kv β 3) is only 70–75%. On this basis, xKv β 4 appears to identify a new Kv β family. This conclusion is further supported by xKv β 4's novel functional effects, as discussed below. Although we screened several embryonic cDNA libraries and did extensive RT-PCR using embryonic mRNA, we have isolated only xKv β 2 and xKv β 4. It is possible that at later stages of development *Xenopus* expresses genes that are orthologous to mammalian Kv β 1 or Kv β 3. Kv β 1 is not a mammalian-specific gene, because Rajeevan et al. (1999) recently isolated a chick Kv β 1.1 ortholog that is 95% identical to mammalian Kv β 1.1. Previous Kv β cloning efforts have focused on adult stages. Thus, it is possible that xKv β 4 reveals a Kv β subfamily that is preferentially expressed during embryonic development.

Consistent with xKv β 2's high primary sequence identity to mammalian forms, the functional effects of xKv β 2 coexpression are similar to those reported for mammalian Kv β 2. Mammalian Kv β 2 coexpression increases the amplitude of Kv1.2 α and Kv1.4 α currents (McCormack et al., 1995; Accili et al., 1997a) and surface expression of Kv1.1 α , Kv1.2 α , and Kv1.6 α subunits (Shi et al., 1996); mammalian Kv β 2 also accelerates Kv1.5 α activation kinetics (Heinemann et al., 1996; Uebele et al., 1996). Similarly, xKv β 2 increases current density and G_{max} and also accelerates activation kinetics when coexpressed heterologously with xKv1.1 α and xKv1.2 α subunits.

The most obvious functional effect of xKv β 4 coexpression is induction of inactivation of Kv1.1 α and Kv1.2 α channels (Fig. 7). In this respect, xKv β 4 is similar to mammalian Kv β 1 and Kv β 3, which have been shown to increase inactivation of coexpressed Kv1 α subunits (Rettig et al., 1994; Heinemann et al., 1995, 1996; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1996; Accili et al., 1997b; Leicher et al., 1998). Induction of inactivation by Kv β 1 subunits is thought to involve an N-terminal ball domain and critically depends on residues that are sensitive to oxidation (Rettig et al., 1994; Wang et al., 1996). In mammalian Kv β 3 as well as in the newly reported xKv β 4, similar residues (cysteine and serine preceding several positively charged amino acids) exist, indicating that induction of inactivation by Kv β 1, Kv β 3, and xKv β 4 may share a common mechanism of action. However, only Kv β 1.1, a Kv β 1 splice variant, has been shown to induce inactivation of both Kv1.1 α and Kv1.2 α channels as does

xKv β 4 (Heinemann et al., 1996). However, coexpression of xKv β 4 also accelerates activation kinetics, an effect not observed on coexpression of either mammalian Kv β 1 or Kv β 3 with Kv1.1 α or Kv1.2 α channels (Heinemann et al., 1995, 1996; Leicher et al., 1998). Furthermore, xKv β 4 coexpression leads to depolarizing shifts in the activation curve (Fig. 4). In contrast, coexpression of Kv β 1 or Kv β 3 subunits typically leads to hyperpolarizing shifts, if any (Pongs et al., 1999). Taken together with xKv β 4's unique primary sequence, these results support the designation of xKv β 4 as a new Kv β family member.

The two xKv β genes have different expression patterns, suggesting that they operate in a nonoverlapping population of neurons. The dorsally restricted expression of xKv β 2 within the spinal cord (e.g., Rohon-Beard cells) is reminiscent of the expression pattern found previously for xKv1.1 (Ribera and Nguyen, 1993), suggesting that xKv1.1 α and xKv β 2 subunits form Kv complexes *in vivo*. The earliest that xKv1.1 α mRNA is detected in Rohon-Beard cells is St 26, several hours after xKv β 2 mRNA is detected. xKv β 2 is transiently detected in somitic tissue between St 24 and 30 (26–35 hr). The earliest that I_{Kv} can be consistently recorded from developing myocytes is St 24 (26 hr). Thus, just as in neurons, Kv β 2 is expressed as soon as would be required to contribute to formation of the first functional potassium channels.

xKv β 4's expression pattern differs both spatially and temporally from that of xKv β 2. Spatially, xKv β 4's expression domain is limited to the nervous system but extends more ventrally within the developing spinal cord. Temporally, xKv β 4 has a pronounced anterior–posterior gradient in its expression pattern, which is less obvious for xKv β 2. The expression pattern of xKv β 4 is similar to that of a recently identified xKv1 α gene, xKv1.3 α (A. Hoffman and A. Ribera, unpublished data), suggesting that xKv1.3 α and xKv β 4 form Kv complexes *in vivo*. However, coexpression of subunit mRNAs or proteins is not a reliable indicator of the final channel stoichiometry, because examination of mammalian brain Kv channel complexes indicates that only a subset of possible channel combinations actually exists (Rhodes et al., 1997; Shamotienko et al., 1997).

In *Xenopus* spinal neurons, developmental regulation of I_{Kv} is synchronized, despite the heterogeneity in neuronal identities as well as patterns of potassium channel gene expression (Ribera, 1996). Accordingly, each neuronal subpopulation must upregulate I_{Kv} via molecular mechanisms that are specific to the potassium channel isoforms expressed by that particular neuronal subtype. However, the different neuronal populations must also temporally coordinate their developmental increases in I_{Kv} density. The results presented here indicate that in those neuronal subpopulations using xKv1 α channels, xKv β mRNAs are present and their coexpression is able to induce relevant developmental changes in I_{Kv} . Because xKv β genes are expressed contemporaneously with Kv1 α subunits, their potential contribution to functional upregulation of I_{Kv} would be regulated at a level subsequent to transcription. In this regard, the sequence and structural similarities between Kv β subunits and aldo-keto reductase enzymes raise the possibility that developmental changes in either cellular redox potentials or concentration of substrate drive Kv β modulation of potassium channel function (McCormack and McCormack, 1994; Gulbis et al., 1999). It is possible to overexpress wild-type and mutant forms of ion channel subunits in the *Xenopus* embryo (Jones and Ribera, 1994; Ribera 1996) and thus test experimentally how Kv β subunits function *in vivo*.

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