Kv1 potassium channel complexes in vivo require Kvβ2 subunits in dorsal spinal neurons

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ABSTRACT

Whereas Kvβ2 subunits modulate potassium current properties carried by Kv1 channel complexes in heterologous systems, little is known about the contributions of Kvβ2 subunits to native potassium channel function. Using antisense approaches and in situ recordings from Xenopus embryo spinal cord neurons, we tested the in vivo roles of Kvβ2 subunits in modulation of voltage-dependent potassium current (IkV). We focused on (1) two different populations of dorsal spinal neurons that express both Kvβ2 and Kv1 α-subunit genes, and (2) the 24 and 48 hr developmental period, during which IkV undergoes developmental regulation. At both 24 and 48 hr, antisense methods produced efficient knock-down of both Kvβ2 protein and IkV. At both times, dominant negative suppression of Kv1 channels also eliminated IkV, indicating that Kv1 channels require Kvβ2 subunits in order to function in dorsal spinal neurons. Even though Kv1 channels determined the IkV of both dorsal neuron types, comparisons of their IkV properties revealed important differences at both developmental stages. The latter results support the notion that different Kv1 α-subunits and/or post-translational modifications underlie the IkVs of the two dorsal neuron types. Overall, the results demonstrate that Kvβ2 subunits function in vivo as obligatory subunits of Kv1 channels in at least two neuron types and two different developmental stages.

KEY WORDS: IkV, spinal cord, Xenopus embryo, native potassium channel, Kv1
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INTRODUCTION

Voltage-gated potassium (Kv) channels play important roles in excitable cells by regulating resting membrane potential, action potential duration, firing frequency and neurotransmitter release (Hille 2001). Functional Kv channel protein complexes contain pore-forming (α) and auxiliary (e.g., β) subunits (Trimmer 1998; Pongs et al. 1999; Li et al. 2006; Torres et al. 2007). The pore-forming α-subunits comprise members of several different Kv subfamilies (Kv1-12). When expressed in heterologous systems, Kv1, Kv2, Kv3, Kv4, Kv7, Kv10, Kv11 and Kv12 α-subunits form functional channels even in the absence of auxiliary subunits (Coetzee et al. 1999; Gutman et al. 2003).

In vivo biochemical and immunohistochemical studies have revealed that the majority of Kv1 channel complexes contain auxiliary subunits that belong to the Kvβ gene family (Kvβ1- Kvβ3; Parcej et al. 1992; Rettig et al. 1994; Scott et al. 1994; Heinemann et al. 1995, 1996; Rhodes et al. 1995, 1996, 1997; Shi et al. 1996; Nakahira et al. 1996; Shamotienko et al. 1997). Kvβ subunits also interact with Kv4 and Kv12 α-subunits (Wilson et al. 1998; Chen et al. 1996, 2000; Yang et al. 2001; but see Tang et al. 1998). Despite the widespread presence of Kvβ2 subunits in native Kv channel complexes, little is known about their physiological roles in vivo.

The limited available information about in vivo Kvβ2 subunit function has been obtained from studies of the Drosophila mutant, hyperkinetic (Hk; Ikeda and Kaplan 1970; Kaplan and Trout 1974; Chouinard et al. 1995) and a genetically engineered Kvβ2 mouse knock-out (McCormack et al. 2002). Drosophila has a
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due to a single gene, *Hk*, that is orthologous to mammalian Kvβ1, Kvβ2 and Kvβ3 genes. *Hk* mutants display an abnormal motor phenotype consisting of rhythmic leg shaking. Interestingly, Kv1 (*Shaker*) currents have reduced amplitudes and altered kinetic properties (Wang and Wu 1996; Yao and Wu 1999). The Kvβ2 knock-out mouse displays seizures, cold swimming-induced tremors, and reduced life spans (McCormack et al. 2002). Despite work from heterologous systems suggesting chaperone-like functions for Kvβ2 genes on Kv1 complexes, the mouse knock-out provided no evidence for abnormal Kv1 channel biosynthesis or trafficking upon elimination of Kvβ2 protein. These results highlight that the *in vivo* roles of Kvβ subunits are poorly understood.

Studies of Kvβ2 subunits expressed heterologously have provided information regarding their interactions and functional effects on α-subunits. Xu et al. (1998) analyzed subunit stoichiometry and found four Kvβ2 subunits per channel; that is, equal numbers of Kvβ2 and Kv1 α-subunits exist in a single Kv1 channel complex. *In vitro*, Kvβ2 subunits produce diverse functional consequences, including effects on voltage-dependence of activation, activation and inactivation kinetics, channel surface membrane expression and current density (Rettig et al. 1994; Heinemann et al. 1996; Morales et al. 1996; Manganas and Trimmer 2000). Because Kvβ2 subunits increase channel complex stability and cell surface insertion of Kv1 α-subunits in heterologous systems, chaperone-like roles have been proposed for Kvβ2 subunits (Shi et al. 1996; Accili et al. 1997; Nagaya and Papazian 1997; Campomanes et al. 2002).
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Because effects seen in heterologous systems do not always recapitulate mechanisms in vivo, we took advantage of unique features of Xenopus embryonic spinal cord neurons to test the in vivo function of Kvβ2 subunits. Voltage-gated potassium currents (I\textsubscript{K\textupsilon}) have been studied in vitro as well as in vivo in spinal neurons of the Xenopus embryo (O'Dowd et al. 1988; Desarmenien et al. 1993; Pineda and Ribera 2008). The Xenopus embryo expresses Kvβ2 mRNA in spinal cord neurons during the same developmental period during which extensive regulation of voltage-gated potassium current occurs (Lazaroff et al. 1999). Further, the developmental changes in I\textsubscript{K\textupsilon} consist of increases in current density and acceleration of activation kinetics (Barish et al. 1986; O'Dowd et al. 1988; Lockery and Spitzer 1992), potassium current properties that are modulated by Kvβ2 subunits when coexpressed heterologously with Kv1 α-subunits. Moreover, dorsal spinal neurons express Kv1.1 α-subunit and Kvβ2 transcripts providing an experimentally accessible and relevant neuronal population for the study of the in vivo roles of Kvβ2 subunits (Ribera and Nguyen 1993; Lazaroff et al. 1999).

To test the in vivo roles of Kvβ2 subunits, we used two different antisense (AS) strategies to knock-down Kvβ2 protein. Western blot analysis indicated that morpholino oligonucleotides (MOs) produced effective knock-down of Kvβ2 protein. We focused on two different populations of neurons in the dorsal spinal cord: Rohon-Beard (RB) primary mechanosensory neurons, and immediately adjacent dorsal non-Rohon Beard cells (non-RB). In the absence of Kvβ2 protein, specific populations of dorsal spinal cord neurons that express the gene
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displayed a near total elimination of $I_{Kv}$. This was true for both dorsal neuron
types at two different developmental stages. However, $I_{Kv}$ properties differed
substantially between RBs and non-RBS and as a function of development. As
for Kvβ2 knock-down, the Kv1 dominant negative efficiently suppressed $I_{Kv}$
channel in the two different neuron types regardless of developmental stage.
Thus, regardless of developmental stage, Kvβ2 protein knock-down reduced $I_{Kv}$
density in spinal neurons as effectively as did dominant negative suppression of
Kv1 channel function. Taken together, the results support the view that Kvβ2
subunits play an essential role in Kv1 channel function. Moreover, because Kvβ2
knock-down effectively suppressed $I_{Kv}$ in both 24 and 48 hr neurons, the
essential role of Kvβ2 in Kv1 channel function is constant and not
developmentally regulated during a period when $I_{Kv}$ properties undergo
substantial changes.
Methods

Xenopus embryos and microinjection

All experimental procedures were approved by the Animal Care and Use Committees of the Center for Comparative Medicine at the University of Colorado Denver at the Anschutz Medical Campus. In vitro fertilization and RNA microinjection were performed as described previously (Jones and Ribera 1994). Embryos were staged on the basis of external morphology (Nieuwkoop and Faber 1967).

Working MO (1-150 pg/nl) or RNA (120-200 pg/nl) solutions were prepared by dilution of stock aliquots with RNAse-free water containing as a lineage tracer RNA encoding green fluorescent protein (GFP; 6 ng/nl; kind gift of Dr. Michael Klymkowsky, University of Colorado, Boulder; Blaine and Ribera 2001). A total volume of 10 nl was injected into one cell of two-cell stage embryos using a gas-driven injection apparatus (2-3 psi for 2 sec; PLI-100, Medical System Corp. Greenvale, NY) with fine-drawn micropipettes (~1-2 μm tip diameter; Sutter P-87 Puller, Sutter Instrument Co., Novato, CA). The day after injection, embryos were examined with epifluorescent illumination and those expressing GFP within the neural tube were kept at room temperature until the desired developmental stage.

Knock-down of Kvβ2: Kvβ2 function was knocked-down by injection of either morpholino (MO) or antisense RNA. MOs were designed and synthesized by GeneTools (Philomath, OR). The Xenopus Kvβ2 (Kvβ2MO) targeted the predicted translation start methionine and had the following sequence: 5' -
Native Kv1 potassium channels require Kvβ2 subunits. The control Kvβ2MO (CtlMO) was designed by inverting the Kvβ2MO sequence: (5’-TACATAggTCTTAgCTggTgTCTgA-3’). Aliquots of MO stock solutions were prepared by resuspending the oligonucleotides in RNAse-free water at a final concentration of 12.5 μg/μl (1.5 mM) and stored at -80°C. For both Kvβ2MO and antisense Kvβ2, dose-response curves were determined to assess specificity of the knock-down.

Antisense Kvβ2 RNA (ASβ2) was synthesized as described previously (Lazaroff et al. 2002). Briefly, the plasmid containing Kvβ2 (pCS2+) was linearized with HindIII and cRNA was synthesized by in vitro transcription with T7 RNA polymerase (Promega, Madison, WI) in the presence of ribonucleotide triphosphates (Pharmacia Biotech, Piscataway, NY). As a control for the antisense, an irrelevant RNA (GFP) was used. We found no differences between Ikv in neurons derived from uninjected or GFP injected blastomeres at either 24 or 48 hr (not shown). In addition, previous work (Lazaroff et al., 2002) demonstrated ASβ2 selectively eliminated effects of Kvβ2 but not Kvβ4 RNA injection into Xenopus oocytes.

Dominant Negative Kv1 α-subunit: The Kv1 α-subunit dominant negative (Kv1DN) was generated as described previously (Ribera 1996). cRNA was synthesized by linearizing the plasmid with XbaI and in vitro transcription with SP6 RNA polymerase in the presence of ribonucleotide triphosphates (Pharmacia Biotech, Piscataway, NY) and cap analogue (Boehringer Manheim, Indianapolis, IN). RNA concentrations were determined spectrophotometrically.
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**Protein extraction**

St 34/35 Xenopus embryos where homogenized in MK lysis buffer (in mM: 50 Tris pH 8.0, 150 NaCl, 0.5% NP40, 0.5% Triton-X100, 1 EGTA, pH 7.4; Klymkowsky Lab On-line Methods; http://spot.colorado.edu/~klym/) containing 1x protease inhibitor (Halt Protease Inhibitor Cocktail Kit; Pierce, Rockford, IL) or 2% SDS in 50 mM Tris pH 7.5. Homogenates were centrifuged and embryo supernatants were treated to remove excess lipid with PHM-L Liposorb absorbent according to the manufacturer instructions (Calbiochem, San Diego, CA). Protein extract aliquots were stored at -80°C until use.

**Western Blots**

20 μg of whole embryo protein extracts were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore, Billerica, MA) by wet electrotransfer (Towbin et al. 1992). Prior to incubation with antibody, membranes were blocked for 2 hr in Tris-buffered saline (TBS; in mM: 136 NaCl, 2.6 KCl, 24.7 Tris, pH 7.4) with 5% nonfat evaporated milk and 0.1% Tween20. Blots were then incubated overnight at 4°C in blocking buffer containing the primary antibody, either anti-Kvβ2 (1:50, Clone 17/70, NeuroMab, Davis, CA; www.neuromab.org; Bekele-Arcuri et al. 1996) or anti-Kv1.1 (1:50, clone K20/78,
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NeuroMab). After rinsing in TBST (TBS containing 0.5% Tween20), blots were incubated with secondary antibody. For standard western blot analysis, a horseradish peroxidase-conjugated anti-mouse secondary antibody was used (1:2000; Bio-Rad Laboratories, Hercules, CA). Blots were then incubated in a chemiluminescent substrate at room temperature for 1-5 min (Pierce, Rockford, Ill) and imaged using a Kodak Image Station 440 CF and Molecular Imaging Software (Carestream Health Inc., Rochester, NY). These experiments were repeated at least three times.

For quantitative measurements, blots were incubated with an Alexa 647-conjugated anti-mouse secondary antibody (1:2000; Invitrogen, Carlsbad, CA) for 2 hr at room temperature (20–22°C) and then scanned using a Typhoon 9400 multi-mode imager (GE Healthcare; Little Chalfont, Buckinghamshire, United Kingdom). Gels were analyzed using ImageQuant Densitometer software (Molecular Dynamics, GE Healthcare; Pittsburg, PA). An image of a representative assay is shown as well as average data for the total of three experiments.

Semi-intact preparations of Xenopus embryos

St 22/23 and St 35/36 Xenopus embryos were dissected using slight modifications of methods previously described for semi-intact preparations of zebrafish embryos (Ribera and Nüsslein-Volhard 1998; Pineda et al. 2005). Briefly, in the presence of Ringer solution (in mM: 145.0 NaCl, 3.0 KCl, 1.8 CaCl₂, 10.0 HEPES, pH 7.2) containing 0.02% Tricaine (Ethyl 3-aminobenzoate
methanesulfonate salt, Sigma-Aldrich, St Louis, MO), the yolky endoderm was removed and embryos were mounted ventral side-down onto glass coverslips using Vetbond Tissue Adhesive (3M Animal Care Products, St Paul, MN). Embryos were then killed in the presence of anesthesia by transection at the level of the hindbrain. Removing the skin and dorsal fin fold exposed the spinal cord. Tricaine was removed by washing the preparation with ≥ 40 ml of recording solution over the course of 15 minutes. Preparations were viewed with differential interference contrast optics on an Axioskop FS2 microscope (Carl Zeiss MicrolImaging GmbH, Hamburg) at a magnification of 640X. RB cells were identified on the basis of: a) superficial location at the dorsal surface of the spinal cord, b) large soma diameter (~20 μm) and c) position relative to the midline (Baccaglini and Spitzer 1978). On the basis of the absence or presence of GFP, cells were identified as internal control (GFP⁻) or MO, AS or Kv1DN (GFP⁺) neurons, as done previously (Blaine and Ribera 2001).

Electrophysiological methods

Conventional whole-cell patch-clamp techniques (Hamill et al. 1981) were used in voltage-clamp mode. Experiments were conducted at room temperature (20–22°C) using an Axopatch 200B amplifier and a Digidata 1440A analog to digital (A/D) interface in conjunction with the pClamp 10.0 software recording package (Molecular Devices Corp., Sunnyvale, CA).

Unpolished electrodes were fabricated from borosilicate glass (Microcaps; Drummond Scientific Co. Broomall, PA) with tip resistances ranging between 2.0
and 3.5 MΩ when filled with pipette solution (in mM: 100 KCl, 10 mM EGTA, 10 HEPES, pH 7.4 with NaOH). After establishment of the whole cell configuration, monoexponential capacitative transients were indicative of adequate spatial control of membrane voltage. Additional criteria were used to assess the quality of the recordings: (1) membrane resistance > 120 MΩ, (2) holding current < 250 pA, and (3) stable access resistance of <12 MΩ.

For recording of the outward potassium currents, the bath solution contained (in mM): 80 NaCl, 3 KCl, 5 MgCl₂, 10 CoCl₂, 5 HEPES and 0.003 TTX, pH 7.4 with NaOH. Currents were elicited by 60 ms depolarizing voltage steps to test potentials ranging between -60 and +100 mV in 10 mV increments from a holding potential of -80 mV; tail currents were recorded at -40 mV after the activating steps. Series resistance was routinely compensated by 75-85% with a lag of 10 μsec. Currents were filtered at 5 kHz and digitized at 25 kHz. Passive leak and capacitative transients were subtracted on line using the P/8 algorithm of the software.

Data Analysis

Data analysis was accomplished using AxoGraph 10 (Axograph Scientific, Sidney, AU), Excel (Microsoft Corp. Redmond, WA) and Origin (OriginLab Corp. Northampton, MA) software. Variability in cell size was accounted for by dividing current amplitudes by the membrane capacitance, which serves as an indicator of membrane surface area (1 pF/cm²; Marty and Neher 1983). Thus, current data are presented as current densities (pA/μm²).
For current density-voltage (I-V) plots, steady state currents were measured by averaging values during a 10 ms interval at the end of each pulse (from 45 to 55 ms). Conductance-voltage (G-V) relationships were constructed by dividing current density by driving force, using the calculated potassium equilibrium potential of -88.3 mV. The Boltzmann equation \( G = \frac{G_{\text{max}}}{1 + \exp\left[\frac{(V_{1/2} - V)}{k}\right]} \); where \( G_{\text{max}} \) = maximal conductance; \( V_{1/2} \) = voltage of half maximal activation and \( k \) = slope factor) was fitted to the data. G-V plots were not corrected for the small voltage errors (< 4 mV) introduced by the uncompensated fraction of the series resistance.

Data Presentation

Results are presented as means ± SEM. Statistical analysis was performed using GraphPad InStat software (GraphPad Software, San Diego, CA). Statistical comparisons were done using the Student’s t-test or ANOVA, for comparisons of two or multiple groups, respectively. ANOVA analysis was followed by Bonferroni correction to consider multiple comparisons. A threshold of \( p \leq 0.05 \) was used to determine statistical significance.
RESULTS

The known molecular determinants of potassium current in the dorsal spinal cord of *Xenopus* embryos consist of Kv1 α-subunits and Kvβ2 subunits (Ribera and Nguyen 1993; Lazaroff et al. 1999). To investigate the functional role of Kvβ2 subunits *in vivo*, we developed a semi-intact preparation that allowed us to identify two different types of dorsal spinal cord neurons. Primary sensory RB and neighboring dorsal non-RB neurons were identified during an experiment on the basis of position and soma diameter (Fig. 1A). RBs had larger soma membrane area, as assessed by cell capacitance, than did non-RB neurons (Fig. 1B). The difference in cell size between RBs and non-RBs permitted their reliable identification *in situ*.

*Knock-down of Kvβ2 protein reduced I_{Kv} density*

We tested the contribution of the Kvβ2 auxiliary subunit to I_{Kv} of RB and non-RB dorsal neurons by using both morpholino and standard antisense RNA strategies to knock-down the Kvβ2 protein in the *Xenopus* embryo. In 24 hr embryos injected unilaterally with 1 ng Kvβ2MO and GFP RNA, we recorded from internal control GFP− RB neurons and GFP+ neurons (see Methods). GFP− RB neurons displayed substantial I_{Kv} (Fig. 2A). In contrast, in GFP+ RB neurons, we found effective suppression of I_{Kv} (Fig. 2A). Similar effects of Kvβ2MO on I_{Kv} were observed in non-RB neurons (Fig. 2B). We also determined the dose-response relationship for the Kvβ2 morpholino (Kvβ2MO) on I_{Kv} density (Fig. 2C). Morpholino injection led to a steep dose-dependent decrease in I_{Kv}...
amplitude in both RB and non-RB cells. For injected doses less than 0.02 ng, the Kvβ2MO blocked between 20-30% of the recorded total $I_{Kv}$ amplitude. Using a dose equal to or higher than 0.05 ng resulted in a surprising almost complete knock-down of $I_{Kv}$. In contrast, injection of the CtlMO at a dose of 1 ng had no effect on $I_{Kv}$ density in either RB or non-RB neurons (Fig. 2D).

In heterologous systems, Kvβ2 subunits increase surface membrane expression and current density produced by Kv1 α-subunits; however, they are not required for formation of functional Kv1 channels (Coetzee et al. 1999; Gutman et al. 2003). Given this precedent, we were surprised by the complete knock-down of $I_{Kv}$ produced by Kvβ2MO and concerned about non-specific effects. To confirm that the Kvβ2MO acted as expected by knocking-down Kvβ2 protein, we used western blot analysis to determine Kvβ2 protein levels in uninjected, CtlMO- and Kvβ2MO-injected embryos (Fig. 3). We used a dose of MO that led to complete knock-down of $I_{Kv}$ (1.0 ng; Fig. 2C). In uninjected embryos, the levels of Kvβ2 protein increased slightly, but significantly, between 24 and 48 hr (Fig. 3A, B; p<0.01). Further, embryos injected with CtlMO had Kvβ2 protein levels similar to those of uninjected embryos, suggesting that the injection itself or MOs in general did not produce non-specific effects on $I_{Kv}$. In contrast, embryos injected with Kvβ2MO had significantly reduced levels of Kvβ2 protein at both 24 and 48 hr ($p \leq 0.001$). Kvβ2MO had no effect on levels of the internal control, β-tubulin (Fig. 3B). These results suggest that the Kvβ2MO led to a specific knock-down of Kvβ2 protein.
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If the results obtained with the Kvβ2MO were directly due to Kvβ2 knock-down rather than possible off-target effects, other methods that result in Kvβ2 knock-down would be expected to produce similar effects (Eisen and Smith 2008). As an alternative to MO-mediated knock-down of Kvβ2 protein, we used traditional RNA antisense (AS) methods to block translation of Kvβ2 subunits. We previously used this method to knock-down Kvβ2 subunits and found that it effectively eliminated endogenous Kvβ2 mRNA in Xenopus embryos and Kv1 currents in embryonic myocytes (Lazaroff et al. 2002).

As found for the Kvβ2MO, antisense Kvβ2 (ASβ2) led to an efficient knock-down of $I_{Kv}$ in a dose-dependent manner in both RB and non-RB neurons (Fig. 4). In 24 hr embryos injected unilaterally with 1 ng ASβ2, internal control RB neurons displayed substantial $I_{Kv}$. In contrast, in GFP+ RB we found effective suppression of $I_{Kv}$ (Fig. 4A). Similar effects of ASβ2 on $I_{Kv}$ were observed in non-RB neurons (Fig. 4B). In comparison to the Kvβ2MO dose-response curve, the ASβ2 dose-response curve was less steep (Fig. 4C). However, at doses of 0.25 ng or greater of ASβ2, $I_{Kv}$ was effectively eliminated in both RB and non-RB neurons. Therefore, two different strategies for blocking Kvβ2 protein translation produced similar and efficient reductions in $I_{Kv}$ density.

Because Kvβ2MO and ASβ2 produced a surprisingly efficient reduction of $I_{Kv}$ amplitude, we used several different tests to assay the specificities of the knock-down methods. As mentioned, both Kvβ2MO and ASβ2 had effects on $I_{Kv}$ that were dose-dependent as expected for a specific action of an antisense agent.
Native Kv1 potassium channels require Kvβ2 subunits (Figs. 2, 4). Moreover, upon injection of sub-saturating doses of either Kvβ2MO or ASβ2, I_k decreased in amplitude (Figs. 2, 4) but did not show changes in kinetic or voltage-dependent properties (Table 1). The latter result is consistent with normal function of the Kvβ2 subunit in the remaining Kv channels that contribute to the residual I_k rather than non-specific actions of either Kvβ2MO or ASβ2 on membrane currents.

In addition, the CtlMO did not affect I_k indicating that MO injection per se did not produce non-specific effects on I_k. (Fig. 2D). Further, as assessed by Western blot analysis, the Kv1.1 protein showed no apparent reduction in mobility or amount in extracts prepared from 48 hr old embryos injected with 1 ng of either MOβ2 or ASβ2 (Fig. 5), demonstrating that the antisense agents did not target other Kv proteins. We also tested whether the Kvβ2MO or the ASβ2 had non-specific effects on general membrane properties. Uninjected, CtlMO, Kvβ2MO and ASβ2 treated neurons had similar membrane input resistances (Fig. 6).

An additional test of Kvβ2MO specificity entailed recording I_k from ventral spinal neurons, a population that does not express the Kvβ2 gene (Lazaroff et al. 1999). As expected if the morpholino targeted Kvβ2 mRNA specifically, Kvβ2MO injection had no effect on I_k density of ventral neurons (Fig. 7).

Overall, the results indicated that both Kvβ2MO and ASβ2 specifically targeted Kvβ2. Moreover, the MO and antisense studies suggest that, in contrast to results obtained from heterologous systems, Kvβ2 subunits are required in vivo for formation of functional Kv channels in both RB and non-RB dorsal cells.
**Dominant negative suppression of Kv1α function also abolished Ikv in RB and non-RB dorsal cells**

The above data suggest that Kvβ2 subunits are required for the formation of functional Kv1 potassium channel complexes in dorsal spinal neurons. In heterologous systems, Kvβ2 subunits associate with Kv1, Kv4 and Kv12 α-subunits. Whereas expression of Kv4 or Kv12 α-subunits in *Xenopus* embryos has not been reported, evidence for Kv1 α-subunit expression in the spinal cord exists. Kv1.2 α-subunit mRNA is expressed in the dorsal ectoderm at the time of neural induction (Ribera 1990). Kv1.1 α-subunit transcripts localize to dorsal regions of the *Xenopus* spinal cord, similarly to Kvβ2 mRNA (Ribera and Nguyen 1993; Lazaroff et al. 1999). On this basis, Kv1 α-subunits are good candidates for targets of Kvβ2 association and modulation.

Overexpression of a dominant negative Kv1 α-subunit (Kv1DN) has served previously as an efficient method for blocking the contribution of Kv1α subunit containing channels to Ikv (Ribera 1996; Lazaroff et al. 2002). We used this approach to block functional Kv1 channels *in situ*. We found that dominant negative suppression of Kv1 α-subunit containing channels produced a near total reduction in Ikv amplitude in both RB and non-RB neurons (Fig. 8), suggesting that Kv1 channels underlie the Ikv of dorsal spinal neurons. Moreover, dominant negative suppression of Kv1 channels reduced Ikv amplitudes as effectively as did Kvβ2MO or ASβ2, supporting the view that both β as well as α-subunits are obligate members of functional Kv1 channel complexes.
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**RB I_{Kv} showed developmental changes in density but not the apparent rate of activation**

On the basis of previous studies of spinal neurons developing in culture, the I_{Kv}s of RB and non-RB neurons should undergo dramatic changes in density and activation kinetics between 24 and 48 hr (Barish 1986; O’Dowd et al. 1988). To test this prediction, we determined how I_{Kv} changed *in vivo* between 24 and 48 hr in RB and non-RB neurons.

RB I_{Kv} density increased ~5 fold between 24 and 48 hr (Fig. 9A, B; 0.20 ± 0.06 pA/µm^2 to 0.90 ± 0.10 pA/µm^2, respectively, at +20 mV; p<0.0001). In contrast, during the same period, the rate of activation, evaluated as the time to half maximum current (t_{1/2}), did not change (Fig. 9C; 2.50 ± 0.23 versus 2.48 ± 0.28 ms for 24 and 48 hr, respectively, at +20 mV). Compared to the average developmental changes noted for I_{Kv} of spinal neurons in culture (e.g., O’Dowd et al. 1988), RB I_{Kv} *in vivo* showed larger increases in current density but no decrease in t_{1/2}.

Consistent with the changes in current density, conductance density increased ~6-fold between 24 and 48 hr (Fig. 9D; 0.30 ± 0.07 pS/µm^2 to 1.60 ± 0.20 pS/µm^2, respectively, p<0.0001). To examine voltage-dependent properties of activation, we determined the maximal conductance (G_{max}) and then plotted normalized conductance as a function of voltage. The Boltzmann equation was fitted to the normalized conductance plots (Fig. 9E) to calculate the voltage of half activation (V_{1/2}) and the slope constant (k) for each developmental stage. Although no differences were found for V_{1/2} (Table 2), a small but significant
Native Kv1 potassium channels require Kvβ2 subunits decrease in voltage sensitivity, as reflected by an increase in the slope factor $k$, was found (Table 2; $p<0.0001$). The small increase in $k$ has no obvious physiological significance and mostly likely reflects distortions in the normalized curve due to non-saturation of the conductance. Overall, these results reveal developmental changes in the maximal conductance of RB $I_{Kv}$.

**Non-RB $I_{Kv}$ showed different developmental changes**

Between 24 and 48 hr, non-RB $I_{Kv}$ density increased ~4-fold from $0.30 \pm 0.10$ pA/µm$^2$ to $1.10 \pm 0.20$ pA/µm$^2$ (Fig. 10A, B; $+20$ mV; $p<0.0001$). However, no statistically significant differences were found between RB and non-RB $I_{Kv}$ densities when compared at the same stage developmental stage (cf., Fig. 9A, B and Fig. 10A, B).

In contrast to RB cells, the $t_{1/2}$ of non-RB $I_{Kv}$ decreased significantly between 24 and 48 hr (Fig. 10C; at $+20$ mV: $3.30 \pm 0.07$ ms to $2.00 \pm 0.40$ ms, respectively; $p<0.03$), reflecting the more rapid apparent activation of non-RB $I_{Kv}$ at the later developmental stage. The developmental decrease in $t_{1/2}$ of non–RB $I_{Kv}$ is consistent with the average changes in $t_{1/2}$ reported for neurons in culture or dorsolateral neurons in situ (O’Dowd et al. 1988; Desarmenien et al. 1993).

Consistent with the changes in current density, non-RB conductance density increased significantly ~3-fold between 24 and 48 hr (Fig. 10D; $0.55 \pm 0.10$ pS/µm$^2$ to $1.60 \pm 0.30$ pS/µm$^2$, respectively, $p<0.03$). Contrary to RB $I_{Kv}$, however, non-RB $I_{Kv}$ showed a negative shift in the $V_{1/2}$ for steady state activation between 24 and 48 hr (Fig. 10E; Table 2), an effect that would lead to
increased current amplitudes at more depolarized potentials. In addition, voltage sensitivity, as assessed by the slope factor $k$, decreased between 24 and 48 hr (Table 2; $p<0.001$). These results indicate that potassium conductance increased and altered its voltage-dependent properties of activation between 24 and 48 hr.

As predicted on the basis of study of spinal neurons in culture (Barish 1986; O'Dowd et al., 1988), the $I_{Kv}$s of RB’s and non-RBs showed substantial developmental regulation between 24 and 48 hr of embryonic development (Figs. 9, 10). However, the changes in each neuron type differed. The $I_{Kv}$ of RB neurons increased in 5-fold in density without any apparent changes in activation kinetics. In non-RB neurons, $I_{Kv}$ also increased in density, ~4-fold. In contrast to RBs, $I_{Kv}$ of non-RB neurons also showed an apparent increase in activation kinetics, as assessed by a decrease in $t_{1/2}$.

*Kv1* α-subunits underlie developmental changes in $I_{Kv}$ of both RB and non RB-neurons

Knock-down of either Kvβ2 or dominant negative suppression of Kv1 channels effectively eliminated the $I_{Kv}$s of RB and non-RB neurons at 24 hr (Figs. 2, 4, 8). Previous results suggest that developmental changes in $I_{Kv}$ in some spinal neurons developing in culture rely upon Kv1 channels (Gurantz et al. 1996; Ribera 1996). Accordingly, we next determined whether Kv1 channels account not only for the initial $I_{Kv}$ recorded at 24 hr from RB and non-RB neurons, but also for the different developmental changes in $I_{Kv}$ in each neuron type.
Native Kv1 potassium channels require Kvβ2 subunits

Similar to results obtained at 24 hr (Fig. 2), Kvβ2MO injection led to efficient elimination of I_{Kv} at 48 hr (Fig. 11A). Kvβ2MO also led to efficient knock-down of I_{Kv} in non-RB neurons at 48 hr (Fig. 11B). However, inward current densities did not differ between uninjected, CtlMO, or Kvβ2MO neurons at 48 hrs (Fig. 11C). Further, injection of ASβ2 produced results for RB (Fig. 11D) and non-RB (Fig. 11E) that were similar to those obtained by injection of Kvβ2MO. These results indicate that at both 24 and 48 hr, knock-down of Kvβ2 subunits eliminated I_{Kv}.

We next tested whether Kv1 channels determined I_{Kv} in dorsal spinal neurons at 48 as well as at 24 hr. Kv1 dominant negative suppression of Kv1 channels reduced I_{Kv} amplitudes of RB and non-RB neurons at 48 hr as efficiently as at 24 hr (Fig. 11D, E). These findings support the view that Kv1 channels underlie I_{Kv} of dorsal spinal neurons at both developmental stages. Taken together, the results indicate that, at both 24 and 48 hr, Kvβ2MO effects were due to elimination of Kv1 channel function.
DISCUSSION

Typically, the effects of Kvβ subunits on Kv1 channel function entail modulation of the kinetics and amplitude of current carried by the pore-forming α-subunit. Our results point to a yet more fundamental role in vivo, whereby Kv1 channel function has an essential requirement for the Kvβ2 subunit in dorsal spinal neurons. Knock-down of Kvβ2 protein, by either MO or RNA antisense methods, produced a near total reduction in I_{Kv} amplitude in dorsal spinal cord neurons. Further, partial knock-down of Kvβ2 protein only reduced the amplitude but not the properties (e.g., t_{1/2}, V_{1/2}) of I_{Kv}, consistent with normal function of Kvβ2 subunits in channels underlying the residual current (Fig. 3, Table 1). Moreover, Kvβ2 knock-down produced effects similar to those of dominant negative suppression of Kv1 channel complexes, as expected if Kvβ2 subunits function as obligatory components of Kv1 channels.

In heterologous systems, Kvβ2 subunits modulate properties of potassium currents (Shi et al. 1996; Accili et al. 1997; Rettig et al. 1994; England et al. 1995a,b; Majumder et al. 1995; Heinemann et al. 1994, 1995, 1996; Morales et al. 1996) that are developmentally regulated in embryonic spinal neurons, e.g., current density and activation kinetics (Barish 1986; O’Dowd et al. 1988; Desarmenien et al. 1993). However, we found that at both 24 and 48 hr, Kvβ2 knock-down led to efficient elimination of I_{Kv}. These findings indicate that rather than play a role in developmental regulation of Kv1 channels, Kvβ2 subunits are always required for Kv1 channel function in two different dorsal spinal cord neurons.
Native Kv1 potassium channels require Kvβ2 subunits

The essential in vivo requirement for Kvβ2 subunits differs from conclusions obtained from study of heterologously expressed subunits. When expressed in *Xenopus* oocytes or cell lines (e.g., HEK293), Kvβ2 subunits can increase the surface and functional density of Kv1 channels. However, in heterologous systems, Kv1 channel formation occurs in the absence of the Kvβ2 subunit. It is possible that when Kv1 α-subunits are expressed at physiological levels, the role of the Kvβ2 subunit becomes essential. In contrast, under the aphysiological and high expression levels associated with heterologous systems, the essential requirement may be masked. Further, we focused on two dorsal neuron types that are known to express Kvβ2 subunits but not other Kvβ subunits. It is likely that, for neurons that express multiple Kvβ subunits, another Kvβ may replace Kvβ2 when the latter is eliminated. Thus, the essential role of Kvβ2 subunits may only be evident when it is absent in cells that express no other Kvβ subunit.

Heterologous system results would predict a reduction in Kv1 α-subunit protein levels, maturation and/or surface expression in the absence of Kvβ2. However, McCormack et al. (2002) found that the Kv1.2 α-subunit underwent normal glycosylation to its mature form in a mouse Kvβ2 knock-out. In addition, Kv1.2 α-subunit protein levels, as assessed by Western blot, were unchanged by knock-out of Kvβ2. Similarly, our results did not show any apparent changes in Kv1.1 protein expression as determined by Western blot analysis in ASβ2 or Kvβ2MO injected embryos (Fig. 5). In the murine model, immunofluorescent
Native Kv1 potassium channels require Kvβ2 subunits

examination of Kv1.1 and Kv1.2 α-subunits in brain sections also showed no
differences between wild type and knock-out with respect to either localization or
amount (McCormack et al. 2002).

The knock-out mice were also examined for behavioral deficits but not for
defects that would be evident at the level of cellular electrophysiology. Our
results suggest that the increased seizure incidence and shorter life spans of
Kvβ2 knock-out mice might be a direct consequence of severe loss of Kv1
channel function. Consistent with this view, Kv1.1 α-subunit knock-out mice also
have seizures (Smart et al. 1998; Wenzel et al. 2007). Similarly, Kv1.2 α-
subunit knock-out mice have seizures and reduced life spans (Brew et al. 2007).

Our results are consistent with those obtained by study of knock-out mice.
We found that Kvβ2 subunits were not required for maturation of Kv1.1 α-subunit
containing channels in the endoplasmic reticulum and Golgi, because no
increase in the immature form was detected after knock-down of Kvβ2 (Fig. 5).
On the basis of our data, we conclude that Kvβ2 subunits affect trafficking of Kv1
channels to the surface membrane or the function of Kv1 channels in the surface
membrane.

The finding that either Kvβ2 knock-down or dominant negative
suppression of Kv1 channel function eliminated $I_{Kv}$ leads to two additional
conclusions. Our results provide evidence for Kvβ2 participation in Kv1 but not
other Kv channels (e.g., Kv4, Kv12; Wilson et al. 1998; Yang et al. 2001).
Previously, we found that Kvβ2 knock-down and Kv1 dominant negative
suppression reduced $I_{Kv}$ to the same extent in embryonic myocytes (Lazaroff et
Native Kv1 potassium channels require Kvβ2 subunits

al. 2002). While the identity of the current that persisted upon Kvβ2 knock-down and Kv1 dominant negative suppression was not determined, Kv2 channels are likely candidates because myocytes express the gene (Burger and Ribera 1996). Because neither previous nor the present study examined cell types that are known to express either Kv4 or Kv12 α-subunits, our results do not exclude the possibility that Kvβ2 subunits might affect function of Kv4 or Kv12 channels in vivo.

Second, recent work has indicated that the I_KVs of dorsal (Type I) and ventral (Type II) spinal cord neurons differ with respect to biophysical and pharmacological properties (Pineda and Ribera 2008). We interpret these data as evidence for Kv1 channel complexes serving as the molecular determinants of I_Kv in dorsal Type I but not ventral Type II spinal neurons. Interestingly, even though Kv1 channels underlie the I_KVs of two different dorsal spinal neuron types, our results demonstrated functional differences between the I_KVs of RB and non-RB neurons. The developmental increases in potassium conductance were similar in RBs and non-RBs (5- and 4-fold, respectively; Figs. 9, 10). However, the developmental changes in activation kinetics differed (Figs. 9, 10). Between 24 and 48 hr, the t_1/2 did not change for RB I_Kv but did for that of non-RBs. From study of spinal neurons in culture, a decrease, rather than no change, in t_1/2 was expected (O'Dowd et al. 1988).

The simplest interpretation of these data is that (1) RBs and non-RBs express different Kv1 α-subunit isotypes and (2) Kvβ2 subunits are obligate members of a diverse range of Kv1 channel complexes at the developmental
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stages relevant to our studies. An alternative more complex interpretation is that RBs and non-RBs express the same Kv1 \(\alpha\)-subunit isotype but then apply different post-translational mechanisms to sculpt functional properties of the native current during development. The latter possibility is especially intriguing given the findings of Desarmenien and Spitzer (1991). These authors found that the developmental change in activation kinetics of \(I_{kV}\) was partially accounted for by a calcium/protein kinase C dependent mechanism. Desarmenien and Spitzer (1991) studied spinal neurons in culture of unknown identity. Our data suggest that the partial dependence on calcium and protein kinase C was a consequence of the necessary population analysis of the heterogeneous spinal cord neuron types that exist in culture, rather than study of specific cell types that is possible in the \textit{in situ} preparations. For example, we found that the \(I_{kV}\) of non-RBs, but not RBs, showed a developmental change in activation kinetics. In addition, it is possible that the calcium/protein kinase C mechanism could target some potassium channel subunit isotypes but not others. The \textit{in situ} preparation that we have developed will allow future testing of these possibilities.

Overall, the results indicate that the “auxiliary” Kvβ2 subunit plays an essential role for Kv1 channel function in dorsal spinal neurons. In addition, Kv1 channel function shows the same absolute requirement for Kvβ2 subunits at both 24 and 48 hr. Thus, Kvβ2 subunits do not mediate the changes in \(I_{kV}\) that occur during this period. Rather, developmental changes in Kv1 \(\alpha\)-subunit isoform expression or post-translational modification underlie maturation of \(I_{kV}\) in dorsal spinal neurons.
Native Kv1 potassium channels require Kvβ2 subunits
GRANTS
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ACKNOWLEDGEMENTS
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REFERENCES


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Chen ML, Hoshi T, Wu CF. Heteromultimeric interactions among K+ channel subunits from *Shaker* and *eag* families in *Xenopus* oocytes. *Neuron* 17: 535-542, 1996.


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Native Kv1 potassium channels require Kvβ2 subunits


Native Kv1 potassium channels require Kvβ2 subunits


Figure Legends

FIG. 1. Semi-intact preparation of the *Xenopus* spinal cord allowed electrophysiological study of two different neuronal populations. A: Using DIC optics, we identified neurons in semi-intact spinal cord preparations of the St 35/36 *Xenopus* embryos. RBs were identifiable on the basis of their characteristic position and large diameter (~20 μm). In the photo, a patch pipette contacts an RB. A non-RB neuron is present to the left of the RB and out of focus (asterisk). B: At both 24 and 48 hrs, RBs had significantly larger cell surface membrane area than neighboring non-RBs (p<0.001,) as assessed by cell membrane capacitance.

FIG. 2. Injection of Kvβ2MO reduced $I_{Kv}$ current density in RB and non-RB neurons in a dose-dependent manner. A: In 24 hr Kvβ2MO-injected (1 ng MO) embryos, internal control GFP−RB neurons (left) displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ RB neurons (right) displayed no or small $I_{Kv}$ amplitudes. B: In Kvβ2MO-injected (1 ng MO) embryos, internal control GFP− non-RB neurons (left) displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ non-RB neurons (right) displayed no or small $I_{Kv}$ amplitudes. C: Kvβ2MO doses ranging between 0.001–1.5 ng were injected into *Xenopus* embryos and $I_{Kv}$ was recorded from RB (black triangles) and non-RB (white triangles neurons) at 24 hrs. The percentage of remaining, unblocked current is shown as normalized current amplitude. N's ranged between 3 and 7. D: In contrast to the Kvβ2MO, the CtlMO (1.0 ng) had
no effect on $I_{Kv}$ density in either RB or non-RB cells. $N$'s ranged between 9 and 16.

FIG. 3. Kvβ2MO injection produced efficient and selective knock-down of Kvβ2 protein. A: Embryos were either uninjected or injected with 1.0 ng of Kvβ2MO orCtlMO. Total protein was extracted from 24 and 48 hr embryos. ~20 µg of total protein were loaded in each lane. Kvβ2 protein was detected as a doublet at 35 at 37 KDa. β-tubulin (50 KDa) was used as an internal control to test for non-specific effects of Kvβ2MO on protein levels. B: The relative amounts of immunoreactivity in each fraction were determined on the basis of fluorescence intensity (see Methods). Kvβ2 fluorescence levels in each lane were normalized to β-tubulin levels and the values are presented in terms of arbitrary units (AU). At both 24 and 48 hr, Kvβ2MO injection (black bars) led to efficient knock-down of Kvβ2 protein versus either uninjected (white bars) orCtlMO-injected embryos (gray bars). Values represent the mean of three independent experiments ± s.e.m. (p<0.0001, for uninjected orCtlMO-injected versus Kvβ2MO-injected embryos).

FIG. 4. Injection of ASβ2 reduced $I_{Kv}$ density in RB and non-RB neurons in a dose-dependent manner. A: In 24 hr ASβ2-injected (1 ng AS) embryos, internal control GFP− RB neurons (left) displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ RB neurons (right) displayed no or small $I_{Kv}$ amplitudes. B: In Kvβ2MO-injected (1 ng AS) embryos, internal control GFP− non-RB neurons (left)
Native Kv1 potassium channels require Kvβ2 subunits displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ non-RB neurons (right) displayed no or small $I_{Kv}$ amplitudes. C: ASβ2 doses ranging between 0.02 – 1.5 ng were injected into Xenopus embryos and $I_{Kv}$ was recorded from RB (black triangles) and non-RB (white triangles) neurons at 24 hrs. The percentage of remaining, unblocked current is shown as normalized current amplitude. $N$'s ranged between 9 and 19.

FIG. 5. Injection of Kvβ2MO or ASβ2 did not affect Kv1.1 protein. Total protein extracts were obtained from 24 and 48 hr uninjected (Ctl) and 48 hr embryos injected with 1.0 ng of either ASβ2 or Kvβ2MO. 20 µg of protein were loaded in each lane. In Ctl 24 hr embryos, Kv1.1 protein appeared as a prominent doublet at ~50 and 55 kDa. Arrows indicate the ~50 kDa immature and the 55 kDa mature forms of the Kv1 protein. In contrast, at 48 hr, control embryos contained much less of the 50kDa form and comparatively more of the 55 KDa form. Protein extracted from ASβ2 or Kvβ2MO injected embryos were similar to 48 hr controls and showed a prominent Kv1.1 band at 55 KDa.

FIG. 6. Kvβ2MO and ASβ2 did not affect other membrane properties. Neither Kvβ2MO nor ASβ2 injections had effects on RB or non-RB input resistance (MΩ) compared to control neurons. $N$'s ranged between 9 and 19.

FIG. 7. Kvβ2 knock-down had no effect on $I_{Kv}$ recorded from ventral neurons, a population that does not express the Kvβ2 gene. A: $I_{Kv}$ did not differ between ventral neurons of Kvβ2MO (right) versus CtlMO (left) injected embryos. The
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The voltage command protocol used is shown at the top of the left panel. For illustrative proposes, only current traces elicited by depolarizations to -60, -30, 0, +30 and +60 mV are shown. B: Mean ventral neuron I(Kv) density did not differ between Kvβ2MO and CtlMO injected embryos. Kvβ2MO and CtlMO data are represented by white (n = 5) and black (n = 4) triangles, respectively.

FIG. 8. Kv1DN reduced I(Kv) density in RB and non-RB neurons to the same extent as did Kvβ2MO or ASβ2. A, B: In 24 hr Kv1DN injected (1ng) embryos, internal control GFP- RB neurons displayed normal I(Kv) amplitudes while GFP+ RB neurons displayed small I(Kv) amplitudes. Similar effects were found in non-RB neurons (B). C: At all voltages, I(Kv) amplitudes in RB and non-RB were reduced by injection of the Kv1DN (symbols are indicated in panels A and B). D: Kv1DN and ASβ2 efficiently reduced I(Kv) density (*, p<0.001 versus Ctl), to the same extent as did MO or AS knock-down of Kvβ2 (cf. Figs. 2, 4).

FIG. 9. Between 24 and 48 hr, RB I(Kv) increased 5-fold in density without changes in activation rate. A: I(Kv) from Xenopus RB neurons increased in amplitude between 24 and 48 hr. The voltage command protocol used is shown at the top of the left panel. For illustrative proposes, only current traces elicited by depolarizations to -60, -30, 0, +30 and +60 mV are shown. B: Mean RB I(Kv) density increased ~5-fold between 24 and 48 hr (p<0.0001, for membrane potentials between 0 and +100 mV). For Panels B, C and E, 24 and 48 hr data are represented by white (n = 9) and black (n = 16) squares, respectively. C: The rate of activation of I(Kv) was assessed by measuring the time to half-
maximum current (t_{1/2}). No statistically significant differences between 24 and 48 hr data were found. D: Between 24 (white bar) and 48 (black bar) hr, RB potassium maximal conductance (G_{max}) increased ~6-fold, from 0.28 ± 0.07 to 1.63±0.16 pS/µm². Values represent the means ± s.e.m. (p<0.0001, 24 versus 48 hr data). E: The steady-state voltage-dependent properties of activation for RB potassium conductance did change between 24 and 48 hr. Curves were fit to a single Boltzmann equation, and V_{1/2} and k values were calculated (Table 2).

FIG. 10. Between 24 and 48 hr, non-RB I_{Kv} displayed increases in density and decreases in t_{1/2}. A: Non-RB I_{Kv} amplitudes increased between 24 (white circles; n = 12) and 48 hr (black circles; n = 11). For illustrative proposes, only current traces elicited by depolarizations to -60, -30, 0, +30 and +60 mV are shown. B: The I-V relationships for I_{Kv} recorded from non-RBs showed that I_{Kv} density increased ~4-fold between 24 and 48 hr. C: In contrast to RB I_{Kv}, the t_{1/2} for non-RB I_{Kv} showed a developmental decrease, reflecting the increased rate of apparent activation (p< 0.001 for voltages between 0 and 80mV). D: Between 24 (white bar) and 48 (black bar) hr, non-RB potassium maximal conductance (G_{max}) increased ~3-fold, from 0.55 ± 0.11 to 1.60±0.30 pS/µm². Values represent the means ± sem (p<0.005, 24 versus 48 hr data). E: The steady-state voltage-dependent properties of activation for non-RB potassium conductance changed between 24 and 48 hr. Curves were fit to a single Boltzmann equation, and V_{1/2} and k values were calculated (Table 2).
Fig. 11. Kv1 channels underlay $I_{Kv}$ of both RB and non-RB neurons of 48 hr *Xenopus* embryos. 

A: In 48 hr Kvβ2MO-injected (1 ng MO) embryos, internal control GFP− RB neurons displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ RB neurons displayed no or small $I_{Kv}$ amplitudes.

B: In 48 hr Kvβ2MO-injected (1 ng MO) embryos, internal control GFP− non-RB neurons displayed normal $I_{Kv}$ amplitudes. In contrast, GFP+ non-RB neurons displayed no or small $I_{Kv}$ amplitudes.

C: CtlMO, Kvβ2MO and ASβ2 injected *Xenopus* embryos did not show effects over peak inward current density (pA/pF). N’s ranged between 6 and 19.

D: Kvβ2MO, ASβ2 and Kv1DN all led to effective suppression of $I_{Kv}$ in RB neurons from 48 hr embryos. N’s ranged between 6 and 19.

D: Kvβ2MO, ASβ2 and Kv1DN all led to effective suppression of $I_{Kv}$ in non-RB neurons from 48 hr embryos. N’s ranged between 6 and 19.
Native Kv1 potassium channels require Kvβ2 subunits

Table 1: Partial knock-down of Kvβ2 has no effect on kinetic or voltage-dependent $I_{Kv}$ properties

<table>
<thead>
<tr>
<th>$I_{Kv}$ Property</th>
<th>$t_{1/2}$ (msec)$^1$</th>
<th>$V_{1/2}$ (mV)</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>2.5 ± 0.2</td>
<td>20.7 ± 1.1</td>
<td>12.3 ± 1.9</td>
</tr>
<tr>
<td>ASβ2, 0.02 ng (n=3)</td>
<td>2.9 ± 1.3</td>
<td>22.7 ± 2.8</td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>MOβ2, 0.02 ng (n=4)</td>
<td>ND</td>
<td>23.8 ± 1.4</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td><strong>Non-RB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=4)</td>
<td>3.3 ± 0.1</td>
<td>21.0 ± 2.2</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>ASβ2, 0.02 ng (n=3)</td>
<td>ND</td>
<td>18.6 ± 2.4</td>
<td>13.7 ± 1.0</td>
</tr>
<tr>
<td>MOβ2, 0.02 ng (n=3)</td>
<td>ND</td>
<td>20.5 ± 2.0</td>
<td>14.0 ± 2.0</td>
</tr>
</tbody>
</table>

ND: not determined (small current amplitudes prevented reliable measurement).

$^1$: evaluated at +20 mV.
Table 2: Steady-State Dependent Properties of Activation

<table>
<thead>
<tr>
<th></th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>$V_{1/2}$ (mV)</td>
</tr>
<tr>
<td></td>
<td>$k$</td>
<td>$k$</td>
</tr>
<tr>
<td>RBs</td>
<td>20.7(\pm)1.2(9)</td>
<td>20.3(\pm)2.0(16)</td>
</tr>
<tr>
<td></td>
<td>12.30(\pm)1.90(9)</td>
<td>25.3(\pm)1.8(16)*</td>
</tr>
<tr>
<td>Non-RBs</td>
<td>21.0(\pm)2.2(6)</td>
<td>2.1(\pm)0.9(7)**</td>
</tr>
<tr>
<td></td>
<td>11.8(\pm)0.4(6)</td>
<td>23.6(\pm)2.5(7)**</td>
</tr>
</tbody>
</table>

*, p<0.0001 vs 24 hr RB $k$

**, p<0.001 vs 24 hr non-RB $V_{1/2}$

***, p<0.001 vs 24 hr non-RB $k$