Homogeneous Development of Electrical Excitability via Heterogeneous Ion Channel Expression

Angeles B. Ribera

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

Synchronous differentiation of delayed-rectifier potassium current regulates electrical excitability and calcium entry in motor, sensory, and interneurons of the developing amphibian spinal cord. Although Kv1 and Kv2 potassium channel transcripts are detectable in these cells, it is not known which transcript contributes to functional expression. Overexpression of a Kv1 dominant-negative subunit indicates that 20% of neurons have only Kv1 potassium currents. In other neurons, non-Kv1 channels function because the dominant-negative subunit either

only partially suppresses or has no effect on current. Thus, diverse embryonic neurons coordinate differentiation of excitability yet rely on heterogeneous potassium channel gene expression.

Key words: potassium channels; *Xenopus* embryos; electrical excitability; neuronal differentiation; gene expression; dominant negative

The delayed-rectifier potassium current (I_{Kv}) plays a pivotal role in maturation of the action potential (Barish, 1986; O'Dowd et al., 1988; Lockery and Spitzer, 1992; Ribera and Spitzer, 1992). I_{Kv} is initially small when impulses are of long duration. Uniform increases in the I_{Kv} density of all primary spinal neurons synchronize conversion of long-duration calcium-dependent action potentials to brief sodium-dependent spikes.

By regulating the duration of the action potential, I_{Kv} also determines the amount of calcium influx associated with impulse generation. In amphibian spinal neurons, spontaneous calcium transients associated with long-duration action potentials provide developmental cues necessary for development of neurotransmitter expression and potassium channel kinetics (Gu and Spitzer, 1995). Thus, mechanisms that regulate I_{Kv} will have an impact on neuronal differentiation (Jones and Ribera, 1994).

Potassium channels are thought to consist of four pore-forming α subunits (MacKinnon, 1991; Liman et al., 1992) as well as auxiliary cytoplasmic β subunits (Rettig et al., 1994). Four major subfamilies of potassium channel α subunits have been identified: Kv1, Kv2, Kv3, and Kv4 (Chandy, 1991; Salkoff et al., 1992). Within each subfamily, several variants may exist and coassemble to form heteromeric channels. However, coassembly of subunits from different subfamilies (e.g., Kv1.1 and Kv2.2) has not been observed.

Developmental regulation of I_{Kv} may be under direct transcriptional control, because a critical period of RNA synthesis has been associated with maturation of I_{Kv} in all embryonic spinal neurons (Ribera and Spitzer, 1989). Candidate, relevant potassium channel α subunit genes are *Xenopus* Kv1.1 and Kv2.2, which are

expressed in embryonic spinal neurons and induce expression of delayed-rectifier channels in heterologous systems (Ribera and Nguyen, 1993; Burger and Ribera, 1996). Early overexpression of Kv1.1 potassium channel RNA in *Xenopus* embryos leads to premature increase in the density of I_{Kv} in all primary spinal neurons (Jones and Ribera, 1994).

Although previous studies are consistent with the hypothesis that Kv1.1 expression underlies the endogenous delayed-rectifier current, the presence of mRNA does not demonstrate conclusively either the synthesis or the activity of the resultant protein. Additionally, functional overexpression of exogenous RNA is possible regardless of the molecular identity of the endogenous current. Thus, overexpression of wild-type functional potassium channel mRNA does not inform about the molecular composition of the native delayed rectifier.

This study tests directly the possibility that Kv1 gene expression underlies endogenous delayed-rectifier function by overexpression of a mutant Kv1 subunit that is incapable of expressing current. In heterologous expression systems, this mutant demonstrates a dominant-negative phenotype, because it efficiently suppresses function of coexpressed wild-type Kv1 channels. *In situ*, this dominant-negative subunit leads to an essentially complete suppression of I_{Kv} in ~20% of primary spinal neurons when it is overexpressed in the developing embryo. However, other neurons appear to be affected only partially by or to be resistant to overexpression of the mutant subunit. Thus, different neuronal subtypes possess molecularly distinct potassium currents but regulate maturation of excitability in a synchronous manner.

MATERIALS AND METHODS

RNA synthesis. The entire coding region of the *Xenopus* Kv1.1 channel gene, flanked with the 5'- and 3'-untranslated sequences of β -globin, was excised from a pSP64T construct (Ribera and Nguyen, 1993) and cloned into the pALTER vector (Promega, Madison, WI). The untranslated sequences confer stability and efficient expression on exogenously introduced RNA (Liman et al., 1992). The resulting recombinant pALTER was linearized with *Xba*I. Capped sense RNA was generated by *in vitro* transcription with SP6 RNA polymerase (Promega) in the presence of ribonucleoside triphosphates (Pharmacia, Piscataway, NJ) and Cap analog (Boehringer Mannheim, Indianapolis, IN). RNA concentrations were

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

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determined spectrophotometrically and confirmed by electrophoresis in agarose-formaldehyde gels.

Site-directed mutagenesis. A tryptophan-to-phenylalanine amino acid substitution in the putative pore region was achieved using the pALTER mutagenesis system according to the manufacturer's instructions (Promega). The mutated residue corresponds to Trp¹³⁴ in the *Shaker B* sequence studied previously by Perozo et al. (1993); in addition, silent mutations caused the introduction of an *Mlu*I site that permitted rapid screening of resultant clones. To verify that results were attributable to the desired mutation, DNA was prepared from three independent clones; sequencing confirmed that the expected mutation was present in each case. All three clones were used for RNA transcription, and identical results were obtained from their respective RNA preparations.

Oocyte injection and recording. Fifty nanoliters of potassium channel RNA solutions (0.05–0.5 mg/ml) containing varying ratios of wild-type and mutant Kv1 RNAs were injected into stage V1, defolliculated *Xenopus* oocytes as described previously (Ribera, 1990; Ribera and Nguyen, 1993). Oocytes were incubated, and the two-electrode voltage-clamp technique (Axoclamp 2A amplifier; Axon Instruments, Foster City, CA) was used to record voltage-activated outward currents induced in the oocytes 2–4 d after injection of RNA.

For the coexpression experiments, RNA mixtures were prepared immediately before injection using a 2 μ l Pipetman (Rainin Instruments, Woburn, MA) for accurate measurement of solution volumes. To keep the amount of wild-type RNA constant in the different solutions, wild-type Kv1.1 RNA was diluted 1:1 either with RNase-free H₂O or with solutions containing varying concentrations of mutant Kv1.1 RNA. The binomial equation predicts the percent of channels expected for each class of multimeric channel (for details, see MacKinnon, 1991). Assuming that each channel has four α subunits, the fraction of channels (F_i) that are of a particular type, i , is calculated using the binomial distribution:

$$F_i = \left(\frac{4!}{(4-i)! i!} \right) F_{WT}^{4-i} F_{MUT}^i \quad (1)$$

where F_{WT} and F_{MUT} are the fractions of wild-type and mutant subunits. The total current recorded will be the sum of current carried by each conducting species.

This calculation of the amplitude of the expected current involves three standard assumptions: (1) the ratio of the RNAs injected will predict linearly their resultant protein ratio; (2) the magnitude of induced current will be dose-dependent on the amount of injected RNA; and (3) the mutant and wild-type subunits will assemble equally well as homo- or heteromeric channels. To maximize the validity of the second assumption, the final concentration of all RNAs in the injection solution was kept at 0.05–0.5 mg/ml; in this concentration range, wild-type RNA causes larger currents as the dose of injected RNA increases. The third assumption is reasonable given that the mutation affects only a single amino acid in the putative pore region and that assembly domains have been located elsewhere (Li et al., 1992; Shen et al., 1993; Babila et al., 1994; Hopkins et al., 1994; Lee et al., 1994; Shen and Pfaffinger, 1995).

Embryo injections. Introduction of RNA into developing embryos was achieved by injection into one side of the animal pole immediately after first cleavage (Jones and Ribera, 1994). Typically, 5 nl of injection solution was pressure-injected (1–3 psi for 2–3 sec) using fine-drawn micropipettes (~10 μ m tip diameter; Narashige, Sea Cliff, NY). The injection solution contained a lineage tracer (20–30 mg/ml rhodamine-conjugated dextran; Molecular Probes, Eugene, OR) and the desired RNA (80 pg/nl). By external morphological criteria, RNA-injected embryos appear to develop normally (Jones and Ribera, 1994).

Cell culture preparation. Embryos were produced by standard *in vitro* fertilization techniques (Moon and Christian, 1989) and staged according to Nieuwkoop and Faber (1967). Neurula-stage cultures were prepared as described previously (Jones and Ribera, 1994; Jones et al., 1995). Cultures were viewed on a Nikon Diaphot microscope (Nikon, Melville, NY) with phase-contrast optics or rhodamine-epifluorescent illumination. Neurons were identified based on morphological criteria (O'Dowd et al., 1988). Each morphologically identified neuron was scored for rhodamine fluorescence. Visualization of the fluorescence did not interfere with cell survival or subsequent differentiation.

c-myc epitope tagging. Six copies of the c-myc epitope were fused to the N terminus of the Kv1.1 sequence by subcloning into the CS2+MT vector (Turner and Weintraub, 1994), which was provided by D. Turner (Fred Hutchinson Cancer Research Center, Seattle, WA); DNA sequencing verified that the Kv1.1 sequence had been inserted in-frame with the

c-myc epitopes. Similar epitope tagging of the wild-type Kv1.1 sequence did not alter the function of expressed channels, as deduced by expression in oocytes. Cultures were prepared as described above, fixed in 1% paraformaldehyde, incubated for 1 hr with affinity-purified anti-c-myc monoclonal antibody (1:5; Oncogene, Cambridge, MA), washed, and then incubated in affinity-purified fluorescein-conjugated goat anti-mouse antibody (1:100; Boehringer Mannheim) for 45 min; all incubations and washes were performed at room temperature. Immunoreactivity was not observed when the primary antibody was omitted or when mutant Kv1.1 RNA lacking the six N-terminal epitope tags was coinjected with dextran. Cultures were viewed either with phase optics or with epifluorescence using appropriate filters to detect selectively either rhodamine or fluorescein signals and were photographed with TMax 400 film. Negatives were scanned into Photoshop (Adobe Systems, Mountain View, CA), and the negative graininess was reduced by "dust and scratch" and "blur" algorithms; "unsharp mask" was applied to enhance edges.

Biophysical methods and analyses. Neurons with short processes (<50 μ m) were selected for recording to avoid space-clamp problems. Before recording, neurons were viewed with epifluorescence to identify them as nonfluorescent (dextran-negative) or fluorescent (dextran-positive); cells with faint fluorescence were not used for electrophysiological analysis. The presence of fluorescent dextran does not alter the electrophysiological properties of the cell (Jones and Ribera, 1994). Whole-cell gigohm recording methods were used (Hamill et al., 1981). An Axopatch 1-C amplifier (Axon Instruments) was used in conjunction with a TL-1 data interface (Axon Instruments), pClamp computer programs (Axon Instruments), and a 386 PC clone for acquisition of data. Electrodes were pulled from borosilicate glass (Drummond Scientific, Broomall, PA) and had resistances ranging between 2 and 4 M Ω when filled with the pipette solution (100 mM KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4). The bath saline consisted of (in mM): NaCl 80, KCl 3, MgCl₂ 5, CoCl₂ 10, HEPES 5, pH 7.4. Sodium currents were blocked by addition of 10⁻⁶ M tetrodotoxin (Sigma, St. Louis, MO); calcium currents were avoided by omission of CaCl₂ from the saline and addition of 10 mM CoCl₂. The capacitive transient was nulled electronically before establishing the whole-cell configuration. Membrane potential was held at -80 mV and stepped to depolarized voltages ranging between -45 and +105 mV in increments of 5 or 10 mV for 20–200 msec. The currents elicited were digitized at 100 μ sec.

Cell capacitance and series resistance were determined from the capacitive current transient recorded after break-in when sampling at 20 μ sec intervals. The cell capacitance was used to evaluate the membrane surface area (1 μ F/cm²) for normalization of current amplitude to density. The series resistance value was used to estimate the associated voltage error. Leak subtraction was accomplished using a modified P/4 protocol with 11 subpulses (pClamp 5.5, Axon Instruments). Calculations of current densities, series and input resistances, series resistance voltage errors, time-to-half-maximum values or half-maximum current, and cell capacitances were performed using computer programs that were provided by D. Gurantz (University of California-San Diego, La Jolla, CA). Data are presented as mean $\pm$ SEM. The degree of statistical significance between mean values was calculated by an unpaired nonparametric test (Mann-Whitney *U* test).

RESULTS

Creation of a dominant-negative potassium channel subunit

For several multimeric proteins, subunits that are mutated in critical regions prevent activity of the assembled protein in a dominant manner and, therefore, can be used to knock out the functional contribution of endogenous proteins (Herskowitz, 1987). A dominant-negative potassium channel Kv1 α subunit would be expected to perturb the function of Kv1 channels (Li et al., 1992; Tu et al., 1995) (Fig. 1). Moreover, because subunits of different subfamilies do not coassemble, potassium channels consisting of Kv2, Kv3, or Kv4 subunits would be resistant to the effects of a Kv1 dominant-negative subunit.

When introduced into the fly Kv1 gene, a tryptophan $\rightarrow$ phenylalanine mutation in the pore region produces a nonconducting potassium channel that still generates gating currents (Perozo et al., 1993). Mutation of the analogous residue in *Xenopus* Kv1.1 creates a

HOMOMULTIMERS **HETEROMULTIMERS**

Figure 1. Five different channel types form after coexpression of wild-type and mutant subunits. Two channels are homomultimers of either wild-type (○) or mutant (⊗) subunits. Assuming that potassium channels consist of four pore-forming α subunits (MacKinnon, 1991; Liman et al., 1992), the other three channels are heteromultimers consisting of one, two, or three mutant subunits. A dominant-negative subunit leads to the formation of nonfunctional channels when it coassembles with itself as a homomultimer or with wild-type subunits, leading to heteromeric channels. The only conducting channel species would be the homomultimer of wild-type subunits.

subunit that does not form functional channels when expressed as a homomultimer (Fig. 2A). In addition, inclusion of the mutant RNA in solutions containing wild-type Kv1.1 RNA leads to a reduction in the amplitude of the expressed current. However, the voltage-dependent properties of activation of currents recorded from oocytes injected with either wild-type RNA or with mixtures of wild-type and mutant RNAs are similar (Fig. 2B). These results suggest that the mutant subunit operates in a dominant-negative manner and prevents expression of functional channels.

Potency of the dominant-negative subunit

Assuming that potassium channel α subunits form tetrameric channels (MacKinnon, 1991; Liman et al., 1992), coexpression of *Xenopus* wild-type and mutant Kv1.1 RNAs in the heterologous oocyte expression system will lead to the formation of wild-type and mutant homomultimers and three classes of heteromultimers with one, two, or three mutant subunits (Fig. 1). The binomial equation predicts the percent of channels expected for each class of multimeric channel (MacKinnon, 1991) (see equation in Materials and Methods). Under conditions in which the absolute amount (rather than concentration) of wild-type Kv1.1 RNA is kept constant, addition of mutant Kv1.1 RNA to injection solutions leads to induction of smaller-amplitude potassium currents (Fig. 2A, Table 1). If the mutant subunit were not dominant, only 6.25% of assembled channels would be expected to be homomultimers of mutant subunits under conditions of 1:1 ratio of wild-type to mutant RNA, and the resultant current, therefore, would be almost twice as large (187.5% of wild-type alone). However, the current amplitude is substantially reduced compared with wild-type alone (Table 1). At several different ratios of wild-type to mutant Kv1.1 RNAs, the magnitude of the reduction in current amplitude is that expected if one or at most two mutant subunits act in a dominant-negative manner to prevent the formation of functional channels (Table 1). (For the purposes of this study, if two- or one-mutant α subunits prevent functional expression of

the tetrameric potassium channel, the subunit is considered to be a dominant-negative one.)

Pharmacological tests also assess the potency of the mutant subunit. In heterologous expression systems such as the oocyte, homomultimeric wild-type channels composed of either Kv1.1 or Kv1.2 subunits have different sensitivities to the blocker tetraethylammonium (TEA; Fig. 3): Kv1.1 channels are 50% blocked by 1 mM TEA, whereas 150 mM TEA produces little reduction in current carried by Kv1.2 channels. However, when wild-type Kv1.1 and Kv1.2 mRNAs are coexpressed, the resultant current has an unusually high sensitivity to TEA, given that channels are expected to consist of either homomultimers of Kv1.1 or Kv1.2 or heteromultimers of Kv1.1 and Kv1.2 (Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990; Covarrubias et al., 1991) (Fig. 3). In contrast, under conditions in which wild-type Kv1.2 and mutant Kv1.1 mRNAs are coexpressed, the sensitivity of the small, resultant current to TEA is not distinguishable from that of Kv1.2 homomultimers (Ribera, 1990) (Fig. 3). These results suggest that channels containing a dominant-negative Kv1.1 subunit are nonfunctional and that the only conducting potassium channels are Kv1.2 homomultimers.

Thus, the mutant subunit behaves in a dominant-negative manner. One, or at most two, mutant subunits suffice for functional removal of Kv1 channels. On this basis, the mutant Kv1.1 subunit should serve as a powerful reagent for knock out of endogenous Kv1 currents.

In situ overexpression of potassium channel subunits

Microinjection of RNA into cleavage-stage embryos is an efficient method for introduction of exogenous potassium channels into embryonic spinal neurons (Jones and Ribera, 1994). Mutant Kv1.1 RNA was injected under conditions that caused, when used previously for overexpression of wild-type Kv1.1 RNA, a two- to threefold increase in potassium current density. Progeny of the original injected blastomeres are identified by inclusion of a fluorescent lineage tracer (rhodamine-conjugated dextran) in the injection solution. *c-myc* epitope tagging of the mutant Kv1.1 sequence assesses the degree of fidelity between the presence of the lineage tracer and protein translated from exogenous RNA. Dextran-positive neurons that meet the criteria for electrophysiological recording are also *c-myc*-immunoreactive (Fig. 4), demonstrating that the lineage tracer reliably traces the disposition of exogenous protein in neuronal tissue.

Heterogeneous effects of overexpression of a dominant-negative Kv1 subunit

By 1 d in culture, the endogenous delayed rectifier has undergone its full extent of maturation and is a large and rapidly activating current (O'Dowd et al., 1988; Ribera and Spitzer, 1989). Neurons derived from uninjected blastomeres exhibit voltage-dependent potassium currents that have densities and kinetic properties expected of the endogenous current (O'Dowd et al., 1988; Ribera and Spitzer, 1989) (Fig. 5). However, fluorescent neurons derived from injected blastomeres often generate small, slowly activating currents (Fig. 5). On average, the dominant-negative subunit leads to both a reduction in density and an increase in the time-to-half-maximum of the delayed-rectifier potassium current of fluorescent neurons (Fig. 6).

Although the average results indicate that the dominant-negative subunit has expected effects *in situ*, examination of the results from individual fluorescent neurons reveals a surprising degree of heterogeneity, ranging from complete ($n = 5/25$) to no

Figure 2. A single amino acid substitution in the putative pore region of the *Xenopus* Kv1.1 potassium channel creates a subunit that behaves in a dominant-negative manner. **A**, *Xenopus* oocytes were injected with wild-type (top), mutant (middle), or a 1:1 ratio of wild-type to mutant Kv1.1 (bottom) RNAs. Injection of wild-type consistently induces expression of a delayed-rectifier potassium current (top), whereas injection of the mutant does not lead to induction of potassium current (middle). Coinjection of wild-type and mutant Kv1.1 RNAs leads to the generation of smaller-amplitude potassium currents (bottom). Scale: 5 μ A, 10 msec. **B**, The steady-state activation properties of the wild-type current (open squares) and the current remaining after coexpression of wild-type and mutant Kv1.1 RNAs (filled squares) are similar. This result is expected if the mutation either leads to a dominant-negative subunit or does not interfere with the voltage dependence of activation.

apparent suppression of endogenous delayed-rectifier potassium currents (Figs. 5, 7A). Considering that all nonfluorescent neurons generate voltage-dependent potassium current, the absence of this current in 20% of fluorescent neurons is of particular significance and indicates that these neurons possess only Kv1 potassium channels.

Fluorescent neurons are characterized by time-to-half-maximum values that are either very large (>5 msec) or within the upper end of values obtained from nonfluorescent neurons (Fig. 7B). Examination of the correlation between the current density and time-to-half-maximum of currents recorded from individual fluorescent neurons (Fig. 7C, D) indicates that they have either unusually slow, small currents or currents that have density and time-to-half-maximum within the normal range. The dominant-negative subunit appears either to affect or to spare both properties of the current simultaneously. Thus, only a subset of neurons is susceptible to the effects of overexpression of dominant-negative Kv1.1 subunits.

DISCUSSION

The major finding of this study is that *in situ* overexpression of a dominant-negative Kv1 potassium channel α subunit heterogeneously affects the endogenous I_{Kv} of primary spinal neurons. This result was not expected, because these neurons have been considered to be homogeneous with respect to electrical excitability:

action potential maturation is regulated synchronously by an I_{Kv} that behaves similarly in all of the neurons (O'Dowd et al., 1988; Ribera and Spitzer, 1992). However, primary spinal neurons are heterogeneous in type (e.g., motor vs sensory) as well as in neurotransmitter sensitivity and content (Bixby and Spitzer, 1984; Spitzer et al., 1993). The present results indicate that heterogeneity is also evident for genes underlying a homologous and synchronized function: I_{Kv} .

Figure 3. Pharmacological analysis of functional channels formed heterologously in oocytes by coexpression of mutant and wild-type Kv1 subunits indicates that the mutant subunit blocks channels in a dominant-negative manner. Coexpression of mutant Kv1.1 with wild-type Kv1.2 (filled triangles) causes the expression of functional channels with sensitivity to TEA that is not different from that obtained by expression of Kv1.2 as a homomultimer (open circles). Open symbols indicate wild-type channels: squares, Kv1.1 homomultimers; circles, Kv1.2 homomultimers; triangles, 1:1 mixtures of Kv1.1 and Kv1.2. Filled symbols represent data from coexpression of mutant Kv1.1 with either wild-type Kv1.1 (squares) or wild-type Kv1.2 (triangles). Values are mean $\pm$ SEM for four to six determinations.

Table 1. Effects of coexpression of wild-type and mutant Kv1.1 subunits on current amplitude

RNA ratio Wild-type:mutant	% Current expected if <i>n</i> subunits are required for block		% Current recorded
	<i>n</i> = 1	<i>n</i> = 2	
1:0	NA	NA	100 $\pm$ 5 (<i>n</i> = 14)
0:1	NA	NA	1 $\pm$ 2 (<i>n</i> = 6)
1:1	12	63	26 $\pm$ 4 (<i>n</i> = 9)
1:2,3	3	30	4 $\pm$ 1 (<i>n</i> = 7)
1:8	~1	3	2 $\pm$ 1 (<i>n</i> = 4)

Figure 4. The fluorescent lineage tracer reliably identifies neurons containing protein translated from exogenous *c-myc*-tagged mutant Kv1.1 RNA. *A*, Two neurons (arrowheads) in culture are viewed with phase-optics (middle), rhodamine (right), or fluorescein (left) epifluorescence. Rhodamine-conjugated dextran identifies cultured cells derived from the injected blastomere; fluorescein labeling identifies cells that are *c-myc*-immunoreactive and, thus, express the exogenous mutant Kv1.1 RNA. Yolk platelets and lipid granules are abundant in somata and possess the endogenous autofluorescence that is visualized in the fluorescein channel. Thus, examination of processes (arrows) that do not contain yolk platelets or lipid granules confirms detection of *c-myc* immunoreactivity. *B*, The percentage of dextran-positive (left) or -negative (right) neurons that are either *c-myc*-positive (filled bar) or *c-myc*-negative (open bar) indicates that dextran is a reliable tracer in neurons of protein translated from exogenous RNA; 50 of 57 dextran-positive neurons are also *c-myc*-positive (88%). Conversely, for 186 dextran-negative neurons, 181 were also *c-myc*-negative (97%). Using the selection criterion for electrophysiological analysis of an intense dextran fluorescence (see Materials and Methods), only the 50 *c-myc*-immunoreactive neurons would have been suitable, indicating that the physiological data appearing in Figures 5–7 are expected to be obtained from the 88% of neurons that are dextran- and *c-myc*-positive.

Although the lack of effect of the dominant-negative subunit in some neurons is not accounted for by differential partitioning of the lineage tracer and exogenous RNA (Fig. 4), endogenous potassium channel subunits may escape coassembly with the ex-

ogenous dominant-negative subunit if they are not processed cotranslationally or targeted to similar subcellular regions (Sheng et al., 1992; Shen et al., 1993; Deal et al., 1994). However, the fluorescence signal from the epitope-tagged form of the mutant

Figure 5. Overexpression of mutant Kv1.1 RNA reduces functional expression of delayed-rectifier potassium current in some, but not all, neurons. Examples of the smallest (top), average size (middle), and largest (bottom) currents recorded from control neurons (non-fluorescent, left) and neurons containing exogenous mutant Kv1.1 (fluorescent, right) are shown. Twenty percent (5/25) of fluorescent cells show complete suppression of current, whereas 20% (5/25) appear to have currents of normal density and activation rate. Scale: 500 pA, 5 msec.

Figure 6. Overexpression of the dominant-negative subunit affects developmentally regulated properties of the whole-cell delayed-rectifier potassium current. *A*, Average current density as a function of voltage for fluorescent (*F*; $n = 25$) and nonfluorescent (*NF*; $n = 18$) neurons. Overall, overexpression of the dominant-negative subunit leads to a reduction in current density. Mean values of $0.4 \pm 0.1 \text{ pA}/\mu\text{m}^2$ ($n = 25$) and $1.0 \pm 0.2 \text{ pA}/\mu\text{m}^2$ ($n = 18$) were obtained for fluorescent and nonfluorescent neurons, respectively ($p = 0.0001$). *B*, Average time-to-half-maximum as a function of voltage for fluorescent (*F*; $n = 20$) and nonfluorescent (*NF*; $n = 18$) neurons; fluorescent neurons that had zero-current densities at +10 mV were excluded. Mean values of 6.1 ± 1.0 and 2.7 ± 0.2 msec were obtained for fluorescent and nonfluorescent neurons, respectively ($p = 0.001$). Overall, overexpression of the dominant-negative subunit leads to an increase in the time-to-half-maximum, indicating that currents activate more slowly.

Figure 7. Overexpression of the dominant-negative subunit affects properties of the whole-cell potassium current in some, but not all, neurons. *A*, Histogram of current densities recorded from fluorescent (filled bars) and nonfluorescent (open bars) neurons at +10 mV. The distribution of values for fluorescent neurons is marked by a new population ($<0.5 \text{ pA}/\mu\text{m}^2$) as well as the continued presence of normal values; these results contrast with previous results obtained after overexpression of wild-type Kv1.1 RNA (Jones and Ribera, 1994), for which current densities are shifted uniformly to larger values. *B*, Histogram of time-to-half-maximum values recorded from fluorescent (filled bars) and nonfluorescent (open bars) neurons at +10 mV. After overexpression of mutant Kv1.1 RNA, neurons have either very large time-to-half-maximum values or values within the normal range. *C*, *D*, Examination of the correlation between the density and time-to-half-maximum of current recorded from individual fluorescent neurons (*D*, filled symbols, bottom graph) indicates that if a fluorescent cell maintains normal (large) current density, it also has a normal (small) time-to-half-maximum value. This result suggests that overexpression of mutant Kv1.1 either affects both current density and time-to-half-maximum simultaneously or has no effect on either parameter. Values used are those obtained at +10 mV, except for five fluorescent neurons in which current was suppressed completely at +10 mV. Three of these neurons had small, slow currents by activation to potentials between 20 and 30 mV. Two additional fluorescent neurons appear in *B* but not in *D*, because their time-to-half-maximum values were >20 msec, even at activation potentials greater than +30 mV.

subunit appears throughout the neuron (Fig. 4). Another possible explanation is that native channels assemble before the time of delivery of exogenous mutant RNA and turn over slowly, thus appearing to be resistant to overexpression of a dominant-negative subunit. However, the exogenous RNA is delivered to early two-cell stage embryos, substantially before the time of initial detection of Kv1.1 mRNA in primary neurons (Ribera and Nguyen, 1993).

An intriguing possibility is that the synchronous differentiation of I_{Kv} is directed by a common β subunit. In support of this hypothesis is the recent finding that coexpression of a *Drosophila* potassium channel β subunit, *Hyperkinetic*, with Kv1 subunits in a heterologous system leads to the induction of larger, more rapidly activating currents (Chouinard et al., 1995). However, vertebrate potassium channel β subunits associate with Kv1 but not Kv2 subunits (Rhodes et al., 1995), suggesting that a common β subunit does not regulate the uniform maturation of I_{Kv} in diverse neuronal subtypes. In addition, previous work demonstrates that overexpression of Kv1 α subunits suffices for induction of larger potassium currents in embryonic spinal neurons (Jones and Ribera, 1994).

Three considerations strongly support the conclusion that functional expression of more than one potassium channel α -subunit gene directs the synchronous developmental regulation of I_{Kv} in *Xenopus* spinal neurons. First, the experimental paradigm indicates that Kv1 channels operate exclusively in at least 20% of neurons, where they account for the entire voltage-dependent outward current. Similarly, Kv1 channels operate in a subset of *Drosophila* neurons (Baker and Salkoff, 1990). Given that overexpression of wild-type Kv1.1 channels causes a uniform two- to threefold increase in current density (Jones and Ribera, 1994), complete suppression of current is consistent with the prediction of the binomial equation (~ 75 – 95% reduction; Table 1), assuming that exogenous and endogenous subunits assemble equally well with each other and that all endogenous subunits are of the Kv1 subfamily. Second, Kv1 channels appear to be either absent or nonfunctional in other neurons that are completely resistant to the dominant-negative Kv1 subunit. This is functional confirmation of the molecular heterogeneity that is evident for neural potassium channel gene expression (Drewe et al., 1992; Tsaour et al., 1992; Ribera and Nguyen, 1993; Burger and Ribera, 1996). Third, time-to-half-maximum values of I_{Kv} in fluorescent neurons that have current densities of normal size fall within the upper range of control (nonfluorescent) values (Fig. 7). This would be expected if non-Kv1 channels have slower kinetics of activation than do Kv1 channels. In the oocyte, *Xenopus* Kv2.2 currents activate more slowly than do Kv1.1 currents (Ribera and Nguyen, 1993; Burger and Ribera, 1996). Thus, a strong candidate for another gene encoding endogenous current in spinal neurons is Kv2.2 (Burger and Ribera, 1996). In *Drosophila* nerve and muscle, Kv2 encodes a majority of their delayed-rectifier currents (Tsunoda and Salkoff, 1995).

In view of the synchronized differentiation of I_{Kv} in primary spinal neurons, it is not obvious what advantages are provided by heterogeneous potassium channel gene expression. During the period of action potential differentiation, the primary role of I_{Kv} is action potential repolarization (Barish, 1986; Lockery and Spitzer, 1992). However, the role of this potassium current in controlling impulse frequency has not been examined. At early stages, impulse frequency encodes differentiation cues (Gu and Spitzer, 1995), whereas at later stages it may be critical for establishment of proper functional wiring or plasticity. The dif-

ferent biophysical properties of individual potassium channel variants may lead to specific firing patterns, each of which is optimally tuned for a specific neuronal subtype, e.g., motor versus sensory. Thus, in the *Xenopus* embryo, ion channel genes may serve as sensitive molecular and functional reporters of early aspects of differentiation of excitable tissues.

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