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Subfamily-Specific Posttranscriptional Mechanism Underlies K^+ Channel Expression in a Developing Neuronal Blastomere

Fumihito Ono,[1,2] You Katsuyama[1], Kouichi Nakajo,[3] and Yasushi Okamura[1,3,4]

[1]*Ion Channel Group, Biomolecular Engineering Department, National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki 305–8566, Japan,* [2]*Department of Medical Physiology, Meiji College of Pharmacy, Kiyose 204–8588, Tokyo, Japan,* [3]*Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153–0041, Japan, and* [4]*Intelligence and Synthesis, Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation*

Na^+ and K^+ channels are the two key proteins that shape the action potentials in neurons. However, little is known about how the expression of these two channels is coordinated. To address this issue, we cloned a *Shab*-related K^+ channel gene from ascidian *Halocynthia roretzi* (TuKv2). In this animal, a blastomere of neuronal lineage isolated from the 8-cell embryo expresses single Na^+ channel and K^+ channel genes after neural induction. Expression of a dominant negative form of TuKv2 eliminated the native delayed rectifier K^+ currents, indicating that the entire delayed rectifier K^+ current of the neuronal blastomere is exclusively encoded by TuKv2. TuKv2 transcripts are expressed more broadly than Na^+ channel transcripts, which are restricted to the neuronal lineages. There is also a temporal mismatch in the expression of TuKv2 transcript and the K^+ current; TuKv2 transcripts are present throughout development, whereas delayed rectifier K^+ currents only appear after the tailbud stage, suggesting that the functional expression of the TuKv2 transcript is suppressed during the early embryonic stages.

To test if this suppression occurs by a mechanism specific to the TuKv2 channel protein, an ascidian *Shaker*-related gene, TuKv1, was misexpressed in neural blastomeres. A TuKv1-encoded current was expressed earlier than the TuKv2 current. Furthermore, the introduction of the TuKv2-expressing plasmid into noninduced cells did not lead to the current expression. These results raise the possibility that the expression of TuKv2 is post-transcriptionally controlled through a mechanism that is dependent on neural induction.

Key words: potassium channel; ascidian*; gene expression; dominant negative; sodium channel; neuronal differentiation; post-transcriptional regulation*

From jellyfish to mammals, voltage-gated K^+ and Na^+ channels play principal roles in determining the properties of neuronal action potentials (Hille, 1992). As a neuron matures, the shape of the action potential becomes sharper because of changes in the relative densities of the two channel types. This suggests that the expression of K^+ and Na^+ channels is coregulated in developing neurons. The mechanism of coordinated expression of the Na^+ and K^+ channels, however, is difficult to address in higher vertebrates, because it requires isolation of ionic currents encoded by each channel gene expressed in the particular neurons. Cloned genes for ion channels are usually studied in heterologous systems, but currents expressed heterologously often have functional properties different from those observed in native environments (McManus et al., 1995). Furthermore, both Na^+ and K^+ channels form large multigene families, and multiple splice variants are often generated from one gene (Pongs, 1992). Several molecular species of Na^+ and K^+ channels commonly exist in one single neuron (Tsunoda and Salkoff, 1995b; Gurantz et al., 1996; Ribera, 1996). The difficulty in identifying the precursors of a particular neuron also hinders the developmental studies of specific channel species in native cells.

Ascidians (also called tunicates) are protochordates, close ancestors to the vertebrates (Conklin, 1905). The ascidian embryos offer an excellent system to study the regulation of voltage-gated ion channels during embryogenesis, because of their simple composition of channel species and stereotyped course of differentiation (Takahashi and Okamura, 1998). Neuronal differentiation occurs in a short time in this animal. Isolated ascidian blastomeres of specific cell fates are amenable to electrophysiological studies (Okado and Takahashi, 1990). Therefore, expressions of ion channels in individual cells can be traced during neural differentiation (Okamura and Takahashi, 1993; Takahashi and Okamura, 1998). We previously showed that a single Na^+ channel gene, TuNaI, accounts for the entire Na^+ current in the neuronal blastomere, *a*4.2 (Okamura and Shidara, 1990; Okamura et al., 1994). The transcription of TuNaI is dependent on a cell-specific contact before the neural tube formation (Okamura et al., 1994). This raises the possibility that Na^+ and K^+ channels are regulated at the transcriptional level by a mechanism dependent on the neural induction.

In the present study, we cloned an ascidian K^+ channel gene that encodes the major delayed rectifier K^+ current of anterior neuronal blastomeres. Unexpectedly, we found that the gene expression pattern of TuKv2 is significantly different from that of the TuNaI gene. We show evidence for a subfamily-specific

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Correspondence should be addressed to Yasushi Okamura, Ion Channel Group, Biomolecular Engineering Department, National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki 305–8566, Japan.

mechanism at the post-transcriptional level that operates to determine the time course of the K^+ channel current expression during the neuronal development.

MATERIALS AND METHODS

Animals. Ascidian *Halocynthia roretzi* was used for all the experiments. Adult animals were purchased in winter from fishermen in northern parts of Japan, Sanriku and Wakkanai. Animals were maintained at 4°C in ultraviolet-sterilized circulating seawater. The spawning of eggs and sperm was induced by keeping the animal at a higher temperature, up to 12°C, under the daylight. The methods of fertilization and of rearing the embryos were described previously (Okamura and Shidara, 1990).

Nomenclature of blastomeres. Ascidian blastomeres were named following the designation system first adopted by Conklin (1905). Blastomeres of the animal hemisphere are designated by small letters, either a or b, and those in the vegital hemisphere by capital letters, either A or B. At the eight-cell stage, one embryo is composed of four pairs of different blastomeres, *a*4.2, *b*4.2, *A*4.1, and *B*4.1. *A*4.2 and *a*4.2 are the anterior cells, and *b*4.2 and *B*4.1 are the posterior cells.

cDNA cloning. Amino acid sequences conserved among Kv2 clones of other animals were selected to perform RT-PCR: YLESCCQ and KWKFFKG. Degenerate primers corresponding to these amino acids were synthesized. Total RNA from ascidian young tadpole was reverse-transcribed using Superscript (Life Technologies, Grand Island, NY) with random hexamers. A partial cDNA clone of TuKv2 (~400 bps long) was isolated after a single round of PCR reaction (30 cycles). Using this partial clone as a probe, longer cDNA clones were screened from a cDNA library that was constructed from tailbud embryos of ascidian *Halocynthia roretzi* in lambda-Zap phage (Stratagene, La Jolla, CA). Oligo-dT-primed cDNAs longer than ~4.5 kb was size-selected on an agarose gel before ligation with phage arm DNAs. The hybridization on nylon filter membranes was performed at 42°C overnight in the solution: 30% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 0.1% BSA. Membranes were washed in 2× SSC, 0.1% SDS at room temperature twice and in 0.1× SSC, 0.1% SDS at 50°C twice. After screening ~100,000 clones, four positive clones were isolated and subcloned into Bluescript following the *in vivo* excision protocol. Two of the four clones contained the longest insert, 4.8 kb. Both clones contained the full coding region of an ascidian Kv2 gene. Deletion clones in both directions were constructed for one of the clones (named as Shb) and sequenced by the deoxy-termination method using an automated sequencer, ABI prism 310 (Applied Biosystems, Foster City, CA). TuKv1 has been isolated by RT-PCR and subsequent library screening as in TuKv2 cloning. Detailed characterization of TuKv1 will be published elsewhere.

Expression in Xenopus *oocytes.* The original TuKv2 clone, Shb, with its natural 5′UTR and 3′UTR did not express robustly, either in *in vitro* translation system or in *Xenopus* oocytes. Therefore we changed 5′UTR and 3′UTR of the native clone. 5′UTR and first three codons of Herpes Simplex virus thymidine kinase derived from plasmid pSP6nucβGal (provided by Dr. Richard Harland, University of California, Berkeley, CA) was cloned into the upstream of TuKv2 so that it is in frame. The distal part of TuKv2 3′UTR was substituted at *Bam*HI site with 3′UTR of rabbit β-globin (Dierks et al., 1981). The constructed clone, called SbV, was *in vitro* transcribed and translated with TNT T7-coupled reticulolysate system kit (Promega, Madison, WI) and run on SDS-PAGE to confirm that a protein of the expected size is synthesized. For injection into *Xenopus* oocytes, plasmid SbV was linearized with *Xba*I and transcribed with T7 RNA polymerase. The plasmid containing TuKv1 insert in Bluescript was linearized with *Xho*I and transcribed with T3 RNA polymerase. RNA was synthesized using Riboprobe system (Promega) in the presence of 0.5 mM ATP, CTP, and UTP, 50 nM GTP, and 0.5 mM capping nucleotide m7G(5′)ppp(5′) (Amersham Pharmacia Biotech, Piscataway, NJ). *Xenopus* oocytes were prepared according to a standard protocol (Vize et al., 1991). Approximately 50 nl of TuKv2 RNA was injected into an oocyte at the concentration of 1 ng/nl. Injected oocytes were cultured for 2–3 d at 18°C in the medium composed of 50% Leibovitz's L-15 medium (Life Technologies), 15 mM Na-HEPES, and 100 μg/ml gentamycin. Electrical recordings of *Xenopus* oocytes were performed in the two-electrode voltage-clamp configuration with Oocyte Clamp OC-725C (Warner Instruments, Grand Haven, MI). The signal was digitized with an analog-to-digital converter, ITC-16, sampled at 10 kHz, filtered at 3–5 kHz, and analyzed using Pulse and Pulsefit program (Heka, Germany). The bath for oocytes was kept at 16°C so that the recording temperature was the same as in recording from ascidian cells.

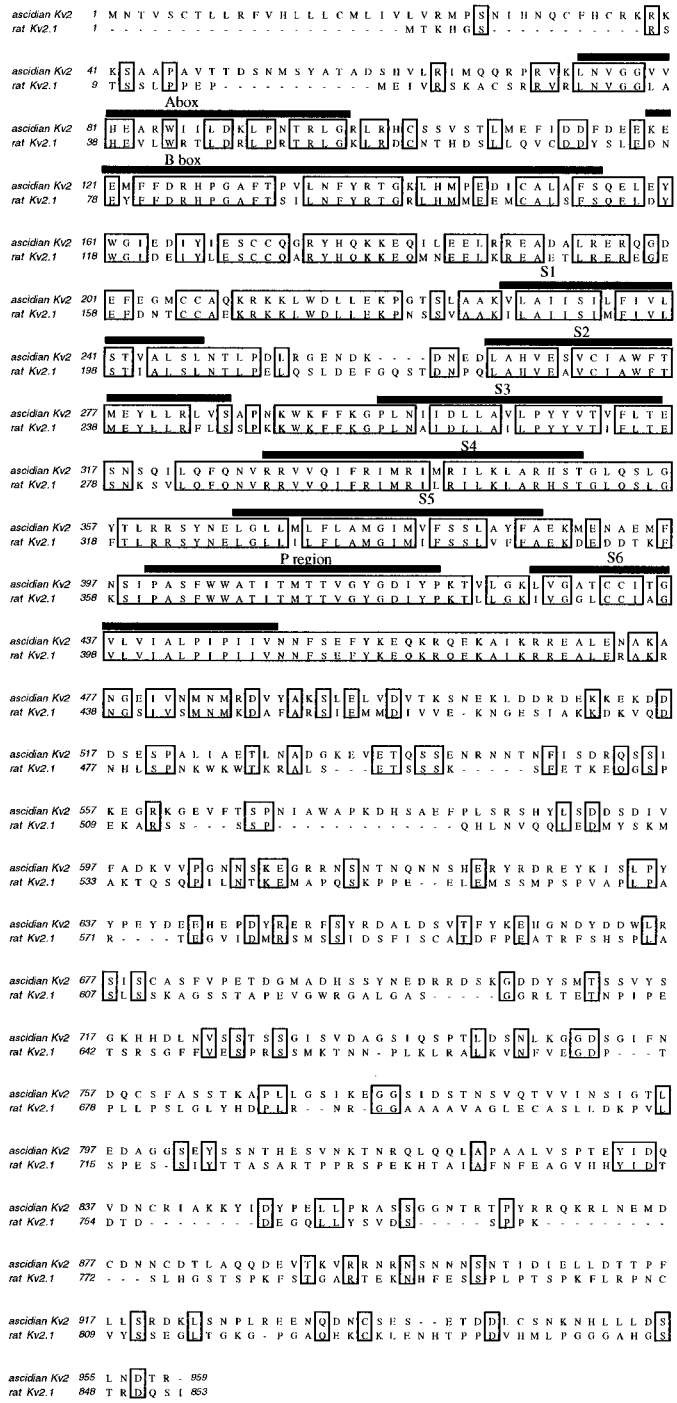

Figure 1. Isolation of a Kv2-related gene, TuKv2. Deduced amino acid sequence of TuKv2 is shown along with that of rat Kv2.1. Identical amino acids are *boxed*. NAB region, putative transmembrane regions, and P region are indicated with *bars*.

The solution for recording (ND-96) contained (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and Na-HEPES 5, pH 7.6. Leak and capacitative currents were not corrected by the leak subtraction method. Currents at the holding potential of −80 mV were <120 nA.

*Electrical measurements of ascidian a*4.2 *cells.* Isolation, cleavage arrest, neural induction, and culture of *a*4.2 cells were performed as previously described (Okamura et al., 1994). Cells were neurally induced either by treatment with 0.2% subtilisin for 1 hr or by cell-contact with *A*4.1 cell (Okado and Takahashi, 1993). Electrical recording was performed in the two-electrode voltage-clamp configuration using AxoClamp2B (Axon

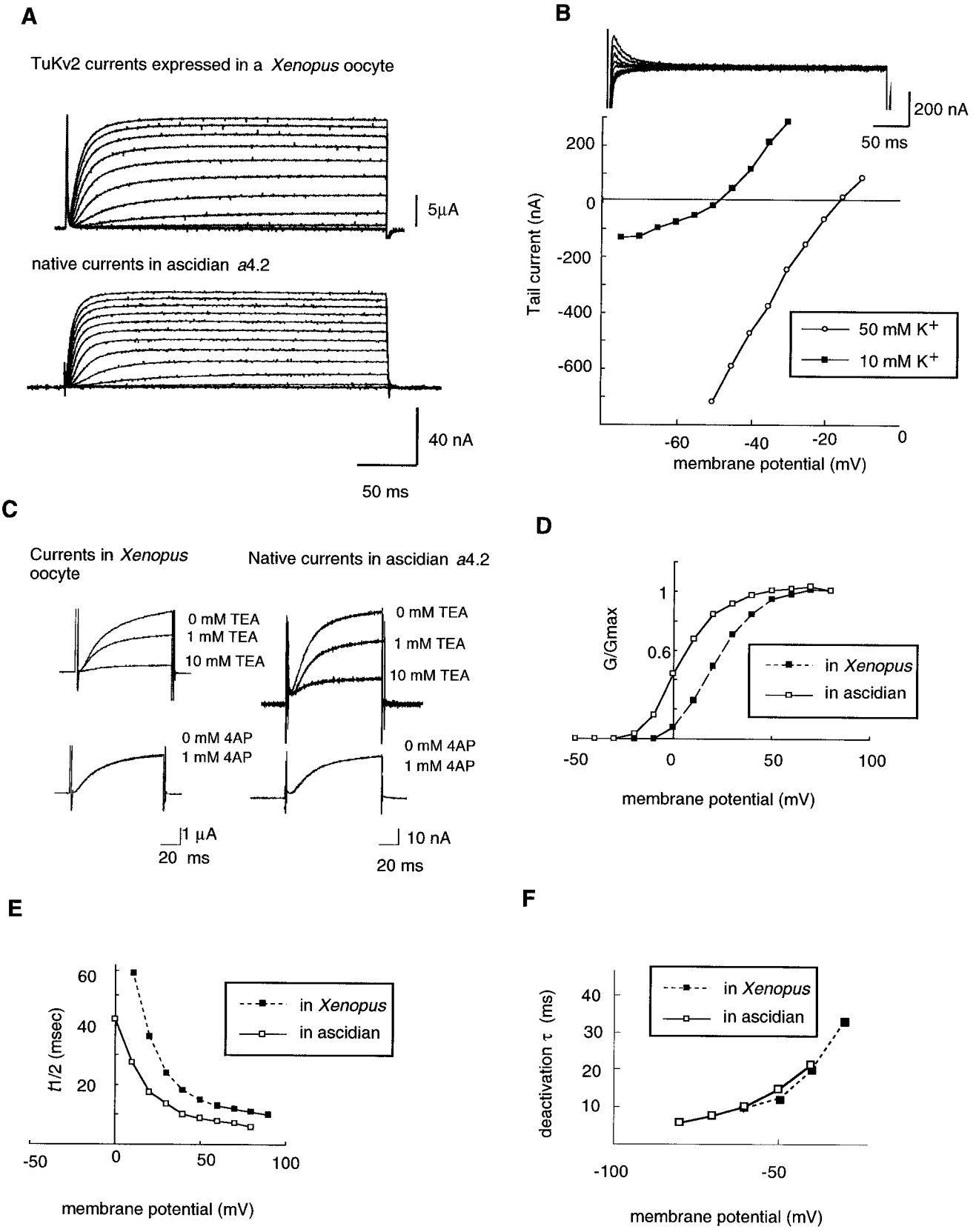

Figure 2. K^+ currents encoded by TuKv2 in *Xenopus* oocytes resemble native delayed rectifier K^+ currents of ascidian *a*4.2 cells. *A*, *Top*, Representative current traces of *Xenopus* oocytes injected with TuKv2 cRNA. *Bottom*, Representative current traces recorded from ascidian neurally induced *a*4.2 cells. Currents were elicited by membrane depolarization pulses ranging from −50 to +80 mV in 10 mV increments after a 50 msec prepulse at −120 mV. Holding potential was −80 mV. *B*, Tail currents of a *Xenopus* oocyte injected with TuKv2 elicited by test pulses after a prepulse at +30 mV for 200 msec in external solutions containing 10 and 50 mM K^+. K^+ was replaced by Na^+ in the 10 mM K^+ solution. The tail current was (*Figure legend continues*)

Instruments, Foster City, CA). The signal was filtered at 3.3 kHz, digitized, sampled at 10 kHz with Clampex (version 6) program (Axon Instruments). Analysis was performed with Clampfit (version 6) program (Axon Instruments). The solution specific for K^+ current recording contained (in mM): tetramethylammonium (TMA)Cl 439, KCl 1, $MgCl_2$ 75, $MnCl_2$ 5, and Na-1,4-piperazinediethane sulfonic acid (PIPES) 5, pH 7.0. The solution for recording Na^+ and K^+ current (standard Ca-free) contained (in mM): NaCl 430, KCl 10, $MgCl_2$ 75, $MnCl_2$ 5, and Na-PIPES 5, pH 7.0. For recording K(Ca) currents, $MnCl_2$ was substituted with $CaCl_2$. The bath solution was kept at 16°C during recording. Cells with large leak currents up to a few nanoamperes caused by cell damages after the insertion of microelectrodes were omitted from analysis. Leak and capacitative currents were not adjusted by the leak subtraction method. Because cell capacitance does not change significantly after 30 hr from fertilization (Okamura and Takahashi, 1993), current densities were estimated only by measuring current amplitudes.

Construction of dominant negative TuKv2. The mutant clone was constructed using QuikChange site-directed mutagenesis kit (Stratagene). Oligoprimers in both directions were synthesized containing sequences that encode phenylalanine instead of wild-type tryptophan. Full-length dominant negative clone, called W404F, was synthesized from the wild-type TuKv2 clone, SbV, following the instruction manual of the kit. The mutation was ascertained by sequencing.

Overexpression in ascidian a4.2 cells. A 3.7-kb-long sequence upstream of ascidian synaptotagmin (Y. Katsuyama and Y. Okamura, unpublished data) was cloned into upstream of the coding region of the wild-type TuKv2 clone (SbV), the dominant negative clone (W404F), and TuKv1. Clones were prepared and purified with Qiagen (Chatsworth, CA) Plasmid Maxi kit and injected into *a*4.2 cells with air pressure just after isolation at the eight-cell stage (6–9 hr after fertilization; Okamura et al., 1994). Cells were neurally induced either by subtilisin or contact to *A*4.1. Time course of current expression is not significantly different between two procedures for neural induction (Okamura and Takahashi, 1993). The injected cells were neurally induced with 0.2% subtilisin and cultured in artificial seawater at 11°C up to the electrical recording at the larval stage (50–55 hr after fertilization). The solution for injection contained 0.02 ng/nl plasmid in the circular form and 1% rhodamine dextran (Sigma, St.Louis, MO). The injection volume was controlled so that each cell emitted about the same intensity of fluorescence.

Northern blot analysis. mRNA was extracted from whole embryos of *Halocynthia roretzi* at sequential stages of development. Five micrograms of RNA of each stage was run on an agarose gel, and transferred onto a blotting membrane. RNA probe was transcribed using Strip-EZ kit (Ambion, Austin, TX) from the linearized plasmid DNA (Shb), which contains the full length sequence of TuKv2. ^{32}P-UTP was included in the reaction mixture and incorporated into the probe. The synthesized probe was hybridized to the membrane overnight at 68°C. The hybridization solution contained 5× SSPE, 50% formamide, 5× Denhardt's solution, 0.5% SDS, and 0.1% BSA. The membrane was washed in 2× SSC, 0.1% SDS, and 0.2× SSC, 0.1% SDS at 68°C. The washed membrane was exposed to a plate and analyzed using a phosphoimager Fuji Bas 2000 (Fuji film Co., Japan).

In situ *hybridization. In situ* hybridization using a digoxigenin probe was performed following the method described previously (Okada et al., 1997). The riboprobe for TuKv2 was synthesized from the clone Shb, which contains the full-length sequence of TuKv2 in pBluescriptII. The plasmid was linearized with *Xho*I and was transcribed with T3 RNA polymerase. The probes for TuNaI were synthesized from plasmids pYT1 and pYR1 (Okamura et al., 1994).

RT-PCR. Cleavage-arrested *a*4.2 blastomeres were prepared as previously described (Okado and Takahashi, 1990, 1993; Okamura et al., 1994). After cleavage arrest and isolation at the eight-cell stage, *a*4.2 cells were separated into two groups: cells in one group were neurally induced by contact with *A*4.1 cells, and cells in the other group were cultured without cell contact. *a*4.2 cells (24–28 cells) of each group were harvested at particular stages of development. Total RNA was extracted from harvested cells following the acid guanidium method. cDNA was synthesized with random hexamers, and PCR was performed following a similar basic protocol described previously (Okamura et al., 1994). Sequence of the 5′ primer for TuKv2 was TCTAGCCGTAAAGGTACTGGC. Sequence of the 3′ primer for TuKv2 was TGAGCTAAATCCTCGTTGTCC. The PCR reaction was performed in a solution containing ^{32}P-dCTP. Primers for TuNaI were the same as the ones used in previous experiments (Okamura et al., 1994). The PCR cycle for TuKv2 was 94°C 1 min, 60°C 1 min, and 72°C 1 min, and the cycle was repeated 29 times. Other pilot experiments were performed to show that the amplification occurs linearly with this cycle number. Amount of template cDNA was normalized by performing PCR (15 cycles) with primers specific to ascidian ribosomal RNA (Wada et al., 1992). Primer sequences for rRNA were TCAATCCTACCTGTGTCCGG and CGTTACCATGACGACCTTCC. Products were run on polyacrylamide gel and analyzed using Fuji Bas 2000 (Fuji film Co.).

GenBank accession numbers for TuKv2 and TuKv1 are AB018545 and AB020853, respectively.

RESULTS

Isolation of full-length ascidian Kv2 cDNA clone

Kv2 genes in many species are known to encode typical delayed rectifier K^+ currents in neurons and muscles (Tsunoda and Salkoff, 1995a; Quattrocki et al., 1994; Burger and Ribera, 1996; Murakoshi and Trimmer, 1999). Therefore we focused on Kv2 subfamily as candidates of K^+ channel genes underlying the delayed rectifier K^+ current in ascidian neural cells. A fragment of the Kv2 gene was obtained from ascidian tadpole cDNA by performing PCR. We screened a cDNA library from tailbud embryos using the PCR fragment as a probe. A full-length cDNA clone that is homologous to genes of the Kv2 subfamily was isolated, and the entire nucleic acid sequence was determined. We named this clone ascidian (or tunicate) Kv2 (TuKv2). The amino acid sequence (959 amino acids) deduced from 2877 bps open reading frame has an ~40% identity with other members of the Kv2 subfamily (Fig. 1). It has N-terminal A and B box (NAB region; Shen and Pfaffinger, 1995) and six transmembrane regions with a high homology to other Kv channel genes. The P region that forms the walls of the channel pore is highly conserved. Multiple alignments with Kv2-related genes from other animals revealed that TuKv2 is more closely related to vertebrate Kv2 channels than to invertebrate Kv2 channels. The amino acid that is most critical for external tetraethylammonium (TEA) sensitivity, residue eight before the beginning of segment S6, is tyrosine in TuKv2. Therefore, we would predict that the channel encoded

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fitted to a single exponential, and the amplitude of the exponential extrapolated to the start of the test pulse was used to describe the tail current. *Inset* is a representative trace recorded in a 10 mM K^+ solution. Similar results were also obtained from another cell. *C*, Drug sensitivities of TuKv2 currents in a *Xenopus* oocyte (*left*) and of native delayed rectifier K^+ currents from a neuronal *a*4.2 cell (*right*). *Left*, *Top*, TuKv2 currents in a *Xenopus* oocyte before TEA application, in 1 mM TEA, and in 10 mM TEA. *Left*, *Bottom*, TuKv2 currents in a *Xenopus* oocyte before 4-AP application and in 1 mM 4-AP. *Right*, *Top*, Native currents from an ascidian neurally induced *a*4.2 cell before TEA application, in 1 mM TEA, and in 10 mM TEA. *Right*, *Bottom*, Native currents from a neurally induced *a*4.2 cell before 4-AP application and in 1 mM 4-AP. Currents were elicited by membrane depolarization pulses to +40 mV after a 50 msec prepulse to −120 mV. Two other cells were tested for each drug and cell type, giving similar results. *D*, *G–V* curve of currents in a TuKv2-injected *Xenopus* oocyte and in a neurally induced ascidian *a*4.2 cell. *Black rectangles,* In *Xenopus* oocytes. *White rectangles,* In ascidian. Conductance values were calculated using the formula $G = I/(V - V_r)$, where G is the conductance, I is the current amplitude, V is the depolarized membrane potential, and V_r is the reversal potential measured by the tail current recordings. *E*, $t_{1/2}$ of the current activation in a TuKv2-injected *Xenopus* oocyte and in a neurally induced ascidian *a*4.2 cell. *Black rectangles,* In *Xenopus* oocytes. *White rectangles,* In ascidian. *F*, Time constant of deactivation (τ) plotted against the membrane potential. Tail currents were fitted to single exponentials. *Black rectangles,* In *Xenopus* oocytes. *White rectangles,* In ascidian. For *E* and *F*, nine *Xenopus* oocytes and six ascidian cells were tested in addition to the cells shown in the figures, and similar results were obtained.

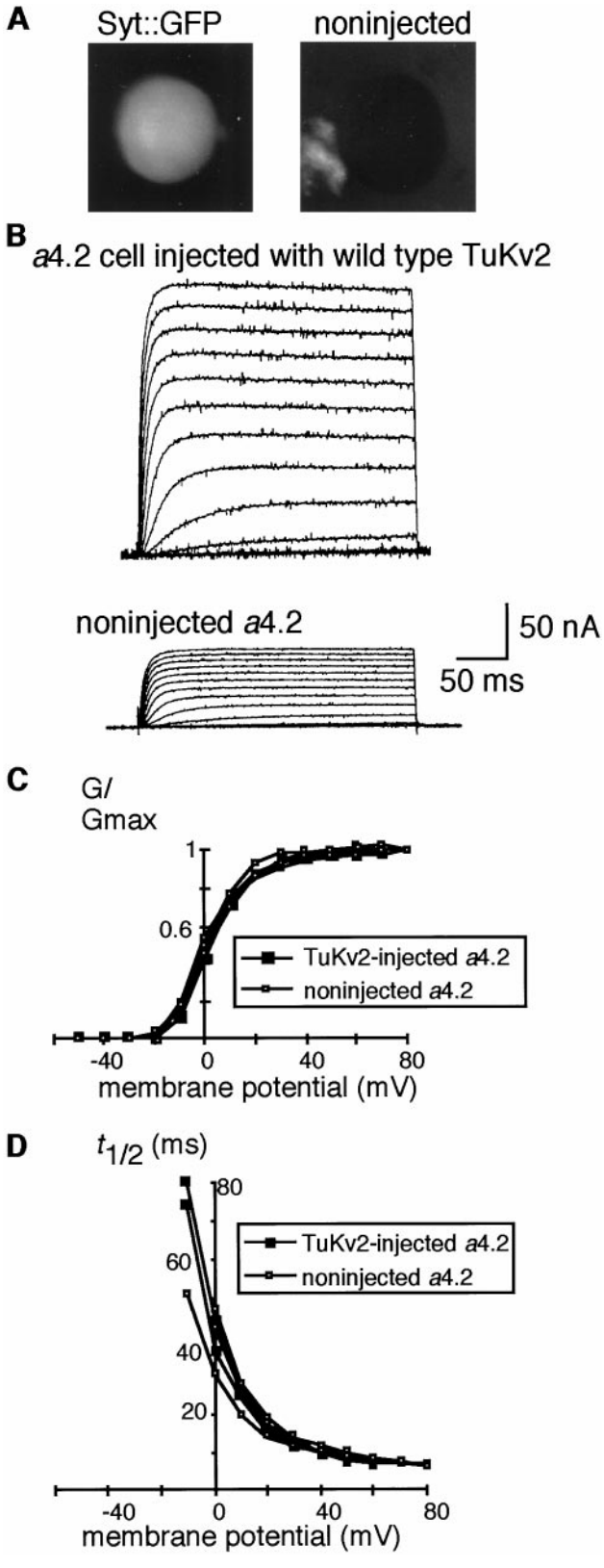

Figure 3. Overexpression of TuKv2 in ascidian neuronal cells leads to an increase in the current amplitude without changing the kinetics. *A*, A neurally induced *a*4.2 cell injected with GFP gene fused to the ascidian synaptotagmin promoter. A cell injected with Syt::GFP emitted fluorescence (*left panel*). A noninjected cell emitted no fluorescence (*right panel*). *B*, Representative traces of neurally induced *a*4.2 cells injected with wild-type TuKv2 (*top panel*) and noninjected *a*4.2 cell (*bottom panel*). Currents were elicited by membrane depolarizations ranging from −50 to +80 mV in 10 mV increments after a 50 msec prepulse to −120 mV. Holding potential was −80 mV. *C*, *G–V* curve of K^+ current in neurally induced *a*4.2 cells injected with wild-type TuKv2 ($n = 3$) and those of noninjected *a*4.2 cells ($n = 3$). Curves of six cells are superimposed. *Black rectangles,* TuKv2 overexpressed cells. *White rectangles,* Noninjected cells. Conductance values were obtained in the same way as in Figure 2*D*. *D*, $t_{1/2}$ of K^+ current in neurally induced *a*4.2 cells injected with wild-type TuKv2 ($n = 3$) and those of noninjected *a*4.2 cells ($n = 3$). Curves of six cells are superimposed. *Black rectangles,* TuKv2 overexpressed cells. *White rectangles,* Noninjected cells. These gating properties were compatible with the previous results (Shidara and Okamura, 1991).

by this gene has a high affinity for TEA (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992).

The delayed rectifier K^+ current encoded by TuKv2 in *Xenopus* oocytes resembles the native ascidian current

To identify the electrical properties of TuKv2, cRNA was expressed in *Xenopus* oocytes. Delayed rectifier K^+ currents started to appear 1–2 d after injection (Fig. 2*A*). The reversal potential of the currents in *Xenopus* oocytes, determined by recording the tail current, shifted positive by ~35 mV with the change in the external K^+ concentration from 10 to 50 mM. This is close to the theoretical Nernst value for K^+ current, demonstrating that TuKv2 is indeed a K^+ current (Fig. 2*B*). Properties of TuKv2-derived K^+ currents in *Xenopus* oocytes were compared with endogenous delayed-rectifier K^+ currents from ascidian neuroblasts. Endogenous currents were recorded from isolated, cleavage-arrested *a*4.2 cells. When *a*4.2 blastomere is isolated and cultured under cleavage arrest, it expresses neuronal excitability after 48 hr (Takahashi and Okamura, 1998). These neurally induced *a*4.2 cells are known to express the TEA-sensitive delayed-rectifier K^+ current (Shidara and Okamura, 1991). Drug sensitivities were compared between the currents in *Xenopus* oocyte and *a*4.2 cell. One and 10 mM TEA significantly reduced the current amplitude both in *a*4.2 cells and in *Xenopus* oocytes (Fig. 2*C*, *top panel*). This property was expected from the half-maximal inhibition dose of TEA (1.3 mM), which was previously reported for endogenous delayed rectifier K^+ currents (Shidara and Okamura, 1991). Both currents were resistant to 1 mM 4-aminopyridine (4-AP; Fig. 2*C*, *bottom panel*). On the other hand, there are some differences in gating properties. The normalized conductance (G/G_{max}) plotted versus membrane potential (*G–V* curve) showed a 20 mV rightward shift in *Xenopus* oocytes (Fig. 2*D*). A similar shift was found in the time to half-maximal activation ($t_{1/2}$), and the TuKv2 current activated more slowly than the endogenous current in ascidian *a*4.2 cells when compared at the same voltages (Fig. 2*E*). This voltage shift was observed only for the activation, but not for the deactivation. The time constant of deactivation (τ) did not show a significant difference (Fig. 2*F*).

TuKv2 exclusively encodes the delayed rectifier K^+ current in ascidian neurally induced *a*4.2 cells

We suspected that the distinct voltage of activation observed in *Xenopus* oocytes and native ascidian cells was caused by different cellular environments, including ionic strength and concentration of divalent cations. Consequently, the channel properties of overexpressed TuKv2 were studied in ascidian neurally induced *a*4.2 cells. A 3.7 kb promoter region of ascidian synaptotagmin (Y. Katsuyama, unpublished data) was fused with a green fluorescent

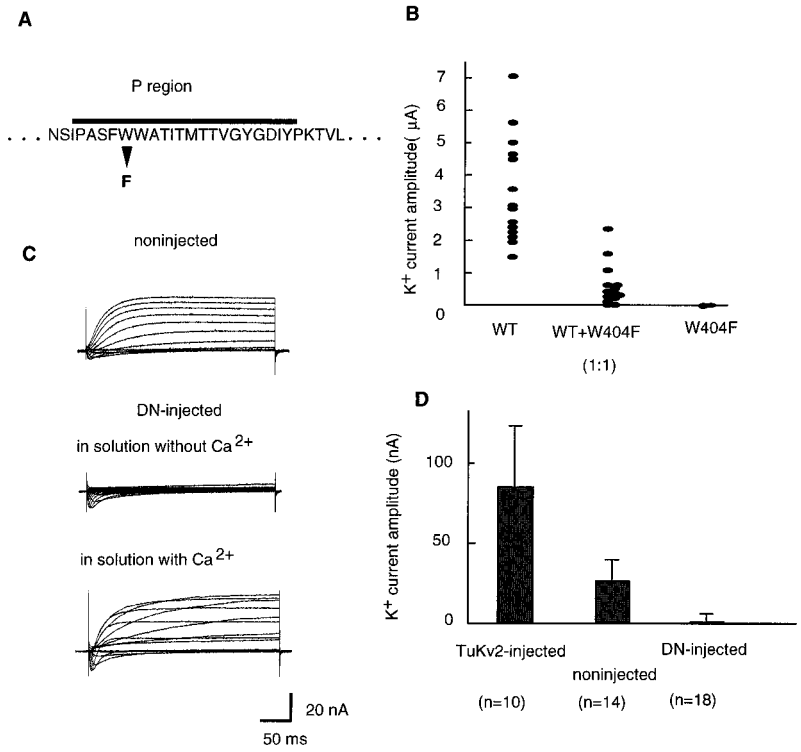

Figure 4. Forced expression of dominant negative TuKv2 in ascidian neuronal cells eliminated the native current. *A*, The amino acid sequence of the P region of TuKv2. The tryptophan was mutated to phenylalanine in the mutant W404F. *B*, Peak amplitudes of K^+ current in *Xenopus* oocytes elicited with a voltage step from −80 to +30 mV. Oocytes were injected with wild-type TuKv2 mRNA alone (*WT; n* = 14), a mixture of wild-type TuKv2 mRNA and W404F mRNA at a ratio of 1:1 (WT+W404F; n = 14), and W404F mRNA alone (W404F; *n* = 7). *C*, Representative traces of a noninjected *a*4.2 cell (*top panel*), an Syt::W404F-injected *a*4.2 cell (*middle panel*) in a solution without Ca^{2+} (standard Ca^{2+}-free). Traces from the same cell shown in the *middle panel* in a solution containing 5 mM Ca^{2+} (*bottom panel*). Currents were elicited by membrane depolarizations ranging from −50 to +60 mV in 10 mV increments after a 50 msec prepulse to −120 mV. Holding potential was −80 mV. *D*, Mean peak amplitudes of K^+ currents at a +10 mV test pulse in neurally induced *a*4.2 cells injected with wild-type TuKv2 (*TuKv2-injected*), no DNA (*noninjected*), and dominant negative TuKv2 (*DN-injected*). Vertical bars are SD. The differences between TuKv2-injected and noninjected cells as well as between noninjected and DN-injected cells are significant statistically (Student's *t* test; $p < 0.01$).

protein (GFP). When this plasmid was injected into neurally induced *a*4.2 cells, GFP fluorescence was detected after 1 or 2 d of culture in all the cells that were injected with the plasmid (*n* = 10; Fig. 3*A*). This promoter was also used to drive expression of TuKv2. When the plasmid Syt::TuKv2 was introduced into neurally induced *a*4.2 cells, the K^+ current was increased 3- to 10-fold compared with noninjected neurally induced cells (Fig. 3*B*). The delayed rectifier K^+ current from cells overexpressing TuKv2 showed *G–V* curve and $t_{1/2}$ similar to those of endogenous currents from noninjected cells (Fig. 3*C*,*D*). These findings suggest that endogenous and overexpressed currents are functionally indistinguishable, suggesting that the endogenous delayed rectifier K^+ current is encoded only by the TuKv2 gene.

It is known that other subfamilies of Kv channel gene such as Kv3 or HERG-related channel also encode delayed rectifier K^+ currents in neuronal cells (Wang et al., 1997). To confirm further that the endogenous delayed rectifier K^+ current in *a*4.2 cells is exclusively encoded by TuKv2, a dominant negative form of TuKv2 was also constructed. Four α-subunits are known to assemble to form one K^+ channel (MacKinnon, 1991). Ionic currents, but not gating currents, of *Drosophila Shaker* K^+ channels in *Xenopus* oocytes disappear completely when a conserved tryptophan (W) in the P region is mutated to phenylalanine (F) (WF mutant; Perozo et al., 1993). This WF mutation makes the channel more readily enter C-type inactivation state (Yang et al., 1997), thereby potentially working as a dominant negative protein (Ribera, 1996). Because genes belonging to different subfamilies of Kv channels do not form heteromultimers (Xu et al., 1995), such a WF mutant of K^+ channel provides a useful molecular tool to suppress functions of a specific subfamily of K^+ channels. We were able to construct a WF mutant of TuKv2 because tryptophan at the corresponding site in the P region is also conserved in TuKv2 (Fig. 4*A*). *Xenopus* oocytes injected with this clone, W404F, expressed no K^+ current. The current expressed by coinjection of the W404F and wild-type TuKv2 at a 1:1 ratio was ~25% of that from control oocytes injected with the same amount of only wild-type TuKv2 (Fig. 4*B*). This is consistent with the idea that the mutated channel works as a dominant negative (Ribera, 1996).

We drove the expression of this W404F mutant in ascidian neuronal cells under the synaptotagmin promoter. Most cells injected with Syt::W404F (26 of 31 cells) showed no delayed rectifier K^+ current, although the Na^+ current remained (Fig. 4*C*, *top* and *middle panels*). Some Syt::W404F-injected cells expressed the K^+ current, but the current amplitude was much reduced compared with noninjected cells (Fig. 4*D*). In some neuronal *a*4.2 cells, Ca-activated K^+ current (K(Ca)) is observed. When the recording solution contained 5 mM Ca^{2+}, some Syt::W404F-injected cells with a barely detectable delayed rectifier K^+ current showed a remarkable amount of K(Ca) current (Fig. 4*C*, *bottom panel*). This indicates that the dominant negative effect of W404F is highly specific to the delayed rectifier K^+ current. Elimination of the K^+ current with W404F therefore suggests that no gene belonging to subfamilies other than Kv2 contributes to the formation of the endogenous current in neuronal *a*4.2 cells, highlighting the simple composition of K^+ currents in these cells.

Transcription of TuKv2 starts much earlier than its functional expression

We analyzed the gene expression of TuKv2 during development. Northern blot analysis with an RNA probe transcribed from the full length TuKv2 clone detected two discrete bands, at 4.8 and 4.3 kb. The longer band, 4.8 kb, corresponds to the insert size of the clone we obtained (Fig. 5). We consider the shorter band a splice variant of TuKv2. Both transcripts were expressed maternally and throughout development, from the gastrula to the larval stage. Previous electrophysiological results (Simoncini et al., 1988; Shidara and Okamura, 1991) and our recent patch-clamp recordings from early isolated blastomeres (Y. Okamura, unpub-

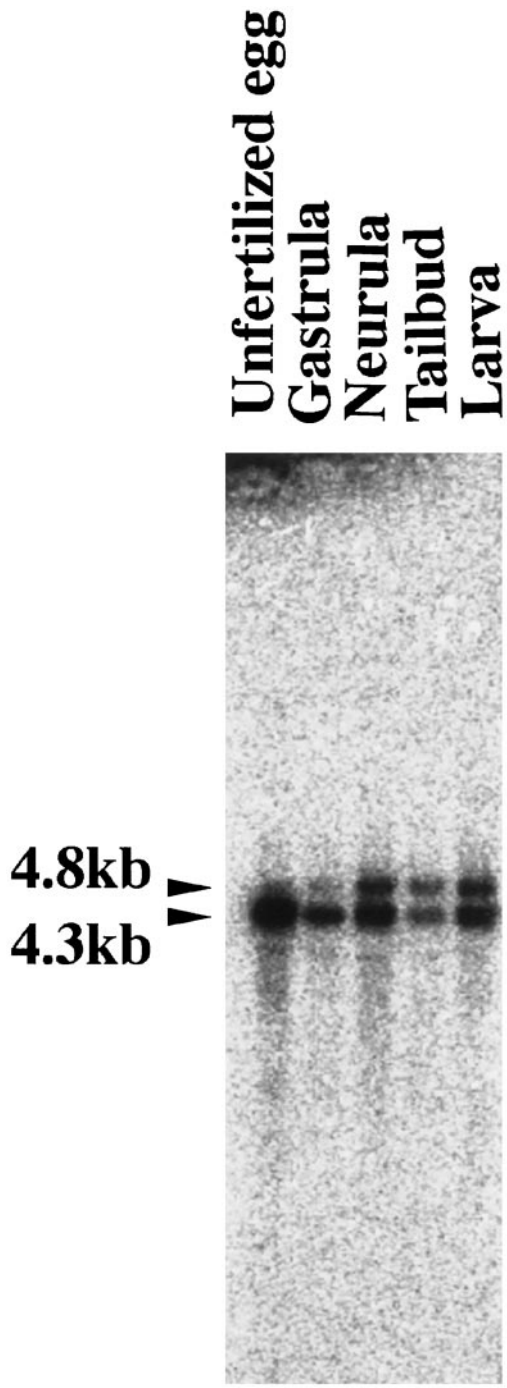

Figure 5. Northern blot analysis of TuKv2. Five micrograms of mRNA from unfertilized eggs (0 hr) or embryos of gastrula (17 hr), neurula (22 hr), tailbud (35 hr), and tadpole larva (55 hr) was loaded. Two bands were detected at 4.8 and 4.3 kb at all stages of development. RNA was quantified by measuring the absorbance by the spectrophotometer before loading. The integrity of RNA was checked by rehybridizing the same blot with a probe specific to ascidian voltage-gated calcium channel (TuCaI; data not shown).

lished data) showed that the delayed rectifier K^+ current is absent in mature unfertilized eggs and in early embryos. Thus, TuKv2 transcripts are present at much earlier stages of development than the functional expression of the delayed rectifier K^+ current.

TuKv2 is regulated spatially in a distinct manner from TuNaI

To examine the tissue specificity of TuKv2 expression, whole-mount *in situ* hybridization analysis was performed. Over long staining periods, the whole embryo, especially of earlier stages, turned opaque, a phenomenon commonly observed among ascidian genes expressed maternally. This is compatible with the Northern blot analysis that revealed significant amounts of maternal transcripts. Such a strong background signal was not detected in the case of TuNaI. In order to discriminate the zygotic expression from maternal transcripts, the exposure to the staining dye was limited to a shorter duration. Signals detected in this manner were associated with nuclei, suggesting that the signals were from the zygotic expression.

Discrete signals were detected in the CNS in the regions derived from the *a*-lineage (Fig. 6*C*; Nishida, 1987). The signal was also detected in motor neurons and in putative peripheral neurons in the papilla (Fig. 6*B*,*C*). However significant expression was also detected in non-neural tissues; the most robust expression was detected bilaterally in the mesenchyme, which gives rise to blood cells and adult body wall muscles (Fig. 6*A*--*C*). In a parallel experiment with TuNaI specific probe, signals were detected in neurons (Fig. 6*D*--*F*) in agreement with previous experiments (Okamura et al., 1994). Some neurons derived from *a*4.2 and motor neurons seem to coexpress TuKv2 and TuNaI (Fig. 6*B*,*C*,*E*,*F*). Other neurons including epidermal neurons, however, express TuNaI but not TuKv2 (Fig. 6*B*,*E*).

Temporally distinct pattern of expression between TuKv2 and TuNaI in cleavage-arrested *a*4.2 cells

The *in situ* hybridization results above demonstrated that TuKv2 mRNA is expressed in some populations of neurons derived from the *a*4.2 cell. RT-PCR analysis was performed to examine if the gene expression of TuKv2 occurs in isolated, cleavage-arrested *a*4.2 cells that consistently show neuronal membrane excitability after neural induction (Okado and Takahashi, 1990, 1993). Ascidian *a*4.2 cells were cleavage-arrested at the eight-cell stage with cytochalasin B and were cultured in contact with *A*4.1 cells (Fig. 5*A*). *A*4.1 cells can induce *a*4.2 cells into the neural cell fate by cell contact during the critical period (Okado and Takahashi, 1993). The two cells were separated after the critical time and cultured in isolation. Isolated neurally induced *a*4.2 cells express Na^+ currents and delayed rectifier K^+ currents when embryos fertilized at the same time and reared in parallel with the blastomeres become larvae and hatch (Okado and Takahashi, 1990; Okamura and Shidara, 1990; Shidara and Okamura, 1991). When another group of sibling *a*4.2 cells were cultured in isolation without neural induction, they follow the epidermal cell fate (Okado and Takahashi, 1990). When the intact control embryos developed into the 32-cell stage, gastrula, neurula, tailbud, and larva, both neurally induced and noninduced *a*4.2 cells were harvested and used for RNA extraction (Fig. 7*A*).

RT-PCR with primers specific to TuKv2 revealed that in neurally induced *a*4.2 cells, TuKv2 transcript was weakly detectable at the 32-cell stage, increased at the gastrula stage, decreased at the neurula and the tailbud stage, and increased again at the larval stage. Thus, its expression had two peaks during development. In contrast, in noninduced *a*4.2 cells, the TuKv2 transcript was detected at the 32-cell stage, decreased with time, and finally was not detectable at the larval stage (Fig. 7*B*). When compared at the larval stage, TuKv2 expression was specific to neurally

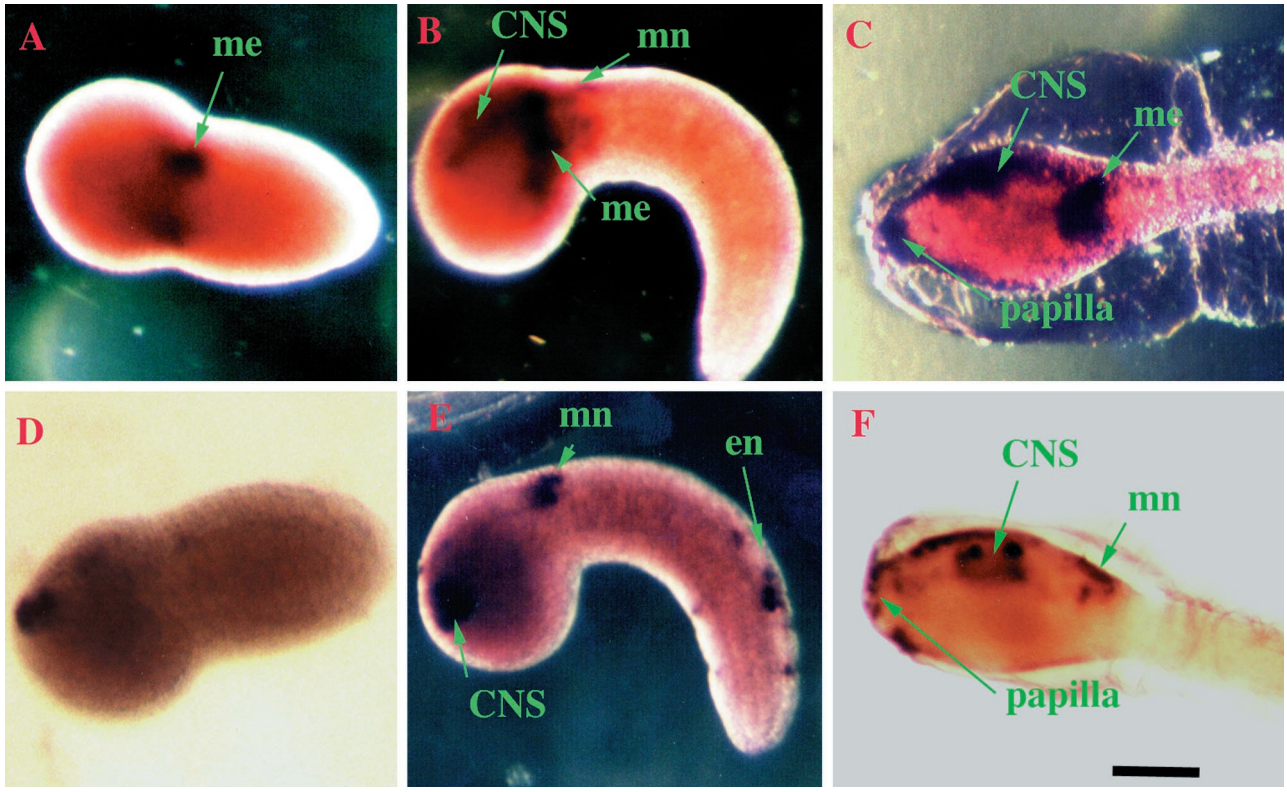

Figure 6. The expression pattern of TuKv2 transcript was distinct from that of TuNaI. Whole-mount *in situ* hybridization was performed on ascidian embryos with riboprobes specific to TuKv2 (*A–C*) and TuNaI (*D–F*). *A* and *D* are dorsal and lateral views of the neurula stage embryos, respectively. *B* and *E* are lateral views of the young tadpole stage embryos. *C* and *F* are lateral views of the trunk regions of swimming larvae. Only the embryo shown in *D* was cleared using a benzylbenzoate–benzylalcohol mixture. *me*; Mesenchyme. *mn*; motor neuron. *en*; epidermal neuron. Scale bar, 100 μm.

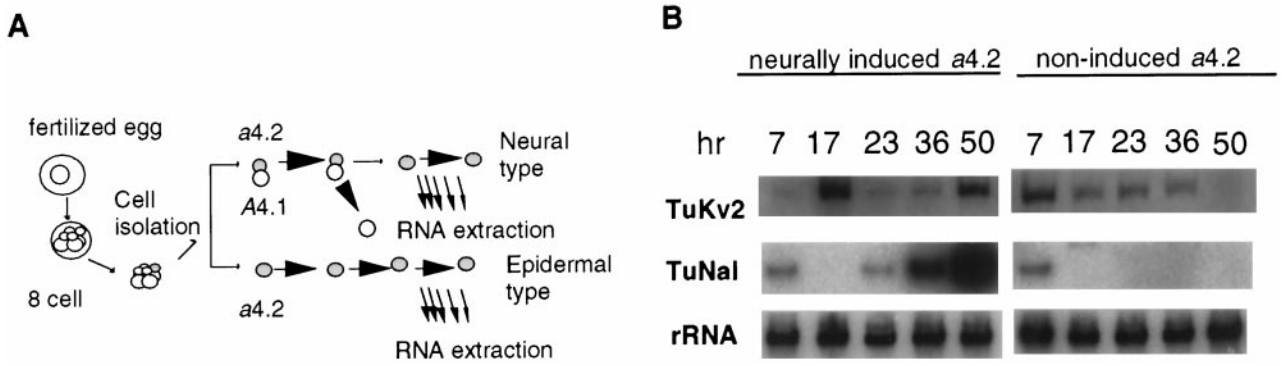

Figure 7. TuKv2 transcripts were detected in isolated neurally induced *a*4.2 cells, and the temporal expression pattern of TuKv2 transcript was distinct from that of TuNaI. *A*, A scheme of experimental design for RT-PCR. Cleavage-arrested *a*4.2 blastomeres were harvested at sequential stages of development with or without neural induction. mRNA was extracted, and RT-PCR was performed. *B*, RT-PCR in neurally induced and noninduced *a*4.2 cells in stages of the 32-cell (about 7 hr after fertilization), gastrula (17 hr), neurula (23 hr), late tailbud (36 hr), and swimming larva (50 hr). Signals show radiolabeled PCR products amplified with primers specific to TuKv2 and TuNaI. PCR was also performed with primers specific to ascidian ribosomal RNA (rRNA) as a control. Despite normalization of quantity of loaded template cDNAs, the intensity of rRNA bands was not perfectly equal among lanes, probably reflecting variability of the volume at pipetting. Developmental changes of the TuNaI and TuKv2 signals were more remarkable than variability of amplification of rRNAs.

induced cells. PCR experiments were repeated twice for the larval stage, and the same results were obtained. This temporal pattern of transcription is significantly different from that of a Na^+ channel gene, TuNaI (Okamura et al., 1994). TuNaI mRNA was detected in the 32-cell stage regardless of neural induction. This early expression was not found in previous RT-PCR studies using whole embryos (Okamura et al., 1994), possibly because of different PCR protocols. This early expression ceased before the gastrula stage. Transcription started again at the neurula stage only in neurally induced *a*4.2, and transcription increased up to the larval stage (Fig. 7*B*). These results also suggest that TuNaI and TuKv2 genes are regulated by separate transcriptional mechanisms.

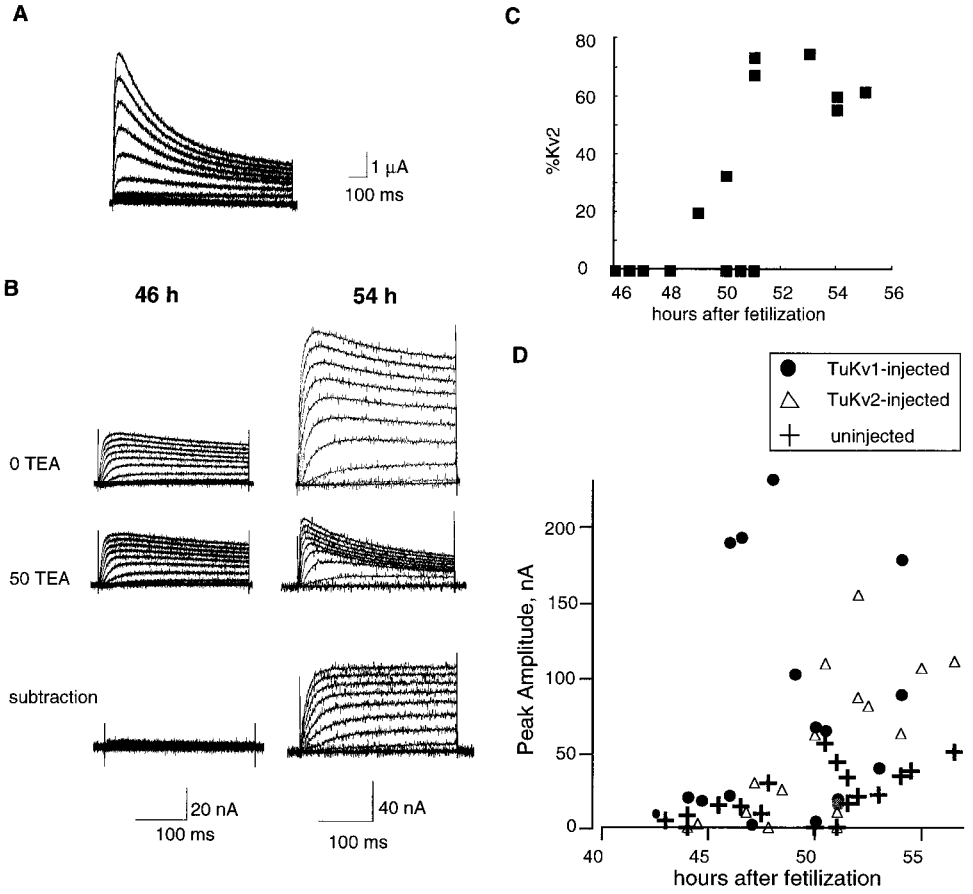

Figure 8. Misexpression of ascidian Kv1 in neurally induced *a*4.2 cell led to the functional expression of Kv1-derived current earlier than the TuKv2-derived current. *A*, Representative current traces of a *Xenopus* oocyte injected with cRNA of TuKv1. Currents were elicited from a holding potential of −70 mV to steps ranging from −80 to +60 mV in 10 mV increments. *B*, Representative current traces from a TuKv1-injected neuronal *a*4.2 cell recorded at 46 and 54 hr after fertilization. *Top panel* shows currents in TEA-free solution. *Middle panel* shows currents in 50 mM TEA. *Bottom panel* shows subtracted currents. Currents were elicited by membrane depolarizations ranging from −50 to +60 mV by 10 mV increments after a 50 msec prepulse to −120 mV. Holding potential was −80 mV. *C*, %Kv2 plotted against the development time. %Kv2 is defined as the maximum TuKv2 current amplitude at +60 mV divided by the sum of the TuKv2 current amplitude and the TuKv1 current amplitude at +60 mV. *D*, Peak amplitudes of K^+ current during a voltage step to +60 mV in *a*4.2 cells injected with TuKv1, TuKv2, and no DNA (noninjected) plotted against the development time. In TuKv1-injected cells, peak amplitudes before the application of TEA are plotted.

Misexpression of ascidian Kv1 in neurally induced *a*4.2 leads to the functional expression of Kv1-derived current earlier than Kv2-derived current

Northern blot analysis and RT-PCR from *a*4.2 cells indicate that TuKv2 transcript is detected at developmental stages when the functional delayed rectifier K^+ current is not detected. The delayed rectifier K^+ current is observed only when *a*4.2 cell is neurally induced and only after 40 hr of development (Shidara and Okamura, 1991). These temporal mismatches between TuKv2 transcripts and the delayed rectifier K^+ current suggest that functional channel expression from early TuKv2 mRNA is suppressed.

To test if TuKv2 channel expression is developmentally regulated by a mechanism specific to the Kv2 family, we misexpressed ascidian Kv1-related gene (TuKv1; Katsuyama et al., unpublished data) in neurally induced *a*4.2 cells. TuKv1 encodes current with a faster inactivation than TuKv2-derived current when expressed in *Xenopus* oocytes (Fig. 8*A*). The magnitude of TuKv1-derived current in *Xenopus* oocyte was as much as that of TuKv2 current (our unpublished data). TuKv1-derived current was insensitive to TEA in *Xenopus* oocyte (data not shown). This pharmacological property makes it easy to discriminate between TuKv1-derived current and TuKv2-derived current. TuKv1 is not expressed endogenously in neurally induced *a*4.2 cells, as evidenced by almost complete elimination of delayed rectifier K^+ currents by expressing a dominant negative form of TuKv2 in this study or by application of TEA (Shidara and Okamura, 1991).

When synaptotagmin promoter was fused to TuKv1 (Syt::Kv1) and was introduced into neurally induced *a*4.2, two currents with different sensitivity to TEA were observed: the endogeneous delayed rectifier K^+ current that can be eliminated by addition of 50 mM TEA and the current encoded by TuKv1 that is resistant to this concentration of TEA. In the cell recorded at 46 hr after fertilization (Fig. 8*B, left*), currents recorded in solutions with and without TEA were identical. In this cell, therefore, K^+ current is mainly based on TuKv1. The low level of expression of TEA-sensitive delayed rectifier K^+ current at this developmental stage is compatible with the data from noninjected cells (Fig. 8*D*) as well as the previous results (Shidara and Okamura, 1991). The reason for the slower inactivation of TuKv1-derived current in the ascidian cell than in *Xenopus* oocyte is not known. We know that TuKv1 and TuKv2 do not coassemble, because TEA-resistant current does not decrease by coinjection with the plasmid of the dominant negative TuKv2 (data not shown). The inactivation of TuKv1 current is variable among *a*4.2 cells, suggesting that other cellular factors, such as the amount of auxiliary subunits (Rettig et al., 1994) or degree of phosphorylation may determine current decay kinetics of TuKv1 currents.

In contrast, currents from cells recorded at 54 hr after fertilization (Fig. 8*B, right*) were sensitive to 50 mM TEA. Subtraction of the two traces between with and without TEA reveals the current encoded by the endogenous TuKv2 in this cell. Current amplitudes of TuKv1 and TuKv2 in each cell were determined in this fashion. The ratio of TuKv2 current amplitude to the sum of TuKv1 amplitude and TuKv2 amplitude (%Kv2) was plotted against the developmental time (Fig. 8*C*). Before 50 hr, the expressed current is almost purely TuKv1-derived. %Kv2 increased as development progressed. This shows that exogenously introduced Kv1 was expressed earlier than the current consisting of the endogenous TuKv2.

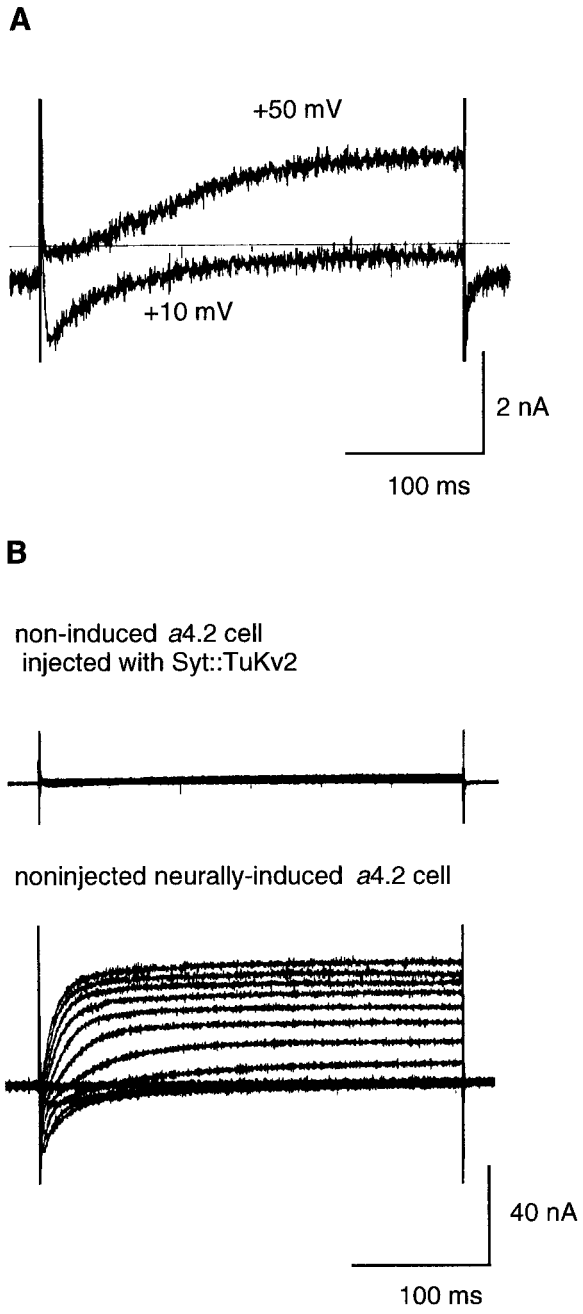

Figure 9. Forced expression of TuKv2 in noninduced *a*4.2 cells did not lead to the current expression at the epidermal differentiation. *A*, Representative traces of a noninduced *a*4.2 cell injected with Syt::TuKv2. Noninduced *a*4.2 cell follows the epidermal cell fate (Okado and Takahashi, 1990). Recording was made in a solution containing 5 mM Ca^{2+}. Current traces were elicited by test pulses at +10 and +50 mV after a 50 msec prepulse at −120 mV. The holding potential was −80 mV. *B*, A comparison of current traces between a noninduced *a*4.2 cell injected with Syt::TuKv2 (*top,* 1 of 3 cells) and a neurally induced *a*4.2 cell (*bottom*, 1 of 3 cells) shown in the same gain scale. Currents were elicited by membrane depolarizations ranging from −50 to +60 mV by 10 mV increments after a 50 msec prepulse to −120 mV. The holding potential was −80 mV.

To rule out the possibility that exogenously introduced genes in general are expressed earlier because of the transcriptional activity of the synaptotagmin promoter or to a large copy number of introduced genes, TuKv2 was exogenously overexpressed in cells from the same batch for TuKv1 expression. The peak amplitude of K^+ current was plotted against the developmental time. In TuKv2-injected cells, in contrast to TuKv1-injected cells, the current amplitude linearly increased with time in the same fashion as the endogenous current of noninjected cells (Fig. 8*D*). At earlier developmental stages, the expression of the misexpressed Kv1 channel is more robust than the overexpressed or endogenous TuKv2 channels. This suggests that the failure of functional TuKv2 expression at earlier stages of development in neurally induced cells is not caused by a general status of the cell, such as immaturity of the transport system. We conclude that there exists a specific mechanism that suppresses the functional expression of only TuKv2 but cannot suppress TuKv1 expression.

Forced expression of TuKv2 in noninduced *a*4.2 cells does not lead to the expression of the delayed rectifier current

RT-PCR experiments of noninduced *a*4.2 cells revealed a significant amount of TuKv2 transcripts at earlier stages of development. Noninduced *a*4.2 cells, however, do not express the delayed rectifier K^+ current at any developmental stage (Okamura and Takahashi, 1993). This mismatch of mRNA and functional currents raises a possibility that the same mechanism that suppresses the expression of the current in neurally induced *a*4.2 cells works also in noninduced cells. Synaptotagmin promoter also drives gene expression in epidermal cells, although at a weaker level than in neuronal cells (Y. Katsuyama, unpublished observation). Syt::TuKv2 was introduced into noninduced *a*4.2 cells. All the injected cells differentiated into epidermal phenotype, as evidenced by a small Ca^{2+} current observed in a solution containing 5 mM Ca^{2+}. No Syt::TuKv2-injected noninduced cell show any expression of the Na^+ current typically observed in neuronal cells ($n = 3$). A small Ca-activated K^+ current, which is also a typical phenotype of epidermally differentiated cells (Hirano et al., 1984), was also observed at higher voltages. This type of current shows a much slower rate of activation than the delayed rectifier K^+ current observed in neurally induced *a*4.2 cells (Fig. 9*A*); at +50 mV the delayed rectifier current reaches the peak amplitude in <30 msec. Furthermore, the amplitude of outward current is much smaller compared with the delayed rectifier K^+ current in neurally induced *a*4.2 cells (Fig. 9*B*). From these results, we conclude that the TuKv2 mRNA leads to the functional expression of the delayed rectifier K^+ current only in neurally induced *a*4.2 cells, and some post-transcriptional mechanism suppresses expression of the delayed rectifier K^+ current in noninduced *a*4.2 cells.

Effect of K^+ channel function on Na^+ channel expression

The electrical excitability of neurally induced *a*4.2 cells is based on a very limited number of genes, only TuKv2 for the delayed rectifier K^+ current and TuNaI for the Na^+ current. Considering different transcriptional regulations between TuKv2 and TuNaI, it is possible that the coordinated expression of K^+ and Na^+ channels is controlled at post-transcriptional level. We determined whether changes in the expression of TuKv2 affect the expression level of TuNaI.

The Na^+ current amplitude was measured with a −10 mV test pulse after hyperpolarizing to −120 mV, which is known to completely remove slow inactivation of ascidian Na^+ channels (Okamura and Shidara, 1990). The recording was made in a Ca^{2+}-free solution, so that the inward current can be assumed to be a pure Na^+ current. At −10 mV, the G/G_{max} for K^+ current

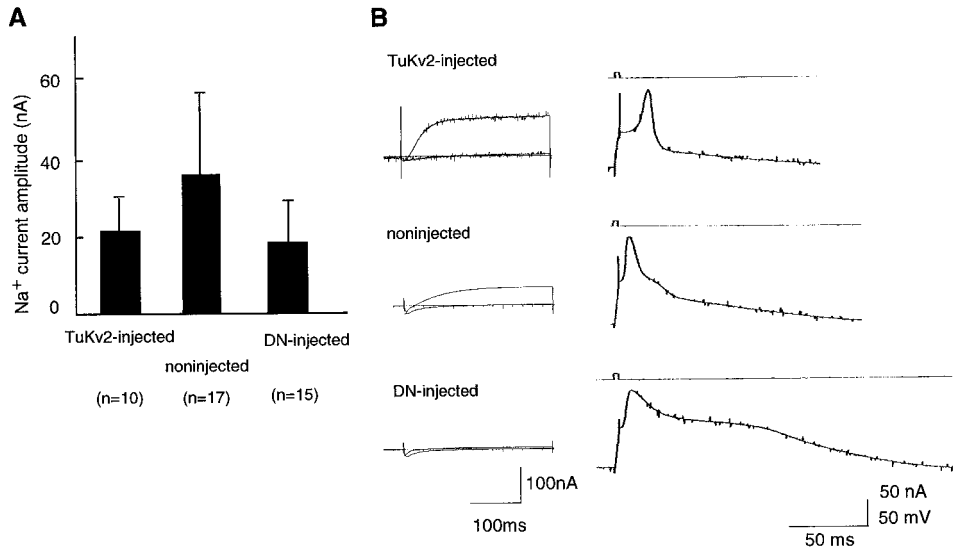

Figure 10. Neither overexpression nor abolition of TuKv2 K^+ channel affected Na^+ channel expression. *A*, Mean peak amplitudes of Na^+ currents at a −10 mV test pulse in ascidian neurally induced *a*4.2 cells injected with wild-type TuKv2 (*TuKv2-injected*; $n = 10$), no DNA (*noninjected*; $n = 17$), and dominant negative TuKv2 (*DN-injected*; $n = 15$). Vertical bars are SD. The holding potential was −120 mV. The difference of mean peak amplitudes was not statistically significant between TuKv2-injected cells and DN-injected cells. *B*, *Left panels,* Representative traces of ascidian neurally induced *a*4.2 cells injected with wild-type TuKv2 (*top*), no DNA (*middle*), and dominant negative TuKv2 (*bottom*). Currents were elicited by voltage steps from a −120 mV prepulse to −10 and +10 mV. *Right panels,* Action potential of each cell to the left. The injected current (10 nA, 3 msec) is shown above each trace. Artifacts are observed at the end of the injected current.

is ~0.2. At 6–8 msec after the start of the test pulse, where the Na^+ current amplitude was measured, the K^+ current amplitude is no more than 1% of the plateau current. Thus, in a cell expressing 100 nA of the K^+ current, its effect on the Na^+ channel amplitude is <0.2 nA. In order to ensure that the K^+ current does not mask the Na^+ current in cells expressing a large amplitude of K^+ current, 50 mM TEA was used to eliminate the K^+ current. The Na^+ current amplitude recorded in this manner remained at the same level in neurally induced *a*4.2 cells overexpressing the dominant-negative TuKv2, compared with cells overexpressing wild-type TuKv2 (Fig. 10*A*). As expected from voltage-clamp data, overexpression of wild-type TuKv2 shortened the width of the action potential, and expression of the dominant negative TuKv2 widened it (Fig. 10*B*). These results indicate that the expression level of the delayed rectifier K^+ channel does not affect Na^+ channel expression.

DISCUSSION

The present study demonstrates that an ascidian Kv2-related gene is regulated by a transcriptional mechanism distinct from that of a Na^+ channel gene. Transcription of TuKv2 occurs much earlier than its functional expression and is not restricted to neuronal lineages. Overexpression of TuKv2 and misexpression of *Shaker*-related K^+ channel gene TuKv1 showed that some post-transcriptional mechanism operates in a subfamily-specific manner to control the expression timing of TuKv2.

Delayed rectifier K^+ current in neurally induced *a*4.2 is exclusively encoded by Kv2 subfamily

Whole-mount *in situ* hybridization revealed that TuKv2 was expressed in the neural region derived from the *a*4.2 blastomere. The expression studies in *Xenopus* oocytes and in native ascidian *a*4.2 cells indicated that TuKv2 encodes the entire macroscopic delayed rectifier K^+ current in ascidian neurally induced *a*4.2 cells.

We introduced a point mutation in the pore region of TuKv2 to produce a nonconductive mutant (Yang et al., 1997). Such a form of TuKv2 completely suppressed the delayed rectifier K^+ current in the neuronal *a*4.2 cells. Dominant negative K^+ channels are presumed to work in a subfamily-specific way because only α-subunits that belong to one subfamily can assemble to form a tetramer (Xu et al., 1995). It is, therefore, deduced that the delayed rectifier K^+ current in the *a*4.2 neuronal cells is exclusively encoded by α-subunits of the Kv2 subfamily. The WF mutation of the *Xenopus* Kv2 channel at the same site of the P-region does not make the channel dominant negative (Blaine and Ribera, 1998), although a similar mutant of *Xenopus* Kv1 does work as a dominant negative (Ribera, 1996). This discrepancy may be attributable to different high order molecular structures between *Xenopus* and ascidian Kv2 channels.

We cannot neglect the possibility that other Kv2-related members are also present in ascidian embryos. Two discrete bands in a Northern blot suggest some TuKv2 molecular diversity. Hybridization of the Northern blot under high or low stringency conditions did not make a significant difference in the results (our unpublished observation), suggesting that the two bands with different sizes represent splice variants. In fact, we have recently isolated another splice variant with the size corresponding to the lower band. This second type has a shorter 5′UTR (our unpublished observation).

The expression of the delayed rectifier K^+ current derived from TuKv2 is post-transcriptionally controlled

A mismatch was found between the TuKv2 transcripts and the functional expression of the delayed rectifier K^+ current during ascidian embryogenesis. First, TuKv2 transcripts are detected in neurally induced *a*4.2 cells before the tailbud stage when the delayed rectifier K^+ current is not detected (Shidara and Okamura, 1991). Second, TuKv2 transcripts are present in noninduced *a*4.2 cells, which never express the delayed rectifier K^+ current at any developmental stage (Okamura and Takahashi, 1993). These findings suggest that the mRNA of α-subunit alone is not sufficient for the functional current expression. This is further indicated by the fact that the forced expression of TuKv2 transcripts in noninduced cell does not lead to the expression of the delayed rectifier K^+ current.

Misexpression of ascidian Kv1-related gene in neurally induced cells showed the earlier expression of the TuKv1 current than the endogenous or overexpressed TuKv2 currents. This implies that the molecular mechanism suppressing the functional expression from TuKv2 transcripts is not through a mechanism common to diverse subfamilies of K^+ channels. There are two possible mechanisms to account for the mismatch between the functional

expression of TuKv2 and its transcription. One possibility is that other subunits or accessory proteins associated with TuKv2 control the functional current expression in ascidian embryos. In other systems, β-subunits not only change electrical properties of voltage-gated K^+ channels but also determine the efficiency of current expression (Shi et al., 1996). Although many of the cloned β-subunits specifically interact with Kv1 channels (Nakahira et al., 1996), some β-subunits can interact with a Kv2 channel (Fink et al., 1996). TuKv2 gives a robust current expression in *Xenopus* oocytes without coinjections of other subunits. This leads to a prediction that there is an endogenous factor in *Xenopus* oocytes that can substitute native subunits expressed in ascidian neuronal cells. This factor might also be related to a recently identified protein, called KchAP, that binds to Kv channels and facilitates the cell surface expression of the mammalian Kv2 channels (Wible et al., 1998).

Another possible mechanism for the post-transcriptional suppression is that the translation is inhibited. In this case again, the masking of mRNA from translation must be subfamily-specific, because overexpression of a *Shaker*-related K^+ channel, TuKv1, led to an earlier functional expression than that of TuKv2. Several proteins are known that bind to mRNA in neuronal cells (Sakakibara and Okano, 1997). TuKv2 mRNA might be inhibited from being translated by factors that bind to mRNA. If a translational control occurs as the critical step for determining the expression timing of TuKv2, this mechanism must be inherent to the ascidian embryos, because the translational efficiencies of TuKv1 and TuKv2 were not significantly different either during *in vitro* translation or during *Xenopus* oocyte expression (our unpublished data). Generating antibodies against TuKv2 in the future can test this idea.

Coordinated expression of K^+ and Na^+ channels

Both the Na^+ and K^+ channels are critical for determining the shapes of the action potentials. There are several lines of evidence that the Na^+ and K^+ channels are coregulated during ascidian neuronal differentiation. When the gap-junctional communication between the *a*4.2 cell and a neighboring cell is forced to persist longer than usual, expression of both the Na^+ and K^+ channels are delayed to a similar extent (Saitoe et al., 1996). Furthermore, this timing of expression of the two channels are constant among *a*4.2 cells that are neurally induced by three different methods, bFGF treatment, subtilisin treatment, and cell contact (Inazawa et al., 1998).

One possible mechanism for this co-regulation is control of transcription through one common transcriptional factor, such as the protein, called REST, which represses a set of neuron-specific genes in mammals (Chong et al., 1995). However, in ascidian *a*4.2 cells, neither the temporal nor spatial transcriptional pattern of the Na^+ and K^+ channels can be explained by the same transcription factor. The temporal pattern of the TuKv2 transcription is different from that of TuNaI, the gene that encodes the Na^+ current in neuronal *a*4.2 cells. In a RT-PCR experiment, the TuNaI transcript was not detected at the gastrula stage, when TuKv2 transcript showed a transient increase. Expression of the TuKv2 and TuNaI transcript is also different in terms of tissue specificity. TuKv2 was also expressed in non-neural tissues such as mesenchymal cells, whereas the expression of TuNaI was restricted to neural tissues (Okamura et al., 1994). Within the neural tissues, TuNaI was more widely distributed than TuKv2, whereas TuKv2 is not expressed in some neurons, including epidermal sensory neurons. Thus, TuKv2 and TuNaI are under different transcriptional controls.

The functional expression of the Na^+ and K^+ channels may be coordinated at the functional level. It is reported that disturbances of ion channel expression lead to changes in status of later cell maturation. Overexpression of the K^+ channels in *Xenopus* neural tissue does not affect the Na^+ channel expression but leads to a decrease in the number of neurons *in vitro* (Jones and Ribera, 1994). In *Drosophila*, one allele of the HERG mutant ts1 is associated with changes in the levels of the functional Na^+ channels, suggesting that the Na^+ channel density is regulated by electrical activity of the cell through the K^+ channel function (Jackson et al., 1984; O'Dowd and Aldrich, 1988; Wang et al., 1997). In ascidian myocytes, it was shown that the electrical activity of the Ca^{2+} channels regulates the expression of the Ca-activated K^+ channels (Dallman et al., 1998). In our previous study (Okamura et al., 1994), however, the decrease in the Na^+ current by injection of antisense oligonucleotides did not affect the level of the K^+ current expression in the ascidian *a*4.2 cells. Furthermore, overexpression of the wild-type and dominant negative K^+ channels did not change the Na^+ channel expression in the present study. Thus a post-translational control between the Na^+ and K^+ channels through electrical activity does not seem relevant in isolated *a*4.2 cells.

However, the coordination of the Na^+ and K^+ channels through electrical activity might be too weak to be detected. The ratio of the K^+ and Na^+ current is variable even in noninjected neuronal *a*4.2 cells from cell to cell (data not shown). The shapes of the action potentials may not be rigorously regulated as long as they can fulfill normal neuronal functions. We also cannot deny the possibility that the lack of an activity-dependent regulation is caused by the isolated conditions of the cell. It has not been addressed whether isolated blastomeres show spontaneous activities during development. It is possible that neurons in developing embryos can tune the ion channel expression in an activity-dependent manner by interacting with each other. This possibility needs to be tested in the future by overexpressing the wild-type and dominant-negative TuKv2 in intact embryos.

REFERENCES

Blaine JT, Ribera AB (1998) Heteromultimeric potassium channels formed by members of the Kv2 subfamily. J Neurosci 18:9585–9593.

Burger C, Ribera AB (1996) *Xenopus* spinal neurons express Kv2 potassium channel transcripts during embryonic development. J Neurosci 16:1412–1421.

Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altshuller YM, Frohman MA, Kraner SD, Mandel G (1995) REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 80:949–957.

Conklin EG (1905) The organization and cell-lineage of the ascidian egg. J Acad Nat Sci Phil 13:1–119.

Dallman JE, Davis AK, Moody WJ (1998) Spontaneous activity regulates calcium-dependent K^+ current expression in developing ascidian muscle. J Physiol (Lond) 511:683–693.

Dierks PA, van Ooyen A, Mantei N, Weissmann C (1981) DNA sequences preceding the rabbit β globin gene are required for formation in mouse L cells of β globin RNA with the correct 5′ terminus. Proc Natl Acad Sci USA 78:1411–1415.

Fink M, Duprat F, Lesage F, Heurteaux C, Romey G, Barhanin J, Lazdunski M (1996) A new K^+ channel β subunit to specifically enhance Kv2.2 (CDRK) expression. J Biol Chem 18:26341–26348.

Gurantz D, Ribera AB, Spitzer NC (1996) Temporal regulation of *Shaker*- and *Shab*-like potassium channel gene expression in single embryonic spinal neurons during K^+ channel development. J Neurosci 16:3287–3295.

Heginbotham L, MacKinnon R (1992) The aromatic binding site for tetraethylammonium ion on potassium channels. Neuron 8:483–91.

Hille B (1992) Ionic channels of excitable membranes. Sunderland, MA: Sinauer.
Hirano T, Takahashi K, Yamashita N (1984) Determination of excitability types in blastomeres of the cleavage-arrested but differentiated embryos of an ascidian. J Physiol (Lond) 347:301–325.
Inazawa T, Okamura Y, Takahashi K (1998) Basic fibroblast growth factor induction of neuronal ion channel expression in ascidian ectodermal blastomeres. J Physiol (Lond) 511:347–359.
Jackson FR, Wilson SD, Strichartz GR, Hall LM (1984) Two types of mutants affecting voltage-sensitive sodium channels in *Drosophila* melanogaster. Nature 308:189–191.
Jones SM, Ribera AB (1994) Overexpression of a potassium channel gene perturbs neural differentiation. J Neurosci 14:2789–2799.
Linsdell P, Moody WJ (1994) Na^+ channel mis-expression accelerates K^+ channel development in embryonic *Xenopus laevis* skeletal muscle. J Physiol (Lond) 480:405–410.
MacKinnon R (1991) Determination of the subunit stoichiometry of a voltage-activated potassium channel. Nature 350:232–235.
MacKinnon R, Yellen G (1990) Mutations affecting TEA blockade and ion permeation in voltage-activated K^+ channels. Science 250:276–279.
McManus OB, Helms LM, Pallanck L, Ganetzky B, Swanson R, Leonard RJ (1995) Functional role of the β subunit of high conductance calcium-activated potassium channels. Neuron14:645–650.
Murakoshi H, Trimmer JS (1999) Identification of the Kv2.1 K^+ channel as a major component of the delayed rectifier K^+ current in rat hippocampal neurons. J Neurosci 19:1728–1735.
Nakahira K, Shi G, Rhodes KJ, Trimmer JS (1996) Selective interaction of voltage-gated K^+ channel β-subunits with α-subunits. J Biol Chem 271:7084–7089.
Nishida H (1987) Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. Dev Biol 121:526–541.
O'Dowd DK, Aldrich RW (1988) Voltage-clamp analysis of sodium channels in wild-type and mutant *Drosophila* neurons. J Neurosci 8:3633–3643.
Okada T, Hirano H, Takahashi K, Okamura Y (1997) Distinct neuronal lineages of the ascidian embryo revealed by expression of a sodium channel gene. Dev Biol 190:257–272.
Okado H, Takahashi K (1990) Induced neural-type differentiation in the cleavage-arrested blastomere isolated from early ascidian embryos. J Physiol (Lond) 427:603–623.
Okado H, Takahashi K (1993) Neural differentiation in cleavage-arrested ascidian blastomeres induced by a proteolytic enzyme. J Physiol (Lond) 463:269–290.
Okamura Y, Shidara M (1990) Changes in sodium channels during neural differentiation in the isolated blastomeres of the ascidian embryo. J Physiol (Lond) 431:39–74.
Okamura Y, Takahashi K (1993) Neural induction suppresses early expression of the inward-rectifier K^+ channel in the ascidian blastomere. J Physiol (Lond) 463:245–68.
Okamura Y, Ono F, Okagaki R, Chong JA, Mandel G (1994) Neural expression of a sodium channel gene requires cell-specific interactions. Neuron 13:937–948.
Perozo E, MacKinnnon R, Bezanilla F, and Stefani E (1993) Gating currents from a nonconducting mutant reveal open-closed conformations in *Shaker* K^+ channels. Neuron 11:353–358.
Pongs O (1992) Molecular biology of voltage-dependent potassium channels. Physiol Rev 72:S69–S88.
Quattrocki EA, Marshall J, Kaczmarek LK (1994) A *Shab* potassium channel contributes to action potential broadening in peptidergic neurons. Neuron 12:73–86.
Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Dolly JO, Pongs O (1994) Inactivation properties of voltage-gated K^+ channels altered by presence of β-subunit. Nature 369:289–294.
Ribera AB (1996) Homogeneous development of electrical excitability via heterogeneous ion channel expression. J Neurosci 16:1123–1130.
Saitoe M, Inazawa T, Takahashi K (1996) Neuronal expression in cleavage-arrested ascidian blastomeres requires gap junctional uncoupling from neighboring cells. J Physiol (Lond) 491:825–842.
Sakakibara S, Okano H (1997) Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. J Neurosci 17:8300–8312.
Shen NV, Pfaffinger PJ (1995) Molecular recognition and assembly sequences involved in the subfamily-specific assembly of voltage-gated K^+ channel subunit proteins. Neuron 14:625–633.
Shi G, Nakahira K, Hammond S, Rhodes KJ, Schechter LE, Trimmer JS (1996) β subunits promote K channel surface expression through effects early in biosynthesis. Neuron 16:843–852.
Shidara M, Okamura Y (1991) Developmental changes in delayed rectifier K^+ currents in the muscular and neural-type blastomere of ascidian embryos. J Physiol (Lond) 443:277–305.
Simoncini L, Block ML, Moody WJ (1988) Lineage-specific development of calcium currents during embryogenesis. Science 242:1572–1575.
Takahashi K, Okamura Y (1998) Ion channels and early development of neural cells. Physiol Rev 78:307–327.
Tsunoda S, Salkoff L (1995a) The major delayed rectifier in both *Drosophila* neurons and muscles is encoded by *Shab*. J Neurosci 15:5209–5221.
Tsunoda S, Salkoff L (1995b) Genetic analysis of *Drosophila* neurons; Shal, Shaw, and *Shab* encode most embryonic potassium currents. J Neurosci 15:1741–1754.
Vize PD, Melton DA, Hemmati-Brivanlou A, Harland RM (1991) Assays for gene function in developing *Xenopus* embryos. Methods Cell Biol 36:361–381.
Wada H, Makabe KW, Nakauchi M, Satoh N (1992) Phylogenetic relationships between solitary and colonial ascidians, as inferred from the sequence of the central region of their respective 18S rRNA. Biol Bull 183:448–455.
Wang XJ, Reynolds ER, Deak P, Hall LM (1997) The seizure locus encodes the *Drosophila* homolog of the HERG potassium channel. J Neurosci 17:882–890.
Wible BA, Yang Q, Kuryshev YA, Accili EA, Brown AM (1998) Cloning and expression of a novel K^+ channel regulatory protein, KChAP. J Biol Chem 273:11745–11751.
Xu J, Yu W, Jan YN, Jan LY, Li M (1995) Assembly of voltage-gated potassium channels. Conserved hydrophilic motifs determine subfamily-specific interactions between α-subunits. J Biol Chem 270:24761–24768.
Yang Y, Yan Y, Sigworth FJ (1997) How does the W434F mutation block current in *Shaker* potassium channels? J Gen Physiol 109: 779–789.