The Journal of Neuroscience, February 1995, 15(2): 1406–1418

Functional Expression of *Shaker* K+ Channels in Cultured *Drosophila* "Giant" Neurons Derived from *Sh* cDNA Transformants: Distinct Properties, Distribution, and Turnover

Ming-Li Zhao,[1] Elizabeth O. Sable,[2,a] Linda E. Iverson,[2] and Chun-Fang Wu[1]

[1]Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242 and [2]Division of Neurosciences, Beckman Research Institute of the City of Hope, Duarte, California 91010

Expression of transgenic *Shaker* (*Sh*) channels has not previously been examined in *Drosophila* neurons. We studied K+ current by whole-cell recording in cultured "giant" neurons derived from germline transformants. Independent lines were generated by using a P-element vector, in which transcription of the 29-4 cDNA, one of the *Sh* splicing variants (Iverson and Rudy, 1990), was under the control of a heat shock (HS)-inducible promoter. Transformants in wild-type and two different *Sh* mutant backgrounds all exhibited an HS-inducible, A-type K+ current that was characterized by a much slower recovery from inactivation and a higher sensitivity to 4-aminopyridine than native K+ currents or *Sh* 29-4 currents expressed in *Xenopus* oocytes. Despite similarities in the kinetic and pharmacological properties of the HS-induced current in all backgrounds examined, host-dependent differences in the peak current amplitude have been consistently observed between multiple lines of 29-4 Sh^{M} and 29-4 Sh^{120} that might reflect differential channel subunit assembly in different hosts. Isolation of the novel 29-4 currents allowed determination of the channel turnover rate in cultured neurons. These currents persisted for up to 3 d or more, comparable with the durations previously reported for Na^+ and Ca^{2+} channels. Surprisingly, the percentage of cells expressing inactivating K+ currents remained approximately the same with or without HS induction, suggesting that some mechanisms exist to restrict functional expression of inactivating K+ channels, including transgenic *Sh* channels and those not encoded by the *Sh* locus, to certain types of neurons.

[Key words: potassium channels, transgenic* Sh *channel, channel distribution, channel turnover, subunit assembly, heat-shock induction, host dependence, 4-aminopyridine, inactivation and recovery,* Drosophila *embryonic neurons]

Potassium channel diversity plays a crucial role in the regulation of neuronal membrane excitability. For example, voltage-gated, transient A-type K+ channels are involved in the initiation and repolarization of action potentials (Hille, 1992). Mutations in the *Shaker (Sh)* locus in *Drosophila* enhance larval neuromuscular transmission (Jan et al., 1977), delay action potential repolarization in the cervical giant fibers (Tanouye et al., 1981), and specifically eliminate or alter an A-type K+ current in embryonic (Zagotta et al., 1988), larval (Wu et al., 1983; Wu and Haugland, 1985), pupal and adult (Salkoff and Wyman, 1981; Salkoff, 1983; Elkins et al., 1986) muscle. In contrast, only a small fraction of transient K+ currents in cultured embryonic neurons (Solc et al., 1987) or dissociated pupal thoracic ganglia (Baker and Salkoff, 1990) is affected by *Sh* mutations, indicating that some A-type channels must be encoded by genes other than *Sh.* Three genes, *Shab, Shal,* and *Shaw*, have been isolated by homology to *Sh* (Butler et al., 1989) and may contribute to A-type channel diversity (Tsunoda and Salkoff, 1993).

Molecular characterization of the *Sh* locus indicated that alternative splicing of *Sh* transcripts produces distinct mRNAs encoding proteins with a constant core region flanked by divergent amino and carboxyl domains (Kamb et al., 1987; Pongs et al., 1988; Schwarz et al., 1988). Each of these RNAs is capable of expressing functional K+ channels, albeit with distinct kinetic properties, in *Xenopus* oocytes (Iverson et al., 1988; Timpe et al., 1988a,b). Coexpression studies in oocytes suggested that *Sh* channels are formed by the assembly of four subunits (MacKinnon, 1991), and additional diversity can be generated by the coassembly of different *Sh* subunits into heteromeric channels (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990).

Genetic and electrophysiological studies in native excitable tissues revealed further complexity in the expression of the *Sh* gene. Nonuniform severity of *Sh* mutant phenotypes in different tissues (Tanouye and Ferrus, 1985; Ganetzky and Wu, 1986) suggests that other tissue-specific factors may influence *Sh* channel function. Allele-specific interactions indicate that A-channels expressed in muscle are heteromultimeric assemblies, composed of nonidentical *Sh* subunits (Haugland, 1987; Haugland and Wu, 1990) and the product of another gene *eag* (Wu et al., 1983; Zhong and Wu, 1991, 1993), which shows some homology with *Sh* proteins (Warmke et al., 1991).

To examine expression of individual *Sh* subunits in a variety of native cell types and genetic backgrounds, we have begun to explore the functional expression of *Sh* cDNAs in transgenic *Drosophila.* Germline transformants were generated using a P-element vector containing *Sh* 29-4 cDNA fused to the heat shock–inducible *hsp70* promoter. Cultured "giant" neurons, which express a variety of channels (Saito and Wu, 1991) in-

Received Feb. 28, 1994; revised July 18, 1994; accepted Aug. 12, 1994.

We thank Mr. W.-D. Yao for collecting part of the data in Figures 6, 9, and 10 and Mr. P. Taft for technical assistance. We also thank Dr. K. Takagawa for expert instruction in the production and characterization of the anti-Sh antibody used for the Western blot shown in Figure 1, and Drs. M. Saito and Y. Zhong for participation in the initial characterization of K+ currents in the transformants. This work was supported by NIH Grants NS18500 and NS26528 to C.-F.W., and NS28135 and NS18858 to L.E.I.

Correspondence should be addressed to Dr. Chun-Fang Wu at the above address.

[a]Present address: Immunex, 51 University Street, Seattle, WA 98101.

cluding those reported in cultured embryonic, larval, and pupal neurons (Byerly and Leung, 1988; Solc and Aldrich, 1988; Baker and Salkoff, 1990), derived from transformants were used to allow whole-cell recording from a large number of central neurons required for this analysis. An A-type K^+ current, arising from expression of *Sh* 29-4 (Iverson and Rudy, 1990), was characterized. The distinct K^+ currents seen enabled us to follow channel turnover process in "giant" neurons for comparison with the stability of Na^+ and Ca^{2+} channels previously reported. Transformants were also studied in wild-type and two different *Sh* mutant backgrounds, Sh^{120} and Sh^M, to investigate the potential influence on K^+ channel expression in different hosts that produce normal, altered, or no *Sh* subunits.

Preliminary accounts of some of the results have appeared previously (Zhao et al., 1992).

Materials and Methods

P-element–mediated germline transformation. *Sh* 29-4 cDNA insert (Iverson and Rudy, 1990, equivalent to *Sh*D; see Schwarz et al., 1988) was excised from BlueScript vector (Stratagene, San Diego, CA) using the restriction endonuclease EcoR1 and subcloned into the *Drosophila* transposable P-element vector, pCasper-hs (a gift from Carl Thummel, University of Utah). The recombinant plasmid was mapped with the restriction endonuclease, BamH1, to determine correct 5′ to 3′ orientation of the cDNA insert relative to the *hsp70* promoter. The resulting pCasper-hs-*Sh* 29-4 and pTurbo (encoding the transposase, a gift from Ernst Hafen, Zoologisches Institut der Universität, Zürich) plasmid DNAs were co-injected into *w f* Sh^{rKO120} or *w f* Sh^M mutant *Drosophila* embryos according to the method of Rubin and Spradling (1982). For simplicity, the allele Sh^{rKO120} will be designated as Sh^{120} in this article hereafter (see Lindsley and Zimm, 1992, for details of the mutations used as markers, such as *w, f*). All surviving adult flies were individually back-crossed to the appropriate host strain and germline transformants were identified on the basis of a change in eye color. Single-pair matings of heterozygous transformants, with pale yellow to orange eyes, were performed and homozygous progeny were identified by their darker orange to red eye color. Identification of the chromosome containing the transgene was determined, for each line, by a series of standard genetic crosses in which transgenic flies were mated to flies carrying balancers for either the X chromosome, FM7a, or autosome 2, M(2)173/sM5,CY, *es* ca^{nd}/TM3, *Sb Ser* (Lindsley and Zimm, 1992). Transformants in which the site of insertion of the P-element was determined to be on an autosome, as identified by the above crosses, were used to introduce the transgene into a background of Sh^+ by a series of genetic crosses. For example, to generate *Sh* 29-4 +b transformants in a wild-type background, *w* Sh^+ virgin females were crossed to male *Sh* 29-4 120b transformants in a *w f* Sh^{120} background (see Table 1 for different lines generated). Heterozygous transformant male progeny, which should all carry the *w* Sh^+ X chromosome, were identified by their orange eye phenotype and back-crossed to *w* Sh^+ virgin females. Heterozygous transgenic progeny generated from the above cross were mated, and their homozygous progeny identified by darker orange eye color. Single-pair matings of homozygous progeny were then performed to establish homozygous lines of each transformant strain in a *w* Sh^+ genetic background. Southern (DNA) blots of EcoR1-digested genomic DNA from transformants were probed with α-P^{32}-labeled *Sh* 29-4 cDNA to confirm that transformants carried DNA inserts corresponding to *Sh* 29-4 cDNA (Sambrook et al., 1989).

Immunoblotting. Three day old adult flies were frozen in liquid nitrogen and vortexed to remove their heads. Twelve heads were placed in a 1.5 ml microfuge tube containing 24 μl of dissociation buffer (5% β-mercaptoethanol, 10% glycerol, 1% SDS, 5 mM Tris-HCl, pH 6.8) and homogenized. Samples were boiled for 2 min and 6 μl of 0.1% bromophenol blue in 10% glycerol was added. Fly debris was pelleted by centrifugation at 10^4 rpm for 2 min in a microfuge and 10 μl of supernatant (equivalent to four fly heads) was loaded onto an 8% SDS-polyacrylamide gel (Laemmli, 1970) along with 10 μl of prestained SDS polyacrylamide low MW markers (Bio-Rad, Richmond, CA). The gel was subjected to electrophoresis in a Bio-Rad Mini-Protean II Cell and proteins were electrophoretically transferred to a nitrocellulose filter (BA85, 0.45 μ pore size, Schleicher and Schuell, Keene, NJ) essentially as described by Towbin et al. (1979) using a Trans-Blot Cell at 100 V for 17 hr. Blot was blocked by incubation in Blotto (5% Carnation Nonfat Dry Milk in 1× phosphate-buffered saline, PBS) containing 0.3% Tween-20 (Baker, Philipsburg, NJ) for 2 hr, then incubated for 3 hr in solution containing the anti-*Sh* monoclonal antibody, 8E8 (specific for the *Sh* type 4 carboxyl domain, K. Takagawa, E. O. Sable, and L. E. Iverson, in preparation), at a concentration of 0.5 ng/μl in Blotto. Blot was rinsed in Blotto containing 0.3% Tween-20 three times (10 min each), and blocked in 0.3% Tween-20, 5% goat serum (Gemini Bioproducts Inc., Calabasaa, CA) in Blotto for 2 hr, then incubated overnight (16 hr) in the secondary antibody solution containing a 1:5000 dilution of an Alkaline phosphatase conjugated goat anti-mouse IgG (Tago, Burlingame, CA) in 0.3% Tween-20 in Blotto. Blot was washed three times in Blotto (10 min each), two times in PBS (10 min each) and two times (10 min each) in alkaline phosphatase buffer (APB, containing 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5). Blot was then developed using 165 μl of 5% nitro blue tetrazolium in 70% dimethylformamide and 82.5 μl of 5% bromochloroindolyl phosphate in 100% dimethylformamide in 25 ml of APB for 30 min. The procedure was carried out at room temperature (22–23°C).

RNA polymerase chain reaction (PCR) analysis. One day old adult flies (20–25) were collected in glass vials and subjected to heat shock (HS) by submerging the vials in a 38°C water bath for 2 hr (non-HS flies were maintained at 18°C). Total RNA was isolated 1 hr following the HS treatment using GlassMax RNA microisolation spin cartridges (Bethesda Research Laboratories) according to instructions provided by the manufacturer. Reverse transcription reactions and PCR amplifications were performed using a 5′ RACE system (Bethesda Research Labs). The antisense primer used for reverse transcription is located 295–314 nucleotides from the 5′ end of the *Sh* 29-4 cDNA (5′-CCATGGCCTCCCATGCCGCC-3′). Primers used for *hsp70-Sh* cDNA templates correspond to nucleotides 19–42 from the 5′ end of the *hsp70* mRNA (5′-GCAAAGTGAACACGTCGCTAAGCG-3′) and to nucleotides 223–244 from the 5′ end of *Sh* 29-4 (5′-CGATCGAAGAAACTGTGGCAGTGGCA-3′). PCR primers were used at 200 ng/50 μl reaction. Reactions were carried out at 94°C for 4 min, 72°C for 5 min, followed by 35 cycles of PCR amplification; 1 min at 94°C, 1.5 min at 60°C, and 1 min at 72°C. PCR products (25 μl of the 50 μl reaction) were subjected to electrophoresis on 1% agarose gels in Tris-borate buffer (Sambrook et al., 1989). To ensure that the PCR amplified product was derived from HS-induced *hsp70-Sh* 29-4 RNA, and not genomic DNA, PCR amplifications were also performed on "cDNA templates" that had been prepared in the absence of reverse transcriptase.

Cell culture. Cultures of *Drosophila* "giant" neurons, derived from cytokinesis-arrested embryonic neuroblasts, were prepared as described previously (Wu et al., 1990; Saito and Wu, 1991). Briefly, embryos from each transgenic line were collected on agar plates for 1–2 hr, then incubated for 3–4 hr at 25°C. Embryos were homogenized in modified Schneider medium (GIBCO, Grand Island, NY) containing 200 ng/ml insulin (Sigma, St. Louis, MO), 20% fetal bovine serum (FBS), 50 μg/ml streptomycin, and 50 U/ml penicillin (all from GIBCO). Cells were dissociated in the above medium containing 1–2 μg cytochalasin B (Sigma), and then plated on glass coverslips. Cultures were maintained in humidified chambers at room temperature (22–23°C). In some cases dissociated cell cultures were washed in culture medium on the second day following plating, otherwise cytochalasin B was removed by rinsing cells in bath solution prior to recording. No evident differences in physiological results were noticed following the two treatments (cf. Saito and Wu, 1991).

Heat shock induction. Cover slips containing cell cultures that were to be subjected to heat shock–induced transcription (HS groups) were placed in sealed plastic petri dishes and incubated at 38.5 ± 0.2°C for 30 min (in a few cases, cell cultures were subjected to 1 hr heat pulses. No electrophysiological difference was detected). Heat shock-treated cell cultures were allowed to recover at room temperature for at least 3 hr. In experiments in which we examined the time course of *Sh* 29-4 expression, the time following HS was measured from the end of heat pulse to the start of electrical recording.

Electrophysiological recording and data analysis. Whole-cell patch-clamp recording was similar to that of Saito and Wu (1991). Patch electrodes were made from glass capillaries (75 μl micropipettes, VWR, Chicago, IL), and in some cases, were coated with Sylgard (184 silicon elastomer, Dow Corning, Midland, MI) to reduce stray capacitance. Tip resistance of patch pipettes, measured in the recording solution, was 3–6 MΩ. Whole-cell recordings were obtained primarily from monopolar or bipolar neurons approximately 10 μm in soma diameter using an

Table 1. Transgenic flies created by the p-element–inducible *Sh* cDNA 29-4 inserted into different *Sh* backgrounds

Host	Line and chromosome inserted
Sh^{120}	120a: X chromosome
	120b: 2nd chromosome
	120c: 2nd chromosome but different site from 120b
Sh^{M}	Ma: X chromosome
	Mb: 2nd chromosome
	Mc: 3rd chromosome
Sh^{+}	+b: 2nd chromosome and message is from 120b
	+c: 3rd chromosome and message is from Mc

EPC-7 (Medical Systems Corp., Greenvale, NY) or Axon-Patch 2B (Axon Instruments, Foster City, CA) patch-clamp amplifier. The seal resistance was usually greater than 8 GΩ, and the junction potential was nulled just prior to rupture of the membrane. Bath solution contained 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 35.5 mM sucrose, buffered with 5 mM HEPES at pH 7.1 to 7.2 (adjusted with NaOH). To isolate voltage-activated outward K^+ currents, 0.2 μM TTX (Sigma) and 0.2 mM Cd^{2+} were added to the bath solution to eliminate inward Na^+ or Ca^{2+} currents. Patch pipettes were filled with a solution containing 144 mM KCl, 1.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 5.0 mM EGTA, buffered with 10 mM HEPES, pH 7.1–7.2 (adjusted with KOH). To determine the pharmacological effects of 4-aminopyridine (4-AP, Sigma), bath solution was replaced at least two times with solution containing the drug at its desired final concentration. At least 30 sec was allowed for equilibration before data collection. An IBM-compatible PC computer, pCLAMP software (version 5.51, Axon Instruments) and A/D, D/A converters (Labmaster, Axon Instruments) were used for voltage pulse generation and data acquisition. Data were filtered at 3 kHz using a four-pole Bessel filter and digitized at 1–5 kHz. Leakage and capacitance currents were digitally subtracted. A series of four pulses to −75 mV from a holding potential of −80 mV were applied immediately after formation of the whole-cell configuration and the current response was used for compensating the effects of leakage resistance and membrane capacitance. To obtain firm, quantitative measurements, cells with a peak current amplitude smaller than 50 pA were excluded from analysis. All figures were constructed using software, CLAMPFIT (pCLAMP 5.51, Axon Instruments), SIGMAPLOT (version 4.0, Jandel Scientific, San Rafael, CA), and SUPERPAINT (version 2.0 for Macintosh, Silicon Beach, San Diego, CA).

Results

The Sh^{120} *mutation expresses reduced amounts of* Sh *subunits;* Sh^{M} *produces no* Sh *gene products*

Individual transformed lines are listed in Table 1. *Sh* 29-4 transformants 120a, 120b, and 120c represent independent lines that were originally generated in a *w f* Sh^{120} host strain. Transformants Ma, Mb, and Mc are independent transformants originally isolated in *w f* Sh^{M} hosts. Transformants in Sh^{+} were derived from one of the transformed lines listed above, then genetically introduced into *w* Sh^{+} hosts as described in Materials and Methods. The two *Sh* mutants, Sh^{120} and Sh^{M}, were chosen for our initial analysis because both mutations show extreme neuronal hyperexcitability at the neuromuscular junction, but voltage-clamp recordings of muscle indicate that Sh^{M} eliminates the A-current and behaves like a null mutation in heterozygotes, while Sh^{120} only reduces A-current amplitude slightly (Wu and Haugland, 1985; Ganetzky and Wu, 1986; Timpe and Jan, 1987; Haugland and Wu, 1990). This implies that Sh^{M} eliminates all *Sh* gene products, while Sh^{120} may affect only a subset of *Sh* subunits that are preferentially expressed in the presynaptic region. Molecular analysis indicates that genomic DNA from Sh^{M} flies contains a 2.2 kb DNA insertion within the constant region of the gene (Kamb et al., 1987). Furthermore, our immunoblotting data, obtained using an anti-*Sh* monoclonal antibody specific to the type 4 carboxyl domain (K. Takagawa, E. O. Sable, and L. E. Iverson, unpublished observations), indicates that Sh^{M} produces no *Sh* polypeptides containing this carboxyl domain while Sh^{120} expresses reduced amounts of *Sh* subunits containing this region (Fig. 1*A*).

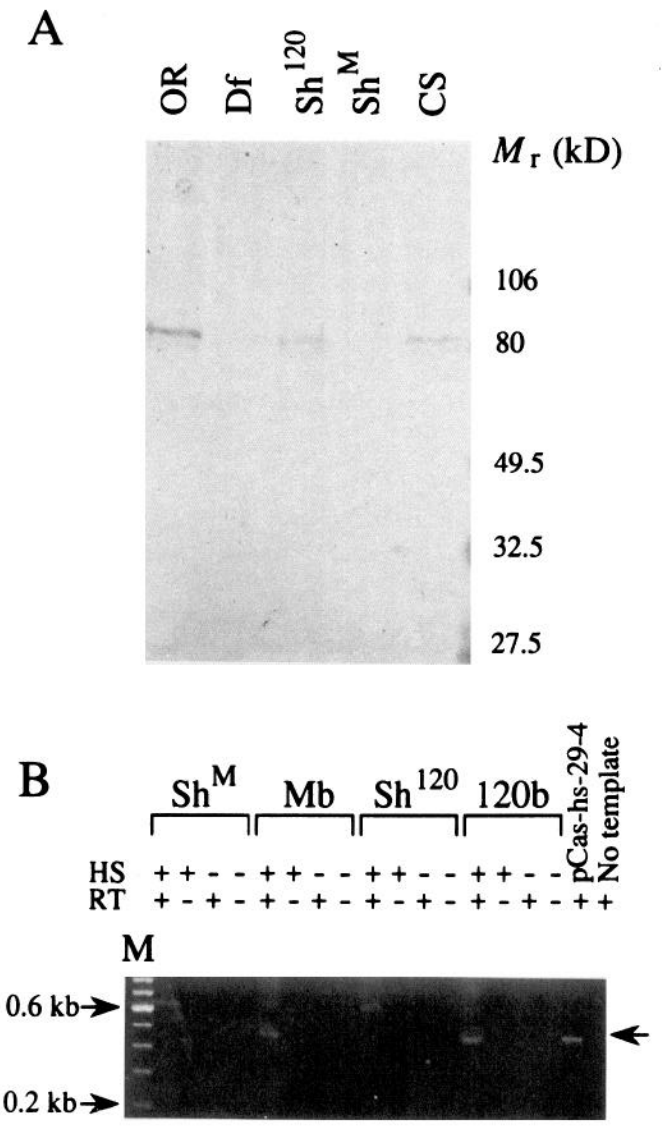

Figure 1. A, Identification of *Sh* polypeptides in protein extracts of adult fly heads. Extracts of two wild-type strains, Canton-S and Oregon-R (*CS* and *OR*), are compared with those of a mutant (*Df*) deficient for much of the *Sh* locus, generated from two X:Y translocations with breakpoints located at different positions within the gene ($W32^{P}/B55^{D}$; Tanouye et al., 1981), and the two mutant strains that were used for generating transformants (Sh^{120} and Sh^{M}). The immunoblot shows bands of approximately 80 kDa only in the wild-type, CS and OR, and the Sh^{120} mutant strains. *B*, Expression of *Hsp70-Sh* 29-4 RNA following HS-induced transcription of the transgene. RNA-PCR analysis of Sh^{M} and Sh^{120} host strains and two transformed lines, Mb and 120b. The *arrow* on the right indicates the 435 bp PCR amplified DNA fragment. *HS+*, RNA prepared from heat-shock treated flies; *HS−*, RNA derived from non-heat-shock treated flies; *RT+*, cDNA prepared in the presence of reverse transcriptase; *RT−*, control cDNA reactions prepared in the absence of reverse transcriptase; *M*, 100 bp DNA ladder; *pCas-hs-29-4*, pCasper-hs-vector containing *Sh* 29-4 cDNA that was used to generate the transformed lines.

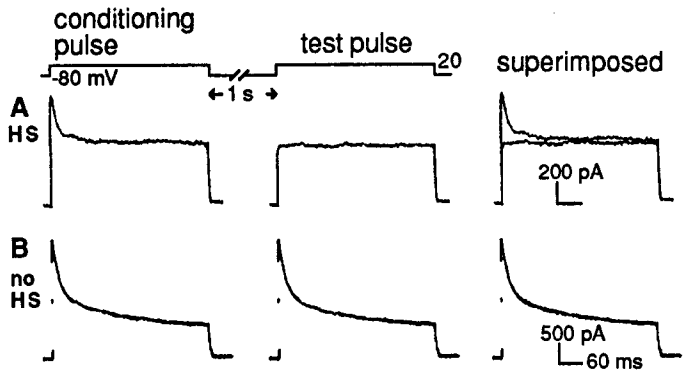

Figure 2. The heat shock (HS)-induced A-type K^+ current in *Sh* 29-4 transformants is distinguished by its slow recovery from inactivation. Voltage-clamp recordings of outward K^+ currents were collected from "giant" neurons derived from transformants in a Sh^{120} background. Currents recorded during the conditioning pulse (*left panel*) and the test pulse (*middle panel*) are shown superimposed in the *right panel*. The conditioning pulse elicited fast-inactivating A-type K+ currents in both the HS-treated neuron (*A*) and the no HS control (*B*). However, the A-current expressed in the HS-treated neuron did not recover from inactivation during the 1 sec interpulse interval and was, therefore, not observed during the test pulse. In this and following figures, conditioning and test pulses were both to +20 mV from a holding potential of −80 mV, unless stated otherwise.

It is difficult to determine quantitatively the amount of *Sh* 29-4 protein induced by HS treatment, because the induction of large amounts of heat shock proteins (such as *hsp70* and *hsp83*) interfered with gel-electrophoresis and immunoblotting of the *Sh* protein. RNA-PCR was used instead for a semiquantitative analysis of the expression of *Sh* 29-4 transcripts in host and transformant lines before and after heat-shock (HS) treatment (Fig. 1*B*). A DNA fragment of the expected MW (435 bp) was clearly observed only when RNA was prepared from transformants (Mb and 120b) that had been subjected to HS treatment (HS+), and only when cDNA was prepared in the presence of reverse transcriptase (RT+). This indicates that significant levels of expression of the transgene does not occur in the absence of HS.

A heat shock (HS)-inducible, A-type K^+ current exhibiting slow recovery from inactivation is expressed in 29-4 transformants in both Sh^{120} *and* Sh^M *backgrounds*

Functional expression of *Sh* 29-4 in "giant" neurons was readily detected by comparing the voltage-dependent outward K^+ currents expressed in the presence or absence of heat shock (HS). Voltage-dependent K^+ currents were isolated from Na^+ currents, Ca^{2+} currents, and Ca^{2+}- and Na^+-dependent K^+ currents (Saito and Wu, 1991) by bath application of TTX and Cd^{2+} throughout the experiments. Figure 2 shows the double-pulse experiments used to isolate a fast-inactivating, slow-recovering A-type K^+ current, in a subset of neurons from transformants following HS induction. Cultured neurons derived from transformants showed considerable variation in the expression of HS-induced K^+ currents (see below). In the case presented in Figure 2*A*, the fast-inactivating component is composed of mostly the HS-induced slow-recovering current, which showed less than 5% recovery from inactivation during a 1 sec interpulse interval. For comparison, the fast-recovering, native A-type K^+ current, expressed in the absence of HS, showed almost complete recovery from inactivation during the same 1 sec interval (Fig. 2*B*).

To determine if slow recovery from inactivation of the HS-inducible K^+ current is dependent on the site of insertion of the transgene, we compared *Sh* 29-4 cDNA expression in two independent transformed lines in the same background, Sh^{120}. Figure 3 shows examples of superimposed double-pulse voltage-clamp traces of neurons derived from 120a (X chromosome insertion) and 120b (2nd chromosome insertion) transformants. Results from a large number of cells indicated that both lines express a fast-inactivating, slow-recovering K^+ current following HS (group a, Fig. 3). In the absence of HS, neither line expressed the slow-recovering component (group c). In addition, a subset of neurons from both lines did not express the slow-recovering component even after HS treatment (group b). These results, together with results from a third independent transformant in a Sh^{120} background (120c, data not shown), indicate that the site of insertion of the transgene has no effect on the properties of the HS-induced K^+ current.

Because the Sh^{120} mutation produces some *Sh* gene products potentially capable of interacting with the HS-induced subunit (Fig. 1), it would be interesting to see whether the novel K^+ current described above could be produced in a host in which the native *Sh* subunits are absent. To explore this possibility we examined expression of *Sh* 29-4 gene product in Sh^M (see Fig. 1), using the same double-pulse protocol. Two independent transformed lines were used for this analysis, Ma and Mb. Strain Ma carries the transgene on the X chromosome, while the transgene in strain Mb is inserted on the 2nd chromosome (see Table 1). Similar to 29-4 expression in Sh^{120} hosts, both Ma and Mb transformants exhibited a fast-inactivating, slow-recovering K^+ current following HS-induction. The slow-recovering K^+ component was limited to a subset of HS-treated neurons (group a, Fig. 3), and it was never observed in no HS controls (group c). Furthermore, a subset of neurons did not express the HS-inducible, slow-recovering A-current even after HS treatment (group b).

Although a variety of voltage-gated K^+ currents that exhibit fast or slow inactivation (including a subset of neurons that express noninactivating, voltage-gated K^+ currents; see Table 2) was seen in transformant neurons, the novel fast-inactivating, slow-recovering A-current has never been detected in control neurons derived from Sh^{120} or Sh^M hosts, Canton-S wild-type and several other mutant strains (unpublished data) with or without HS-induction. The same is true for transformants in the absence of HS induction. This provided the first line of evidence that a K^+ current expressed following HS-induced transcription of the transgene could be distinguished from all other K^+ currents expressed in these neurons. The expression is dependent neither on the insertion site (X or 2nd chromosome) nor on the *Sh* mutant background (Sh^{120} or Sh^M).

Kinetics analysis of inactivation and recovery from inactivation

A group of neurons in the transformant lines following HS treatment displayed inactivating, A-type K^+ currents with kinetic properties clearly distinct from control neurons without HS treatment. All neurons examined that showed an inactivating component in their outward K^+ currents, with or without HS treatment, were pooled to correlate the rate of inactivation and that of recovery from inactivation (Fig. 4). For the sake of simplicity, the fraction of recovery (r) following a 1 sec interpulse interval is plotted against the fast time constant for inactivation ($\tau 1$. In general, two time constants are required for a satisfactory fit of the time course of inactivation. The slow time constant, $\tau 2$, con-

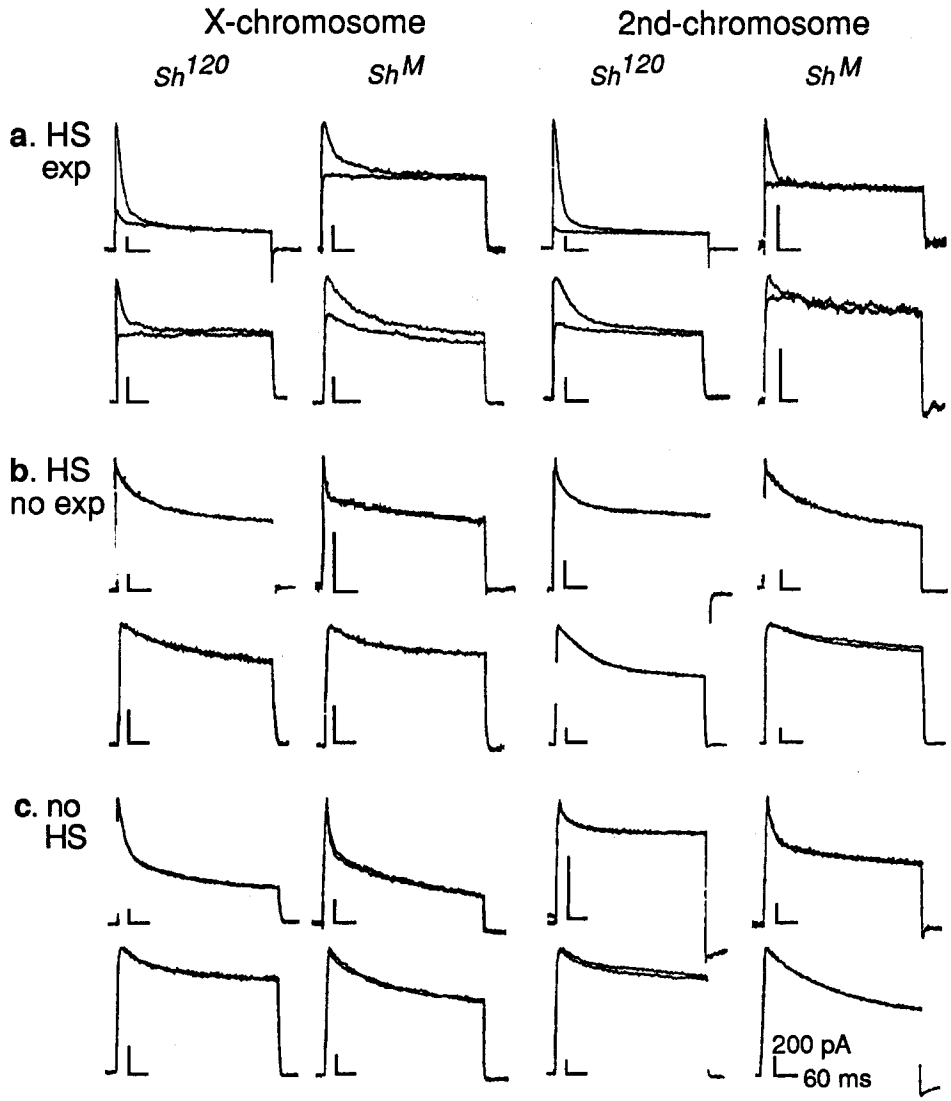

Figure 3. Slow recovery from inactivation of the HS-induced A-currents is independent of the insertion site and the *Sh* mutant background. Superimposed traces of currents elicited by double-pulse paradigm (1 sec interpulse interval; see Fig. 2) in "giant" neurons derived from 120a (Sh^{120}, X chromosome), 120b (Sh^{120}, 2nd chromosome), Ma (Sh^{M}, X chromosome), and Mb (Sh^{M}, 2nd chromosome) transformants. Two examples for each case are shown. The slow-recovering HS-induced component is indicated by the difference between the two superimposed traces, and is expressed only in a subset of HS-treated neurons (*a*). *b*, HS-treated neurons with no expression of slow-recovering current. *c*, No HS control neuron.

Table 2. Percentage of cells displaying K^+ currents of different inactivation and recovery properties in transformant lines of different hosts

Sh 29-4 insertion host	Treatment	Lines	Total cells examined	% Neurons with inactivating current		% Neurons with non-inact. current only
				Normal recovery	Slow recovery	
Sh+	HS	+b	204	53.0	34.3	12.7
		+c	111	62.2	25.2	12.6
	No HS	+b	15	86.0	0	14.0
		+c	9[a]	89.0	0	11.0
Sh^{120}	HS	120a	174	49.4	26.4	24.1
		120b	63	35.0	49.2	15.8
	No HS	120a	38	79.0	0	21.0
		120b	9[a]	78.0	0	22.0
Sh^{M}	HS	Ma	50	42.0	30.0	28.0
		Mb	125	54.4	32.0	11.2
	No HS	Ma	31	84.0	0	16.0
		Mb	5[a]	60.0	0	40.0

[a] Smaller sample size that should weigh less in estimating the percentage.

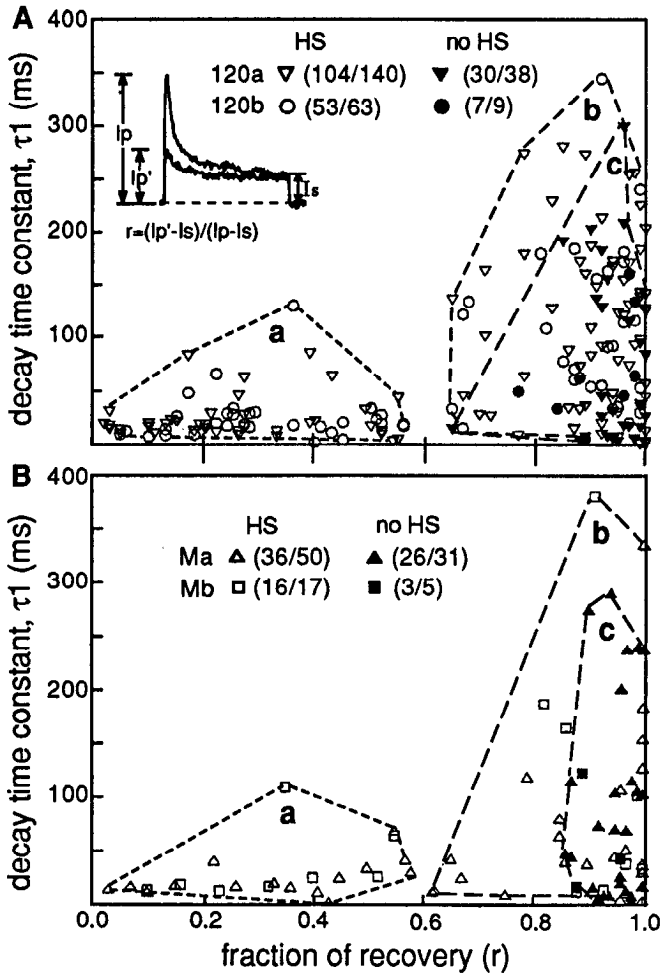

Figure 4. HS-induced K^+ currents in all Sh^{120} and Sh^M transformants are characterized by slower recovery from inactivation than native K^+ currents and relatively fast inactivation. The fraction of current (r) that recovered from inactivation during the 1 sec interval between conditioning and test pulses is plotted against the fast time constant for inactivation ($\tau 1$) for transformants in Sh^{120} (*A*) and Sh^M (*B*) backgrounds. The method used for calculating $r = (I_{p'} - I_s)/(I_p - I_s)$ is shown in the *inset* (*A*), where I_p is the peak current elicited by the conditioning pulse, $I_{p'}$ is the peak current elicited by the test pulse, and I_s is the steady-state current remaining at the end of each pulse. Current decay was fit using a double exponential function, $I = I_s + A_1 \exp(-t/\tau 1) + A_2 \exp(-t/\tau 2)$, where $\tau 1$ and $\tau 2$ are the fast and slow decay time constants for the two inactivating components A_1 and A_2, respectively. *Open symbols*, HS-treated neurons; *solid symbols*, no HS control neurons. *Group a*, HS-induced *Sh* 29-4 expression; *group b*, HS-treated, but no expression of slow recovering component; *group c*, no HS control neurons. See text for details of the grouping.

tributes less to inactivation development and is not included in the analysis.) The formula used for calculating r is shown in the inset in Figure 4*A*. Data derived from 120a and 120b transformants in a Sh^{120} background (Fig. 4*A*) are compared with data from Ma and Mb transformants in Sh^M hosts (Fig. 4*B*). The three regions labeled a, b, and c in Figure 4 are equivalent to the three types of neurons depicted in Figure 3 (groups a, b, and c), and represent (a) HS treatment resulting in expression of the slow-recovering component, (b) HS treatment, no expression of slow-recovering component, and (c) no HS treatment. One striking feature of the plots is that a natural separation of the data points occurs in the region around $r = 0.6$ (see also Fig. 6). Unlike control neurons (solid symbols; no HS), HS treatment of all 120a, 120b, Ma, and Mb transformants (open symbols) resulted in a subpopulation of neurons expressing K^+ currents that are contained within the fast-inactivating ($\tau_1 < 150$ msec), slow-recovering ($r < 0.6$) region (group a). The consistency of these results suggests that, hereafter, $r < 0.6$ may be used as the criterion for positive identification of neurons that express the novel A-type K^+ current with slow recovery resulting from HS-induced *Sh* 29-4 expression (although it is still possible that currents in group b neurons contain small fractions of HS-induced *Sh* 29-4 currents and currents in group a neurons contain some native currents).

Kinetics of inactivation and recovery from inactivation of HS-inducible K^+ current are similar in mutant or Sh^+ *backgrounds*

The kinetics of recovery from inactivation of the HS-inducible K^+ current in Sh^{120} hosts was determined by varying the interpulse interval in a double-pulse voltage-clamp paradigm. Figure 5 shows the fraction of current that has recovered from inactivation plotted as a function of the interval between the two voltage pulses (see inset in Fig. 5*B*). Recovery from inactivation of the no HS control groups (solid symbols, equivalent to group c in Fig. 4*A*) and the HS-treated but no expression groups (open symbols, equivalent to group b in Fig. 4*A*) are shown in Figure 5*A*. These currents recovered very quickly from inactivation, exhibiting 50% recovery at an interpulse interval of less than 200 msec. In contrast to the controls shown in Figure 5*A*, it is clear that recovery from inactivation of the HS-induced K^+ current was significantly slower than that of native K^+ currents (open symbols, Fig. 5*B*). On average, this component required an interpulse interval of about 4 sec to attain 50% recovery from inactivation. For comparison, data from Figure 5*A* are replotted in Figure 5*B* (solid diamonds). For both sets of recovery time course, two time constants are used in order to obtain a satisfactory fit.

The much slower rate of recovery of 29-4 currents expressed in transformants compared to 29-4 currents expressed in *Xenopus* oocytes (about five times slower; see Iverson and Rudy, 1990) raised the possibility that neurons derived from *Sh* mutants used as host strains (Sh^M and Sh^{120}) may be missing a factor that affects recovery kinetics and is, fortuitously, provided by the oocytes. If this factor is a different *Sh* subunit, then 29-4 transformants in a Sh^+ background should express K^+ currents in response to HS induction that show faster recovery from inactivation. To examine this possibility we analyzed recovery from inactivation of 29-4 K^+ currents expressed in two transformed lines in Sh^+ hosts (see Table 1). Sh^+ transformants were originally generated in Sh^{120} (+b from 120b, circles, Fig. 6) or Sh^M (+c from Mc, squares) hosts then genetically introduced into Sh^+ backgrounds as described in Materials and Methods. Consistent with expression of 29-4 in *Sh* mutant hosts, the two transformants in Sh^+ hosts both expressed fast-inactivating, slow-recovering A-currents in a subset of neurons following HS induction that satisfies the criterion $r < 0.6$ (group a, Fig. 6*A*). The slow-recovering K^+ current is not detectable in the absence of HS induction (group c, Fig. 6*A*), and a subset of neurons derived from both transformants did not express the slow-recovering component even in the presence of HS-treatment (group b, Fig. 6*A*).

Figure 6*B* shows the fast recovery from inactivation typical of controls (solid symbols, equivalent to groups b and c in Fig.

6*A*), with half-recovery occurring at less than 200 msec. In contrast, the slow-recovering component (open symbols, Fig. 6*B*) did not reach half-recovery until about 5 sec. The two distinct recovery processes of K^+ currents expressed in Sh^+ transformant neurons were very similar to that observed for Sh^{120} transformants. For comparison, the same curves used to fit the data shown in Figure 5*B* are plotted in Figure 6*B* and follow the data points closely. The result demonstrates that the difference in recovery rate observed for 29-4 currents expressed in transformants relative to 29-4 currents expressed in *Xenopus* oocytes is not due simply to the absence of a particular *Sh* subunit. Rather, this result implies that if the oocyte is providing a factor that can modulate recovery kinetics of 29-4 currents, then this putative factor is apparently absent in the "giant" neurons used here.

The HS-induced K^+ current exhibits a similar voltage-dependent activation but a higher sensitivity to 4-AP than native A-currents

The HS-inducible 29-4 K^+ current is characterized by its distinct inactivation and recovery kinetics. To determine if its voltage dependence and pharmacological properties are also distinguishable from the native A-currents in "giant" neurons, we examined the voltage-dependent activation of the HS-inducible K^+ current in 29-4 transformants in Sh^{120}, Sh^M and Sh^+ hosts in comparison with native A-currents in wild-type neurons. Figure 7 shows examples of the HS-inducible and native A-currents in 29-4 Sh^+ transformants and Sh^+ controls, extracted by the double-pulse paradigm with 1 sec and 10 msec interpulse intervals, respectively. Normalized current–voltage relations of the fast-inactivating, slow-recovering components extracted from all transformant strains are compared with the inactivating A-type currents in Sh^+ (Fig. 7*C*). Despite considerable variation, the voltage range in which activation of the HS-induced A-currents occurred was similar in all hosts and substantially overlapped with that of the native A-currents.

The drug 4-aminopyridine (4-AP) is known to block *Sh* K^+ currents in *Drosophila* muscle (Salkoff and Wyman, 1981; Wu and Haugland, 1985) as well as all K^+ currents expressed from *Sh* cRNAs injected into *Xenopus* oocytes (Iverson et al., 1988; Timpe et al., 1988b; Iverson and Rudy, 1990). The efficiency of 4-AP block of the HS-inducible A-current expressed in transformants was determined. The inactivating component of the outward K^+ current was estimated by using the same approximation ($I_p - I_s$) shown in Figure 4*A*. Superimposed double-pulse recordings (interpulse interval of 1 sec) of two 120a neurons, with or without HS induction, are shown in Figure 8*A*. At 1 mM, 4-AP blocked about 80% of the HS-induced A-current, which showed only partial recovery after washing out the drug (upper traces). The same concentration of 4-AP applied to no HS controls reduced the native A-current by about 25%, and this current showed complete recovery after wash out (lower traces). Dose–response relations for 4-AP block of inactivating currents ($I_p - I_s$) in cells that expressed HS-induced A-type K^+ channels in 29-4 Sh^{120}, Sh^M and Sh^+ transformants are shown in Figure 8*B* (open symbols). 4-AP sensitivity of native A-currents expressed in no HS controls are also shown for comparison (solid symbols, Fig. 8*B*). The data indicate that the HS-induced A-currents from all 29-4 transformants are more sensitive to 4-AP than native currents, and also more sensitive to 4-AP block than K^+ current produced by expression of 29-4 in *Xenopus* oocytes (Timpe et al., 1988b; Iverson and Rudy, 1990). Taken together, these results suggest that pharmacological sensitivity may be more variable than activation in different cellular environment, and this variability may arise from factors other than *Sh* subunits.

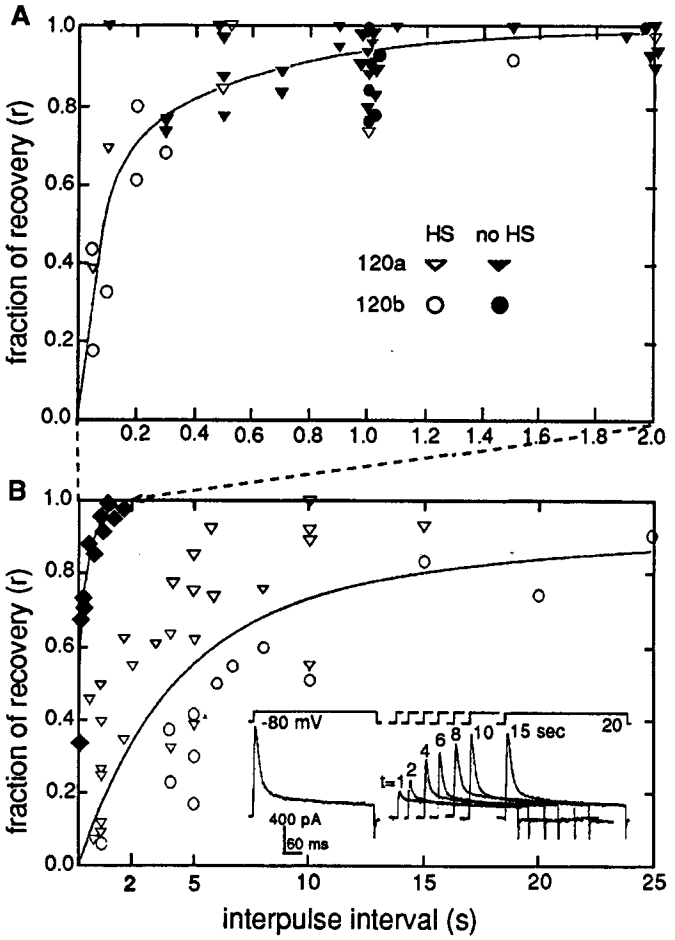

Figure 5. Kinetics of recovery from inactivation of HS-induced and native A-currents. The time course of recovery from inactivation of two different lines of Sh^{120} transformants was determined in double-pulse experiments by varying the duration of the interpulse interval. *A*, Fast recovering A-currents in no HS control neurons (*solid symbols*) or HS-treated, but no expression (*open symbols*). *B*, Recovery from inactivation of HS-induced current (*open symbols*). Note the different time scales used in *A* and *B*. The averaged data and curve in *A* is reproduced in *B* (*solid diamonds*) for comparison at the same time scale. *Inset* shows data obtained from an HS-treated neuron expressing the slow-recovering A-current that required more than 15 sec to recover completely from inactivation at −80 mV.

Temporal regulation and host dependence of expression efficiency of 29-4 K^+ current in "giant" neurons

Because the distinct properties of fast inactivation and slow recovery from inactivation allowed isolation of the HS-induced K^+ current from native K^+ currents in "giant" neurons, we were able to address a number of questions related to channel expression in these cells. First, do the amplitude of the HS-induced A-current and the percentage of cells expressing this novel current vary in different backgrounds? Second, since these "giant" neurons express a variety of K^+ currents (Saito and Wu, 1991), does expression of the HS-induced A-current occur only in a subpopulation of neurons expressing certain types of native K^+ currents or does its expression occur only at the expense of particular types of native K^+ currents? Third, to our knowledge,

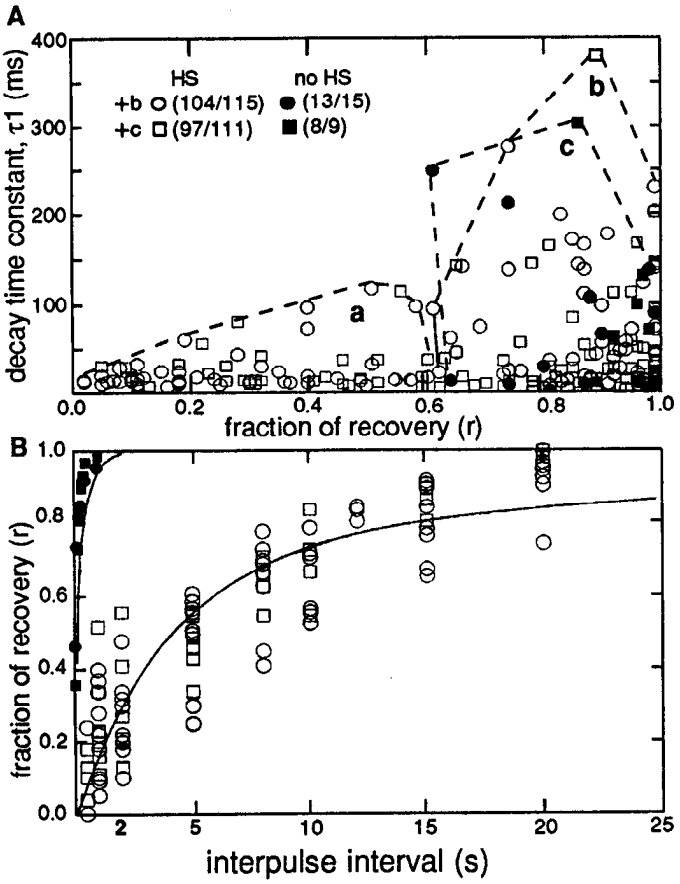

Figure 6. The HS-induced A-current expressed in Sh^+ hosts also exhibits extremely slow recovery kinetics. *A*, The fraction of current (r) recovered during the 1 sec interval was plotted against the fast time constant for inactivation ($\tau 1$); r and $\tau 1$ for neurons from two different lines (*+b* and *+c*) were determined using the same equation described in Figure 4. *Open symbols*, HS-treated neurons; *solid symbols*, no HS controls. *Group a*, HS-treated neurons expressing 29-4 currents; *group b*, HS-treated but no 29-4 expression; *group c*, no HS control neurons. *B*, The kinetics of recovery from inactivation of A-currents in Sh^+ hosts was determined using the same paradigm in Figure 5*B* (*inset*). Fast recovery of native K^+ currents in no HS control neurons (*solid symbols*, neurons from *group c* in *A*) are compared to the slow recovering A-currents expressed in HS-treated neurons (*open symbols*, neurons of *group a*). The curves shown in Figure 5*B* are replotted here for comparison.

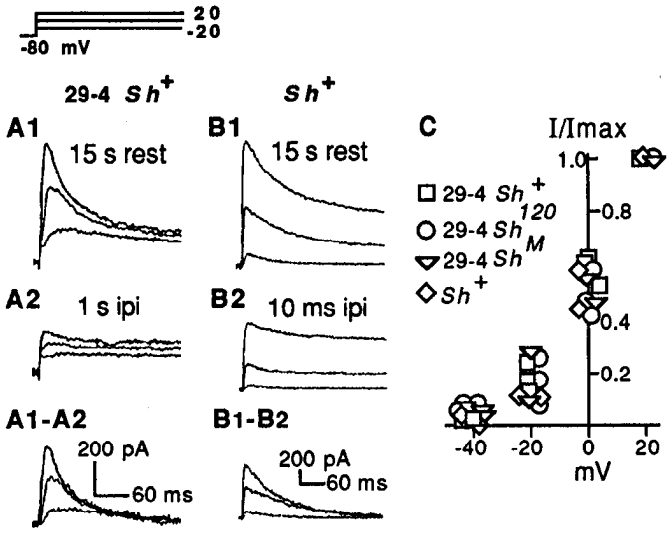

Figure 7. Voltage dependence of activation of HS-induced A-current is independent of *Sh* host background and is similar to that of native A-type K^+ currents. Identical conditioning and test pulses to −20, 0, and 20 mV were applied with a 15 sec rest interval between double-pulse cycles. The inactivating component is isolated by subtracting the current elicited by the test pulse (*A2, B2*) from the current elicited by the conditioning pulse (*A1, B1*). The interpulse interval (*ipi*) was 1 sec for transformants and 10 msec for Sh^+ controls. Current extracted for a 29-4 transformant neuron in a Sh^+ host (*A1-A2*) and a Sh^+ control neuron (*B1-B2*) are shown. Extracted currents for each neurons were normalized to the inactivating current elicited by the +20 mV pulse (I_{max}), and plotted as a function of the test pulse potential (*C*). *Squares*, HS-induced A-currents isolated by subtraction for 29-4 Sh^+ transformants; *circles*, 29-4 Sh^{120} transformants; *triangles*, 29-4 Sh^M transformants; *diamonds*, native A-type currents in Sh^+ control neurons. For the sake of clarity, some data points are displaced along the voltage axis.

there has been no report on the turnover process of native K^+ channels. If the time course of expression of the novel A-currents following HS-induction is indicative of the stability of these channels in neurons, it can then be compared with the stability of native voltage-gated Na^+ and Ca^{2+} channels reported in other organisms. A detailed statistical analysis, generated from the complete data base accumulated in this study, may provide important clues to the answers to these questions.

All cells examined that displayed inactivating K^+ currents, either HS-inducible or native types, were pooled in the plots shown in Figure 9. The data base is equivalent to Figures 4 and 6*A*, but the fraction of recovery (r) is plotted against the amplitude of the inactivating components ($I_p - I_s$; see Fig. 4*A* inset). Among those cells that exhibited the novel, slow-recovering A-currents, the current amplitude appeared to be smaller in Sh^M than in Sh^{120} or Sh^+ hosts (compare group a in Fig. 9*A–C*). To determine if expression of the HS-inducible current occurred preferentially in those cells lacking native A-currents, or if it simply displaced certain components of the native A-currents, all the data from Figure 9 was included in Table 2, along with data from cells that expressed only noninactivating outward K^+ currents. Surprisingly, the percentage of neurons exhibiting only noninactivating K^+ currents remained roughly the same with or without HS treatment in all transformants. In contrast, there was an obvious decrease in the percentage of neurons expressing native A-currents (with fast recovery kinetics) in transformant neurons following HS treatment (Table 2). Consistent with the above observation, the data clearly indicate that the total inactivating component ($I_p - I_s$) in cells expressing *Sh* 29-4 currents was no greater than the inactivating component in control groups without HS treatment (compare groups a and c in Fig. 9). This suggests that there may be an upper limit on the density of A-currents in these neurons, and that HS-induced expression of the novel A-current occurs at the expense of native A-currents.

To examine the stability of the 29-4 channels in different genetic backgrounds, we carried out an analysis of distribution of the HS-induced A-current detected in neurons at various times after HS. The inactivating component ($I_p - I_s$) displaying slow recovery kinetics was used to estimate the amplitude of HS-inducible A-currents in all neurons ($r < 0.6$, group a neurons in Figs. 4, 6*A*, 9), and is plotted against the time following HS induction for all three genetic backgrounds in Figure 10*A–C*. Figure 10*D* shows the percentage of cells expressing the novel current within each time bin. Although the 29-4 currents in Sh^M hosts were consistently lower in amplitude (Fig. 10*B*), they appeared to persist for more than 2 d (Fig. 10*D*). The amplitude

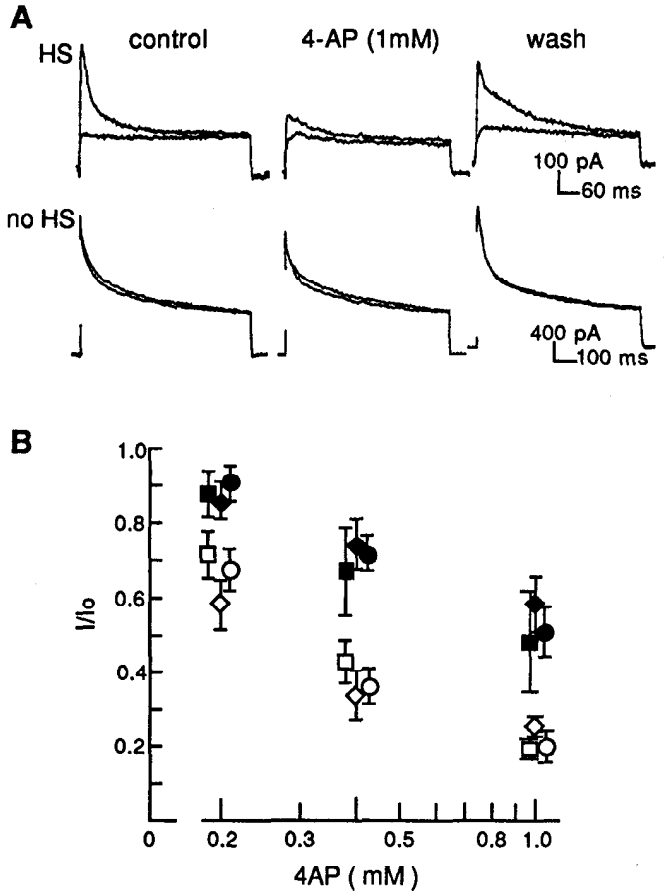

Figure 8. Sensitivity of HS-induced A-currents to 4-AP is distinct from that of native A-type currents. *A*, Superimposed current records, obtained using the double-pulse protocol (Fig. 2) are shown for HS-treated (*upper traces*) and no HS control (*lower traces*) neurons derived from the 29-4 transformants, 120a. Current records were obtained prior to (*left*) and during (*middle*) bath application of 1 mM 4-AP, and after washing out the drug (*right*). *B*, Dosage–response relations for 4-AP block of the inactivating currents ($I_p - I_s$) in control (*solid symbols*) and HS-treated neurons (*open symbols*). *Diamonds*, 29-4 Sh^+; *circles*, 29-4 Sh^{120}; *squares*, 29-4 Sh^M. Each data point represents mean ± SEM determined from six to eight neurons.

of the novel A-currents expressed in Sh^{120} or Sh^+ hosts varied considerably. However, the maximum amplitude in Sh^+ hosts appeared to peak early, around 6 hr after HS, and then gradually declined to a residual level within 18 hr (Fig. 10*C*). A similar time course was also reflected in the percentage of cells expressing the HS-inducible current in Sh^+ hosts (Fig. 10*D*). In contrast, both the amplitude and percentage distributions of the HS-inducible A-current in Sh^{120} hosts showed a more gradual rise and decline, reaching peak amplitude and percentage between 8 and 12 hr after HS and showed little decline in 57 hr (Fig. 10*A*,*D*).

Discussion

This article reports the first examination of functional expression of transgenic *Shaker* K^+ channels in neurons of *Drosophila* germline transformants. Whole-cell patch-clamp recording was performed on cultured "giant" neurons derived from cytokinesis-arrested embryonic neuroblasts (Wu et al., 1990). Their large size facilitates electrophysiological studies of basic properties of *Drosophila* central neurons. Previous studies suggest that undifferentiated neuroblasts in gastrulae are already determined; even after cell-division arrest, they continue to differentiate a variety of phenotypes. Different arborization patterns (Wu et al., 1990), ionic channels (Saito and Wu, 1991) and enzymes for transmitter synthesis (Huff et al., 1989) are expressed in subsets of these multinucleated "giant" neurons. A rich variety of cell types are present in the *Drosophila* nervous system and it is not known how different ionic channels, such as *Sh* K^+ channels, contribute to neuronal diversity. The "giant" neuron culture system enabled us to obtain a large data base for analyzing the pattern of functional expression of a particular *Sh* cDNA, 29-4, under the control of an HS-inducible promoter. Transformants were generated in wild-type and two different *Sh* mutant backgrounds to examine the potential interaction between transgenic and different host *Sh* subunits.

Recovery from inactivation and sensitivity to 4-aminopyridine are significantly different between Sh *29-4 channels expressed in transformants and native K^+ channels*

In spite of the heterogenous K^+ currents present in neurons, positive identification of *Sh* 29-4 currents was feasible because the HS-induced A-current recovered much slower from inactivation than native inactivating K^+ currents expressed in no HS control groups of transformants (group c, Figs. 3, 4, 6*A*), or in neurons from wild-type, Sh^{120} and Sh^M host strains with or without HS treatment (data not shown). The difference is striking, less than 0.2 sec (Fig. 5*A*) versus longer than 4 sec (Fig. 5*B*) at half-recovery (see also Fig. 6*B*).

Sensitivity to block by 4-AP and recovery from inactivation of the HS-induced A-current were similar in several independent transformed lines and in all host strains (Figs. 4, 6*A*, 8). These observations indicate that the novel slow-recovering A-current does not depend on particular sites of chromosomal insertion of the transgene. Furthermore, similarity in 4-AP sensitivity and recovery kinetics of the HS-induced A-current in different *Sh* mutant backgrounds indicates that these properties depend on *Sh* 29-4 subunits. Apparently, native *Sh* subunits either do not coassemble with or are dominated by 29-4 subunits in the channel assembly.

It should be noted that recovery from inactivation of *Sh* 29-4 channels expressed in transformant neurons is very different from that of *Sh* 29-4 channels expressed in oocytes, although the fast time constant ($\tau 1$) of inactivation is similar in the two preparations (Iverson and Rudy, 1990). Analysis of mutant and chimeric *Sh* channels in oocytes indicated that these channels undergo two distinct types of inactivation, fast (N-type, Hoshi et al., 1990) or slow (C-type, Iverson and Rudy, 1990; Hoshi et al., 1991) inactivation. In oocytes, all *Sh* channels with type 4 carboxyl domains, including 29-4, recover quickly from inactivation, showing complete recovery during interpulse intervals of 1 sec (Iverson and Rudy, 1990). However, *Sh* channels containing the type 4 carboxyl domain can be induced to enter the C-type inactivated state, which is associated with slow recovery, by maintained depolarizations of several seconds (Iverson and Rudy, 1990). Nevertheless, native A-currents in "giant" neurons did not show slower recovery after more prolonged depolarization at 20 mV for 0.75 sec (see, for example, Fig. 8*A*, lower traces).

It is previously reported that all *Sh* channels expressed in oocytes exhibit similar 4-AP sensitivity (Timpe et al., 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). However, the pharmacological sensitivity of 29-4 channels expressed in transformants is substantially higher than that of 29-4 channels

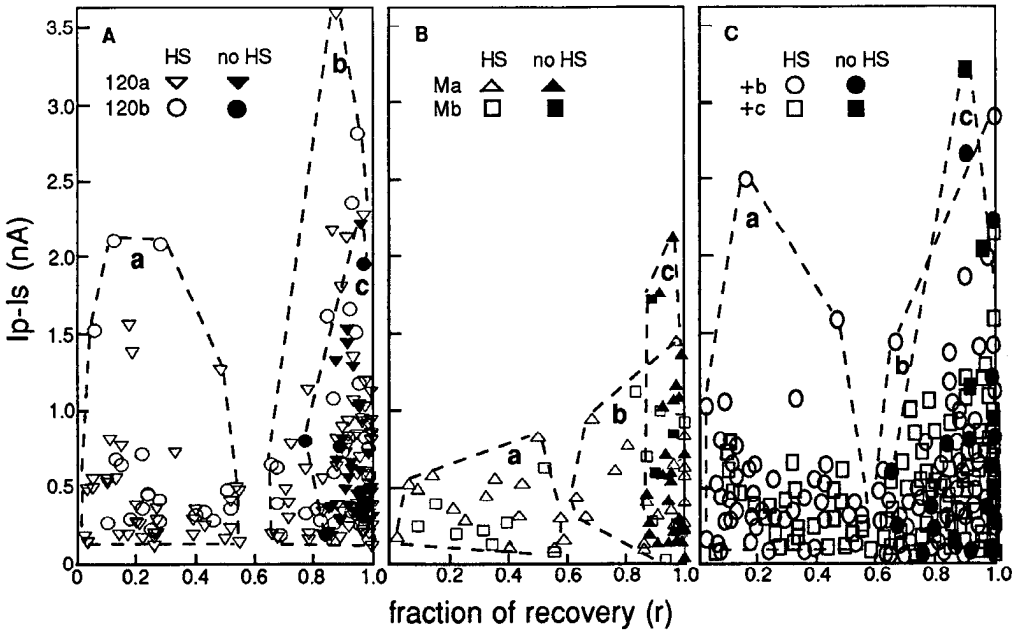

Figure 9. HS-induced expression of *Sh* 29-4 does not increase the amplitude of transient, inactivating K^+ currents. Inactivating K^+ current ($I_p - I_s$) in individual neurons is plotted against the fraction of recovery (r), determined as described in Figure 4. The identical samples of neurons shown in Figures 4 and 6 are analyzed and presented here. Transformant neurons in three different host backgrounds, Sh^{120} (*A*), Sh^{M} (*B*) and Sh^{+} (*C*) are compared. *Group a*, HS expression; *group b*, HS but no expression; *group c*, no HS controls.

expressed in oocytes. This effect is specific to slow recovering channels expressed from the transgene, because the 4-AP sensitivity of fast-recovering A-type currents ($r > 0.6$) expressed in HS-treated transformant neurons resembled that of native currents (data not shown).

Recordings from a large number of sampled neurons indicate that, while the native A-currents varied considerably in the rate of inactivation, the novel *Sh* 29-4 currents were restricted to neurons with a faster decay rate (Figs. 4, 6*A*). In contrast, the voltage range of activation overlapped considerably for the native and *Sh* 29-4 A-currents. The time constant ($\tau 1$) of inactivation and voltage dependence of activation were similar for *Sh* 29-4 channels expressed in both *Xenopus* oocytes (Iverson and Rudy, 1990) and transformant neurons (Fig. 7). One possibility is that, recovery from inactivation and sensitivity to 4-AP are more sensitive to modification by different cellular environments. It will be interesting to examine whether this is true for other excitable tissues in 29-4 transformants.

Tissue-specific expression and influence of cellular factors

Transgenic A-type currents in *Sh*B transformants have been previously studied in cultured embryonic muscle cells (Zagotta et al., 1989). In contrast to our observations in neurons, it was found that the transformant current shows more rapid inactivation and recovery than the native muscle A-type currents, and thus resembles the *Sh*B current expressed in *Xenopus* oocytes. The significance of this difference may become clear when further studies on expression of *Sh*B (4-4, Iverson and Rudy, 1990) in neurons and 29-4 (*Sh*D) in muscle are conducted. Nevertheless, previous studies suggest that factors, such as endogenous homologous subunits or channel-associated proteins, are present in oocytes that can participate in the assembly or modulation of channels (Rudy et al., 1988; Buller and White, 1990; Blumenthal and Kaczmarek, 1993; Saal et al., 1993; Isom et al., 1994). The significantly slower recovery rates of 29-4 channels in transformants relative to 29-4 channels in oocytes is likely due to differences of such factors in the two systems. It is also possible that *Sh* 29-4 subunits contribute only a minor component to neuronal *Sh* channels. Consistent with this observation, the *Sh* translocation, W32, which separates the type 29 5′ domain from the remainder of the gene (Kamb et al., 1987), results in elimination of all *Sh* K^+ currents in muscle (Timpe and Jan, 1987), but exhibits only weak effects on adult giant fiber axons (Tanouye et al., 1981; Tanouye and Ferrus, 1985). In addition, only probes containing sequences from this 5′ domain hybridizes in muscle (Tseng-Crank et al., 1991).

Moreover, the voltage range of activation and the rate of inactivation for *Sh* 29-4 channels in "giant" neurons resemble those of native A-currents in embryonic, larval and pupal muscles (see review, Wu and Ganetzky, 1992). It is possible that the *Sh* 29-4 splicing product contributes a subunit to muscle A-channels.

Percentage of neurons expressing inactivating K^+ currents is not increased by HS-induced expression of Sh *29-4*

Only one A-type K^+ channel has been identified in *Drosophila* muscle, that encoded by the *Sh* locus (see review, Wu and Ganetzky, 1992). In contrast, *Drosophila* neurons appear to express a diverse array of voltage-dependent K^+ channels, including inactivating and noninactivating types, and many neurons lack inactivating A-type currents (see for review, Wu and Ganetzky, 1992; Saito and Wu, 1993). If expression of *Sh* 29-4 is additive to native K^+ currents, then one might expect to observe a higher percentage of neurons expressing inactivating currents following HS treatment. The data presented in Table 2 demonstrates clearly that this is not true. HS treatment consistently failed to increase the percentage of neurons exhibiting inactivating currents. Furthermore, amplitude distributions of the total inactivating currents ($I_p - I_s$) in cells expressing HS-induced 29-4 channels are no greater than that of native K^+ currents (see Fig. 9).

The lack of slow-recovering A-currents in a subset of HS-treated neurons (group b, Figs. 4, 6*A*) further suggests that some mechanism exists that limits the number of A-channels in these neurons. The significance of this finding is not known. If we assume that HS induces ectopic expression of *Sh* 29-4 messages in all cells within the transformant, the lack of the slow-recovering A-currents in these neurons would indicate restriction in translation of *Sh* 29-4 messages, or absence of other regulatory

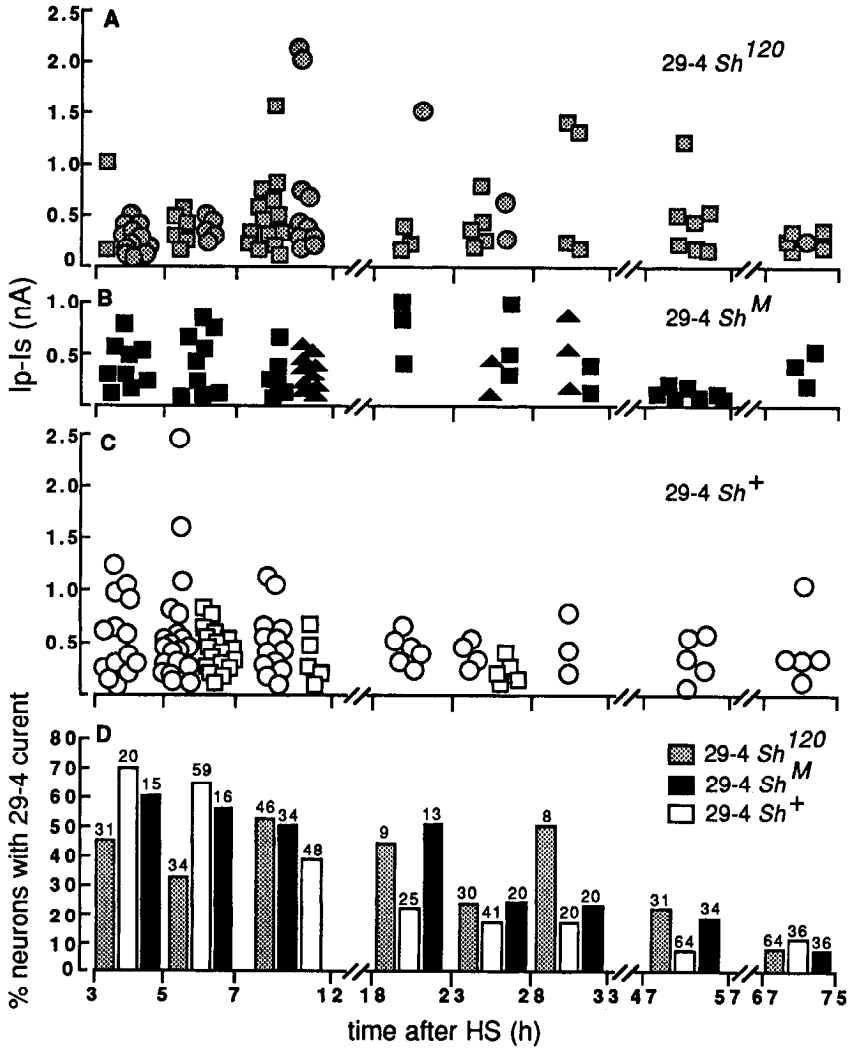

Figure 10. Amplitude distributions and the percentage of neurons expressing the HS-induced, slow-recovering A-current are indicative of differential temporal regulation of *Sh* 29-4 channels in neurons. Amplitude of the HS-induced A-current ($I_p - I_s$) in all group of neurons in Figure 9 is plotted against the time following HS treatment. *A*, 29-4 Sh^{120} transformants (compare Fig. 9*A*); *B*, 29-4 Sh^M transformants (compare Fig. 9*B*); and *C*, 29-4 Sh^+ transformants (compare Fig. 9*C*). *D*, The percentage of neurons expressing the slow-recovering A-current is plotted against time after HS. The percentage is determined as the number of neurons expressing the novel, slow-recovering A-current among the total population of sampled neurons (*n*) subjected to HS treatment.

factors, such as channel associated proteins, required for the formation of functional channels from the translated subunits.

An extension of this idea is that the cells lacking inactivating currents (Table 2) may be missing a factor that is important for functional expression of all A-channels, including those containing *Sh* and non-*Sh* subunits. Since the same phenomenon was observed in all host backgrounds, such a factor must be derived from genes other than *Sh* and may be related to posttranslational mechanisms such as channel assembly, anchoring sites or functional modification.

Temporal regulation of the HS-induced A-current may depend on Sh *mutant background*

Amplitude distributions of inactivating currents in Sh^M hosts expressing 29-4 channels appeared smaller than in Sh^{120} and Sh^+ hosts (Figs. 9, 10). Since it is likely that the inactivating currents ($I_p - I_s$) in neurons exhibiting slow recovery ($r < 0.6$) is mainly mediated by 29-4 channels (Fig. 9), several conclusions can be drawn from the amplitude distributions at different times following HS (Fig. 10). In Sh^+ hosts (Fig. 10*C*), the maximum amplitude of the 29-4 currents appeared at 5–7 hr after HS and decreased rapidly with time. In contrast, 29-4 currents in Sh^{120} hosts (Fig. 10*A*) peaked at 8–12 hr after HS and decreased at a much slower rate. A similar difference in decay rate can be seen in the percentage of neurons expressing 29-4 currents at various times after HS (Fig. 10*D*). Although data for temporal expression in Sh^M hosts (Fig. 10*B,D*) are somewhat limited, the turnover rate appears closer to Sh^{120} than Sh^+ hosts. These results are indicative of host-dependent effects on *Sh* 29-4 expression in neurons. Whether *Sh* subunits contributed by the hosts affect the function and/or the stability of the novel currents attributable to *Sh* 29-4 subunits must await further investigation.

Sh 29-4 currents remain functional for days in "giant" neurons. Since the turn over rates of the induced *Sh* 29-4 mRNA is still not known, we can not rule out the possibility of persistent synthesis of the *Sh* 29-4 subunit. However, if indicative of the useful life of K^+ channels in *Drosophila* neurons, this temporal range is directly comparable to the turnover time reported for

other voltage-dependent channels (Schein, 1976; Schmidt and Catteral, 1986).

References

Baker K, Salkoff L (1990) The *Drosophila Shaker* gene codes for a distinctive K^+ current in a subset of neurons. Neuron 2:129–140.

Blumenthal EM, Kaczmarek LK (1993) The minK protein exists in functional and nonfunctional forms when expressed in *Xenopus* oocytes: implications for channel function and modulation. Soc Neurosci Abstr 19:710.

Buller AL, White MM (1990) Functional acetylcholine receptors expressed in *Xenopus* oocytes after injection of *Torpedo* β, γ and δ subunit RNAs are a consequence of endogenous oocyte gene expression. Mol Pharmacol 37:423–428.

Butler A, Wei A, Baker K, Salkoff L (1989) A family of putative potassium channel genes in *Drosophila*. Science 243:943–947.

Byerly L, Leung HT (1988) Ionic currents of *Drosophila* neurons in embryonic cultures. J Neurosci 8:4379–4393.

Christie MJ, North RA, Osborne PB, Douglass J, Adelman JP (1990) Heteropolymeric potassium channels expressed in *Xenopus* oocytes from cloned subunits. Neuron 4:405–411.

Elkins T, Ganetzky B, Wu C-F (1986) A *Drosophila* mutation that eliminates a calcium-dependent potassium current. Proc Natl Acad Sci USA 83:8415–8419.

Ganetzky B, Wu C-F (1986) Neurogenetics of membrane excitability in *Drosophila*. Annu Rev Genet 20:13–44

Haugland FN (1987) A voltage clamp analysis of membrane potassium currents in larval muscle fibers of *Shaker* mutants of *Drosophila*. PhD thesis, University of Iowa, Iowa City.

Haugland FN, Wu C-F (1990) A voltage-clamp analysis of gene-dosage effects of the *Shaker* locus on larval muscle potassium currents in *Drosophila*. J Neurosci 10:1357–1371.

Hille B (1992) Ion channels of excitable membrane. Sunderland, MA: Sinauer.

Hoshi T, Zagotta WN, Aldrich RW (1990) Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. Science 250: 533–538.

Hoshi T, Zagotta WN, Aldrich RW (1991) Two types of inactivation in *Shaker* K^+ channels: effects of alterations in the carboxy-terminal region. Neuron 7:547–556.

Huff R, Furst A, Mahowald AP (1989) *Drosophila* embryonic neuroblasts in culture: autonomous differentiation of specific neurotransmitters. Dev Biol 134:146–157.

Isacoff EY, Jan Y-N, Jan LY (1990) Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes. Nature 345:530–534.

Isom LL, De Jongh KS, Catterall WA (1994) Auxiliary subunits of voltage-gated ion channels. Neuron 12:1183–1194.

Iverson LE, Rudy B (1990) The role of the divergent amino and carboxyl domains on the inactivation properties of potassium channels derived from the *Shaker* gene of *Drosophila*. J Neurosci 10:2903–2916.

Iverson LE, Tanouye MA, Lester HA, Davidson N, Rudy B (1988) A-Type potassium channels expressed from *Shaker* locus cDNA. Proc Natl Acad Sci USA 85:5723–5727.

Jan Y-N, Jan LY, Dennis MJ (1977) Two mutations of synaptic transmission in *Drosophila*. Proc R Soc Lond [Biol] 198:87–108.

Kamb A, Iverson LE, Tanouye MA (1987) Molecular characterization of *Shaker*, a *Drosophila* gene that encodes a potassium channel. Cell 50:405–413.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lindsley DL, Zimm GG (1992) The genome of *Drosophila melanogaster*. San Diego: Academic.

MacKinnon R (1991) Determination of the subunit stoichiometry of a voltage-activated potassium channel. Nature 350:232–235.

McComack K, Lin J-W, Iverson LE, Rudy B (1990) *Shaker* K^+ channel subunits form heteromultimeric channels with novel functional properties. Biochem Biophys Res Commun 171:1361–1371.

Pongs O, Kecskemethy N, Muller R, Krah-Jentgens I, Baumann A, Kiltz HH, Canal I, Llamazares S, Ferrus A (1988) *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. EMBO J 7:1087–1096.

Rubin GM, Spradling AC (1982) Genetic transformation of *Drosophila* with transposable element vectors. Science 218:348–353.

Rudy B, Hoger JH, Lester HA, Davidson N (1988) At least two mRNA species contribute to the properties of rat brain A-type potassium channels expressed in *Xenopus* oocytes. Neuron 1:649–658.

Ruppersberg JP, Schroter KH, Sakmann B, Sewing S, Pongs O (1990) Heteromultimeric channels formed by rat brain potassium-channel proteins. Nature 345:535–537.

Saal D, Chung S, Kaczmarek LK (1993) Expression and modulation of the $K_v1.5$ potassium channel in a mouse fibroblast cell line. Soc Neurosci Abstr 19:710.

Saito M, Wu C-F (1991) Expression of ion channels and mutational effects in "giant" *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts. J Neurosci 11:2135–2150.

Saito M, Wu C-F (1993) Ionic channels in cultured *Drosophila* neurons. In: Comparative molecular neurobiology (Pichon Y, ed), pp 366–389. Basel: Birkhauser.

Salkoff L (1983) Genetic and voltage-clamp analysis of a *Drosophila* potassium channel. Cold Spring Harbor Symp Quant Biol 48:221–231.

Salkoff L, Wyman R (1981) Genetic modification of potassium channels in *Drosophila Shaker* mutants. Nature 293:228–230.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schein SJ (1976) Calcium channel stability measured by gradual loss of excitability in pawn mutants of *Paramecium aurelia*. J Exp Biol 65:725–736.

Schmidt JW, Catterall WA (1986) Biosynthesis and processing of the subunit of the voltage-sensitive sodium channel in rat brain neurons. Cell 46:437–445.

Schwarz TL, Tempel BL, Papazian DM, Jan Y-N, Jan LY (1988) Multiple potassium channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. Nature 331:137–142.

Solc CK, Aldrich RW (1988) Voltage-gated potassium channels in larval CNS neurons of *Drosophila*. J Neurosci 8:2556–2570.

Solc CK, Zagotta WN, Aldrich RW (1987) Single-channel and genetic analyses reveal two distinct A-type potassium channels in *Drosophila*. Science 236:1094-1098.

Tanouye MA, Ferrus A (1985) Action potentials in normal and *Shaker* mutant *Drosophila*. J Neurogenet 2:253–271.

Tanouye MA, Ferrus A, Fujita SC (1981) Abnormal action potentials associated with the *Shaker* complex locus of *Drosophila*. Proc Natl Acad Sci USA 78:6548–6552.

Timpe LC, Jan LY (1987) Gene dosage and complementation analysis of the *Shaker* locus in *Drosophila*. J Neurosci 7:1307–1317.

Timpe LC, Jan Y-N, Jan LY (1988a) Four cDNA clones from the *Shaker* locus of *Drosophila* induce kinetically distinct A-type potassium currents in *Xenopus* oocytes. Neuron 1:659–667.

Timpe LC, Schwarz TL, Tempel BL, Papazian DM, Jan Y-N, Jan LY (1988b) Expression of functional potassium channels from *Shaker* cDNA in *Xenopus* oocyte. Nature 331:143–145.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Tseng-Crank J, Pollock JA, Hayashi I, Tanouye MA (1991) Expression of ion channel genes in *Drosophila*. J Neurogenet 7:229–239.

Tsunoda S, Salkoff L (1993) *Shal* encodes most transient K^+ currents in embryonic *Drosophila* neurons; *Shaw* may also be present. Soc Neurosci Abstr 19:704.

Warmke J, Drysdale R, Ganetzky B (1991) A distinct potassium channel polypeptide encoded by the *Drosophila eag* gene. Science 252: 1560–1562.

Wu C-F, Ganetzky B (1992) Neurogenetic studies of ion channels in *Drosophila*. In: Ion channels, Vol 3 (Narahashi T, ed), pp 261–314. New York: Plenum.

Wu C-F, Haugland FN (1985) Voltage clamp analysis of membrane currents in larval muscle fibers of *Drosophila*: alteration of potassium currents in *Shaker* mutants. J Neurosci 5:2626–2640.

Wu C-F, Ganetzky B, Haugland FN, Liu A-X (1983) Potassium currents in *Drosophila*: different components affected by mutations of two genes. Science 220:1076–1078.

Wu C-F, Sakai K, Saito M, Hotta Y (1990) Giant *Drosophila* neurons differentiated from cytokinesis-arrested embryonic neuroblasts. J Neurobiol 21:499–507.

Zagotta WN, Brainard MS, Aldrich RW (1988) Single-channel analysis

of four distinct classes of potassium channels in *Drosophila* muscle. J Neurosci 8:4765–4779.

Zagotta WN, Germeraad S, Garber SS, Hoshi T, Aldrich RW (1989) Properties of *Sh*B A-type potassium channels expressed in *Shaker* mutant *Drosophila* by germline transformation. Neuron 3:773–782.

Zhao M-L, Sable EO, Saito M, Iverson LE, Wu C-F (1992) Host-dependent *Shaker* cDNA expression in cultured *Drosophila* "giant" neurons by germline transformation. Soc Neurosci Abstr 18:78.

Zhong Y, Wu C-F (1991) Alteration of four identified K^+ currents in *Drosophila* muscle by mutations in *eag*. Science 252:1562–1564.

Zhong Y, Wu C-F (1993) Modulation of different K^+ currents in *Drosophila*: a hypothetical role for the *eag* subunit in multimeric K^+ channels. J Neurosci 13:4669–4679.