

# Expression of *Drosophila* Shaker potassium channels in mammalian cells infected with recombinant vaccinia virus

(heterologous expression/rat basophilic leukemia cells/rat pheochromocytoma cells)

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**ABSTRACT** A recombinant vaccinia virus containing a *Drosophila* potassium channel (Shaker H4) cDNA was constructed by homologous recombination between wild-type vaccinia virus DNA and a transfer plasmid. The new virus was used to infect four types of mammalian cells in culture. Electrophysiological recording 24–72 hr after infection revealed the expression of voltage-gated transient potassium channels in all four cell types. The properties of the induced currents were identical to those previously observed following injection of the Shaker H4 transcript into oocytes. Vaccinia promises to be an effective vehicle for the heterologous expression of transmembrane ion channels in a variety of cell types.

One of the most important benefits realized from the molecular cloning of cDNAs encoding ion-channel and transmitter-receptor peptides has been the opportunity to achieve functional expression of the clones in foreign cells (1). Microinjected *Xenopus* oocytes and stably transfected cell lines constitute the two usual systems for such heterologous expression at present. Important applications of heterologous expression include confirmation of a clone's identity, studies of pharmacological and kinetic diversity among proteins encoded by homologous cloned mRNAs, structure-function studies that measure changes in function introduced by manipulations of the amino acid sequence, and the isolation of new cDNA clones.

Both oocytes and stably transfected cell lines present unique technical advantages for such studies. We have begun to explore another type of transient expression system with the hope that it will prove to be a useful complement to the more widely used methods. We describe here the use of vaccinia virus (VV) to achieve the transient expression of a voltage-gated ion channel in cultured cells. Vaccinia presently constitutes an important tool for heterologous expression of both soluble and membrane-bound proteins in animal cells (2–4). Because VV has a broad host range, it can be used to study cell-specific processing or modulation of a given gene product (5). Because VV carries its own polymerases and replicates in the cytoplasm, its transcripts are not likely to be subjected to fortuitous splicing.

We have constructed a recombinant VV in which the coding region of a *Drosophila* transient potassium channel cDNA, Shaker H4 (6), was placed downstream of the VV 7.5-kDa promoter and inserted into the nonessential VV thymidine kinase gene. We have infected a variety of cells in culture with the recombinant virus (VV:H4) and have observed robust expression of ion channels that are very similar to both the Shaker H4 potassium channel as it is expressed

in oocytes and the channels responsible for transient potassium currents in *Drosophila* muscle cells (7).

## MATERIALS AND METHODS

**Viruses and Cells.** The WR strain of VV (wild type; VV:WT) and the recombinant virus (VV:H4) were propagated separately in African green monkey BSC-40 cells and purified as previously described (8). Thymidine kinase-negative L cells (Ltk<sup>-</sup>) and BSC-40 cells were laboratory stocks (G.T. lab). Rat basophilic leukocytes (RBL-1 cells) and NIH mouse 3T3 fibroblasts were obtained from the American Type Culture Collection. Monkey kidney CV-1 cells were obtained from S. Subramani (University of California, San Diego), and rat adrenal pheochromocytoma PC-12 cells were obtained from L. Greene (Columbia University). Cells were maintained in temperature- and CO<sub>2</sub>-controlled incubators under appropriate culture conditions.

**Construction of the Recombinant Virus.** The Shaker H4 cDNA (2.2 kilobases) was excised from the multiple cloning site of pBluescript (Stratagene) by partial digestion with *Bam*HI followed by complete digestion with *Cla* I. The *Bam*HI–*Cla* I fragment (containing one internal *Bam*HI site) was purified by agarose gel electrophoresis and subcloned into the vaccinia recombination plasmid pVV3 (8), which had been digested to completion with *Bam*HI and *Cla* I. This placed the initiation codon of the H4 cDNA 17 base pairs downstream from the end of the 5' VV flanking sequence containing the vaccinia 7.5-kDa promoter. The resultant plasmid (pVV3:H4) was amplified in *Escherichia coli* and purified by two cycles of cesium chloride centrifugation. The pVV3:H4 plasmid DNA was mixed with VV:WT genomic DNA and transfected by calcium phosphate precipitation into Ltk<sup>-</sup> cells that had been infected with VV:WT virus 3 hr earlier. A recombinant virus containing the H4 cDNA inserted within the viral thymidine kinase gene was identified and purified by marker rescue (9, 10).

**Viral Infections.** Infections for marker rescue and propagation of the virus were carried out in 100-mm tissue culture dishes as described (8). Infections for electrophysiology experiments were carried out on nearly confluent sheets of cells in T-250 culture flasks. Cells were rinsed with phosphate-buffered saline containing 1 mM MgCl<sub>2</sub> (PBS/Mg<sup>2+</sup>) and were infected with either wild-type virus or the recombinant VV:H4 at a multiplicity of infection of 1–5 per cell for 20–30 min in PBS/Mg<sup>2+</sup> at room temperature. The virus-containing medium was replaced by Eagle's minimum essential medium plus 10% fetal bovine serum, and the cells were

Abbreviation: VV, vaccinia virus.

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incubated at 37°C. One hour after infection, the cells were dispersed with trypsin, replated into several 35-mm tissue culture dishes ( $\approx 10^4$  cells per dish), and allowed to grow at 37°C overnight prior to recording.

**Electrophysiology.** Individual tissue culture dishes containing infected cells were rinsed three times with bath solution (110 mM NaCl/2.5 mM KCl/1.0 mM CaCl<sub>2</sub>/2.0 mM MgCl<sub>2</sub>/10 mM potassium Hepes/10 mM glucose/5 mM pyruvate, pH 7.2) and placed on the stage of an inverted microscope equipped with Hoffman modulation contrast optics. Voltage-activated currents were measured by whole-cell patch-clamp recording (Axoclamp-1A; Axon Instruments, Burlingame, CA). Pipets were pulled in two stages from Kimax borosilicate glass tubing (outer diameter, 1.8 mm) and heat-polished to a final diameter of  $\approx 1 \mu\text{m}$ . Pipets were filled with intracellular solution (145 mM potassium glutamate/8 mM NaCl/3 mM MgCl<sub>2</sub>/2 mM ATP/0.3 mM GTP/0.5 mM EGTA/10 mM sodium Hepes, pH 7.2) and had a final resistance of 3–10 M $\Omega$ . Stimulation, data acquisition, and analysis were performed with the aid of a microcomputer using pCLAMP software (Axon Instruments). Series resistance was compensated 10–80%. Digital leak subtraction was sometimes used. All experiments were performed at room temperature (19–22°C).

## RESULTS

**Cultured Cells Infected with VV:H4 Recombinant Virus Express Transient Outward Currents.** Voltage-clamp recordings of whole-cell membrane currents were made from four types of cultured cells 24–72 hr after infection with the recombinant VV:H4. Fig. 1 compares waveforms of voltage-clamp currents elicited by depolarizing voltage steps from a holding potential of  $-100 \text{ mV}$  for one cell of each type. VV:H4-infected cells displayed a transient outward current that was active at membrane potentials above  $-40 \text{ mV}$ . Control cells, either noninfected or infected with VV:WT, exhibited no such currents (data not shown). The waveforms of the VV:H4-induced currents were the same in each of the four cell types, even though each cell type displayed different intrinsic properties. For example, rat basophilic leukemia RBL-1 cells (Fig. 1B) had high input resistance ( $>100 \text{ M}\Omega$ ) and there appeared to be no other currents activated in the voltage range used to elicit the vaccinia-induced currents. Both control and VV:H4-infected PC-12 cells, however, exhibited a large (1–2 nA), slowly activating ( $t_{1/2} \approx 20 \text{ ms}$ ) outward current whose range of activation overlapped partially with that of the VV:H4-induced transient outward current (Fig. 1D). Changing the holding potential to  $-30 \text{ mV}$  completely inactivated the transient currents, leaving only the maintained outward current (Fig. 1E). The waveform of the transient current at each voltage was obtained by subtracting the family of traces recorded when the holding potential was  $-30 \text{ mV}$  from those recorded when the holding potential was  $-100 \text{ mV}$ . The subtraction currents (Fig. 1F) resembled the transient outward currents recorded from infected RBL-1 cells. In both CV-1 (Fig. 1A) and NIH 3T3 cells (Fig. 1C), the VV:H4-induced transient outward currents were the only time- and voltage-dependent currents observed in the voltage range tested; however, both of these cell types exhibited large leakage currents (with or without infection). Removal of currents other than the transient outward current induced by infection could be accomplished by a number of methods (see Fig. 1 legend). Clearly, the RBL-1 cells presented the most favorable background for studying the kinetics of the VV:H4-induced currents.

The overall efficiency of expression, meaning the fraction of nominally infected cells expressing transient outward currents, was  $\approx 50\%$  for PC-12, RBL-1, and CV-1 cells. The efficiency was lower ( $\approx 10\%$ ) in the NIH 3T3 cells. We presume that this difference arose from a lower rate of

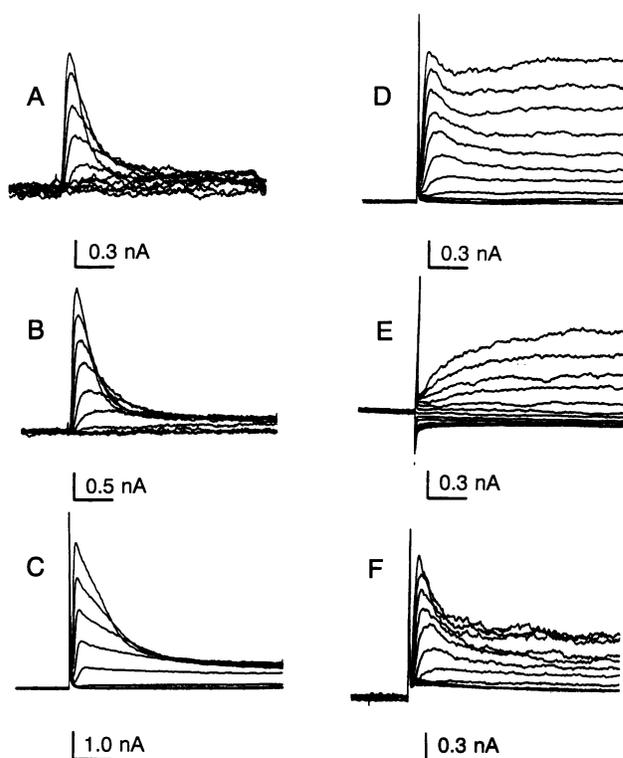


FIG. 1. Currents elicited by depolarizing voltage steps in cells infected with VV:H4 virus. (A) CV-1 cell. (B) RBL-1 cell. (C) NIH 3T3 cell. (D–F) PC-12 cell. Each trial comprised a family of 80-ms steps to voltages from  $-80 \text{ mV}$  to  $+30 \text{ mV}$  in  $10\text{-mV}$  increments from a holding potential ( $V_H$ ) of  $-100 \text{ mV}$  (A, B, and D) or  $-30 \text{ mV}$  (E). Leak and capacitive currents in A and B were eliminated by digital subtraction. Traces in C are the result of subtraction of records at  $V_H = -30$  from records at  $V_H = -100$  to remove leakage currents (individual records not shown). Transient outward currents in the PC-12 cell (F) were isolated from the maintained outward currents (E) by similar subtraction from traces in D. Current amplitude scale is indicated within each panel; time scale is the same (10 ms) for each. Temperature, 19–21°C.

infection of the 3T3 cells, since the magnitudes of the currents expressed in “positive” 3T3 cells were among the largest of all the cell types. Furthermore,  $>80\%$  of cells displaying morphological characteristics of viral infection, such as a slightly rounded profile, were found to be positive for expression of Shaker-like currents. Based on the single-channel conductance of the Shaker H4 channel expressed in oocytes (6), and the amplitudes of macroscopic currents we recorded from cells of known diameter, we estimate the density of functional channels induced by VV:H4 infection to be in the range of 1–5 per  $\mu\text{m}^2$  of plasma membrane.

**Transient Currents in all Four Cell Types Show the Same Voltage Dependence of Activation and Inactivation.** Fig. 2A shows the normalized peak current amplitudes of the transient outward currents elicited by a family of depolarizing voltage steps from a holding potential of  $-100 \text{ mV}$  for one cell of each type studied. The values superimpose, indicating that the voltage dependence of activation was the same in each cell. The threshold for activation ( $-40$  to  $-30 \text{ mV}$ ) is the same as that reported for the Shaker H4 cDNA expressed in oocytes (6) and is also the same as that reported for one type of transient potassium current (A current) recorded from embryonic *Drosophila* muscle cells in culture (11).

Just as the process of activation of the A-type current is voltage-dependent, so is the process of inactivation. As described above, no transient outward current was elicited when depolarizing voltage steps were delivered from a holding potential of  $-30 \text{ mV}$ ; the channels were completely

inactivated. As shown in Fig. 2B, the degree of inhibition produced by a short conditioning prepulse is a steep function

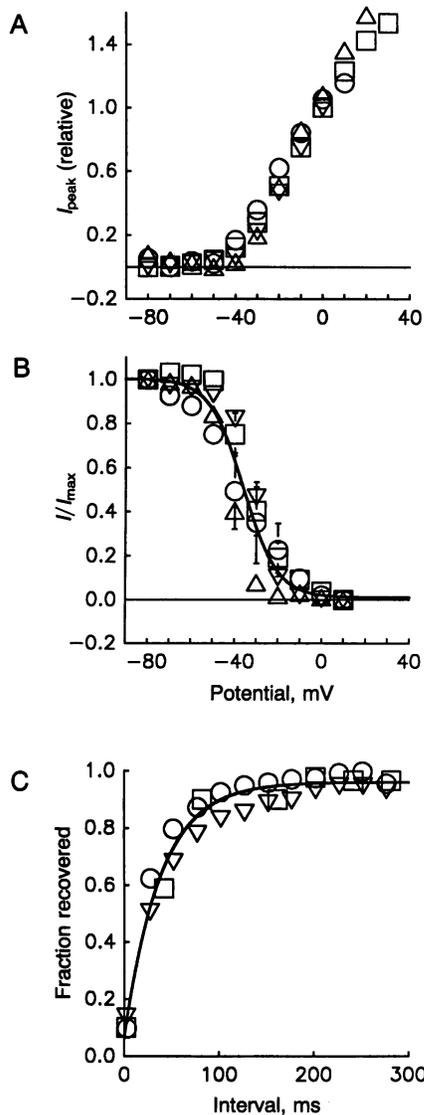


FIG. 2. Voltage dependence of activation and steady-state inactivation of transient outward currents elicited by infection with VV:H4. Cell types:  $\square$ , RBL-1;  $\circ$ , CV-1;  $\nabla$ , NIH 3T3;  $\triangle$ , PC-12. (A) Voltage dependence of activation. Voltage steps (80-ms duration) to levels shown on the abscissa were delivered from a holding potential of  $-100$  mV; the peak current, measured after leak subtraction, is plotted on the ordinate. Peak current at each voltage is normalized relative to the peak current at 0 mV. Data are from one cell of each type, as indicated in the key above. (B) Steady-state inactivation. A 100-ms prepulse to a value between  $-80$  mV and  $+10$  mV preceded each test pulse to  $+20$  mV. The ratio of peak current during the test pulse relative to that recorded with a prepulse to  $-100$  mV ( $I / I_{\text{max}}$ ) is plotted as a function of the prepulse voltage. Points are mean values from more than one cell of each type, except for PC-12, which is from a single cell. Error bars are SEM, shown where they exceeded symbol size. Continuous line is the best fit of a Boltzman distribution,  $P(V) = I / I_{\text{max}} = \{1 + \exp[(V - V_{1/2})/z]\}^{-1}$ , to all the points shown.  $P(V)$  is the fraction of activatable current remaining following a prepulse to voltage  $V$ . The voltage for half-inactivation,  $V_{1/2} = -36$  mV; the voltage for an  $e$ -fold change,  $z = 8$  mV. (C) Recovery from inactivation. The voltage was held at  $-100$  mV except for paired steps to  $+20$  mV for 80 ms; the interval between steps was varied between 2 and 295 ms. The ratio of the peak current during the second step to that during the first step is plotted against the interval between steps. The time between trials was 2 sec, which allowed for full recovery of the currents. Data are from single cells. The continuous line is the best fit of a single exponential to all the data shown;  $\tau = 38$  ms.

of its voltage. Recovery from inactivation occurred rapidly only at hyperpolarized membrane potentials. Fig. 2C shows the time course of recovery at  $-100$  mV from inactivation induced by an 80-ms voltage step to  $+20$  mV. Both the voltage for half-inactivation ( $-36$  mV; Fig. 2B) and the time constant for recovery from inactivation ( $\tau = 38$  msec; 90% recovery in 200 ms; Fig. 2C) agree with the values obtained by expression of the H4 cDNA in oocytes (6) and with those reported for *Drosophila* A-current channels (11, 12).

**Kinetics of Activation and Inactivation Are Voltage-Dependent.** It is apparent (e.g., Fig. 1) that the VV:H4-induced currents peaked sooner and also decayed more

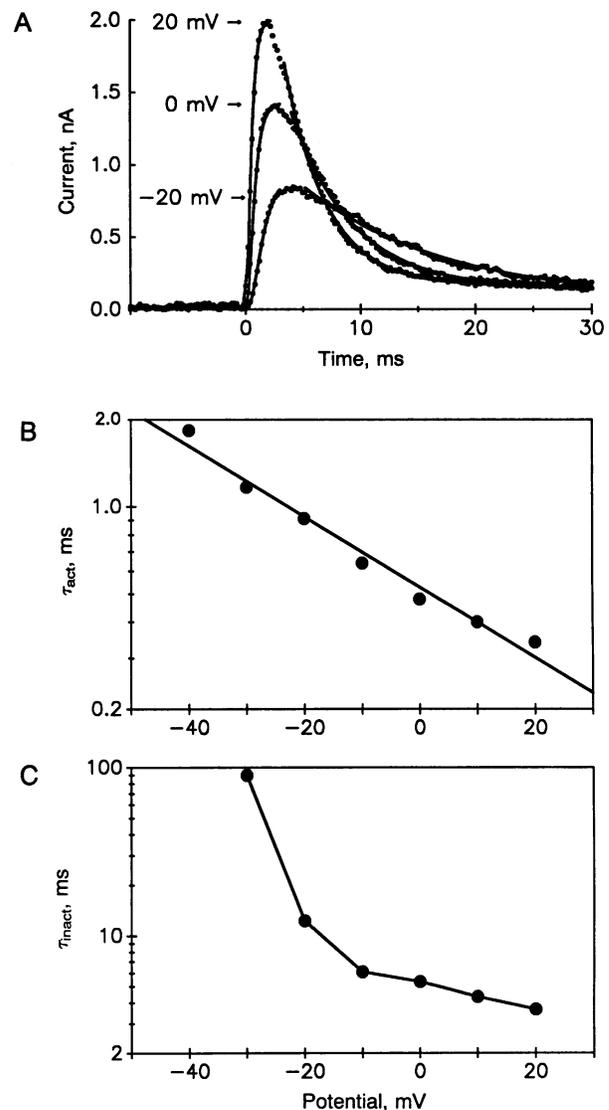


FIG. 3. Voltage dependence of activation and inactivation phases of transient outward currents in VV:H4-infected RBL-1 cell. (A) Current traces (dots) elicited by depolarizing voltage steps to  $-20$ ,  $0$ , and  $+20$  mV from a holding potential of  $-100$  mV are superimposed on separate functions for the rising and falling phases (solid lines), determined with nonlinear fits. Rising phases are fit by a third-order exponential,  $A[1 - \exp(-t/\tau_{\text{act}})]^3$ ; falling phases are fit by single exponentials. (B) Voltage dependence of  $\tau_{\text{act}}$ , plotted on a semilogarithmic scale. The line is a least-squares fit to the data points and corresponds to an  $e$ -fold change for 22 mV. Data are from a complete family of voltage steps for the cell of A. (C) Voltage dependence of inactivation. Time constants,  $\tau_{\text{inact}}$ , from exponential fits to falling phases of the current traces are plotted against test potential on a semilogarithmic scale. The rate of inactivation is not a single-exponential function of voltage.

rapidly with larger depolarizations. Due to the favorable membrane properties of the RBL-1 cells and the high levels of expression, we were able to study the waveforms in detail (Fig. 3). The rising (activation) phases were well fit by a third-order exponential process (Fig. 3A). One interpretation of such a time course (13) is that three or more independent events (such as charge movement within each subunit of a multimeric channel protein) are required for the channel to open. The activation time constant,  $\tau_{act}$ , varied between 1.9 ms at  $-40$  mV and 0.34 ms at  $+20$  mV and depended exponentially on voltage ( $e$ -fold per 22 mV; Fig. 3B). The decay phase at each voltage was fit by a single exponential (see Fig. 3A legend). The inactivation time constant,  $\tau_{inact}$ , varied from 90 ms at  $-30$  mV to 3.6 ms at  $+20$  mV and did not display a simple exponential dependence on voltage (Fig. 3C). Tail currents (Fig. 4) reversed near the calculated equilibrium potential for potassium, implying a high selectivity for potassium ions. The tail currents decayed with a single exponential time course ( $\tau \approx 2$  ms) that was not strongly voltage-dependent.

**Induced A-Type Currents Are Blocked by 4-Aminopyridine and Charybdotoxin.** One of the hallmarks of A-current potassium channels is their sensitivity to 4-aminopyridine. Fig. 5A shows the development of and recovery from block by 4-aminopyridine of the VV:H4-induced currents in an RBL-1 cell. Currents induced by VV:H4 infection of the other cell types were similarly blocked. Currents were also blocked by 10 nM charybdotoxin (Fig. 5B), but recovery was incomplete. Charybdotoxin blocks A currents expressed in oocytes after injection of Shaker H4 transcript (14), but it does not block A currents in *Drosophila* muscle (15, 16).

## DISCUSSION

This study demonstrates that VV is an efficient tool for the heterologous expression of a voltage-gated ion channel in animal cells. There are several useful systems for heterologous expression of cloned cDNAs, including injection of *in vitro* transcripts into *Xenopus* oocytes and stable transfection of cDNA constructs into cell lines. Like oocytes, vaccinia has the disadvantage of being a transient expression system, but the period of viability of the infected cells is more than sufficient for electrophysiological investigations. For certain experiments requiring cultured cells (such as fluorescence

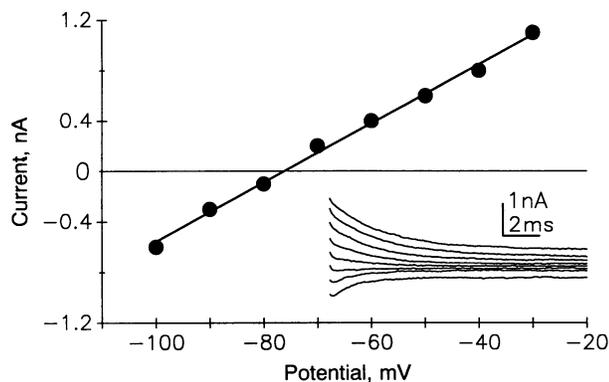


FIG. 4. Reversal potential of VV:H4-induced currents in an RBL-1 cell. A 4-ms step from a holding potential of  $-100$  mV to an activation potential of  $+20$  mV was followed by a return to a family of test potentials from  $-100$  to  $-20$  mV. The tail currents (Inset) elicited by this protocol were fit with single exponentials. The amplitude of the zero-time intercept of the exponential fits is plotted against the test potential. The currents reversed at  $-78$  mV. Leakage currents were removed by subtraction of current traces elicited by pulses from a holding potential of  $-30$  mV. No transient outward current was activated by the latter protocol.

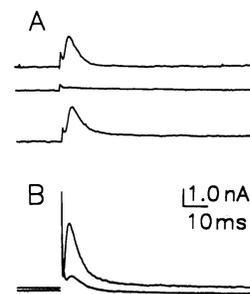


FIG. 5. Pharmacological block of VV:H4-induced transient outward currents in an RBL-1 cell. (A) Currents recorded during a voltage step from  $-100$  mV to  $0$  mV before (top trace), during (middle trace), and after (bottom trace) exposure to  $10$  mM 4-aminopyridine pressure-ejected from a nearby pipet. (B) Currents recorded before (upper trace) and during (lower trace) bath exposure to  $10$  nM charybdotoxin. The charybdotoxin block was only partly reversible, even after a 10-min wash.

microscopy), the virus may provide an alternative to construction of a stably transfected cell line.

A principal advantage of vaccinia over other transient expression systems is the broad range of mammalian cell types that can be infected, including primary cells (G.T., unpublished work). Thus, VV may prove useful for expression of polypeptides requiring cell-specific posttranslational processing or when additional, cell-specific subunits are required for complete functional reconstitution. Like the oocyte, VV can be used to coexpress multiple foreign gene products in a single cell (5, 17–19) and may prove useful in attempts to study intracellular signal pathways. The large fraction of expressing cells is also a considerable advantage as compared to the usual transient transfection techniques.

The four cell types chosen for this study are from different origins; only the PC-12 cells are derived from excitable tissue. Transient potassium currents are not usually seen in any of the cell types, except as rare ( $<2\%$  of cells) constituents in PC-12 cells (20). We observed no striking differences in the kinetics of the VV:H4-induced currents among the four cell types; all appeared similar to those seen following injection of H4 transcript into *Xenopus* oocytes (6). It is possible, however, that single channel recording from VV-infected cells may yet reveal differences in kinetics or conductance that are not apparent in the macroscopic currents. Aside from the sensitivity to charybdotoxin, the properties of the Shaker H4 channels strongly resemble those of transient potassium channels observed in fly muscle (7). Our observation that charybdotoxin blocks A-type currents expressed in cultured mammalian cells infected with VV:H4 demonstrates that charybdotoxin sensitivity is not an artifact of expression in *Xenopus* oocytes, but it does not explain the lack of charybdotoxin blockade of A-current channels in *Drosophila* (15, 16). One possibility is a difference in post-translational processing between *Drosophila* and vertebrate cells.

We are encouraged that the virus-induced expression of A-type currents in PC-12 cells did not alter the properties of the endogenous voltage-gated currents. This result supports the general feasibility of studying more complex interactions between native membrane constituents and those expressed heterologously by VV infection.

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1. Lester, H. A. (1988) *Science* **241**, 1057–1063.
2. Moss, B. (1985) in *Virology*, ed. Fields, B. N. (Raven, New York), pp. 685–703.
3. Piccini, A., Perkus, M. E. & Paoletti, E. (1987) *Methods Enzymol.* **153**, 545–563.
4. Thomas, G., Herbert, E. & Hruby, D. (1986) *Science* **232**, 1641–1643.
5. Thomas, G., Thorne, B. A., Thomas, L., Allen, R., Fuller, B. & Thorne, J. (1988) *Science* **242**, 226–230.
6. Iverson, L., Tanouye, M. A., Lester, H. A., Davidson, N. & Rudy, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5723–5727.
7. Zagotta, W. N., Brainard, M. S. & Aldrich, R. W. (1988) *J. Neurosci.* **8**, 4765–4779.
8. Rice, C. M., Franke, C. A., Strauss, J. H. & Hruby, D. E. (1988) *J. Virol.* **56**, 227–239.
9. Villarreal, L. P. & Berg, P. (1977) *Science* **196**, 183–186.
10. Hruby, D., Thomas, G., Herbert, E. & Franke, C. (1986) *Methods Enzymol.* **124**, 295–309.
11. Solc, C. K., Zagotta, W. N. & Aldrich, R. W. (1987) *Science* **236**, 1094–1098.
12. Zagotta, W. N. & Aldrich, R. W. (1989) *J. Gen. Physiol.*, in press.
13. Hodgkin, A. L. & Huxley, A. F. (1952) *J. Physiol. (London)* **117**, 500–544.
14. MacKinnon, R., Reinhart, P. H. & White, M. M. (1988) *Neuron* **1**, 997–1001.
15. Elkins, T., Ganetzky, B. & Wu, C. F. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8415–8419.
16. Zagotta, W. N., Hoshi, T. & Aldrich, R. W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7243–7247.
17. Fuerst, T. R., Niles, E. G., Studier, W. & Moss, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8122–8126.
18. Fuerst, T. R., Earl, P. & Moss, B. (1987) *Mol. Cell. Biol.* **7**, 2538–2544.
19. Coupar, B. E. H., Andrew, M. E. & Boyle, D. B. (1988) *Gene* **68**, 1–10.
20. Hoshi, T. & Aldrich, R. W. (1988) *J. Gen. Physiol.* **91**, 73–106.