

# Do voltage-dependent K<sup>+</sup> channels require Ca<sup>2+</sup>? A critical test employing a heterologous expression system

(Shaker/baculovirus/rolling rock)

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**ABSTRACT** Removal of Ca<sup>2+</sup> from the solution bathing neurons is known in many cases to alter the gating properties of voltage-dependent K<sup>+</sup> channels and to induce a large, nonselective “leak” conductance. We used a heterologous expression system to test whether the leak conductance observed in neurons is mediated by voltage-dependent K<sup>+</sup> channels in an altered, debased conformation. Voltage-dependent K<sup>+</sup> channels were expressed in an insect cell line infected with a recombinant baculovirus carrying the cDNA for *Drosophila* Shaker “A-type” K<sup>+</sup> channels. These expressed channels respond to low Ca<sup>2+</sup> identically to voltage-dependent K<sup>+</sup> channels in native neuronal membranes; upon removal of external Ca<sup>2+</sup>, Shaker K<sup>+</sup> currents disappear and are replaced by a steady, nonselective leak conductance. However, control cells devoid of Shaker channels were free of any voltage-dependent conductances and did not generate a leak when external Ca<sup>2+</sup> was removed. These results show that Ca<sup>2+</sup> is essential for proper function of voltage-dependent K<sup>+</sup> channels and is required to stabilize the native conformations of these membrane proteins.

Voltage-dependent K<sup>+</sup> channels play a central role in the generation, propagation, and integration of electrical signals in many types of cells. These channels all display high selectivity for K<sup>+</sup> over Na<sup>+</sup> and strong voltage dependence of opening and closing rates. The long-recognized functional similarities among voltage-dependent K<sup>+</sup> channels are now being rationalized in terms of homologies among members of a very large molecular family (1–5). Because membrane voltage is the physiological signal used to switch these channels between open and closed conformations, relatively little attention has been given to the roles of physiological ligands in their proper functioning. In particular, Ca<sup>2+</sup>, which is crucially involved in the action of Ca<sup>2+</sup>-activated K<sup>+</sup> channels and many membrane-transducing systems, is not generally viewed as being directly involved in the operation of voltage-dependent K<sup>+</sup> channels (6).

Recent experiments, however, have shown that Ca<sup>2+</sup> in the external medium has profound effects on the operation of voltage-dependent K<sup>+</sup> channels in squid neurons (7, 8). In particular, it was shown that Ca<sup>2+</sup> is required for the channel to close at normal rates. In addition to this effect on channel gating, Ca<sup>2+</sup> is also needed to maintain the K<sup>+</sup> selectivity of the channel's conduction pathway; when Ca<sup>2+</sup> is removed from the medium bathing the neuron, the voltage-dependent K<sup>+</sup> currents disappear, and a large, nonselective “leak” conductance appears (8). This effect is reversible; upon reintroduction of external Ca<sup>2+</sup>, the voltage-dependent K<sup>+</sup> currents return concomitantly with the loss of the membrane leak. These observations prompted Armstrong and Lopez-Barneo (8) to propose that Ca<sup>2+</sup> is an essential cofactor

needed to maintain the native structure of voltage-dependent K<sup>+</sup> channels. According to this picture, Ca<sup>2+</sup> removal partially denatures the channel so that its pore loses the ability to discriminate among small ions and its voltage-dependent “gate” is unable to close.

One problem that makes this interpretation equivocal is that the neuronal membrane is electrically complicated, providing a home for many different types of ion channels. The experiments in neurons do not allow us to conclude with certainty that the leak induced by Ca<sup>2+</sup> removal is in fact mediated by the specific set of voltage-dependent K<sup>+</sup> channels under study, rather than by other membrane proteins present. The experiments here are designed to test this idea rigorously; we examine the effect of Ca<sup>2+</sup> removal in a non-neuronal cell line in which a heterologous voltage-dependent K<sup>+</sup> channel is specifically expressed by infection with a recombinant baculovirus. The results clearly demonstrate that the nonselective leak induced by removing Ca<sup>2+</sup> has its origin in a massive functional alteration of the expressed K<sup>+</sup> channels.

## METHODS

Techniques for growing and maintaining the insect cell line Sf9 in culture have been described (9), as have the procedures for manipulating the *Autographa californica* nuclear polyhedrosis virus. Cells were grown in monolayer at 26°C. These experiments employed a recombinant virus, Shaker 3A1, containing cDNA derived from the *Drosophila* Shaker H4 (10) “A-type” K<sup>+</sup> channel placed directly behind the polyhedrin promoter (11). Shaker currents were assayed 1 or 2 days after infection of Sf9 cells with this recombinant virus, exactly as described (11).

Whole-cell electrophysiological recording of Sf9 cells was carried out in a perfusion chamber mounted on the stage of an inverted microscope, at 21–23°C, by using an Axopatch 1C amplifier. “Stubby-tip” patch pipettes of 0.7- to 1.8-MΩ resistance were fashioned on a Sachs-Flaming microelectrode puller (Sutter Instruments, Novato, CA) and lightly polished on a microforge. After achieving a high-resistance whole-cell recording, we lifted the cell off the bottom of the chamber and moved it to the perfusion chamber's inflow port to ensure high-fidelity changes of solution. The dead time of the perfusion system was 5 s, and solution changes were complete in about 15 s. Data were filtered at 5 kHz and collected at 14 kHz on a 386-based acquisition system.

The “internal” solution, used to fill the patch pipette and thus dialyze the cytoplasm, was 70 mM KCl/50 mM KF/10 mM Hepes/2 mM MgCl<sub>2</sub>/5 mM EGTA, pH 7.2. The “normal-Ca<sup>2+</sup>” external solution was 140 mM NaCl/10 mM Mes/2 mM MgCl<sub>2</sub>/2.5 mM CaCl<sub>2</sub>, pH 6.4. In some experiments KCl was added by replacement for NaCl. “Zero-Ca<sup>2+</sup>” external solutions had no added CaCl<sub>2</sub> and contained an additional 6 mM NaCl for osmotic balance. These nomi-

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nally zero- $\text{Ca}^{2+}$  solutions had  $\text{Ca}^{2+}$  levels of 3–6  $\mu\text{M}$ , as determined by elemental analysis.

## RESULTS

The Shaker locus of *Drosophila* codes for a family of transiently activating, or A-type  $\text{K}^+$  channels (5, 10, 12). These channels are strongly voltage-dependent and are known to be molecular cousins of a variety of voltage-dependent  $\text{K}^+$  channels, including delayed rectifiers (1–4, 13–15). In these experiments, we study Shaker  $\text{K}^+$  channels expressed in a cell line, Sf9, from the armyworm caterpillar *Spodoptera frugiperda*, following transient, lytic infection by a recombinant baculovirus (11). In this system, Shaker currents evoked by voltage steps are similar in behavior to those seen in *Drosophila*.

Fig. 1 shows that these Shaker  $\text{K}^+$  channels exhibit the same requirement for external  $\text{Ca}^{2+}$  as was originally seen for delayed rectifier  $\text{K}^+$  channels of squid (8). Upon removal of external  $\text{Ca}^{2+}$ , the time-dependent transient portion of the current disappears, and a large leak conductance appears. This leak may be most easily observed as an increase of inward current at the holding potential of  $-85$  mV, but it is also apparent as a steady outward current at the command potential of 50 mV. As in squid neurons, the effect is reversible upon restoration of external  $\text{Ca}^{2+}$ . Removal of the 2 mM  $\text{Mg}^{2+}$  normally present in all experiments is without effect on either the leak or the  $\text{K}^+$  currents (data not shown).

Both the voltage-dependent  $\text{K}^+$  currents and the leak conductance follow similar time courses in response to  $\text{Ca}^{2+}$  removal (Fig. 2). After presenting a cell with  $\text{Ca}^{2+}$ -free bathing solution, Shaker currents diminish with a half-time of 20–30 s, and the leak grows in parallel. When  $\text{Ca}^{2+}$  is restored, however, the leak disappears almost immediately, and the  $\text{K}^+$  currents return more slowly, taking about a minute to recover to a steady level, as with squid  $\text{K}^+$  channels.

The results above argue that heterologously expressed shaker  $\text{K}^+$  channels exhibit an external  $\text{Ca}^{2+}$  requirement identical to that originally observed in native neuronal delayed rectifier channels. The baculovirus-infected Sf9 system offers an advantage unavailable with channels naturally expressed in their native neuronal membranes: the ability to manipulate the membrane content of  $\text{K}^+$  channels. In particular, we can ask whether the leak appearing upon  $\text{Ca}^{2+}$  removal in the above experiments is specifically mediated by the expressed Shaker channels or by other  $\text{Ca}^{2+}$ -requiring membrane components in parallel. In Fig. 3, we present a

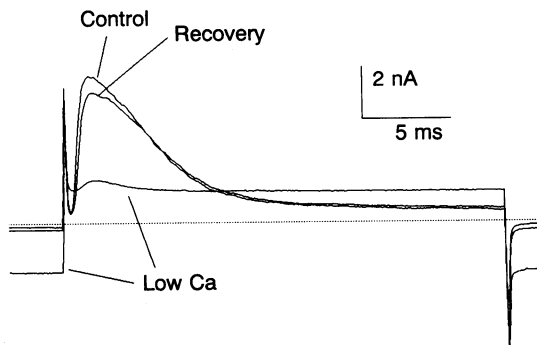


FIG. 1. Sensitivity of Shaker currents to  $\text{Ca}^{2+}$  removal. Whole-cell recordings were taken from an Sf9 cell 2 days after infection by Shaker recombinant virus. Currents were elicited by 25-ms voltage pulses from a holding potential of  $-85$  mV to 50 mV. Pulses were applied every 5 s throughout the experiment. Current traces are shown in normal- $\text{Ca}^{2+}$  medium (Control), after 50 s in zero- $\text{Ca}^{2+}$  medium (Low Ca), and 100 s after return to normal  $\text{Ca}^{2+}$  medium (recovery).

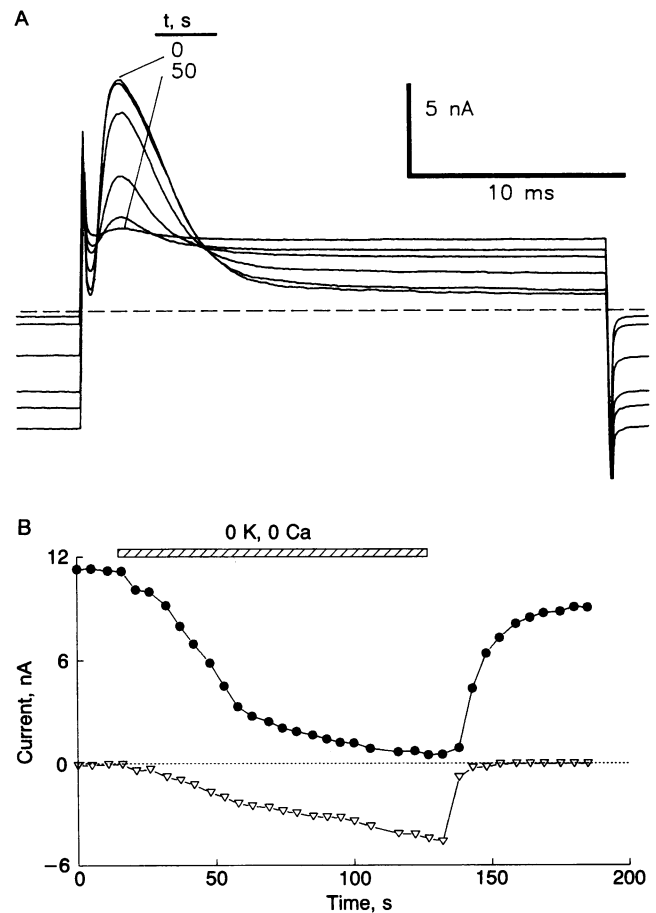


FIG. 2. Time course of the effect of  $\text{Ca}^{2+}$  removal. Recordings were collected under conditions of Fig. 1. (A) Successive current traces taken at 10-s intervals after switching to zero- $\text{Ca}^{2+}$  medium. (B) Time course of low- $\text{Ca}^{2+}$  effect and recovery. Transient current (●) was measured as the difference between peak and steady-state current at 50 mV; leak current (▽) was measured as the holding current at  $-85$  mV.

control experiment in a cell infected with wild-type virus, which does not carry the Shaker coding sequence. As previously reported (11), wild-type infected or uninfected Sf9 cells have high electrical resistance and show no discernible voltage-dependent channel activity. Removal of  $\text{Ca}^{2+}$  from the external medium has little effect on the low background conductance of these cells devoid of Shaker channels. We

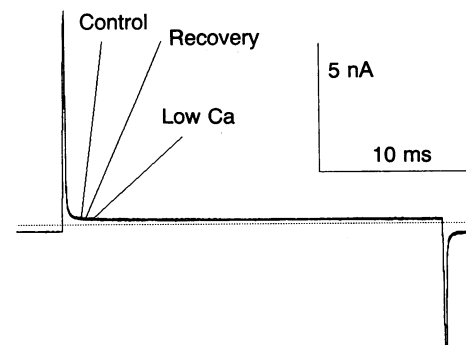


FIG. 3. Lack of a low- $\text{Ca}^{2+}$  effect on control Sf9 cells. Recordings in normal- $\text{Ca}^{2+}$  medium (Control) and zero- $\text{Ca}^{2+}$  medium (Low Ca) were taken as in Fig. 1, except that an Sf9 cell infected with wild-type virus, and thus devoid of Shaker channels, was used. In this experiment, the leak current at 50 mV was 0.12 and 0.15 nA in normal- $\text{Ca}^{2+}$  and zero- $\text{Ca}^{2+}$  media, respectively.

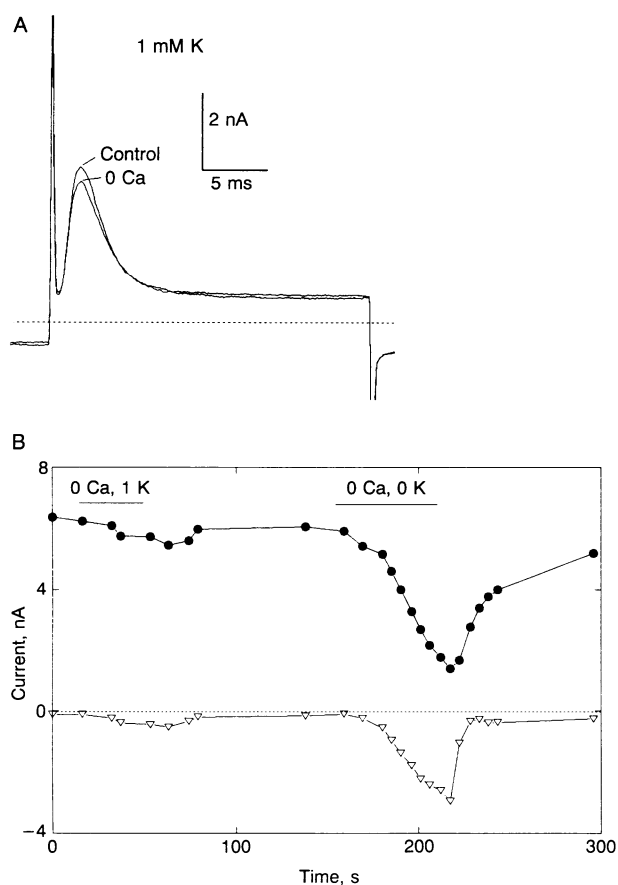


FIG. 4. Protection of Shaker channel by K<sup>+</sup>. An Sf9 cell was exposed to zero-Ca<sup>2+</sup> medium as in Fig. 1, except that 1 mM K<sup>+</sup> was present in the external medium. (A) Raw current records in normal Ca<sup>2+</sup>, 70 s after exposure to zero-Ca<sup>2+</sup> medium and 60 s after return to normal-Ca<sup>2+</sup> medium. (B) Response to zero Ca<sup>2+</sup> of the transient (●) and leak (▽) currents with 1 mM K<sup>+</sup> or zero K<sup>+</sup> in the external medium. The cell was switched from normal-Ca<sup>2+</sup> medium to zero-Ca<sup>2+</sup> medium with K<sup>+</sup> concentrations as indicated. Transient and leak currents were measured as in Fig. 2.

therefore conclude that the leak appearing in cells infected with Shaker recombinant virus is mediated by the Shaker K<sup>+</sup> channels and not by parallel conductance pathways present in the Sf9 plasma membrane.

The above experiments were carried out in K<sup>+</sup>-free external solutions, as were the earlier studies in squid neurons. If we now include as little as 1 mM K<sup>+</sup> in the external medium, the Shaker channels are much less sensitive to Ca<sup>2+</sup> removal. Fig. 4 shows this remarkable protective effect of K<sup>+</sup>. The raw data traces of Fig. 4A show that with 1 mM external K<sup>+</sup> present removal of Ca<sup>2+</sup> has almost no effect on the size of the K<sup>+</sup> current or the background leakage conductance, on this time scale. (We have noticed that with prolonged maintenance of these conditions, a leak develops and transient current diminishes slowly.) This protective effect of external K<sup>+</sup> is further emphasized by the experiment in Fig. 4B, in which Ca<sup>2+</sup> is removed in the presence of 1 mM K<sup>+</sup>, with only a very weak response; upon subsequent removal of Ca<sup>2+</sup> in the absence of K<sup>+</sup>, the characteristic replacement of K<sup>+</sup> current by nonspecific leak ensues at once. This protective effect of K<sup>+</sup> is highly selective for this cation, since all these experiments are carried out in the presence of 140 mM Na<sup>+</sup>.

## DISCUSSION

These results show that external Ca<sup>2+</sup> is required at millimolar levels for the proper functioning of the Shaker A-type

K<sup>+</sup> channel. Removal of Ca<sup>2+</sup> results in a profound alteration in this channel's behavior: a loss of both K<sup>+</sup> selectivity and voltage-dependent gating. In these low-Ca<sup>2+</sup> conditions, the channel acts as a nonselective pore, which is no longer influenced by transmembrane voltage. Because these are such dramatic alterations in channel function, we suggest that underlying structural changes are involved: that Ca<sup>2+</sup> is a specific cofactor whose binding to an externally facing site is necessary to stabilize the proper conformation of the Shaker channel.

This conclusion merely confirms a similar inference drawn about a different voltage-dependent K<sup>+</sup> channel, the squid neuron delayed rectifier (8). Nevertheless, these experiments are important because of the simplicity of the baculovirus/Sf9 system. Here, the K<sup>+</sup> channel is specifically introduced into the membrane of an electrically inexcitable, non-neuronal cell, and any new properties thereby conferred upon the Sf9 membrane may be attributed to the K<sup>+</sup> channel itself. Since this low-Ca<sup>2+</sup> effect is observed in Shaker A-type channels from *Drosophila*, delayed rectifiers from squid, and voltage-dependent K<sup>+</sup> channels from human T lymphocytes (16) and mammalian pituitary cells (C.M.A., unpublished results), we propose that the involvement of Ca<sup>2+</sup> is a fundamental mechanistic property of the family of voltage-dependent K<sup>+</sup> channels and not simply a bizarre quirk of one particular channel protein. Because many types of cells carry K<sup>+</sup> channels, this effect may account for the widely recognized effect of low-Ca<sup>2+</sup> medium in leading to "membrane damage" of many *in vitro* cell preparations (17).

A striking observation here is that external K<sup>+</sup> strongly protects the channel against the effects of low Ca<sup>2+</sup>. With 1 mM K<sup>+</sup> present in the external solution, removal of Ca<sup>2+</sup> causes only a small decrease in Shaker current and a small increase of leak, at least on the 1- to 2-min time scale used here. The effect is specific for K<sup>+</sup>, as all experiments were done in the presence of >100-fold higher concentrations of Na<sup>+</sup>. This specificity suggests that the site of K<sup>+</sup> action is in the conduction pore itself. With this picture in mind, we would need to postulate the existence in the pore of a specific K<sup>+</sup>-binding site occupied in the submillimolar range of external K<sup>+</sup> concentration; such a proposal would not be unusual, because high-affinity binding to K<sup>+</sup>-specific sites is known to form the basis of K<sup>+</sup> conduction in the high-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (18). These experiments do not tell us whether K<sup>+</sup> and Ca<sup>2+</sup> act at the same site, as has been proposed on the basis of the effects of these ions on channel gating (7, 8). Our results are consistent with such an idea, but they are also in harmony with a picture in which K<sup>+</sup> and Ca<sup>2+</sup> bind to the channel at different sites and stabilize its K<sup>+</sup>-selective conformation.

Although the Ca<sup>2+</sup>-dependent changes observed in the behavior of voltage-dependent K<sup>+</sup> channels are dramatic, the underlying structural changes need not be immense. We could speculate, for instance, that Ca<sup>2+</sup> is required to maintain a tight and rigid association of the four subunits thought to make up this type of channel (19). In the absence of Ca<sup>2+</sup>, the structure might then allow more conformational flexibility in the pore structure. Such a "melting" of the structure would have disastrous consequences for the high ionic selectivity of a K<sup>+</sup> channel, since an increase in the effective radius of the narrow selectivity regions of only 0.4 Å would largely eliminate the selectivity of a K<sup>+</sup> pore against Na<sup>+</sup> (20). The stabilization and "tightening" of correctly folded protein conformations by Ca<sup>2+</sup> has numerous precedents in water-soluble proteins (21–24).

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