Revisiting the Role of Ca^{2+} in *Shaker* K^+ Channel Gating

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ABSTRACT *Shaker* K^+ channels were expressed in outside-out macropatches excised from *Xenopus* oocytes, and the effects on gating of removal of extracellular Ca^{2+} were examined in the complete absence of intracellular divalent cations. Removal of extracellular Ca^{2+} by perfusion with EDTA-containing solution caused a small negative shift in the channel's voltage-activation curve and led to an increased nonselective leak, but did not otherwise alter or disrupt the channels. The results contradict the proposal that Ca^{2+} is an essential component required for maintenance of ion selectivity and proper gating of K_v-type K^+ channels. The large nonselective leak in Ca^{2+}-free conditions was found to be a patch-seal phenomenon related to F^- ion in the recording pipette.

INTRODUCTION

From the earliest years of squid axon biophysics, extracellular divalent cations have been recognized as modulators of voltage-gated ion channels. Frankenhaeuser and Hodgkin (1957) first described the shifts in Na^+ and K^+ channel voltage-activation curves that arise from changes in external Ca^{2+}. Raising Ca^{2+} stabilizes the closed channel and thereby produces a positive-going shift of voltage activation, as classically explained by surface-potential theory (reviewed in Hille, 1992). According to this mechanism, Ca^{2+} ions reduce the local electrostatic potential near the membrane surface and thus alter the electric field near the channels' voltage sensors. In simple form, surface-potential mechanisms predict that extracellular divalent cations should shift the voltage dependence of channel activation and deactivation equally. Because of many experimental violations of this and other expectations, it has been suggested that divalent cations may additionally play a direct role in gating of voltage-dependent Na^+ and K^+ channels (Armstrong and Matteson, 1986; Grissmer and Cahalan, 1989; Spires and Begenisich, 1992, 1994; Armstrong and Cota, 1999; Armstrong, 1999). In the case of K_v-type K^+ channels, it has been specifically argued (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990) that Ca^{2+} acts as an essential gating cofactor, that channels can close only if Ca^{2+} (or perhaps another divalent cation) resides within the K^+-conduction pore; moreover, extracellular Ca^{2+} was proposed to be essential for the preservation of native K^+ channel ion selectivity. This proposal emanated from studies on the dramatic effects on K^+ channels of removing Ca^{2+} from the extracellular medium. Armstrong and Lopez-Barneo (1987), working with native delayed-rectifier K^+ currents in squid neurons, showed that upon external Ca^{2+} removal, the time- and voltage-dependent K^+ currents disappeared, and a large, nonselective leak developed simultaneously. This effect was reversible, as long as integrity of the neuron was maintained under these low-Ca^{2+} conditions; reintroduction of external Ca^{2+} led to rapid disappearance of the nonselective leak and a slower reappearance of voltage-dependent K^+ currents. Similar results were obtained with *Shaker* K^+ channels heterologously expressed in a non-neuronal cell line (Armstrong and Miller, 1990).

The past decade has witnessed profound advances in mechanistic understanding of K^+ channels, in both gating (Liu et al., 1997; Yellen, 1998; Cha et al., 1999) and ion selectivity (Doyle et al., 1998; Jiang and MacKinnon, 2000). No role for Ca^{2+} in the fundamental workings of K^+ channels has even been intimated in these recent studies, however. For this reason, we reexamined the effect of external Ca^{2+} on K^+ channels, now exploiting superior methods that have come into standard use over the past decade. In particular, we studied voltage-dependent gating of *Shaker* channels expressed at high density in excised outside-out macropatches pulled from *Xenopus* oocytes, a system with well-defined aqueous solutions on both sides of the membrane that lends itself easily to rapid and reliable changes of extracellular solutions. Our results in this cleaner system differ in important ways from those obtained previously. We find that normal *Shaker* K^+ currents are maintained in the complete absence of divalent cations. As in previous work, a leak develops when extracellular Ca^{2+} was removed, but this effect is much larger and more robust with F^- ion in the intracellular pipette solution, a condition used in all previous studies of extracellular Ca^{2+} on K^+ channels; when intracellular F^- is replaced by Cl^-, only small leaks of variable magnitude grow in response to Ca^{2+} removal. We conclude that divalent cations are not required for proper closing and selectivity of voltage-dependent K^+ channels and that the leak observed previously does not represent conversion of healthy to debased K^+ channels, but rather is a consequence of using F^- as an intracellular anion.

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MATERIALS AND METHODS

In vitro transcription and oocyte preparation

This study employs variants of the *Shaker* B channel (Schwarz et al., 1988) containing a point mutation (F425G) that increases charybdotoxin affinity (Goldstein and Miller, 1992); in some experiments, an inactivation-removed construct (Δ6–46) was used (Hoshi et al., 1990). cDNA plasmid in pBluescript KS was linearized by *Not*I or *Fsp*I digestion, and cRNA was transcribed with T7 RNA polymerase (Promega Corp., Madison, WI). Oocytes were isolated from *Xenopus laevis* frogs and gently agitated for 70–90 min at room temperature in collagenase (2 mg/ml; Worthington Biochemical Corp., Lakewood, NJ) in Ca^{2+}-free solution containing (mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, and 5 Hepes, pH 7.5. The oocyte preparation was then rinsed thoroughly and stored at 17°C in ND96-gentamicin solution containing (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, pH 7.6, and 0.1 mg/ml gentamicin. Defolliculated oocytes were selected the following day and injected with 27 or 50 nl of cRNA (0.3–0.6 mg/ml).

Patch recording

Shaker currents were recorded 2–5 days after cRNA injection in the excised outside-out configuration using fire-polished electrodes (1.5–2.5 MΩ) with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Electrical contact to the bath recording solution was made via a 200 mM KCl, 1 mM EDTA, 10 mM Hepes, pH 7.4, agarose bridge. The patches were pulled from *Xenopus* oocytes in a large chamber and then moved to a small perfusion chamber (chamber volume, 100 μl) to achieve a high rate of solution change. The flow rate of the chamber was 30 μl/s, and ≥95% solution replacement was achieved within 5 s. The recorded current signal was filtered at 5 kHz and sampled at 20 kHz using an analog-to-digital converter (DigiData 1200) interfaced with a personal computer, and pClamp 8 software (Axon Instruments) was used for acquisition and analysis. The standard pulse protocol employed a holding potential of −90 mV followed by repeated test pulses to +30 mV for 25 ms at 3-s intervals. Voltage-activation curves were examined with inactivation-removed *Shaker* B, with 30-ms command pulses from −80 to +10 mV in 5-mV increments followed by a 15-ms tail pulse to −80 mV at 1-s interpulse intervals. Activation curves were calculated using standard tail-current analysis (Liman et al., 1991), by fitting data to a Boltzmann function, $I/I_{max} = 1/\{1 + \exp[-zF(V - V_o)/RT]\}$, to determine V_o (half-maximal activation voltage) and z (slope factor). The data were obtained from 3–10 patches for each condition of experiments.

Recording solutions

Two intracellular pipette solutions were tested for external Ca^{2+} removal experiments: 1) 100KCl contained (mM) 100 KCl, 1 EDTA, 10 Hepes (pH 7.4 with KOH), and 2) 50KF contained (mM) 50 KF, 50 KCl, 1 EDTA, 10 Hepes (pH 7.4 with KOH). External bath solutions are listed in Table 1.

TABLE 1 External bath solutions (mM)

Name	[KCl]	[NaCl]	[$CaCl_2$]	[EDTA]
1Ca10K	10	90	1	0
0Ca10K	10	90	0	1
1Ca0K	0	100	1	0
0Ca0K	0	100	0	1

All solutions also contain 10 mM Hepes-NaOH, pH 7.4.

RESULTS

Shaker currents survive removal of Ca^{2+}

To test the effect of external Ca^{2+} removal from voltage-dependent K^+ channels, we employed excised outside-out patch recording from *Shaker*-expressing *Xenopus* oocytes with pipette solutions containing 1 mM EDTA and no added divalent cations. In an initial set of experiments using 10 mM extracellular K^+ (Fig. 1), patches were formed in the presence of 1 mM Ca^{2+}, and the recording chamber was subsequently perfused with 0Ca10K solution (Table 1), which contains 1 mM EDTA and has no added divalent cations. *Shaker* currents are maintained for up to 1 min in the complete absence of extracellular divalent cations, with peak current ~30% higher than in the 1 mM Ca^{2+} control. Under conditions of Fig. 1 *A*, with 100 mM KCl in the intracellular solution, small leaks with variable magnitudes appeared upon Ca^{2+} removal and promptly disappeared upon reintroduction of Ca^{2+}. If the pipette solution contained 50 mM KF, however, a much larger leak developed following Ca^{2+} removal (Fig. 1, *B* and *C*). This leak was rapidly reversible in response to reintroduction of Ca^{2+}, but patches rarely survived more than a few minutes of zero-Ca^{2+} exposure with F^- ion in the pipette solution. Typically, the leak became increasingly severe if the patch was subjected to a second round of zero-Ca^{2+} exposure, and recovery was not as complete as in the first exposure (Fig. 1 *C*). In all cases, normal inactivating *Shaker* currents were clearly maintained on top of the leak. These observations are in general agreement with previous experiments of this kind (Armstrong and Miller, 1990), although in the previous experiments, large leaks were not observed under zero-Ca^{2+} conditions with extracellular K^+ present.

We then repeated these experiments with K^+-free external solutions (Fig. 2), a condition shown previously to produce a dramatic sensitivity of K^+ channels to Ca^{2+} removal (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). Surprisingly, under these conditions, *Shaker* currents did not disappear in response to zero-Ca^{2+} exposure; instead, familiar *Shaker* channel gating was observed on top of leaks that reversibly appeared upon Ca^{2+} removal (Fig. 2, *A* and *B*). As in the external 10 mM K^+ condition, much larger nonselective leaks developed when F^- was present in the pipette solution.

In Fig. 3, uninjected oocytes were tested to determine whether the large leak arising from Ca^{2+}-removal is specifically mediated by the expressed *Shaker* channels. In these patches devoid of *Shaker* channels, removal of Ca^{2+} from the external medium gave rise to small and variable nonselective leaks with 100 mM KCl in the pipette, and again a much larger leak appeared with 50 mM KF internal solution. Therefore, we conclude that the expressed *Shaker* channels are not involved in leak development and that internal KF is somehow responsible for the large leak.

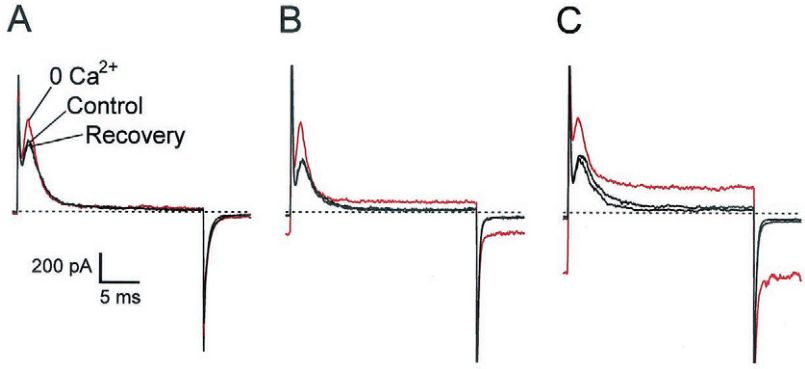

FIGURE 1 *Shaker* K^+ channels survive zero Ca^{2+}. Outside-out patches bearing *Shaker* channels were examined in 1Ca10K and 0Ca10K solutions, according to standard pulse protocol (Materials and Methods). Current traces are shown in bath solutions containing 1 mM Ca^{2+} (black trace, control), after 60 s (*A* and *C*) or 30 s (*B*) in zero-Ca^{2+} (red trace, 0 Ca^{2+}), and 30 s after return to 1 mM Ca^{2+} (black line, recovery). Intracellular pipette solutions contained 100 mM KCl (*A*) or 50 mM KF plus 50 mM KCl (*B* and *C*). (*C*) A second exposure to zero-Ca^{2+} in the same patch as in *B*. Perfusion times cited in all figure legends are approximate.

In zero-Ca^{2+} solutions, the leak made it difficult to examine *Shaker* channels in outside-out patches for more than a few minutes. To circumvent this constraint, we preincubated inactivation-removed *Shaker*-expressing oocytes in 0Ca0K solution for 30 min and subsequently formed patches in this same EDTA-containing solution. As illustrated in Fig. 4, familiar *Shaker* currents were still observed from such patches never exposed to Ca^{2+}. We note, however, that the initial seals formed and patches pulled in EDTA-containing solutions were typically leakier than those formed in 1 mM Ca^{2+} (see inward holding current before the pulse and outward tail currents riding on the inward leak after the pulse).

External Ca^{2+} ions affect gating of voltage-dependent K^+ channels

Increasing concentrations of extracellular Ca^{2+} in the range of 10–100 mM are well known to progressively inhibit voltage-dependent ion channels by shifting the activation curve in the positive direction along the voltage axis (Frankenhaeuser and Hodgkin, 1957; Campbell and Hille, 1976; Armstrong and Matteson, 1986; Hille, 1992; Armstrong, 1999). We considered it worthwhile to carry out similar experiments in the low-Ca^{2+} conditions employed above. Families of currents from macropatches containing inactivation-removed *Shaker* channels were compared in 1Ca10K and 0Ca10K solutions. Fig. 5 shows that reduction of Ca^{2+} from 1 mM to below 1 μM leads to a small (9 mV) negative-going shift in the voltage-activation curve, equivalent to ~1.3 kcal/mol stabilization of the open channel. This is similar to the magnitude of Ca^{2+}-dependent gating shifts seen in the classical work cited above, and it naturally accounts for the increase in peak current arising from removal of Ca^{2+} from inactivating *Shaker* channels (Fig. 1 *A*).

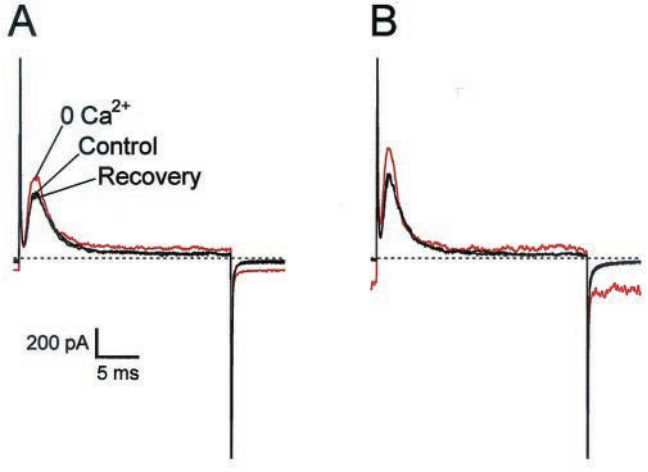

FIGURE 2 Zero-Ca^{2+} exposure in the absence of extracellular K^+. *Shaker* channels were exposed to a 30-s zero-Ca^{2+} challenge in zero-K^+ bath solution, with other conditions as in Fig. 1. Intracellular pipette solutions were 100 mM KCl (*A*) or 50 mM KF (*B*).

DISCUSSION

Previous experiments using whole-cell recording on inactivating *Shaker* K^+ channels expressed in insect cells and on native delayed rectifiers in squid neurons reported disappearance of K^+ current concomitant with the development of a nonselective leak following removal of external Ca^{2+};

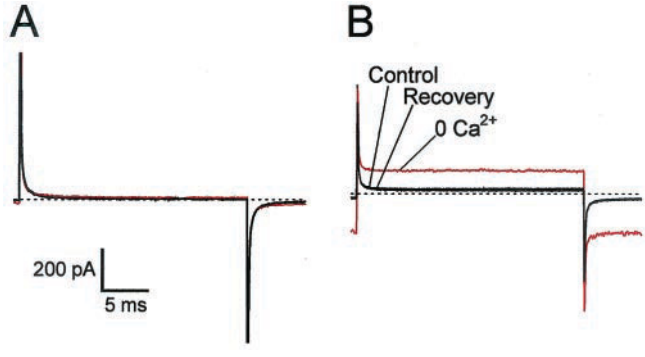

FIGURE 3 *Shaker* channels are not involved in leak development. Excised outside-out patches were formed from uninjected *Xenopus* oocytes in 1Ca0K solution and were subjected to solution changes to 0Ca0K for 60 s, as in Fig. 2. Recording pipette contained 100 mM KCl (*A*) or 50 mM KF (*B*).

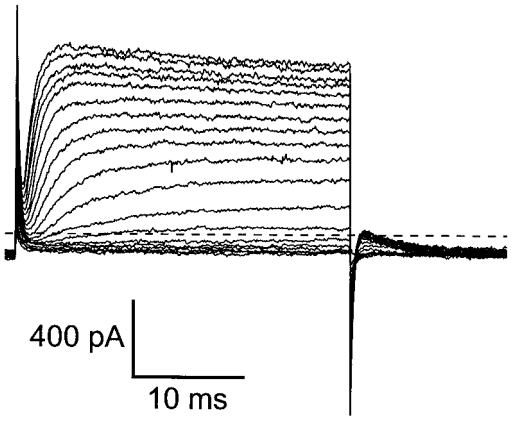

FIGURE 4 *Shaker* channel gating is preserved in patches never exposed to Ca^{2+}. Inactivation-removed *Shaker*-expressing oocytes were preincubated in 0Ca0K for 30 min, and outside-out patches were then formed in the same solution. Current responses to a family of voltage pulses were recorded in 0Ca0K, as detailed in Materials and Methods.

these results were taken to mean that external Ca^{2+} is required for both channel closure and maintenance of K^+ selectivity (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). We have now repeated these kinds of experiments in a better-defined system: *Shaker* K^+ channels in outside-out macropatches pulled from *Xenopus* oocytes. To ensure zero-divalent cation conditions, an efficient perfusion chamber was used, Mg^{2+} was omitted from all solutions, and 1 mM EDTA was included in Ca^{2+}-free

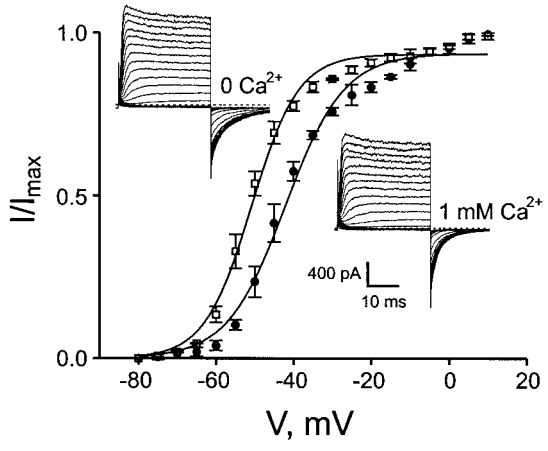

FIGURE 5 Complete removal of Ca^{2+} shifts voltage-dependent gating of *Shaker*. Families of currents from macropatches containing inactivation-removed *Shaker* channels were compared in 1 mM Ca^{2+} and 0 Ca^{2+} conditions, with 100 mM KCl pipette solution. Currents were recorded as in Fig. 4, first in 1Ca10K and then after 60 s in 0Ca10K. Voltage-activation curves were calculated from tail-current analysis, with data normalized to 10 mV. Each point represents data from three separate patches (±SE), and solid curves are Boltzmann fits to the data. Gating parameters were $V_o = -42 \pm 2$ mV, $z = 3.5 \pm 0.3$ in 1 mM Ca^{2+}, and $V_o = -51 \pm 1$ mV, $z = 4.3 \pm 0.1$ in 0 Ca^{2+}.

solutions. We find that normal *Shaker* K^+ currents persist for at least 1 min in divalent-cation-free solutions on both sides of the membrane, a time scale many orders of magnitude longer than that of gating, permeation, or block; moreover, *Shaker* currents were maintained after 30 min of exposure of whole oocytes to EDTA-containing external solutions. (In this case, however, the K^+ channels would be exposed to intracellular Mg^{2+}, on the order of 0.1–1 mM.) In all cases, removal of Ca^{2+} produced a nonselective leak in the excised patches.

These results are in striking contrast to previous experiments in several ways. First, we never observed disappearance of voltage- and time-dependent K^+ currents following Ca^{2+} removal, as seen in the earlier experiments (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). Second, we find that the low-Ca^{2+}-induced leak observed both here and in previous work is unrelated to expression of *Shaker* K^+ channels; patches pulled from uninjected oocytes become just as leaky upon Ca^{2+} removal as do *Shaker*-containing patches. We also show here that this large, prominent leak is dependent on the presence of F^- ion inside the patch pipette, a condition used in all previous experiments on this effect and, indeed, in many classical perfused squid axon studies (Armstrong, 1971). (This is an ironic situation, as intracellular F^- was originally introduced as a membrane stabilizer, and in our experience excised patches are more stable with F^- in the pipette if Ca^{2+} is present in the bath solution.) Without intracellular F^- present, we find that the low-Ca^{2+} leak is smaller and less experimentally troublesome. The large F^--dependent leak is likely to be a patch-seal phenomenon, possibly reflecting a layer of insoluble CaF_2 associated with the glass-membrane interface.

These experiments present direct clashes with previous experiments in primary results, and we have no explanation for the conflict. We do, however, consider the system employed here cleaner and better defined than in previous work. In any case, the current results are fatal to the idea that extracellular Ca^{2+} removal leads to a fundamental structural change in K_v-type channels, in which a debased, Ca^{2+}-starved channel becomes locked into an open state, concomitant with loss of K^+ selectivity in its permeation pathway.

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