Cysteines in the *Shaker* K^+ Channel Are Not Essential for Channel Activity or Zinc Modulation

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ABSTRACT We investigated whether the cysteine residues in *Shaker* potassium (K^+) channels are essential for activation and inactivation gating or for modulation of activation gating by external zinc (Zn^{2+}). Mutants of the *Shaker* K^+ channel were prepared in which all seven cysteine residues were replaced (C-less). These changes were made in both wild-type *Shaker* H4 channels and in a deletion mutant (Δ6–46) lacking N-type ("fast") inactivation. Replacement of all cysteines left most functional properties of the K^+ currents unaltered. The most noticeable difference between the C-less and wild-type currents was the faster C-type inactivation of the C-less channel which could be attributed largely to the mutation of Cys^{462}. This is consistent with the effects of previously reported mutations of nearby residues in the S6 region. There were also small changes in the activation gating of C-less currents. Modulation by external Zn^{2+} of the voltage dependence and rate of activation gating is preserved in the C-less channels, indicating that none of the cysteines in the *Shaker* K^+ channel plays an important role in Zn^{2+} modulation.

INTRODUCTION

All voltage-gated ion channel proteins described to date have several cysteine residues, some of which are highly conserved. In many other proteins, cysteines are essential for the maintenance of structure and for functions such as enzymatic activity and the coordination of metal ions. For example, cysteines are important for the catalytic activity of nitrilase (Kobayashi et al., 1993) and for agonist binding to the nicotinic acetylcholine receptor (Mishina et al., 1985; Kao and Karlin, 1986). Cysteines play important roles in the coordination of zinc in zinc finger proteins, such as transcription factors (Hanas et al., 1983; Pan and Coleman, 1989), replication proteins (Zavitz and Marians, 1993), and glucocorticoid receptors (Freedman et al., 1988), and zinc enzymes, such as alcohol dehydrogenase and aspartate transcarbamoylase (see Vallee and Auld (1990) for review). Although cysteines have been shown to be critical for certain protein functions, their importance in voltage-gated ion channel function is not known.

We investigated whether the cysteine residues in *Shaker* K^+ channels are essential for activation and inactivation gating or for modulation of activation gating by external zinc (Zn^{2+}). Zn^{2+} has been demonstrated to slow the activation gating of K^+ current in squid axons (Gilly and Armstrong, 1982) and squid neurons (Spires and Begenisich, 1992). We speculated that cysteine residues might be important in the actions of Zn^{2+}, because Zn^{2+} is known to interact with cysteines in other proteins. We used site-directed mutagenesis to introduce conservative replacements for the seven cysteines in the *Shaker* H4 K^+ channel pseudo-subunit. Since K^+ channels assemble as tetramers (MacKinnon, 1991), substitutions for a single cysteine would be expected to yield a change in each of the four subunits of the channel. Mutant *Shaker* K^+ channels lacking some or all of the 28 cysteines were expressed in *Xenopus* oocytes or a mammalian cell line (HEK293) and were studied by electrophysiology.

Replacement of all cysteines did not substantially alter most functional properties of the macroscopic currents. Both wild-type channels and those lacking cysteines showed a positive shift and slowing of activation gating upon perfusion with external Zn^{2+}. The most dramatic effect of replacement of all of the native cysteines was the roughly 20-fold acceleration of the rate of C-type inactivation. This could be attributed mostly to mutation of the cysteine at position 462. In addition to establishing that native cysteines are not essential for *Shaker* K^+ channel function, this study provides a foundation for the introduction of cysteines into a cysteine-less channel background.

Preliminary results were reported previously (Boland et al., 1993).

MATERIALS AND METHODS

Mutagenesis and RNA synthesis

Mutations were introduced into the *Shaker* H4 K^+ channel cDNA (Kamb et al., 1988) in a Bluescript KS^+ vector (Stratagene, La Jolla, CA). In some cases, we deleted the coding sequence for amino acids 6–46 to remove fast N-type inactivation (Hoshi et al., 1990; Yellen et al., 1991); this deletion mutant is called ShΔ. Oligonucleotide-directed mutagenesis followed the *dut⁻ ung⁻* selection protocol of Kunkel (1985) using 21–26 nucleotide mutagenic primers (Bio-Rad mutagenesis kit). Cysteines was replaced by serine (Cys^{96}, Cys^{301}, and Cys^{308}), valine (Cys^{245}, Cys^{286}, and Cys^{505}), or alanine (Cys^{462}). The success of the mutation was confirmed by DNA sequencing of the full-length insert using the chain termination method of Sanger et al. (1977). Mutant regions of the insert were subcloned into the GW1-CMV vector (British Biotech) for expression in mammalian cells (Choi et al., 1991). The success of the transfer was confirmed by DNA sequencing.

The Bluescript plasmid containing the cDNA encoding for wild-type or mutant channels was linearized using *Hin*dIII. RNA was synthesized in vitro using T7 polymerase and was purified by use of the RNAid kit (Bio-101, La Jolla, CA) prior to injection into oocytes.

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Expression of channels in oocytes

Oocytes were harvested from *Xenopus laevis* (Xenopus I, Wisconsin) that were previously injected with human chorionic gonadotropin. Oocytes were defolliculated by gentle agitation for 1.5–2 h in 1 mg/ml collagenase in Ca-free Ringer's solution; after the first hour, the solution was replaced with fresh enzyme. Subsequently, oocytes were extensively washed with Ca-free Ringer's and stage V and VI oocytes were collected using a dissecting microscope. Oocytes were injected the same day or the following day with about 50 nl of RNA dissolved in water. Oocytes were maintained at 14°C in a Ringer's solution containing 2 mM sodium pyruvate, 1 mM theophylline, and 50 units/ml penicillin/streptomycin. The solution was changed daily. For patch recordings on oocytes, the vitelline membrane was removed manually after 5–10-min incubation of the oocyte in a hypertonic solution of (in mM): 220 *N*-methyl-D-glucamine, 220 aspartic acid, 2 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 EGTA, pH 7.4 with *N*-methyl-D-glucamine.

Expression of channels in cultured mammalian cells

For expression in mammalian cells, we used the human embryonic kidney 293 cell line (HEK293; ATCC CRL-1533, Rockville, MD). Cultures grown in Dulbecco's modified Eagle's medium F12 (GIBCO-BRL) with 10% fetal bovine serum (Sigma) until 40–60% confluent, and were split 1:3 1 day prior to transfection. Cells for transfection were collected by treatment with 0.05% trypsin, 0.53 mM EDTA, washed once, and resuspended in HEPES-buffered saline at 2×10^6 cells/ml. Electroporation was done at 400–475 V (Electroporator; Invitrogen, San Diego, CA) in 0.4-cm cuvettes containing 200 μl of diluted cells, 5–56 μg of channel expression plasmid, 1 μg of SV40 T antigen expression plasmid (gift of Steve Moss, Johns Hopkins), and 5 μg of plasmid DNA for expression of β-galactosidase. Cells were plated onto protamine-coated coverslips and maintained in Dulbecco's modified Eagle's medium F12 with 10% fetal bovine serum at 37°C and 5% CO_2. The efficiency of transfection was monitored by staining for β-galactosidase reaction product and ranged between 30 and 70%.

Current recording

Whole oocyte currents were recorded 1–2 days after RNA injection by two electrode voltage clamp using an OC-725B amplifier (Warner Instrument Corp., Hamden, CT). Whole cell currents or excised patches from cultured cells were recorded 1–3 days after electroporation. Excised patches from oocytes were recorded 2–4 days after RNA injection. Cultured cells and macropatches were voltage-clamped, and currents were recorded using an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA). Currents were sampled at 5 kHz and filtered at 3 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA).

Solutions

The composition of the external solution was (in mM): 155 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. The internal solution was (in mM): 160 KCl, 1 EGTA, 0.5 $MgCl_2$, 10 HEPES, pH 7.4. For recording external tetraethylammonium blockade, TEACl was added as a replacement for NaCl. $ZnCl_2$ (99.999% pure, Aldrich Chemical, Milwaukee, WI) solutions were diluted from a 1 mM working solution.

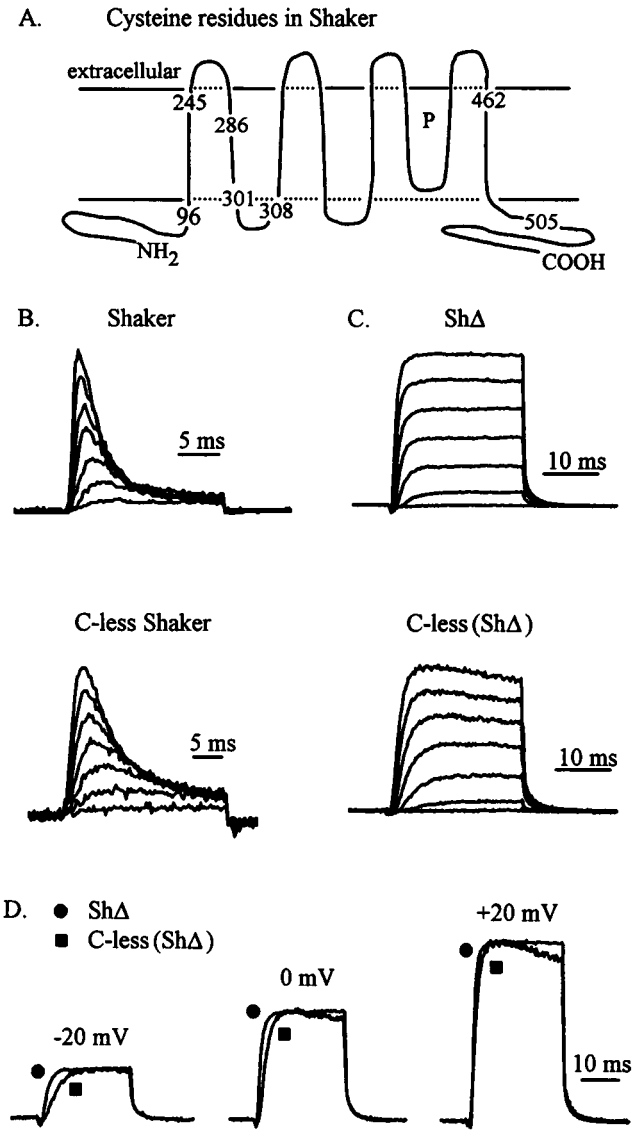

FIGURE 1 Expression of K^+ channels lacking cysteines. (*A*) Schematic of the putative transmembrane topology for a *Shaker* K^+ channel subunit, showing the locations of native cysteine residues. Residue numbers are based on the original numbering of the *Shaker* H4 sequence in Kamb, et al. (1988). (*B*) *Shaker* (*upper*) and C-less *Shaker* (*lower*) K^+ current activation families. Currents were recorded in an outside-out membrane patch (*upper*) or two-electrode voltage-clamp (*lower*) from a frog oocyte. Currents were evoked by a series of voltage steps between −30 and +30 mV in 10 mV increments. The holding potential was −80 mV. (*C*) Whole-cell currents recorded in HEK293 cells showing activation families for ShΔ (*upper*) and C-less(ShΔ) (*lower*) channels with N-type inactivation removed. Voltage steps were between −40 and +20 mV in 10 mV increments from a holding potential of −80 mV. Maximum currents shown are: *Shaker*, 655 pA; C-less *Shaker*, 2.1 μA; ShΔ, 5.0 nA; C-less(ShΔ), 4.1 nA. (*D*) Whole-cell currents recorded in HEK293 cells; voltage pulses were from −80 mV to the indicated test potential with tail pulses to −55 mV. To compare the activation time courses of ShΔ (*circles*) and C-less(ShΔ) (*squares*), currents were scaled to match at the peak. Maximum currents at +20 mV are: ShΔ, 5.2 nA and C-less(ShΔ), 8.3 nA.

RESULTS

Gating properties of K currents

We compared the gating properties of K^+ currents expressed from the C-less *Shaker* mutant and wild-type *Shaker*. Fig. 1 *A* indicates the locations of the seven cysteine residues in the *Shaker* K^+ channel sequence using a working model of the transmembrane topology of voltage-activated K^+ channels. Fig. 1 *B* compares the K^+ currents expressed from *Shaker*

and C-less *Shaker* channels, both of which exhibit a fast N-type inactivation. There was little difference in the rate of N-type inactivation following the replacement of all cysteines in the K^+ channel. In whole oocytes at a test potential of +20 mV, the time constant of inactivation was 5.5 ± 1.0 ms for *Shaker* and 5.8 ± 1.2 ms for C-less *Shaker* (n = 3 for each; single exponentials were fit by eye). Fig. 1 *C* compares currents from wild-type and C-less channels in the ShΔ mutant that lacks N-type inactivation (Hoshi et al., 1990). These, too, appear similar, though the C-less(ShΔ) currents show a slow decline of current due to a change in C-type inactivation, which is discussed below.

Activation gating was slightly altered in the C-less channels. This is seen best in Fig. 1 *D*, with current traces from ShΔ and C-less(ShΔ) scaled to match at the peak. The time course of activation gating is slowed in the C-less channels, and this is reflected as a depolarizing shift in the steady-state activation curve (see Table 1). C-less(ShΔ) channels and wild-type ShΔ channels have similar rates of deactivation though, as seen by the overlap of the tail currents (Fig. 1 *D* and Table 1).

Other properties of K^+ currents

We looked at two other well-described properties of K^+ channels, blockade of the ionic pore by external tetraethylammonium (TEA) and the selectivity of the pore for K^+ over Na^+. The results (Table 1) indicate that C-less channels were not different from wild-type K^+ channels in either property. We also compared the amplitudes of ShΔ and C-less(ShΔ) currents expressed in oocytes after injections of standardized quantities of RNA. As indicated in Table 1, C-less (ShΔ) currents were about threefold smaller than wild-type ShΔ currents. It was not possible to compare directly the expression of K^+ channels in transfected mammalian cells since we cannot know how much DNA enters the cell during the electroporation, but C-less currents were generally smaller than wild-type currents in mammalian cells incubated with the same concentrations of DNA during electroporation.

TABLE 1 Comparison of ShΔ and C-less(ShΔ) K^+ currents

K^+ current property	ShΔ	C-less(ShΔ)
Tau activation (ms), +20 mV[a]	0.9 ± 0.1 (14)	1.2 ± 0.1 (15)
Tau deactivation (ms), +20 mV[b]	2.5 ± 0.6 (8)	2.3 ± 0.4 (11)
$V_{1/2}$ activation (mV)[c]	−27 ± 2 (8)	−23 ± 2 (10)
Reversal potential (mV)[d]	−85 ± 2 (6)	−84 ± 4 (3)
Block by external 30 mM TEA (%)[e]	51 ± 3 (5)	50 ± 1 (6)
Amplitude at −20 mV (μA)[f]	5.8 ± 1.9 (11)	2.1 ± 0.5 (6)

[a–e] Whole-cell patch clamp recordings from transfected HEK293 cells. [a,b] Time constants of activation and deactivation are from single exponentials, fit by eye, to [a] the current elicited by a test depolarization to +20 mV or [b] the tail current at −55 mV following a +20 mV depolarization. [c] The half-activation voltage is from steady-state activation curves constructed from tail current amplitudes following a family of test depolarizations. [d] Reversal potential was extrapolated from tail currents recorded with 160 mM K internal and 5 mM K external at 23°C. The calculated equilibrium potential for K was −88 mV. [e] Block by TEA was measured at 0 mV. [f] Whole-oocyte currents recorded by two-electrode voltage-clamp, 24 h after injection of oocytes from the same frog. RNA was diluted to achieve similar concentrations, as determined by visual and photographic comparison of samples run on an ethidium-bromide stained agarose gel. The number of determinations is given in parentheses.

Zinc modulation

We examined the effects of external Zn^{2+} on ShΔ and C-less-(ShΔ) currents. Zn^{2+} has been demonstrated to slow the activation gating of K^+ current in squid axons (Gilly and Armstrong, 1982) and squid neurons (Spires and Begenisich, 1992), and Zn^{2+} is known to interact with cysteine residues in other proteins (see Introduction). As shown in Fig. 2 *A*, wild-type ShΔ currents were modulated by external Zn^{2+}. The time course of activation was substantially slower in the presence of Zn^{2+}; concentrations as low as 30 μM were effective. This effect is manifested as a depolarizing shift in the steady-state activation curves, which are measured from the amplitude of tail currents following a family of test pulses (Fig. 2 *A*). C-less(ShΔ) currents were also modulated by external Zn^{2+}, and a characteristic slowing of activation gating and depolarizing shift of the activation curve were observed (Fig. 2 *B*). For comparison, activation curves in 300 μM Zn^{2+} were shifted in the depolarizing direction by 18 ± 2 mV for wild-type ShΔ currents (n = 6), and by 14 ± 3 mV for C-less(ShΔ) currents (n = 3). Compared to control currents elicited by depolarizations to 0 mV, the time constant for current activation was slowed by a factor of 5.1 ± 0.7 for ShΔ currents (n = 8) and 3.0 ± 0.5 for C-less(ShΔ) currents

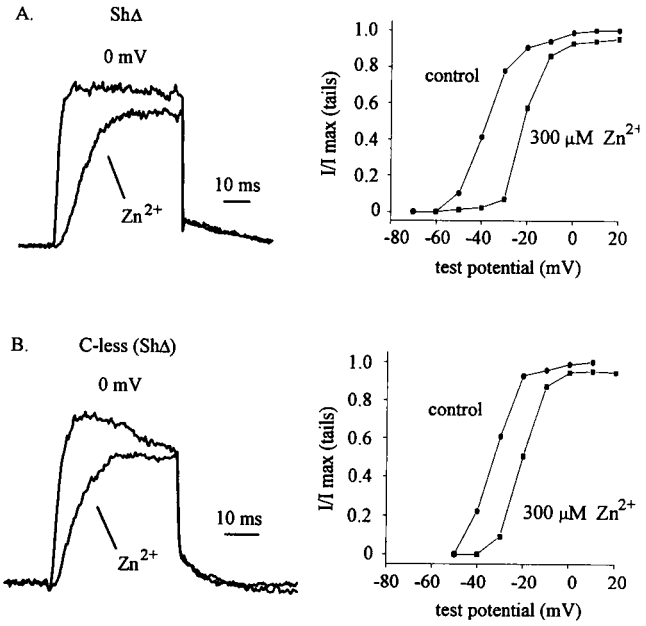

FIGURE 2 Effect on external zinc on activation gating. Currents were recorded in outside-out membrane patches from transfected HEK293 cells expressing ShΔ or C-less(ShΔ) channels. (*A*) Control current and current modulated by 300 μM Zn^{2+} for voltage pulses from −80 to 0 mV with tail currents to −55 mV. The peak control currents shown are: ShΔ, 504 pA; C-less(ShΔ), 990 pA. (*B*) Activation curves constructed from the amplitude of the tail currents at −55 mV, following a family of depolarizations, in the absence or presence of 300 μM Zn^{2+}. Current amplitudes were normalized to the maximum current; same cells as in (*A*).

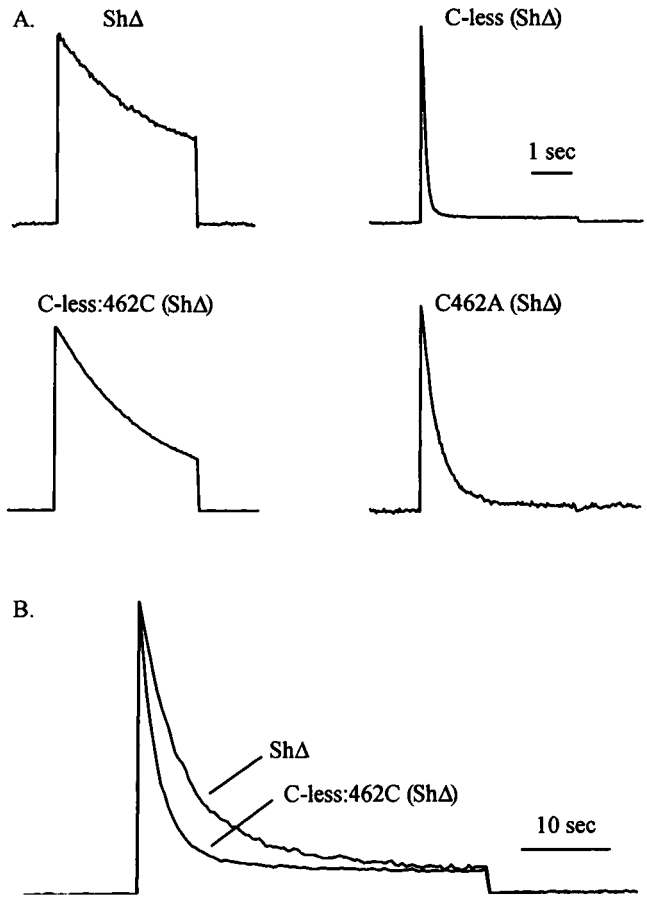

FIGURE 3 C-type inactivation in cysteine substitution mutants. Whole-cell currents were recorded in transfected HEK293 cells expressing different K^+ channels, all lacking N-type inactivation. Depolarizing pulses were from −80 mV to 0 mV. (*A*) Voltage pulses were 4 s in duration. Leak currents were very small and were not subtracted. Peak currents shown are: ShΔ, 2.8 nA; C-less:462C(ShΔ), 8.7 nA; C-less(ShΔ), 12 nA; C462A(ShΔ), 5.3 nA. (*B*) Voltage pulses were 40 s in duration. Currents were leak-subtracted by DC correction and then scaled to match at the peak. Maximum currents are: ShΔ, 3.6 nA; C-less:462C(ShΔ), 4.2 nA. Note that the rate of inactivation observed in the whole-cell recording is 1.5 to 2 times slower than inactivation in outside-out patches; this is true even when the whole-cell currents are small and series resistance errors after compensation are less than 5 mV.

(n = 4). Zn^{2+} had no noticeable effects on the deactivation kinetics for ShΔ or C-less(ShΔ) currents.

External Cd^{2+} also shifted the activation curves and slowed activation kinetics of ShΔ and C-less(ShΔ) currents, although 300 μM Cd^{2+} was less effective than 300 μM Zn^{2+}. Increasing concentrations of external Ca^{2+} also slowed activation gating; addition of 7 mM Ca^{2+} had effects similar to 1 mM Zn^{2+}. In addition, modulation by 300 μM Zn^{2+} was preserved in 11 mM external divalents (10 Ca^{2+}, 1 Mg^{2+}). Zn^{2+} modulation of activation gating was also seen in wild-type *Shaker* and C-less *Shaker* currents with intact N-type inactivation (data not shown).

C-type inactivation

The main difference we observed between C-less K^+ channels and wild-type K^+ channels was an enhanced rate of inactivation during a long pulse or during high frequency stimulation. The different inactivation rates were observed in channels with N-type inactivation removed, and thus correspond to differences in a second inactivation process known as C-type inactivation. Mutations in the carboxy-terminal half of the protein, particularly in the pore and S6 regions, are known to influence the rate of C-type inactivation (Hoshi et al., 1991, LaBarca and MacKinnon, 1992; Lopez-Barneo et al., 1993).

Fig. 3 compares whole-cell currents recorded from HEK293 cells transfected with different K^+ channels made in ShΔ with N-type inactivation removed. C-less currents inactivated about 24-fold more rapidly than ShΔ currents. We suspected that the replacement of Cys^{462} might account for the change in inactivation rates, since mutations at the adjacent residue 463 also accelerate inactivation (Hoshi et al., 1991). To test this idea, we made two complementary constructs; one with replacement of only the suspected cysteine (C462A(ShΔ)), and one with replacement of all but this cysteine (C-less:462C(ShΔ)). Replacement of C462 alone resulted in an acceleration of inactivation, much like that seen for the C-less channel. However, the C-less:462C(ShΔ) channels showed currents with inactivation only slightly faster than wild-type (Fig. 3). The C-less:462C(ShΔ) currents inactivated with a single exponential whereas ShΔ channels always had more than one component. Inactivation of both currents was incomplete, leaving about 5–10% of the maximum current (Fig. 3 *B*). The inactivation time constants for these four channels are compared in Table 2.

In wild-type ShΔ channels, C-type inactivation is sensitive to external TEA. At concentrations of TEA that block the channel, C-type inactivation is slowed, as though TEA binding prevents access to the inactivated state (Choi et al., 1991). C-less(ShΔ) channels, though they have a faster inactivation rate than wild-type channels, exhibit this characteristic slowing of C-type inactivation by external TEA (six of six cells tested). In one cell tested with long pulses, C-type inactivation of C-less(ShΔ) K^+ channels was slowed about twofold by a half-blocking concentration of TEA (Fig. 4). This effect is quantitatively the same as that for wild-type ShΔ (Choi et al., 1991).

TABLE 2 Comparison of C-type inactivation in cysteine substitution mutants

Channel Construct	Tau inactivation
ShΔ	2.8 ± 0.4 s (4)
C-less:462C(ShΔ)	2.5 ± 0.3 s (2)
C-less(ShΔ)	119 ± 11.4 ms (9)
C462A(ShΔ)	352 ± 29.0 ms (4)

Inactivation of whole-cell currents recorded in HEK293 cells with voltage steps from −80 to 0 mV was fit by eye with a single exponential. ShΔ currents had more than one component of inactivation and the fastest component was fit assuming decay to 20% of the peak amplitude. For slowly decaying currents (ShΔ, C-less:462C(ShΔ)), voltage pulses were 40 s in duration. For quickly decaying currents (C-less(ShΔ), C462A(ShΔ)), pulses were 4 s in duration. The number of determinations is given in parentheses.

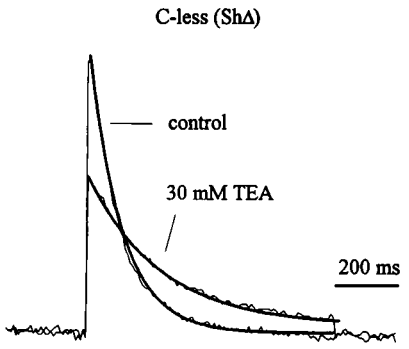

FIGURE 4 External TEA block of K^+ channels lacking cysteines. Currents recorded in an outside-out patch from an HEK293 cell following transfection with C-less(ShΔ) DNA. Depolarizing pulses were from −80 to 0 mV. Peak control current is 167 pA. Single exponentials fit to the decay of the currents had time constants of 99 (control) and 222 ms (30 mM external TEA; fit with decay to 9% of the peak current).

DISCUSSION

C-less channels retain most functions of wild-type *Shaker* K^+ channels

Shaker K^+ channels lacking all native cysteines could be expressed in both frog oocytes and mammalian cells, and the properties of the channels in the two systems are not different. C-less channels have slightly altered activation gating; activation is slower and activation curves are shifted in voltage dependence. The effect of cysteine replacements on activation gating are mild, however, when compared to other mutations which have been shown to alter K^+ channel activation (Lopez et al., 1991; McCormack et al., 1991).

Compared to wild-type ShΔ K^+ channels, C-less channels have similar rates of deactivation. C-less channels retain their high selectivity for K^+ over Na^+. C-less channels are blocked by extracellular TEA, with a half-blocking concentration equal to that of wild-type channels. These basic properties of wild-type *Shaker* K^+ channels were not changed in the mutant C-less channels, irrespective of whether the channels retained N-type inactivation, indicating that the replacement of all native cysteines does not significantly alter N-type inactivation.

C-less K^+ channels are modulated by external Zn^{2+}

The activation kinetics of delayed rectifier K^+ currents in squid axon (Gilly and Armstrong, 1982) and squid neuron (Spires and Begenisich, 1992) are greatly slowed by low concentrations of external Zn^{2+}. Since Zn^{2+} affects channel opening but not channel closing, and does so even in the presence of high concentrations of external Ca^{2+} and Mg^{2+}, the effect of Zn^{2+} is unlikely to be due to membrane surface charge screening (Gilly and Armstrong, 1982). In the present study also, activation gating of ShΔ and C-less(ShΔ) K^+ channels are slowed by external Zn^{2+}, while deactivation gating is unaffected. Much larger changes in external Ca^{2+} (from 3 to 10 mM) produce a smaller effect on activation gating than does 300 μM Zn^{2+}; even in 10 mM Ca^{2+}, the effect of 300 μM Zn^{2+} is still apparent (data not shown).

The nature of the Zn^{2+} interaction with K^+ channels is not known, but these data suggest that cysteine residues are not critical for the interaction of Zn^{2+} with activation gating in *Shaker* K^+ channels. This is in contrast to the importance of cysteines in metal coordination sites of zinc finger proteins (Hanas et al., 1983; Freedman et al., 1988; Pan and Coleman, 1989; Zavitz and Marians, 1993) and zinc enzymes (Vallee and Auld, 1990). Our results are consistent, however, with the results of Spires and Begenisich (1992), who found that the sulfhydryl reagent *p*-chloromercuriphenylsulfonic acid did not affect the modulation by Zn^{2+} of delayed rectifier K^+ currents in squid neurons. Both studies support the idea that cysteine residues are not critical for the interaction of Zn^{2+} with K^+ channels.

C-less K^+ channels exhibit enhanced C-type inactivation

C-less K^+ currents differed from wild-type currents primarily in their rate of C-type inactivation, which occurs by a mechanism distinct from that of N-type inactivation produced by the "ball and chain" mechanism (Hoshi et al., 1991; Choi et al., 1991). For long voltage pulses, C-less currents inactivated about 20-fold more rapidly than wild-type currents. K^+ channels with cysteines replaced at all but one position, Cys^{462}, showed C-type inactivation similar to that of the wild-type channels. Conversely, channels with a single cysteine replacement at residue 462 showed more rapid C-type inactivation, like that of the channels with all cysteines replaced. Thus, the enhanced rate of C-type inactivation in C-less K^+ channels can be attributed primarily to replacement of the cysteine at residue 462, which is not surprising, since nearby residues in the S6 regions have been shown to influence the rate of C-type inactivation (Hoshi et al., 1991).

Although C-type inactivation of C-less K^+ channels is accelerated compared to wild-type channels, the mechanism of inactivation in C-less channels retains a characteristic functional property. External TEA block of C-less(ShΔ) channels reduced the rate of C-type inactivation, as has been previously shown for wild-type ShΔ currents (Choi et al., 1991).

What is the role of cysteines in the function of K^+ channels?

Two cysteines (Cys^{286} and Cys^{462} in *Shaker* H4) are completely conserved across the four families of cloned voltage-activated K^+ channels and might, therefore, be expected to serve critical functions. We have shown, however, that replacement of all cysteines in the *Shaker* K^+ channel does not substantially alter most of the basic functions of the channel. It is not known whether these mutations affect protein assembly or stability, other properties commonly influenced by cysteines through the formation of disulfide bridges.

The N-type inactivation of mammalian K^+ channel clones RCK4 and Raw3 has been shown to depend upon a cysteine residue in the "ball" region of the amino terminus (Ruppersberg et al., 1991). N-type inactivation is prevented by oxidation of Cys^{13} (probably by the formation of a disulfide bridge) and is restored by exposure to a reducing environment. Whereas there may be an important role for this cysteine residue in the mechanism of N-type inactivation of certain K^+ channels, there is no corresponding cysteine in the "ball" region of *Shaker* K^+ channels. Moreover, replacement in *Shaker* K^+ channels of the cysteine residues most likely to be exposed to the cytoplasm (e.g., Cys^{96}, Cys^{505}), did not dramatically alter the functional properties of the channels; C-less channels retained the wild-type N-type inactivation process.

The most dramatic change observed upon replacement of cysteines in the *Shaker* K^+ channels is a greatly accelerated rate of C-type inactivation, which can be attributed largely to the mutation at Cys^{462}. Mutations of a homologous cysteine residue in DRK1 K^+ channels also slow the rate of C-type inactivation (Zhang et al., 1993). The consequences of replacement of Cys^{462} are not specific to this residue, however. Mutations of the adjacent residue 463 (Hoshi et al., 1991) and of residues in the pore region (Lopez-Barneo et al., 1993) also modify the rate of C-type inactivation. These observations suggest that several regions near or comprising the external mouth of the pore are important in the process of C-type inactivation.

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REFERENCES

Boland, L. M., M. E. Jurman, and G. Yellen. 1993. None of the cysteines in the *Shaker* potassium channel is essential for channel activity or for zinc modulation. *Soc. Neurosci. Abstr.* 23:1062.

Choi, K. L, R. W. Aldrich, and G. Yellen. 1991. Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K channels. *Proc. Natl. Acad. Sci. USA.* 88:5092–5095.

Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto. 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature (Lond.).* 334:543–546.

Gilly, W. F., and C. M. Armstrong. 1982. Divalent cations and the activation kinetics of potassium channels in squid giant axons. *J. Gen. Physiol.* 79:965–996.

Hanas, J. S., D. J. Hazada, W. F. Bogenhagen, F. Y.-H. Wu, and C.-W. Wu. 1983. *Xenopus* transcription factor A requires zinc for binding to the 5S RNA gene. *J. Biol. Chem.* 258:14120–14125.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science (Wash. DC).* 250:533–538.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1991. Two types of inactivation in *Shaker* K^+ channels: effects of alterations in the carboxy-terminal region. *Neuron.* 7:547–556.

Kamb, A., J. Tseng-Crank, and M. A. Tanouye. 1988. Multiple products of the Drosophila *Shaker* gene may contribute to potassium channel diversity. *Neuron.* 1:421–430.

Kao, P. N., and A. Karlin. 1986. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *J. Biol. Chem.* 261:8085–8088.

Kobayashi, M., H. Izui, T. Nagasawa, and H. Yamada H. 1993. Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues. *Proc. Natl. Acad. Sci. USA.* 90:247–251.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488–492.

Labarca, P., and R. MacKinnon. 1992. Permeant ions influence the rate of C-type inactivation in *Shaker* K channels. *Biophys. J.* 61:A378.

Lopez, G. A., Y. N. Jan, and L. Y. Jan. 1991. Hydrophobic substitution mutations in the S4 sequence alter voltage-dependent gating in *Shaker* K^+ channels. *Neuron.* 7:327–336.

Lopez-Barneo, J., T. Hoshi, S. H. Heinemann, and R. W. Aldrich. 1993. Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. *Receptors and Channels.* 1:61–71.

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

McCormack, K., M. A. Tanouye, L. E. Iverson, J.-W. Lin, M. Ramaswami, T. McCormack, J. T. Campanelli, M. Mathew, and B. Rudy. 1991. A role for hydrophobic residues in the voltage-dependent gating of *Shaker* K^+ channels. *Proc. Natl. Acad. Sci. USA.* 88:2931–2935.

Mishina, M., T. Tobimatsu, K. Imoto, K. Tanaka, Y. Fujita, K. Fukada, M. Kurasaki, H. Takahashi, Y. Morimoto, T. Hirose, S. Inayama, T. Takahaski, M. Kuno, and S. Numa. 1985. Location of functional regions of acetylcholine receptor α-subunit by site-directed mutagenesis. *Nature (Lond.).* 313:364–369.

Pan, T., and J. E. Coleman. 1989. Structure and function of the Zn(II) binding site within the DNA-binding domain of the GAL4 transcription factor. *Proc. Natl. Acad. Sci. USA.* 86:3145–3149.

Ruppersberg, J. P., M. Stocker, O. Pongs, S. H. Heinemann, R. Frank, and M. Koenen. 1991. Regulation of fast inactivation of cloned mammalian I_K(A) channels by cysteine oxidation. *Nature (Lond.).* 352:711–714.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Spires, S., and T. Begenisich. 1992. Chemical properties of the divalent cation binding site on potassium channels. *J. Gen. Physiol.* 100:181–193.

Vallee, B. L., and D. S. Auld. 1990. Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry.* 29:5647–5659.

Yellen, G., M. E. Jurman, T. Abramson, and R. MacKinnon. 1991. Mutations affecting internal TEA blockade identify the probable pore-forming regions of a K^+ channel. *Science (Wash. DC).* 251:939–942.

Zavitz, K. H., and K. J. Marians. 1993. Helicase-deficient cysteine to glycine substitution mutants of *Escherichia coli* replication protein PriA retain single-stranded DNA-dependent ATPase activity. Zn^{2+} stimulation of mutant PriA helicase and primosome assembly activities. *J. Biol. Chem.* 268:4337–4346.

Zhang, H.-j., R. D. Zuhlke, and R. H. Joho. 1993. Mutations of a conserved cysteine residue in S6 of Kv2.1 (DRK1) alter inactivation and deactivation kinetics. *Soc. Neurosci. Abstr.* 23:1062.