Oxidation of an Engineered Pore Cysteine Locks a Voltage-Gated K^+ Channel in a Nonconducting State

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ABSTRACT We report the use of cysteine-substituted mutants in conjunction with in situ oxidation to determine the physical proximity of a pair of engineered cysteines in the pore region of the voltage-gated K^+ channel Kv2.1. We show that the newly introduced cysteine I379C, located near the outer end of the narrow ion-conduction pathway, renders the K^+ channel sensitive to oxidation by H_2O_2, but only if the native cysteine at position 394 in S6 remains in place. Conservative substitutions in S6 for cysteine 394 abolish H_2O_2 sensitivity in the Kv2.1 mutant I379C. Comparative immunoblot analysis of wild-type and I379C Kv2.1-expressing HEK293 cells demonstrates the presence of subunit dimers for I379C, but not for wild-type Kv2.1. At the single-channel level, the probability of opening of I379C channels, unlike wild-type, is reduced in the presence of H_2O_2; however, oxidation of I379C does not alter unit current. These findings imply that cysteine 379, located near the outer end of the narrow ion-conduction pathway, participates in disulfide bridge formation, locking the channel in a nonconducting state from which it cannot undergo conformational transitions required for opening.

INTRODUCTION

Voltage-gated K^+ channels are ubiquitous membrane proteins that are important for the fine tuning of electrical properties in excitable cells (Rudy, 1988; Hille, 1992). K^+ channels are formed by four identical or similar subunits, each comprising six putative transmembrane segments, S1–S6 (MacKinnon, 1991; Liman et al., 1992). A major part of the ion-conduction pathway of voltage-gated K^+ channels is formed by the region between S5 and S6 (Hartmann et al., 1991; Kavanaugh et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991), and the S4-S5 linker and parts of S6 may participate in forming the inner channel mouth (Choi et al., 1993; Lopez et al., 1994; Slesinger et al., 1993). Recently, we and others have used cysteine-scanning mutagenesis to probe amino acid side-chain accessibility in the ion-conduction pathway of a voltage-gated K^+ channel (Kürz et al., 1994 and 1995; Lü and Miller, 1995; Pascual et al., 1995). Of 28 cysteine-substituted mutants in the S5–S6 linker of Kv2.1 (Frech et al., 1989), 14 expressed K^+ channels with ion selectivity, voltage sensitivity, and current amplitudes similar to wild type (Kürz et al., 1995). In 5 of the 14 mutants the engineered cysteine side chain was accessible to externally applied thiol-specific reagents of different size or charge (Fig. 1).

Substituted cysteine residues in a protein may create the possibility of disulfide formation between a newly introduced cysteine and a thiol group already present, provided the distance between the α carbons of the two half-cystines is 4–7.5 Å (Richardson and Richardson, 1989), but distances up to 15 Å appear possible due to thermal backbone motions (Careaga and Falke, 1992). In homomultimeric proteins, disulfide bridges may form between native or newly engineered cysteines if the thiol groups in different subunits face each other at the appropriate distance. This latter strategy has been used successfully to map contact points between identical subunits of some transmembrane signal transducers of *Escherichia coli* (Lynch and Koshland, 1991; Careaga and Falke, 1992; Pakula and Simon, 1992). Here, we report that the Kv2.1 mutant I379C carrying a cysteine near the outer entryway to the narrow ion-selective pore is sensitive to H_2O_2, presumably by in situ oxidation and disulfide formation, which locks the channel in a closed state and prevents the necessary conformational transitions for channel opening.

METHODS

Oocyte injection and electrophysiology

Kv2.1 K^+ channel mutants were generated and cRNA was synthesized as described (Zühlke et al., 1994 and 1995). Approximately 150 pg in vitro-synthesized cRNA was injected into *Xenopus laevis* oocytes; after 1–4 days cells were subjected to a standard two-electrode voltage-clamp protocol (Van Dongen et al., 1990). Oocytes were held at −80 mV, and 400-ms test pulses to 40 mV were applied every 20 s. Initial current levels were 5–10 μA. Experiments were done in ND96 [96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 $CaCl_2$, and 5 mM HEPES (pH 7.4)] at room temperature (22 ± 1°C). The SH-reagent MTSET (2-trimethylammonium-ethyl) methanethiosulfonate bromide was used as described (Kürz et al., 1995). Both voltage-sensing and current-passing electrodes were filled with 3 M KCl and had resistances of 0.2–1.0 MΩ. The pCLAMP system was used to generate the voltage-pulse protocol and for data acquisition. Signals were filtered at 500 Hz and digitized at 1–2 kHz. Linear capacitive and leakage currents were subtracted online using a P/4 protocol. Membrane potentials were not corrected for series resistance errors.

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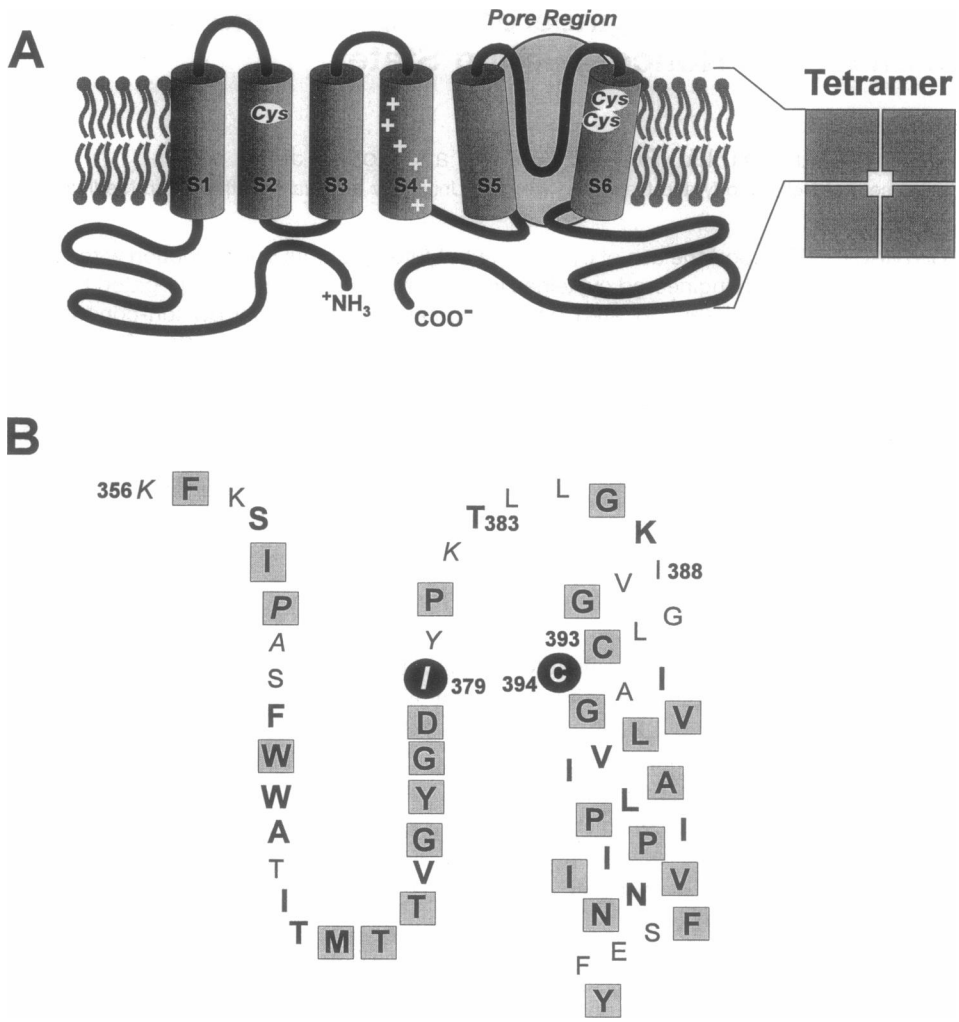

FIGURE 1 Amino acid sequence in the pore and S6 region of Kv2.1. (*A*) A single Kv2.1 subunit is depicted with its six transmembrane segments, the pore region, and the three cysteines present in the hydrophobic core. (*B*) The lower part of the figure shows the amino acid sequence in the pore region and in S6. Positions generally conserved in voltage-gated K^+ channels are shown in bold letters; absolutely invariant positions are highlighted by squares. Italicized positions, when mutated to cysteine, are accessible to SH-specific reagents from the external vestibule. Cysteine 379 in the pore and cysteine 394 in S6 required for H_2O_2 sensitivity are surrounded by a filled circle; the invariant cysteine 393 is involved in gating and ion permeation (Zühlke et al., 1994).

Single-channel recording

Experiments were essentially performed as described previously (Moorman et al., 1990). Oocytes were kept in (mM) 100 KCl, 1.0 $MgCl_2$, 5.0 HEPES (pH 7.4); the pipette contained (mM) 96 NaCl, 2.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.0 HEPES (pH 7.4), and 0.1% H_2O_2 when indicated. Test pulses of 330 ms from −100 mV to 0 mV were applied every 3 s. Analog signals were filtered at 1 kHz (Axopatch 200A, pCLAMP 6.0), digitized at 5 kHz, and stored on optical disk for offline analysis. Signals were digitally filtered at 500 Hz, and passive leak and capacitance currents were subtracted using the mean of traces without channel openings. Single-channel data were analyzed using Fetchan and pSTAT (pCLAMP 6.0).

Kv2.1 expression in HEK293 cells and Western blot analysis

Human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transiently transfected using a liposome-mediated transfection procedure with a plasmid encoding wild-type or mutant Kv2.1 under the control of the cytomegalovirus promotor. HEK293 cells were seeded at subconfluent densities on 35-mm petri dishes and allowed to settle overnight. Cells were then treated with 15 μl DOTAP (Boehringer Mannheim) in 1.5 ml DMEM/0.5% FBS containing 2.5 μg plasmid DNA, according to the manufacturer's recommendation. Cells were incubated for 48 h at 37°C. Before harvesting, lipofected cells were exposed for 5 min to either 1 mM DTT or 0.1% H_2O_2 in phosphate-buffered saline (PBS). Cells were washed twice in PBS, suspended directly in 200 μl 20-mM *N*-ethylmaleimide in standard SDS-containing lysis buffer, and sonicated for 2 s (until the suspension was no longer viscous). Aliquots (30 μl) were adjusted to different concentrations of DTT, heated to 100°C for 5 min, and subjected to electrophoresis through 5% polyacrylamide before transfer to nitrocellulose. The Kv2.1-specific antibody KC (a kind gift of Dr. James Trimmer) was used at a dilution of 1:250. The second-stage antibody was a 1:2000 dilution of donkey anti-rabbit Ig antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized by chemiluminescence (ECL Western blotting protocol, Amersham).

RESULTS

The amino acid sequence in the pore region and S6 of Kv2.1 is depicted in Fig. 1 *B*. We showed previously that the side chains at positions K356, P361, I379, Y380, and K382, when mutated to cysteine, were accessible to SH-specific reagents from the external vestibule (Kürz et al., 1994 and 1995). During the course of this work, we noticed that the mutant I379C displayed varying levels of current when expressed in *Xenopus laevis* oocytes. Currents measured by a standard two-electrode voltage clamp were often increased severalfold after application of 1 mM dithiothreitol

(DTT). This observation raised the question whether the introduced cysteine at position 379 might have undergone spontaneous oxidation and disulfide formation either with another cysteine in the same subunit or with the same cysteine of a different subunit. Formation of a disulfide bridge could a) prevent gating (i.e., movement of S4); b) induce a subtle structural change in or near the conduction pathway with concomitant complete loss of ion permeation (i.e., the channel could be locked in a nonconducting state); or c) hinder passage of ions and reduce unit conductance without affecting gating processes. Application of the reducing agent DTT might therefore open the in situ-formed disulfide bridge and restore function in the previously nonconducting channel. Hence, we predicted that in situ oxidation might convert the functional, conducting K^+ channel mutant I379C to a nonconducting state. This prediction turned out to be correct. Exposure of the Kv2.1 mutant I379C to 0.1% H_2O_2 for 5 min gradually reduced K^+ currents to greater than 95% (Fig. 2 *A*). The effect was highly specific: wild-type Kv2.1 (isoleucine at position 379) and mutant I379A were not affected (Fig. 2 *A*), and none of the other tested cysteine-substituted mutants was sensitive to H_2O_2 (Table 1). Current reduction after H_2O_2 application developed over several minutes and was reversible by addition of 1 mM DTT. This cycle could be repeated several times (data not shown).

The H_2O_2 sensitivity of this mutant suggested that the newly introduced cysteine at position 379 near the outer end of the narrow conduction pathway in Kv2.1 was involved in disulfide formation, either with the same cysteine residue of another subunit or with one of the native cysteines already present in the same or a different channel subunit. Three native cysteines are in the hydrophobic core region (S1–S6) of Kv2.1, one in S2 (C232), and two in S6 (C393 and C394) (Fig. 1 *A*). The fact that these cysteines are not essential for Kv2.1 function in *Xenopus* oocytes (Zühlke et al., 1994) enabled us to test whether one of them was required to render the I379C mutant H_2O_2-sensitive. We introduced the I379C mutation in the cysteineless Kv2.1 triple mutant C232S/C393S/C394S. In contrast to the I379C single mutant, the quadruple mutant was no longer sensitive to 0.1% H_2O_2 (Fig. 2 *B* and Table 1). This mutant still reacted with the extracellularly applied thiol-specific reagent (2-trimethylammoniumethyl) methanethiosulfonate (MTSET), suggesting that the cysteine side chain at position 379 was in place and accessible from the outer channel vestibule, as in the case of the single mutant I379C (Kürz et al., 1995).

To determine which of the cysteines in S2 or S6, in addition to the one at position 379, might be needed to render the I379C mutant sensitive to H_2O_2, we converted each of the three native cysteines to a serine, both singly and in combination, keeping the newly introduced cysteine at position 379. Only the double mutant I379C/C394S was no longer affected by H_2O_2; in contrast, mutants I379C/C232S, I379C/C393S, and I379C/C232S/C393S, were still sensitive to H_2O_2 (Fig. 3). We also tested four additional I379C-derived double mutants: C394A, C394T, C394V, and

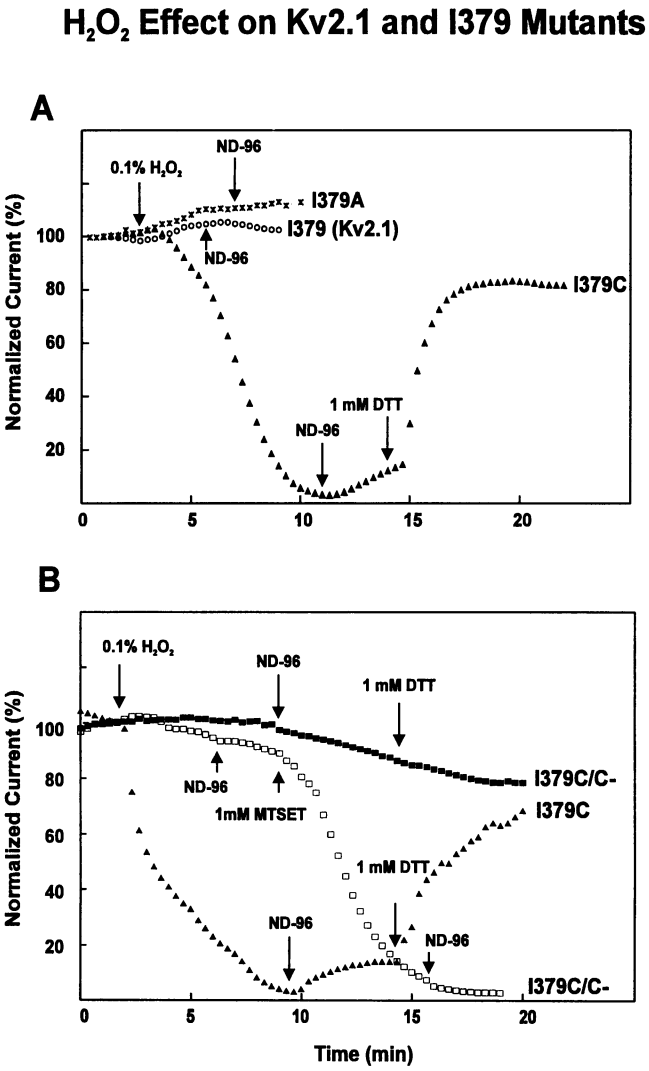

FIGURE 2 Effect of H_2O_2 on wild-type and mutant Kv2.1 K^+ channels. (*A*) Kv2.1-expressing *Xenopus laevis* oocytes were superfused with 0.1% H_2O_2 in ND96. For all traces the beginning of H_2O_2 perfusion is indicated by the same arrow; a subsequent change to ND96 or 1 mM DTT in ND96 is marked by an arrow near the corresponding trace. Outward current through mutant I379C channels is almost completely lost after 5 min; the effect may be reversed by application of 1 mM DTT. Wild-type Kv2.1 (I379) and mutant I379A are not affected by H_2O_2. (*B*) A cysteine in the hydrophobic channel core is required for H_2O_2 sensitivity. The I379C mutant in a cysteine-less Kv2.1 K^+ channel (C232S/C393S/C394S) is not sensitive to H_2O_2; however, the cysteine at position 379 is still accessible to MTSET from the outside. Peak currents (5–10 μA) are normalized to the beginning of the experiment (100%).

C394N. Only mutant I379C/C394A yielded a functional K^+ channel and, like I379C/C394S, it was resistant to H_2O_2 (Fig. 3). The other three double mutants (T, V, or N in S6) did not result in measurable channel activity.

Our experiments showed that cysteines at positions 379 and 394 were necessary to render Kv2.1 K^+ channels sensitive to H_2O_2. This suggested that an intramolecular disulfide was formed between the cysteine side chain at position 379, near the end of the ion-conduction pathway, and cys

TABLE 1 Effect of H_2O_2 on cysteine-substituted Kv2.1 K^+ channels

Mutant	% Remaining current	*n*
Kv2.1 (I379)	95 ± 2.4	3
P361C	89 ± 18	5
F364C	88	2
A367C	99.5	1
T368C	100.3 ± 6.3	3
I369C	88.2 ± 12.6	3
M371C*	113.6 ± 9.4	3
T373C*	88.6	2
I379C	11 ± 5.9	5
I379C/C-less	106 ± 12	4
I379A	95	2
Y380C	74 ± 10	3
Y380C/C-less	93 ± 2.5	4
T383C	121	1

Remaining peak currents 5 min after application of 0.1% H_2O_2 (mean ± SD). Oocytes were held at −80 mV, and 400-ms test pulses to 40 mV were applied every 20 s. Initial current levels were 5–10 μA. (To compensate for lower expression levels, 20–30-fold higher RNA concentrations were injected for mutants marked with *.)

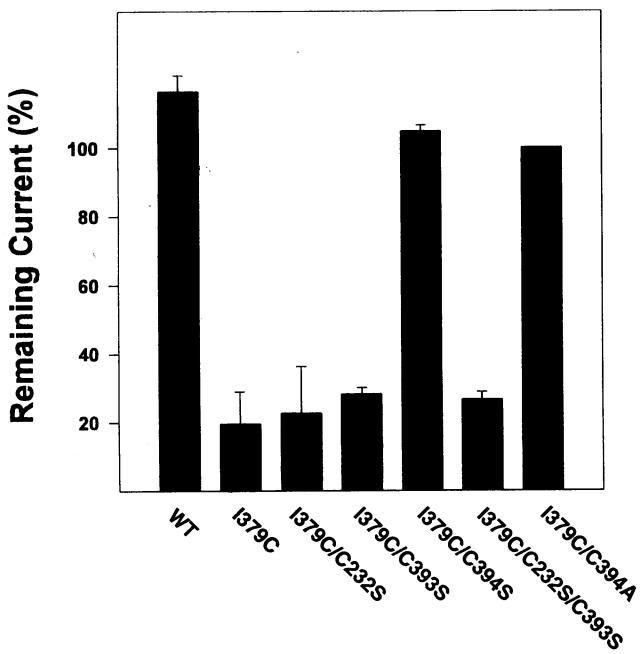

FIGURE 3 Cysteines at positions 379 and 394 in Kv2.1 are required for H_2O_2 sensitivity. Substitution of a serine for cysteine at position 394 in S6 confers H_2O_2 resistance on the sensitive Kv2.1-mutant I379C. Oocytes were held at −80 mV, and 400-ms test pulses to 40 mV were applied every 20 s. The bars show remaining peak current 5 min after application of 1% H_2O_2 (mean ± SD; n = 3). Initial current levels were 10–20 μA.

teine 394 in S6 of Kv2.1, although other interpretations were possible. Position 379 is in a narrow region of the pore (Kürz et al., 1994 and 1995; Aiyar et al., 1995; Lü and Miller, 1995; Pascual et al., 1995). Cysteines at this position in different subunits could be close enough to generate dimers by intersubunit disulfide formation. To determine if oxidation of I379C mutant channels might lead to subunit dimerization, we expressed wild-type Kv2.1 and the mutant I379C in human embryonic kidney cells (HEK293). Cell membranes were solubilized, and the size of the channel subunits under reducing and nonreducing conditions was determined by Western blot analysis (Fig. 4). For wild-type and mutant I379C, the majority of Kv2.1-specific, immunoreactive material migrated as two bands, both under reducing and nonreducing conditions, corresponding in size to approximately 95 kD and 115 kD (marked by arrowheads in Fig. 4). An additional band at approximately 215 kD, corresponding in size to a dimer, was only detected in case of the mutant I379C, but was not visible for wild-type Kv2.1. After exposure of wild-type-expressing HEK293 cells to 0.1% H_2O_2, the bulk of immunoreactive Kv2.1-specific material did no longer enter the stacking gel unless the concentration of DTT in the sample was above 1 mM (Fig. 4 *A*). At higher DTT concentrations, wild-type channels exclusively migrated as monomers in clear contrast to the mutant I379C, which still showed a band corresponding to subunit dimers under partially reducing conditions (Fig. 4 *A*). Exposure of I379C-expressing HEK293 cells to 1 mM DTT before solubilization did not completely reduce the spontaneously formed dimers in a sample that had not been treated with H_2O_2 (Fig. 4 *B*).

What is the mechanism underlying H_2O_2-induced current reduction? Are unit conductance, probability of opening (P_o), or both affected? To address these questions, we studied the effect of H_2O_2 on membrane patches containing single wild-type or mutant-I379C channels. Single-channel activities and amplitude histograms of wild-type and I379C-mutant channels are shown in Fig. 5. No significant difference between unit currents at 0 mV in the absence (0.31 ± 0.02 pA; three patches, mean ± SD) or presence (0.29 ± 0.01 pA; three patches) of 0.1% H_2O_2 in the recording pipette could be detected for wild-type channels (Fig. 5 *A*). Visual inspection of the current traces suggested that P_o was not substantially changed in the presence of H_2O_2, explaining why superfusion of wild-type Kv2.1-expressing oocytes with H_2O_2 did not have an effect on the macroscopic current. The effect of H_2O_2 on mutant I379C was very different. The unit current at 0 mV for mutant channels (0.21 ± 0.03 pA; six patches, mean ± SD) was smaller than that for wild-type; but, importantly, it was not altered in the presence of 0.1% H_2O_2 (0.19 ± 0.03 pA; three patches) in the recording pipette (Fig. 5 *B*). In marked contrast to Kv2.1, the probability of opening was, however, dramatically reduced in the presence of H_2O_2, although the small unit current made it difficult to accurately determine the open times. Exposure to 0.1% H_2O_2 initially led to altered I379C-mutant channels, which exhibited infrequent, brief openings mainly visible during the first few traces after seal formation (Fig. 5 *B*). After a few minutes, only null-traces were detected in I379C-expressing membrane patches, in contrast to wild-type Kv2.1-expressing patches, where single-channel activity remained unaltered. These findings indicate that a major change in the channel's gating underlies the macroscopic current reduction after oxidation. Noise

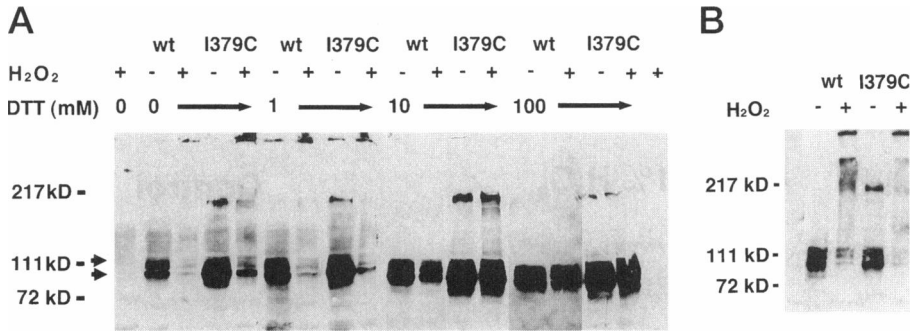

FIGURE 4 Expression of wild-type and I379C-mutant Kv2.1 in HEK293 cells. (*A*) Wild-type and I379C Kv2.1-expressing HEK293 cells were solubilized and subjected to immunoblot analysis using a Kv2.1-specific antibody. Cells were either directly lysed (−) or first treated with 0.1% H_2O_2 (+) for 5 min before harvesting. Identical aliquots were adjusted to the indicated concentration of DTT before electrophoresis. The monomer positions are shown by arrowheads, and relevant molecular size standards are indicated. The first and last lane show the results with untransfected HEK293 cells. Both for wild-type and I379C Kv2.1, exposure to H_2O_2 shifts the immunoreactive material to the top of the stacking gel. Kv2.1 dimer is, however, only visible for I379C. (*B*) Kv2.1-expressing HEK293 cells were pretreated with 1 mM DTT for 5 min, then either directly lysed (−) or first exposed to 0.1% H_2O_2 for 5 min before harvesting. Identical aliquots were subjected to electrophoresis in the absence of DTT from the sample. A remaining fraction of in situ-formed dimer is still visible in I379C-expressing cells. Exposure to H_2O_2 leads to extensive cross-linking for mutant and wild-type Kv2.1.

analysis of untreated and H_2O_2-treated cell-attached patches expressing wild-type and mutant channels confirmed that unit conductances were not substantially changed after exposure to H_2O_2 under conditions that would have resulted in 90% reduction of whole-cell currents in case of I379C (data not shown).

DISCUSSION

We showed that the newly introduced cysteine at position 379 in the pore region rendered the voltage-gated K^+ channel Kv2.1 sensitive to oxidation by H_2O_2 (Figs. 1 and 2). Only when a cysteine was present at this position near the outer end of the narrow ion-conduction pathway was the mutant K^+ channel sensitive to H_2O_2, and only if the native cysteine at position 394 in S6 remained unchanged (Fig. 3). None of the other nine cysteine-substituted pore mutants was sensitive to H_2O_2 (Table 1). Introduction of subtle mutations (alanine or serine) at position 394 in S6 converted the H_2O_2-sensitive I379C mutant to a H_2O_2-resistant form, in spite of the cysteine at position 379 near the outer entryway of the pore. These results suggested that cysteine 379 formed either an intramolecular disulfide bond with cysteine 394 in S6 of the same subunit, an intermolecular bridge with cysteine 394 of another subunit, or disulfide formation took place between two cysteines 379 of adjacent or opposite subunits. Western blot analysis indicated the presence of subunit dimers, but not trimers or tetramers, in HEK293 cells expressing mutant I379C K^+ channels (Fig. 4). The dimer band was clearly specific for mutant I379C, but the band's intensity did not simply depend on or increase with exposure to 0.1% H_2O_2 as one might have expected if dimerization of subunits were solely caused by H_2O_2-induced oxidation. Our initial observation that K^+ currents were sometimes increased 10-fold in I379C mutant-expressing oocytes after addition of 1 mM DTT suggested spontaneous in situ disulfide bond formation. It is possible that a fraction of I379C mutant channels was already oxidized and cross-linked in HEK293 cells before exposure to H_2O_2. Treatment of wild-type or mutant-expressing HEK293 cells for 5 min with 0.1% H_2O_2 shifted most of the Kv2.1-immunoreactive material to the top of the stacking gel when DTT was absent from the sample. This probably reflects extensive cross-linking of channel subunits to other proteins or to each other after H_2O_2-induced oxidation of cysteines located near the cytoplasmic N- and C-terminal tails of the channel subunit. After treatment with 10 mM DTT, wild-type Kv2.1 migrated as monomer in marked contrast to I379C which showed a fraction of the immunoreactive material corresponding to subunit dimers. This dimer band was specific for I379C and was consistently detected. Addition of 1 mM DTT, which restored approximately 80% of macroscopic K^+ current in channel-expressing oocytes, did not suffice to generate a majority of monomer, both for wild-type Kv2.1 and for the mutant I379C. Because these experiments were performed in two different expression systems, which may have somewhat different reactivities to DTT, the concentration ranges of the DTT effects on channel function in oocytes and channel cross-linking in HEK293 cells may overlap, but they need not exactly coincide. In our opinion, this clearly demonstrates the possibility of dimer formation for the mutant I379C, but not for wild-type Kv2.1.

Taken together, our findings suggest that cysteine 379 near the outer end of the pore participates in dimer formation either with another cysteine 379 of an adjacent or opposite subunit or with cysteine 394 in S6 of a different subunit. It could also be possible that the functional effects are due to the formation of an intrasubunit disulfide between positions 379 and 394, while the cross-linking visualized by gel electrophoresis is without functional significance. We formally cannot distinguish between these possibilities, but we consider the first one most likely for the following reasons: a) we never detected trimers or tetramers under

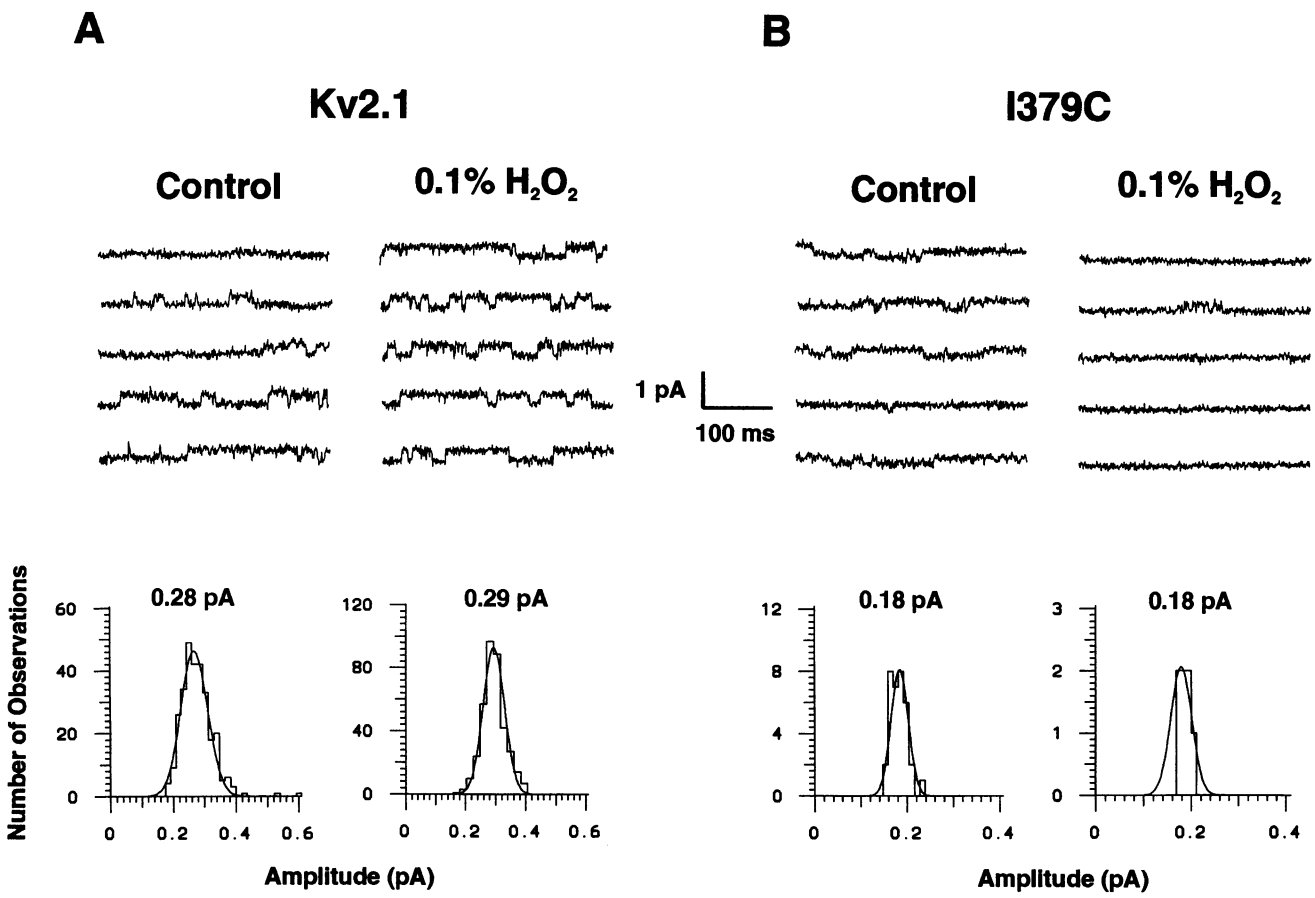

FIGURE 5 Oxidation of I379C channels decreases open probability but not unit current. The top panels show traces of single-channel recordings of wild-type Kv2.1 (*A*) and mutant I379C (*B*) before and after exposure to 0.1% H_2O_2. In the absence of H_2O_2, I379C-mutant channels show few null traces and long openings (tens of milliseconds) similar to wild type. After exposure to 0.1% H_2O_2, openings of I379C are initially infrequent and brief, and they disappear completely after a few minutes. The traces shown are from five consecutive 330-ms test pulses from −100 mV to 0 mV immediately after formation of a stable seal (upward deflections correspond to channel openings). The bottom panels show the amplitude histograms corresponding to the patches above. Each number represents the mean unit current for that particular patch. Unit currents of I379C channels are smaller than those of wild-type Kv2.1 channels. Exposure to H_2O_2 decreases the probability of opening for I379C, but does not change current amplitude.

partially reducing conditions, a possible outcome if C379 were cross-linked to C394 of a different subunit; b) we showed previously that several thiol-specific reagents have easy access to the cysteine at position 379, but that none of these reagents has an effect on cysteine 394 of the wild type (Kürz et al., 1995), presumably because this position is buried inside the protein (Zühlke et al., 1994). Hence, we consider it unlikely that the cysteine at position 379 near the outer entryway to the pore is exposed to the pore lumen and accessible to a buried residue in S6. The interpretation of intersubunit disulfide formation between cysteines at position 379 is in agreement with recent work demonstrating that the Shaker mutant M448C, which corresponds to the mutant I379C in Kv2.1, can undergo intersubunit disulfide formation after in situ oxidation (Liu et al., 1996).

If the cysteine in S6, although required for the channel's H_2O_2 sensitivity, does not participate in disulfide bond formation, then relatively subtle changes at this position, e.g., substituting an alanine or serine for cysteine, would have to perturb substantially the close apposition of the pore cysteines and be propagated from S6 to position 379 in the pore to prevent disulfide formation. It may seem surprising that small changes in S6 prevent disulfide formation between cysteines located near the outer entryway to the pore. Decreasing the side-chain volume at position 394 from 38 $Å^3$ (cysteine) to 25 $Å^3$ (serine) or 19 $Å^3$ (alanine) corresponds to a diameter reduction of 0.6 Å and 0.8 Å, respectively (assuming spherical side chains). If this side chain were in a region of tight protein packing it might be possible that neutral changes of subangstrom dimensions could distort the pore to the extent that disulfide bridge formation between subunits is no longer possible. Our additional substitutions of threonine, valine, or asparagine for cysteine 394 in S6 led to loss of K^+ channel function, in agreement with the idea that this side chain is within the sphere of influence of position 379 near the outer end of the ion-conduction pathway.

We showed previously that the invariant cysteine 393 in S6 of the K^+ channel Kv2.1 is involved in gating and ion permeation (Zühlke et al., 1994). Particularly, the chemical nature, not the side-chain volume, governs the rate constants

of deactivation and inactivation; in contrast, the side-chain volume influences the Rb^+/K^+ conductance ratio. We postulated that the side chain at position 393 is in a region of tight protein packing in S6, is involved in conformational changes during open-closed-state transitions, and also contributes to the control of ion permeation. Here, we report that the side chain at position 379 near the outer end of the narrow ion-conduction pathway is within the sphere of influence of position 394, which is adjacent to the side chain involved in gating and ion permeation. The residue in Shaker K^+ channels corresponding to position 394 in Kv2.1 is involved in C-type inactivation (Hoshi et al., 1991).

Our findings point to a possible mechanism that may be responsible for H_2O_2 sensitivity. Disulfide bridge formation between subunits may prevent necessary conformational transitions for opening by locking the channel in a closed state, from which it does not frequent the open state. Homotetrameric I379C K^+ channels can form two disulfide bridges in the pore after cysteine oxidation. Our single-channel recordings show that openings of I379C channels become brief and infrequent soon after exposure to H_2O_2 (immediately after seal formation), and, within a few minutes, channel openings can no longer be detected (Fig. 5). These results suggest the existence of three different "gating modes," corresponding to three different states of oxidation: 0, 1, or 2 disulfide bridges. Although the long openings of fully reduced, native channels were difficult to resolve because of the small unit current, it is clear that immediately after H_2O_2 exposure only infrequent and brief channel openings could be detected, showing no change in unit current (Fig. 5). These infrequent, brief openings could reflect the activities of modified I379C-mutant channels after the formation of a first disulfide bridge. This initial phase is temporary and is followed by a period of channel inactivity when openings can no longer be detected, presumably after the second disulfide bridge has been formed.

Gating currents corresponding to voltage-induced conformational transitions precede K^+ channel openings (Bezanilla et al., 1991). Depending upon the particular closed state in which the channels are "frozen," gating currents might be partially or completely absent in the mutant I379C as a result of exposure to H_2O_2. Wild-type Kv2.1 and the mutant I379C show similar gating currents in the absence and presence of H_2O_2, suggesting that intersubunit cross-linking does not prevent the movement of gating charges (E. Stefani; personal communication). It has been suggested that Shaker K^+ channels undergo a structural alteration near the outer mouth of the pore upon C-type inactivation (Yellen et al., 1994; Liu et al., 1996). Although this particular conformational change appears to be absent in Kv2.1 (Kürz et al., 1995), our experiments indicate that subunit movement, involving the outer end of the pore (position 379), is required for channel opening. Restriction of this movement by oxidation-induced disulfide bridge formation appears to prevent the necessary conformational changes for transitions from a closed to an open state.

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