Contribution of the NH₂ terminus of Kv2.1 to channel activation

JUAN M. PASCUAL,1 CHAR-CHANG SHIEH,2 GLENN E. KIRSCH,2 AND ARTHUR M. BROWN2

1Center for Molecular Recognition, Columbia University, New York, New York 10032; Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030; and 2Department of Physiology and Biophysics and Rammekamp Center, MetroHealth Campus, Case Western Reserve University, Cleveland, Ohio 44109

Pascual, Juan M., Char-Chang Shieh, Glenn E. Kirsch, and Arthur M. Brown. Contribution of the NH₂ terminus of Kv2.1 to channel activation. Am. J. Physiol. 273 (Cell Physiol. 42): C1849–C1858, 1997.—Opening and closing of voltage-operated channels requires the interaction of diverse structural elements. One approach to the identification of channel domains that participate in gating is to locate the sites of action of modifiers. Covalent reaction of Kv2.1 channels with the neutral, sulfhydryl-specific methylmethanethiosulfonate (MMTS) caused a slowing of channel gating with a predominant effect on the kinetics of activation. These effects were also obtained after intracellular, but not extracellular, application of a charged MMTS analog. Single channel analysis revealed that MMTS acted primarily by prolonging the latency to first opening without substantially affecting gating transitions after the channel first opens and until it inactivates. To localize the channel cysteine(s) with which MMTS reacts, we generated NH₂- and COOH-terminal deletion mutants and a construct in which all three cysteines in transmembrane regions were substituted. Only the NH₂-terminal deletion construct gave rise to currents that activated slowly and displayed MMTS-insensitive kinetics. These results show that the NH₂-terminal tail of Kv2.1 participates in transitions leading to activation through interactions involving reduced cysteine(s) that can be modulated from the cytoplasmic phase.

electrophysiology; site-directed mutagenesis; chemical modification; cysteine

Voltage-dependent ion channels visit different conformational states driven by changes in membrane potential. Voltage-gated K⁺ channels reach the open (active) state after depolarization and the closed (deactive) state on repolarization. Additionally, they close (inactivate) in the presence of sustained depolarizations by alternative mechanisms. The transition rates between these states can be profoundly affected by mutations in several channel domains. Extensive work has identified regions involved in inactivation. Deletion of residues 6–46 of Shaker K⁺ channels abolishes the fast inactivation observed in these channels (7), whereas mutations in the sixth hydrophobic domain (S6) alter the rate of a separate slow inactivation process (8). Amino acid substitutions in the pore-forming (P) region of Kv2.1, a channel that inactivates slowly, speed or delay inactivation (6; J. M. Pascual and A. M. Brown, unpublished observations). On the other hand, evolutionarily conserved residues in the S4 segment play a key role in activation gating and have been well characterized as sensing elements required for voltage-dependent activation (13, 17). A substantial part of the charge movement that leads to channel opening can be accounted for by voltage-facilitated translocation of basic S4 residues relative to the protein core (15, 31). Analysis of the rates of reaction of hydrophilic methanethiosulfonate derivatives with substituted cysteines showed that S4 residues are subject to chemical modification from the extracellular or the cytoplasmic phase, depending on the state of the channel gates (11, 30). These experiments support the notion that, instead of being buried in the rest of the protein core, the voltage sensor is exposed on both sides of the membrane and is available to modulation from the cytoplasmic phase, as proposed by Perozo and Bezanilla (20). Although other S4-interacting residues have been identified in transmembrane areas of the channel (16), considerable effort will be required to identify the rest of the structural components that participate in voltage-dependent gating and to elucidate how they regulate ion flow through the pore.

We set out to identify channel regions involved in K⁺ channel activation outside S4. Our search was motivated by the findings of Caputo et al. (4) using the sulfhydryl-specific reagent p-hydroxymercuri phenylsulfonic acid (PHMPS) on squid axon K⁺ channels. PHMPS causes a pronounced slowing of activation gating, yet there are no cysteines in the S4 region of cloned voltage-gated channels (including the squid K⁺ channel from optic lobe; D. Patton and F. Bezanilla, personal communication; 21), suggesting that PHMPS reacts with a target located elsewhere in the channel itself or in a closely associated subunit. Our approach to localizing the sulfhydryl-reactive domain(s) associated with gating modification combined cysteine elimination by genetic substitution or deletion with a functional assay for the loss of sensitivity to sulfhydryl reagents. We have used the sulfhydryl modifier methylmethanethiosulfonate (CH₃SO₂SCH₃, MMTS) extracellularly applied on Kv2.1 expressed in Xenopus oocytes. MMTS was chosen because 1) it reacts specifically with cysteiny1 sulphydryls, attaching a thiomethyl group via disulfide bonding (24), 2) it is uncharged, small, and lipid soluble, potentially having access to membrane-embedded and cytoplasmic regions, even when applied extracellularly, and 3) several charged, hydrophilic MMTS analogs are available (1).

In whole cells, MMTS reacted with Kv2.1, producing an irreversible slowing of activation kinetics, which was due to a prolongation of the latency to first opening, as shown by single channel analysis. This effect could be prevented by deleting the first 139 amino acids from the NH₂ terminus of the channel. Additionally, MMTS...
increased channel conductance by modification of a separate site that could be regenerated by the reducing agent dithiothreitol \([\text{HSCH}_2\text{CH(OH)CH(OH)CH}_2\text{SH}], \text{DTT}\). The results support the involvement of the NH\(_2\) terminus of Kv2.1 in voltage-dependent gating and its susceptibility to modulation from the cytoplasm.

**MATERIALS AND METHODS**

Recombinant DNA. Deletion of the 347 COOH-terminal amino acids (residues 506–853; \(\Delta C\)) of Kv2.1 was achieved by the excision of a BamHI–I–BglI cassette followed by the ligation of an adapter sequence coding for the amino acids RPPPEPER followed by a stop codon. Incorporation of this epitopic sequence did not affect unitary channel conductance or gating, as detailed by Pascual et al. (19). Substitutions C232A, C393S, and C394S, located in membrane-associated regions, were sequentially introduced on a full-length Kv2.1 clone by oligonucleotide-mediated mutagenesis using a phage-subcloned, single-stranded Kv2.1 DNA cassette. The NH\(_2\)-terminal deletion construct (\(\Delta N\)) was generated by digestion of Kv2.1 DNA with ClaI, which released a fragment encoding the first 120 amino acids and most of the 5'-untranslated and polylinier regions. Self-ligation of the remaining segment yielded a clone in which the first in-frame methionine was at position 140, therefore lacking the first 139 amino acids. Substitutions C163S and C164S were also performed by oligonucleotide-directed mutagenesis. All constructs were verified by restriction analysis and dideoxy sequencing extending over mutant and ligation areas.

Capped runoff cRNA transcripts were prepared after plasmid linearization with Not I, quantitated, and stored in 100 mM KCl at \(-80^\circ\text{C}\), as described by Pascual et al. (19). Stage V and VI oocytes were injected with 46 nl of sufficiently concentrated cRNA to achieve 3–10 \(\mu\)A of whole cell current at 40 mV. cRNA was injected (in ng/\(\mu\)l) as follows: 2 Kv2.1, 0.5 \(\Delta N\), 120 \(\Delta C\), 120 AN, 50 transmembrane core cysteine, and 5 all other mutants. Cells were maintained in culture for 1–5 days before electrophysiological analysis.

Whole cell recording. Cells were transferred to a 250-\(\mu\)l recording chamber and perfused at 5 ml/min with a solution containing (in mM) 51 KOH, 60 NaOH, 120 methanesulfonic acid, \(15\) \(\text{NaCl}\), 5 \(\text{MgCl}_2\), 2 NaHCO\(_3\), 10 NaOH, 10 N-2-hydroxyethylpipera-

zin-N'-2-ethanesulfonic acid (HEPES), and 2 CaCl\(_2\), adjusted to pH 7.2. The KOH concentration allowed the ethylammonium analog of MMTS (MTSEA, CH\(_3\)SO\(_2\)-

\[\text{SCH}_2\text{CH}_2\text{NH}_3\]) to achieve 3–10 \(\mu\)A of whole cell current at 40 mV. cRNA was injected (in ng/\(\mu\)l) as follows: 2 Kv2.1, 0.5 \(\Delta C\), 120 \(\Delta N\), 50 transmembrane core cysteine, and 5 all other mutants. Cells were maintained in culture for 1–5 days before electrophysiological analysis.

Whole cell recording. Cells were transferred to a 250-\(\mu\)l recording chamber and perfused at 5 ml/min with a solution including (in mM) 51 KOH, 60 NaOH, 120 methanesulfonic acid, \(15\) \(\text{NaCl}\), 5 \(\text{MgCl}_2\), 2 NaHCO\(_3\), 10 NaOH, 10 N-2-hydroxyethylpipera-

zin-N'-2-ethanesulfonic acid (HEPES), and 2 CaCl\(_2\), adjusted to pH 7.2. The KOH concentration allowed the measurement of large-amplitude inward tail currents on membrane repolarization. A 3 M KCl–agar bridge connected the recording chamber to an Ag-AgCl reference electrode. The ethylammonium analog of MMTS (MTSEA, CH\(_3\)SO\(_2\)-

\[\text{SCH}_2\text{CH}_2\text{NH}_3\]) was a gift of Arthur Karlin. MMTS was purchased from Sigma Chemical (St. Louis, MO) and DTT from US Biochemical (Cleveland, OH). Because methanethio-

sulfonates hydrolyze rapidly at neutral pH and room temperature (A. Karlin, personal communication), MMTS and MT-

SEA were dissolved in perfusion solution immediately before application to the oocyte and used for a maximum of 5 min. Low-resistance (0.3–0.7 M\(\Omega\)) agarose-cushion electrodes were manufactured from beveled glass micropipette tips filled with a 0.5% agarose-3 M KCl gel and backfilled with 3 M KCl. Currents were leak and linear capacitance subtracted using an on-line P/4 routine. Similar results were obtained in experiments performed without subtraction. Cells exposed to MMTS were monitored for a decrease in membrane resistance, which was often observed while the reagent was being perfused. Cells in which the leak at \(-80\) mV reached 10% of the voltage-gated ionic conductance at 40 mV or in which the leak persisted after withdrawal of MMTS were discarded from the analysis. Macroscopic tail currents were elicited by instant repolarizing voltage pulses to \(-80\) mV after prolonged depolarizing prepulses of sufficient duration (0.5–1.5 s) to activate most channels. Tail current amplitudes were measured by extrapolation of monoexponential fits back to the time of the voltage jump. Steady-state activation gating was analyzed by fitting current-voltage (I–V) relationships obtained from tail current measurements in whole oocytes. Tail current amplitudes were fitted to the Boltzmann equation:

\[
I/I_{\text{maximum}} = \frac{1}{1 + \exp[(V - V_n)/K]^{-1}}
\]

where \(I\) represents normalized tail current amplitude, \(V_n\) is prepulse potential, \(V_s\) is activation midvoltage, and \(k\) is a slope parameter expressed in F/RT units. Experiments were carried out at 11–23°C. Values are means \(\pm\) SE. A two-tailed \(t\)-test was used to assess the significance of the difference between means.

GIANT PATCH RECORDING. Patch pipettes of \(\sim 100\) \(\mu\)m diameter were polished to \(-0.5\) M\(\Omega\) of resistance when filled with (in mM) 120 NaCl, 2.5 KCl, 2 CaCl\(_2\), and 10 HEPES, pH 7.2. Bath solution containing (in mM) 100 KCl, 10 EGTA, and 10 HEPES, pH 7.2, was used to zero the membrane resting potential. Inside-out patches were excised from mechanically devitellinized oocytes, and their intracellular aspect was exposed to bath solution. An Axopatch-1D amplifier (Axon Instruments, Foster City, CA) was used. Currents were low-pass filtered at 0.5–1 kHz (–3 dB, 4-pole Bessel filter) and digitized at a sampling rate of 2 kHz. Capacitance was compensated. Leak currents and series resistance were negligible and were left uncompensated. Typically, initial giant patch peak currents measured from oocytes expressing Kv2.1 reached 1–4 nA and decayed monoexponentially after repetitive depolarization to a steady level with a time constant of \(~3\) min at a holding potential of \(-80\) mV, as described by Pascual et al. (19). Exceptionally, a few patches did not exhibit rundown. The effects of internal MTSEA application to giant patches did not depend on rundown velocity or magnitude.

Single channel recording. Cell-associated single channel activity was recorded using the equipment and solutions described for giant patch experiments, except pipettes were fire polished to a resistance of 5–10 M\(\Omega\). Channels were activated with test pulses of 0–40 mV from a holding potential of \(-60\) mV. Currents were low-pass filtered at 1 kHz before digitization at 4 kHz. Records were corrected for linear capacitative and leak currents by subtracting the smoothed average of records lacking channel activity (10). Single channel currents were idealized using TRANSIT (27). This algorithm uses a \(dl/dt\) threshold to identify transitions between open and closed levels during a first data pass. In a second pass, idealized openings were constructed using a half-amplitude threshold criterion. Amplitudes and open and closed interval durations of idealized data were collected into distribution histograms. Amplitude histograms were fitted with Gaussian functions using a maximum likelihood estimate. Slope conductances were determined from least-squares fits of current amplitude–voltage data in a test potential range of 0–60 mV. Mean open times (\(t_{\text{open}}\)) were calculated by fitting open time histograms to a single-exponential decay function using a maximum likelihood estimate. Open times <0.4 ms were excluded from fitting. The distribution histograms of closed interval durations were fitted with two-exponential decay functions from which mean closed times within a burst (\(t_{\text{closed}}\)) or between bursts (\(t_{\text{interburst}}\)) were determined. Bursts were defined by establishing a critical closed interval of 1 ms, and their mean durations (\(t_{\text{burst}}\)) were obtained by fitting the duration histograms to a single exponential. The time constant of the first latency (waiting time from the start of the test pulse to first opening)
was deduced from monoexponential fits to cumulative distribution histograms.

RESULTS

Currents arising from the expression of Kv2.1 in oocytes activate over several milliseconds following a sigmoidal time course, a feature of delayed rectifier channels, and display a slow inactivation process appreciable at depolarized voltages (>-20 mV; Fig. 1A). The channels deactivate on return to hyperpolarized potentials, generating inward tail currents, which are noticeable because of the high extracellular K+ concentration used in this experiment (see MATERIALS AND METHODS). Activation of MMTS-treated channels required larger depolarizations and followed a slower time course than Kv2.1 control currents (Fig. 1B). A parallel phenomenon was a prolongation of the time course of channel deactivation, as indicated by the slowing of tail current decays. These effects developed slowly, over several minutes of MMTS application, and could not be reversed by washout, as expected if MMTS reacted covalently with sulhydryl groups associated with the channel.

To test the accessibility of the site(s) modified by MMTS, we attempted to reverse the reaction by reduction with DTT. In control experiments, high (50 mM) concentrations of DTT had no effect on the Mg2.1 currents. We reasoned that if MMTS reacted with a single, readily accessible site, DTT would regenerate the cysteiny1 sulhydryl, gradually restoring currents to their premodification appearance. However, application of 5 mM DTT to MMTS-treated cells increased current amplitudes only slightly, with no effect on activation or deactivation kinetics (Fig. 1C). This result suggests that access of the slightly larger DTT to the MMTS-modified site that causes alteration of channel kinetics is restricted and/or that penetration of DTT through the bilayer to accumulate in the cytoplasmic phase proceeds slowly. Scaled MMTS-modified channel currents superimposed on post-DTT current traces (not shown), indicating that gating follows an identical time course under both conditions. This result is consistent with a dual action of MMTS: 1) a predominant effect on channel kinetics, irreversible under our experimental conditions, and 2) a small decrease in conductance that arises from modification of an additional site that can be subsequently reduced by DTT.

Effect of MMTS on activation kinetics. To describe the changes in channel kinetics after MMTS in quantitative terms, we measured the time course of current activation in whole oocytes. As noted above, activation of Mg2.1 currents after a 40-mV depolarizing test pulse proceeds following a sigmoidal time course (Fig. 1A). This phenomenon is compatible with the contention that the channels must traverse several closed states before opening. We fitted the 5–95% amplitude interval of these currents with the sum of two exponentials of similar amplitudes and time constants (τ) of 11 ms (τfast) and 24 ms (τslow) (Table 1). This procedure eliminated the foot of the activation process from the fit, which arises from channels that open early during depolarization. On reaction with MMTS (Fig. 1B), the slower exponential component of activation was substantially affected, becoming prolonged by fourfold (τslow,MMTS = 95 ms), whereas the fast component was only marginally slowed (τfast,MMTS = 16 ms; Table 1). This effect persisted

![Fig. 1. Modification of Kv2.1 currents by sulphydryl reagents. A: whole oocyte control currents elicited by a series of depolarizing potentials in 10-mV steps from holding potential of -80 mV. B: application of 4 mM methylmethanethiosulfonate (MMTS) over 5-min prolonged time course of activation and delayed deactivation tail currents. C: subsequent superfusion of 5 mM dithiothreitol (DTT) over 5 min caused slight increase in current amplitudes but had no effect on kinetics. Reactions were monitored to completion.](Image)

<table>
<thead>
<tr>
<th>Current</th>
<th>Vm (mV)</th>
<th>k (F/RT)</th>
<th>n</th>
<th>τfast (ms)</th>
<th>τslow (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv2.1</td>
<td>-1 ± 1</td>
<td>11 ± 1</td>
<td>12</td>
<td>11 ± 2</td>
<td>24 ± 3</td>
<td>6</td>
</tr>
<tr>
<td>Kv2.1 + MMTS</td>
<td>ND</td>
<td>16 ± 2</td>
<td>95 ± 10</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔN</td>
<td>53 ± 3</td>
<td>13 ± 2</td>
<td>4</td>
<td>13 ± 4</td>
<td>97 ± 32</td>
<td>4</td>
</tr>
<tr>
<td>ΔN + MMTS</td>
<td>52 ± 3</td>
<td>14 ± 2</td>
<td>6</td>
<td>14 ± 6</td>
<td>151 ± 16</td>
<td>4</td>
</tr>
<tr>
<td>ΔC</td>
<td>3 ± 2</td>
<td>9 ± 1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys-less core</td>
<td>-21 ± 2</td>
<td>8 ± 1</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C232A</td>
<td>-15 ± 2</td>
<td>9 ± 1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C393S</td>
<td>-5 ± 1</td>
<td>8 ± 1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C393S/C394S</td>
<td>-6 ± 1</td>
<td>10 ± 1</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C163S/C164S</td>
<td>8 ± 3</td>
<td>10 ± 1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Whole oocyte tail currents were induced by repolarizing test pulses after prolonged depolarizations that activated most channels. Tail peak amplitudes were estimated from monoeponential fits to current decay traces and extrapolation to start of repolarization test pulse. Midactivation voltages (V1/2) and slope parameters (k) were obtained from fitting tail current amplitude-voltage data to a Boltzmann equation as described in MATERIALS AND METHODS. Time course of activation was analyzed by fitting 5–95% portion of current amplitudes with sum of 2 exponentials, from which fast (τfast) and slow (τslow) time constants were obtained. ΔN, NH2-terminal deletion construct; ΔC, COOH-terminal amino acid deletion; Cys-less, cysteineless. ND,steady-state activation of currents could not be achieved because of membrane instability after methylmethanethiosulfonate (MMTS) treatment. *Measurements obtained from 1 batch of oocytes in which membrane tolerated prolonged depolarizations (1.5 s) after MMTS application.
in oocytes expressing Kv2.1 and treated with DTT after reaction with MMTS (not shown), as if the site that caused conductance reduction after S-methylation could be modified and regenerated independently of the site(s) that mediated the effects of MMTS on channel kinetics. Another action of MMTS was a prolongation of the time course of deactivation, as measured by fitting tail currents with single-exponential functions. As shown in Fig. 1, after MMTS treatment, Kv2.1 channels deactivate more slowly (deactivation $\tau = 19$ ms) than in control conditions (deactivation $\tau = 7$ ms), indicating that open channels remain open longer before they close. This effect also persisted after DTT application (deactivation $\tau = 18$ ms).

We also determined the voltage dependence of channel activation (I-V relationship) in the unmodified Kv2.1 (Fig. 2A). The $V_r$ for steady-state activation of Kv2.1 currents, obtained by fitting I-V data to a Boltzmann equation (see MATERIALS AND METHODS), is $-1$ mV, with a $k$ of $11 F/RT$ (Table 1). In contrast, activation of MMTS-treated Kv2.1 channels proceeded too slowly to reach steady state at the end of test potentials of sufficient duration to fully activate Kv2.1 in control conditions (Fig. 1). Prolongation of test pulses impaired clamp performance in these cells and prevented accurate steady-state measurements. Therefore, we could not measure the shift of the activation midpoint of these channels.

Sidedness of MMTS reaction. We began to search for the structural element(s) responsible for the kinetic changes induced by MMTS by investigating the pathway from which MMTS gained access to its target in the channel. The neutral character of MMTS makes it potentially membrane permeant. Therefore, reaction with Kv2.1 could take place 1) from the extracellular solution, 2) from the membrane phase with MMTS molecules that partition in the lipid bilayer, or 3) after diffusion through the membrane and intracellular accumulation before the molecules gained access to cytoplasm-exposed areas of the channel. From a simple perspective, if the reaction occurred from the extracellular phase with a solvent-exposed site, we would expect it to proceed at a relatively fast rate, whereas subsequent partition in the bilayer and/or penetration through protein-protein interfaces would impose a diffusional barrier that could be expected to slow the observed rate of reaction. Similarly, the reducing environment provided by intracellular compounds such as glutathione would also retard access of MMTS to Kv2.1 if the reaction took place in the cytoplasmic phase.

A transient increase in oocyte resting conductance during MMTS perfusion prevented accurate determination of channel modification rates. Nevertheless, high (4 mM) MMTS concentrations applied for at least 2 min were required to drive the reaction to completion. This result stands in contrast with that obtained for the reaction of MMTS with 2-mercaptoethanol, which appears to proceed two to three orders of magnitude faster, as determined by Stauffer and Karlin (25). Therefore, MMTS modified Kv2.1 at a much slower rate, as if access to the reactive cysteine(s) was restricted by a diffusional barrier or as if MMTS was being quenched by other reactive groups in the oocyte interior. However, an alternative possibility was that the reaction rate-limiting step was not access to the site but disulfide formation. This would be the case if, for example, the target site(s) was extracellularly accessible but had a poor intrinsic reactivity because of infrequent ionization of the cysteinyl sulfhydryl. Support for either of the former hypotheses vs. the latter was provided by experiments using MTSEA. MTSEA is hydrophilic and mostly charged at pH 7.2, being less permeant through the lipid bilayer than MMTS. Therefore, fast reaction with MTSEA is indicative of solvent exposure (1). Whereas extracellular application of MTSEA (up to 5 mM) and other charged MMTS analogs had no effect on Kv2.1 currents (19), exposure of the intracellular side of giant patches containing numerous channels to 2 mM MTSEA resulted in an irreversible slowing of activation kinetics that developed nearly instantaneously and resembled the effects of MMTS applied to saturation by superfusion (Fig. 2). Therefore, the site responsible for modulation of Kv2.1 kinetics appeared to be accessible to MTSEA from the cytoplasmic but not from the extracellular phase.

Locating a target domain for MMTS. We continued the search for the channel region mediating the effects of MMTS by assigning the 15 cysteines present in the Kv2.1 clone to 3 primary structural domains: 4 in a COOH-terminal domain, 3 in a transmembrane core region (including membrane-related segments S1–S6), and the remaining 8 in an NH2-terminal domain. To identify on which of the channel domains MMTS reacted to modify gating, we engineered three cysteine-deficient constructs (Fig. 3): 1) a COOH-terminal deletion that eliminates the last 347 residues (amino acids 506–853), which included all COOH-terminal cysteines ($\Delta C$), 2) a transmembrane core cysteineless construct combining substitutions C232A (located in S2) and C393S and C394S (both in S6), and 3) an NH2-terminal deletion by excision of the first 139 amino acids ($\Delta N$).
This deletion mutant lacked the first six cysteines. Several other single and multiple cysteine substitutions were also studied. A list of these mutants, together with some of their kinetic properties, is given in Table 1.

All mutant constructs produced robust (several µA) voltage-gated K^+ currents in oocytes suitable for detecting changes in kinetics after MMTS exposure. Activation of COOH-terminal deletion and transmembrane core cysteineless channels resembled Kv2.1, except for a negative shift (V_e = −20 mV) in the voltage dependence of activation of cysteineless core channels, which could be largely accounted for by the substitution C232A carried by that construct (Table 1). Nevertheless, as illustrated in Fig. 3, A and B, activation induced by strong depolarizing pulses was prolonged for both of these mutants after reaction with MMTS, indicating that the target cysteine(s) associated with the gating machinery remained unaltered by these mutations and therefore must be located in the NH2 terminus or, perhaps, in an intimately associated subunit that interacts with the NH2 terminus of the channel. Indeed, deletion of the first 139 residues generated channels that activated over a prolonged time interval, displaying kinetics that were unaffected by MMTS application (Fig. 3C, Table 1). The time course of activation of unmodified and MMTS-treated DN currents overlapped that measured for MMTS-modified Kv2.1 channels (Table 1). Analysis of DN steady-state activation gating also supported the conclusion that the effects of MMTS on Kv2.1 are mediated by reaction with the NH2 terminus. The V_e of DN currents was 53 mV, with k of 13 F/RT (Table 1). After reaction with MMTS, both parameters remained unchanged (Table 1, Fig. 4), despite a reduction in channel conductance, as described below. Replacement of residues C163 and C164 (located before S1 and left in place in the DN deletion construct) with serine had no effect on the time course of activation, affecting only marginally its voltage dependence (Table 1), and was not analyzed further. Nevertheless, MMTS still reacted with DN channels, causing a slight (15%) decrease in current amplitude, an observation that is consistent with the hypothesis...
stated above of an additional site that is responsible for the reduction in conductance noticed after modification and appears to remain intact in the deletion mutant. Compensating for current reduction by multiplying amplitudes by a scaling factor (ranging from 1.12 to 1.19 in 10 cells tested) allowed accurate superimposition of \( D^N \) current onset traces obtained before and after MMTS application (Fig. 3, inset), supporting the insensitivity of \( D^N \) channel gating to MMTS. Therefore, both MMTS targets appear to modulate different aspects of channel function (kinetics and conductance) from separate loci.

Conduction through mutant and modified channels. Measurement of instantaneous I-V relationships allowed the assessment of ion conduction in open Kv2.1 and \( D^N \) channels before and after MMTS treatment. As shown in Fig. 5, Kv2.1 currents display outward rectification in asymmetric solutions (51 and 60 mM extracellular KOH and NaOH, respectively) (see MATERIALS AND METHODS; 19). Kv2.1 and \( D^N \) channel currents showed a similar degree of rectification, a phenomenon that is consistent with the integrity of the permeation pathway in \( D^N \) channels. Figure 5 also illustrates the effects of MMTS modification on ion conduction through \( D^N \) channels. After MMTS reaction, outward currents were slightly more reduced than inward currents, without shifting reversal potential, resulting in a smoothing of the rectifying profile of \( D^N \) channels.

Figure 6 and Table 2 illustrate the effects of MMTS modification on currents through single channels and their correspondence with the whole cell measurements described above. After MMTS reaction, the unitary slope conductance of Kv2.1 measured in a range of positive potentials significantly decreased from 10 to 8.2 pS (\( P < 0.05 \)). This result correlated with and accounted for the current reduction (\(-15\%\)) obtained from the outward limb of macroscopic I-V relationship measurements. On the other hand, the conductance of single \( D^N \) channels (9.2 pS) was not significantly different from Kv2.1, in support of the notion that the NH2-terminal deletion does not interfere with K+ permeation.

Mechanism of gating modification by MMTS. Analysis of single channel kinetics yielded information about changes in Kv2.1 gating phenotype after reaction with MMTS (Fig. 6, Table 2). Channel kinetics were studied in the presence of 400-ms depolarizing pulses to minimize transit to the inactivated state, which occurs at a rate of 0.3 s\(^{-1}\), as measured in whole cell recordings. Five parameters were used to characterize gating of individual Kv2.1 channels: 1) latency to first opening after a depolarizing step, 2) burst duration (defined as a family of related openings interrupted only by brief closings; \( t_{burst} \)), 3) interburst interval (\( t_{interburst} \)), 4) open time (\( t_{open} \)), and 5) closed time (including all time intervals that the channels spend closed except parameters 1 and 3, \( t_{closed} \)). Average values for these parameters are given in Table 2 for Kv2.1, MMTS-treated Kv2.1, and \( D^N \) channels, and representative recordings are shown in Fig. 6.

Remarkably, only the first latency appeared to be significantly prolonged (\(-4\)-fold) in MMTS-exposed and \( D^N \) channels compared with Kv2.1. None of the other parameters measured diverged significantly from Kv2.1.
values (Table 2). These results indicate that the conformational changes underlying the transitions responsible for the behavior of the channel after the first opening are unaffected by MMTS or by the deletion of the NH₂-terminal domain. Figure 6D plots the cumulative probability of first opening vs. time of Kv2.1, MMTS-treated, and ΔN channels after a voltage step to 40 mV. As also observed in Shaker channels (2, 9), the sigmoidal appearance of the three plots resembles the time course of macroscopic activation and further indicates the existence of several closed states that can be visited before opening. Additional support for the similarity of functional effects between MMTS modification and NH₂-terminal deletion on the kinetics of activation is provided by their similar first latency distributions, which stands in contrast to the steeper distribution obtained for Kv2.1 (Fig. 6D).

As exemplified by noninactivating Shaker channels (2, 9), the latency of activation of delayed rectifiers is voltage dependent. At depolarized test potentials, the latency to first opening decreases as the channels travel faster through the activation pathway. Figure 6E illustrates the voltage dependence of the latency to first opening of Kv2.1 channels. As expected, depolarizing test pulses of increasing magnitude shortened the delay of first Kv2.1 openings. A similar voltage dependence was appreciated in the latencies of MMTS-treated Kv2.1 and ΔN channels, despite their prolonged values, suggesting that, over the potential range studied, the process by which MMTS and the deletion of the NH₂ terminus affected activation has little intrinsic voltage dependence.

An additional effect of MMTS was a slowing of deactivation, which was observed in tail current measurements (Fig. 1). This phenomenon suggests that reaction with MMTS also increases the stability of the open state at negative potentials, perhaps by slowing transitions leading away from the activated state.

Effect on inactivation gating. Inactivation of Kv2.1 currents proceeds slowly over several seconds and can be well approximated with a single-exponential function with a time constant of 3.3 s. MMTS decreased the

Table 2. Single channel conductance and average kinetic parameters in Kv2.1 and mutants

<table>
<thead>
<tr>
<th></th>
<th>γ, pS</th>
<th>t_{open}, ms</th>
<th>t_{closed}, ms</th>
<th>t_{burst}, ms</th>
<th>t_{interburst}, ms</th>
<th>Latency, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv2.1</td>
<td>10.0 ± 0.2</td>
<td>13.9 ± 0.7</td>
<td>0.90 ± 0.10</td>
<td>22.4 ± 1.5</td>
<td>17.5 ± 2.3</td>
<td>21.7 ± 3.1</td>
</tr>
<tr>
<td>Kv2.1 + MMTS</td>
<td>8.2 ± 0.7</td>
<td>13.0 ± 2.0</td>
<td>0.88 ± 0.04</td>
<td>24.4 ± 3.0</td>
<td>20.3 ± 3.6</td>
<td>88.6 ± 11.4</td>
</tr>
<tr>
<td>ΔN</td>
<td>9.2 ± 0.3</td>
<td>15.8 ± 1.4</td>
<td>0.78 ± 0.04</td>
<td>27.4 ± 2.7</td>
<td>12.3 ± 1.0</td>
<td>69.2 ± 6.7</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4–8 independent measurements. Cell-attached single channel activity was recorded in presence of depolarizing potentials from negative holding potentials as described in MATERIALS AND METHODS. Conductance (γ) measurements were obtained from slope of outward current-voltage relationships. Open (t_{open}), closed (t_{closed}), burst (t_{burst}), and interburst times (t_{interburst}) and first latencies were monoeXponentially distributed and obtained as described in MATERIALS AND METHODS.
rate of inactivation of Kv2.1 channels, as revealed by long depolarizing pulses (Fig. 7). Inactivation time constants increased for Kv2.1 and transmembrane core cysteineless currents after application of MMTS. Similar results were obtained in oocytes expressing ΔC channels (not shown). In contrast, ΔN currents displayed little inactivation under prolonged depolarizations (up to 20 s) and were only scaled down by MMTS (Fig. 7). Therefore, these experiments do not indicate whether the effects on Kv2.1 inactivation were also mediated through modification of the NH2 terminus or arose from reaction with cysteine(s) located in another domain. Nevertheless, the observation that the ΔN deletion abolished inactivation suggests a central role of the NH2 terminus in the slow inactivation of Kv2.1, as previously noted (28).

DISCUSSION

Effects of Kv2.1 sulfhydryl modification. Reaction of Kv2.1 with cysteine-specific MMTS caused a pronounced and irreversible slowing of channel kinetics. We centered our studies on the modulation of activation by MMTS, because 1) all Kv2.1 cysteines are located outside S4, a central component of the voltage sensor, indicating that other channel regions not yet identified must be involved in the effects of MMTS and may, therefore, participate in gating, and 2) as summarized by Caputo et al. (4), most studies on chemical modification of native excitable membranes using sulfhydryl reagents report (somewhat discrepant) results on K+ channel inactivation, with less emphasis on activation. The findings presented suggest that MMTS modulates activation of Kv2.1 by reacting with one or more cysteines located at the NH2 terminus. Sequential cysteine-elimination mutagenesis results together with functional assay show that the presence of the hydrophilic NH2-terminal domain, which has been shown to be intracellular (29), is required for MMTS susceptibility. An alternative possibility is that the reactive cysteine(s) is located in an accessory or modulatory subunit that is present in the oocyte and associates with Kv2.1. However, there is no evidence that Kv2.1 interacts with other subunits when expressed in oocytes. Therefore, we favor the proposal that the effects of MMTS are due to direct reaction with the NH2 terminus of the channel. On the basis of the slowness of the reaction when MMTS is extracellularly applied and the finding that a charged MMTS homolog (MTSEA) readily reacts when applied to the intracellular but not the extracellular side of the membrane, we infer that the NH2-terminal target is exposed to and preferentially accessible from the cytoplasmic phase.

VanDongen et al. (28) reported that extensive deletions at the NH 2 terminus of Kv2.1 were associated with marked changes in whole cell current kinetics that were concomitant with a drastic decrease in current expression levels, raising the concern that the structural integrity of the channel had been globally affected. The single channel analysis results reported here, however, disprove that possibility. NH2-terminal deletion and chemical modification by MMTS cause specific and similar changes in a set of channel properties but preserve all others, indicating that 1) the overall structure of the protein is most likely unchanged in both cases and 2) the effects of deleting the NH2-terminal domain resemble those obtained by modification of reduced cysteine(s) located in it. Because single channel conductance and open probability after first opening are largely preserved, we conclude that the reduced whole cell current levels observed with the ΔN construct arise from a reduction in the number of channels that populate the plasma membrane.

The mechanism responsible for the delay of activation observed in Kv2.1 channels after reaction with MMTS and the slow activation of ΔN channels appears to be an increase in the latency to first opening, which was invariant over the potential range studied. Open and closed times after first opening and before inactivation were virtually unaffected by MMTS. Therefore, in the presence of depolarizing potentials, alterations at the NH2-terminal domain influence only transitions in the activation pathway before first opening (2, 8) but...
have little influence after opening and until the channel sojourns in the inactivated state. The fact that in MMTS-treated channels the latency to first opening was prolonged regardless of voltage may suggest that MMTS modulates a voltage-insensitive channel transition, but it is also possible that MMTS slows the rates of interconversion between two or more states in voltage-dependent segments of the activation pathway of the channel. In either case, the modification does not seem to affect the stability of the fully activated channel while depolarization is maintained. On the other hand, the observed slowing of tail currents under repolarizing potentials induced by MMTS reaction indicates that at negative potentials the open state of the channel has been stabilized. Further channel kinetic analyses are required to characterize the steps in the activation pathway of Kv2.1 and the manner in which MMTS affects their rates of interconversion by reacting with the NH2 terminus.

A separate effect of MMTS was a small decrease in current amplitude, which was verified by single channel conductance measurements and could be restored by reaction with the reducing agent DTT. The conductance of Kv2.1 and \( \Delta N \) channels was similar in control conditions (10.0 vs. 9.2 pS), MMTS reduced the single channel conductance of Kv2.1 by 15%, whereas a similar reduction was observed after analysis of MMTS-treated \( \Delta N \) whole cell currents, despite the lack of an effect on channel kinetics. Additional support for the similarities in structure and function of both channel pores is provided by their analogous instantaneous I-V profiles. Taken together, these results indicate that a minimum of two cysteines are involved in MMTS action on Kv2.1: one associated with the NH2 terminus and accessible from the cytoplasm and another outside the NH2 terminus, which can react with DTT after modification by MMTS and possibly lines the conduction pathway. A likely candidate for the latter residue is C393, which is located in S6 and has been proposed to lie near the pore (33).

Evidence for a dual effect of oxidizing agents on delayed rectifier currents was also provided by the experiments of Caputo et al. (4) on native squid axon channels using PHMPS. PHMPS causes a slowing of channel kinetics and a reduction in single channel conductance that are remarkably similar to our findings with cloned Kv2.1. In addition, a decrease in open probability accompanies these effects. Although the latter phenomenon was not observed in Kv2.1, we propose that the NH2-terminal domain of squid K+ channels may be responsible for the modulatory effects of PHMPS on channel kinetics and that it plays a similar role in activation, and we infer that participation of the NH2-terminal domain in activation gating is a generic feature of Kv2-related channels. In contrast, cysteine replacement (3), MMTS exposure (14), or deletions (7, 26) at the NH2 terminus of Shaker channels do not affect activation gating, suggesting a divergent functional specialization.

Mutational evidence argues against the involvement of disulfide bonding between NH2-terminal residues and the rest of the channel protein as the mechanism underlying gating modulation. According to that view, hypothesized by Ruppersberg et al. (22), oxidation of cysteine residues at the NH2-terminal segment involved in fast inactivation of A-type K+ channels would uncouple the inactivating domain from its receptor by preventing the formation of cystine. Although a significant fraction of the cysteines in solved structures is found bridging to remote sites in the primary amino acid sequence (5), in our experiments with Kv2.1, point or combined elimination of all cysteines in regions downstream of the NH2 terminus did not have significant effects on channel activation or on the ability of MMTS to affect gating. These results suggest that disulfide bonding to residues located elsewhere in the channel protein is not required for the participation of the NH2 terminus in gating. The observation that MMTS and MTSEA produce similar effects, despite their differently charged attaching groups (0 and +1, respectively), raises the possibility that the interference of modified cysteines with the gating process is caused by steric hindrance in a region subject to a conformational rearrangement during gating.

**Contribution of the NH2-terminal domain to K+ channel function.** The two hallmark aspects of voltage-gated channel function are selective ion conduction and control of gating by membrane potential. The developments brought about by mutational and functional analysis in the past 10 years have led to the notion that both properties are specified in evolutionarily conserved protein motifs. Whereas ion selectivity appears to arise from interactions with amino acids in the P region, voltage-dependent gating requires the presence of the amphipatic domain S4. Conversely, the modulation of these properties and processing of channel subunits by the cell reside in less conserved regions, allowing for a wide phenotypic diversity.

The NH2-terminal domain of voltage-gated K+ channels is variable in length and amino acid composition. Nevertheless, key roles are specified in it. The compatibility of NH2-terminal (together with other) regions allows subunit association to form functional tetrameric channels (23, 29). Heteromeric assembly of subunits with compatible recognition domains is a mechanism used by cells to diversify K+ currents. Fast channel closing by N-type inactivation is provided by specialization of the first residues of numerous K+ channels into a tethered pore-blocking particle (7). The presence of intracellularly exposed cysteine residues associated with the blocking particle potentially allows for modulation by the redox state of the cytoplasmic phase, a phenomenon that may have physiological relevance (22). Additionally, the NH2 terminus of Shaker-related channels provides a recognition region for modulatory (\( \beta \)) subunits that affect the kinetics of channel inactivation (32).

Our findings illustrate a different aspect of the regulation of K+ channel activity by the NH2-terminal domain: modulation of activation. Recent results that voltage-sensing residues in S4 are accessible from the cytoplasmic phase (11, 31) and that charge transfer by
phosphorylation offsets voltage-dependent parameters of channel gating (20) indicate that the membrane electric field sensor is susceptible to modulation from the cytoplasmic compartment. Our conclusion that the participation of the NH2 terminus of Kv2.1 in gating can be altered by chemical modification from the cytoplasmic phase is best explained in the context of a gating apparatus involving several domains in addition to S4 and subject to intracellular modulation.

We are indebted to Arthur Karlin for generosity and suggestions on the manuscript. We thank Mykle Akabas, Francisco Bezanilla, Arthur Karlin, David Patton, and Antoninus VanDongen for discussion of the results and Wei-Qiang Dong for oocyte culture and injection.

J. M. Pascual was supported in part by National Institutes of Health Neurological Sciences Academic Development Award NS-01698. C.-C. Shieh was funded in part by a grant-in-aid from the National Institute of Neurological Disorders and Stroke Grants NS-29473 to G. E. Kirsch and NS-23887 to A. M. Brown.

A preliminary account of these results has been reported in abstract form (18).

Address for reprint requests: J. M. Pascual, Department of Neurology, Box 164, Neurological Institute of New York, 710 W. 168th St., New York, NY 10032.

Received 29 April 1997; accepted in final form 25 July 1997.

REFERENCES


