Conformational Changes in Kir2.1 Channels during NH$_4^+$-induced Inactivation

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We have shown previously that NH$_4^+$ binding to the external pore of a Kir2.1 channel induces channel inactivation possibly through conformational changes. In this study, we performed further biophysical analyses of the NH$_4^+$-induced inactivation modeled by a refined kinetic scheme. Also, we investigated the conformational change hypothesis by examining whether the chemical modification of single-cysteine substitution of amino acids located at the internal pore alters the kinetics of the NH$_4^+$-induced inactivation. In addition, we examined whether the mutation of amino acids located at various parts of a Kir2.1 channel influences the NH$_4^+$-induced inactivation. Kir2.1 channels were expressed in Xenopus oocytes and studied using patch-clamp techniques. The gating of the NH$_4^+$-induced inactivation was affected by mutation of several amino acids located at various regions of the Kir2.1 channel. These results suggest that amino acids from different parts of a Kir2.1 channel are involved in the channel closure. Furthermore, internal chemical modification of several cysteine mutants resulted in the block of inward currents and changes in the on and off rate for the NH$_4^+$-induced inactivation, suggesting that the internal pore mouth is involved in the closure of a Kir2.1 channel. Taken together these results provide new evidence for conformational changes affecting the NH$_4^+$-induced inactivation in the Kir2.1 channel.

Ion channels are membrane proteins that interact closely with permeant ions. Therefore, it is conceivable that the structures of these proteins may assume different conformations during different functional states, such as in the opening and closing of ion channels. In inward rectifier K$^+$ channels, the most studied gating mechanism is the membrane voltage ($V_m$)$^{1,2}$-dependent channel block by internal Mg$^{2+}$ and polyamines (1–4). This $V_m$-dependent block results in inward rectification, which contributes to the physiological functions of these channels. However, little is known whether structural changes are involved in the functions of inward rectifier K$^+$ channels. It has been shown that the gating of these channels depends on permeant ions (5, 6), and most recent evidence supports that gating may be attributed to conformational changes resulting from the interaction between permeant ions and the backbone carbonyls in the selectivity filter of the cloned Kir2.1 channels (7). Our previous study demonstrated that external NH$_4^+$ induces the Kir2.1 channel into fast inactivation during hyperpolarization (8). We showed that the NH$_4^+$-induced inactivation is not because of the NH$_4^+$ block of Kir2.1 channels. Furthermore, studies in the R148Y mutant suggest that one or both of the two binding sites located at the external pore mouth are involved in the NH$_4^+$-induced inactivation. Because the binding site is located outside the electrical field (9), and yet the inactivation is $V_m$-dependent, we propose that a $V_m$-dependent process occurs within the pore to effect channel closure.

Even though evidence supports the involvement of conformational changes in the gating of the Kir2.1 channel, there remain doubts challenging the hypothesis because of the lack of an intrinsic $V_m$ sensor in these channels. In this study we further analyzed the biophysical properties of the NH$_4^+$-induced inactivation. We found that its gating shares several similarities with that of a cloned Cl$^-$ channel, CIC-0. The gating in both channel types depends on $V_m$, concentrations of permeant ions, and is described by a Boltzmann distribution with a non-zero offset (10–12). It has also been demonstrated that an intrinsic $V_m$ sensor is not required in the gating of the CIC-0 channel. The $V_m$ dependence of gating arises from an intrinsically $V_m$-dependent conformational change induced by the $V_m$-independent binding of Cl$^-$ to the channel (12). Based on the model for the gating in the CIC-0 channel, we propose a non-equilibrium kinetic scheme to account for the NH$_4^+$-induced inactivation in the Kir2.1 channel.

Also, we further suggest that if global conformational changes are indeed involved in the NH$_4^+$-induced inactivation, several amino acids lining the pore of a Kir2.1 channel should participate in the $V_m$-dependent process preceding to channel closure. We tested this hypothesis using two approaches. First, we examined whether the mutation of amino acids located at different parts of the Kir2.1 channel influences the gating of the NH$_4^+$-induced inactivation. Second, we investigated whether the kinetics of the NH$_4^+$-induced inactivation is altered by MTSET modification of cysteine mutants whose mutation is located at the internal pore. Our results show that the mutation of several amino acids located at different part of the Kir2.1 channel indeed changed the gating of the NH$_4^+$-induced inactivation. In addition, chemical modification at the internal pore mouth reduced the kinetics of the NH$_4^+$-induced inactivation.

Kinetic studies have provided us with bountiful information on how the inward rectifier K$^+$ channels operate to serve their functions. However, we have very little information on their underlying structures mainly because of technical limitations. The NH$_4^+$-induced inactivation provides us with a model to
study the structural-functional relationship within the Kir2.1 channels.

EXPERIMENTAL PROCEDURES

Molecular Biology and Preparation of Xenopus Oocytes—Site-directed mutations were generated in the wild-type channel (IRK1 clone) using the Altered Sites II in vitro mutagenesis systems (Promega, Madison, WI). The cysteine mutants were constructed in the IRK1J clone. Purification of cDNA, and in vitro T7 or SP6 transcription reactions (mMessage mMMachine; Ambion, Dallas, TX) were performed as described previously (13). Xenopus oocytes were isolated by partial ovariectomy from frogs anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester). The incision was sutured, and the animal was monitored during the recovery period before it was returned to its tank. Following the last oocyte collection, frogs were anesthetized with 0.1% of tricaine and sacrificed by decapitation. All surgical and anesthetic procedures conformed to national ethics committee guidelines.

Electrophysiology Techniques—Currents were recorded from inside-out patches at room temperature using the giant and single-channel patch-clamp techniques (14, 15) with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). The resistance of the electrode pipette ranged from 0.15 to 0.25 megohms for giant patch recordings and from 1 to 3 megohms for single-channel recordings when filled with the electrode solutions. The [NH₄⁺] solutions contained the following (in mM): NH₄Cl (10–300), N-methyl-d-glucamine (0–100), EDTA (5), and HEPES (5), at pH 7.4. In the experiments of ionic strength, both the external and internal solutions contained the following (in mM): NH₄OH (15), sucrose (200), EDTA (5), and HEPES (5), at pH 7.4. The 100 mM [K⁺] solution contained the following (in mM): KCl+KOH (100), EDTA (5), and HEPES (5), at pH 7.4. Rundown of channel activity was delayed by treating inside-out patches with L-α-phosphatidylinositol-4,5-bisphosphate (Sigma) (13, 16). MTSET (Toronto Research Chemicals, North York, Ontario, Canada) was made as a stock in water each day, stored at −20 °C, and diluted in bath solution immediately before application.

The command V_m and data acquisition functions were processed using a Pentium computer, a DigiData board, and pClamp6 software (Axon Instruments, Foster City, CA). Data sampling rates were 2.5–5 kHz, and the data were filtered at 0.5–1 kHz with an 8-pole low pass filter (Frequency Devices, Rochester, NY). In the experiments of V_m-dependent inactivation, the holding potential was 0 mV, prepulses ranged from −300 to +300 mV, and the test V_m was −120 mV. Single-channel currents were recorded at −140 mV from a holding potential of 0 mV. The open and closed events obtained from voltage steps have exactly the same distributions as those obtained from the steady state (8, 17). Capacitive currents were corrected using the built-in capacitance neutralization in the Axopatch 200A amplifier.

Data Analysis—Instantaneous currents were determined by fitting monoeponential functions to the currents at the test pulses and by extrapolating them to their beginnings. Histograms of the duration of time that channels remained open and closed were constructed with square root-log ordinates (13). The histograms were fit to monoeXponential functions with the maximum log likelihood method, and, in general, biexponential functions did not provide significantly better fits (p > 0.05) than monoeXponential functions, as judged by the maximal likelihood ratio test (4). Substates were observed, but they did not occur frequently. Transitions between the open state and substate, as well as between the closed state and substate, were not included in data analysis.

The time course of current inhibition of mutants by MTSET followed single exponential decay. Time constants for MTSET modification were obtained by fitting the time courses of current inhibition. The apparent second-order rate constants for MTSET modification were then calculated as the reciprocal of the respective time constants divided by the concentration of MTSET. Results are presented as mean ± S.E.

RESULTS

Biophysical Properties of the NH₄⁺-induced Inactivation—Previously, we have examined NH₄⁺-induced inactivation with the voltage protocol described in Fig. 1A. In this study we analyzed further the gating properties of the NH₄⁺-induced inactivation. Fig. 1B illustrates the representative currents recorded in 10 and 100 mM symmetrical [NH₄⁺] respectively. Inward currents inactivated during strong hyperpolarization and the rate of the inactivation was higher in 100 mM [NH₄⁺] than that in 10 mM [NH₄⁺]. Note that some residual capacitive currents were not corrected in the currents recorded in 10 mM [NH₄⁺]. The degrees of inactivation, however, were actually the same for both 10 and 100 mM [NH₄⁺] (see Fig. 2A). Instantaneous tail currents were recorded after prepulses to various test voltages. The steady-state open probability was quantified by normalizing these tail currents to the maximal one obtained following the most depolarizing prepulse potential (normalized I). Fig. 2A shows that normalized I was smaller at more negative V_m. Changes in symmetrical [NH₄⁺] did not affect the normalized I-V_m relationship. The normalized I-V_m relationship was fitted with a Boltzmann distribution containing a non-zero offset. The effective gating charge is around 0.7, and the non-zero offset is about 0.2 for all [NH₄⁺] tested. Because the time course of inactivation could be fitted to a monoeXponential function, the rate of inactivation was calculated as the reciprocal of the time constant (τ). The rate of inactivation depended on V_m and [NH₄⁺] (Fig. 2B).

The single Kir2.1 channel demonstrates one open and one closed state in our previous study where we proposed that the
FIG. 2. Kinetics of the NH$_4^+$-induced inactivation in various [NH$_4^+$]. A and B, the $V_m$ dependence of normalized I and rate of the inactivation in 10 (●, $n = 5$), 30 (○, $n = 5$), 50 (▲, $n = 5$), 100 (◇, $n = 5$), 200 (■, $n = 4$), and 300 mM [NH$_4^+$] (□, $n = 7$). Solid curves are the fit of data to a Boltzmann equation, $p_{\min} + (1 - p_{\min})(1 + \exp(-zF(V - V_{0.5})/RT))$. C and E, $V_m$ dependence of $k_{on}$ and $k_{off}$. Values of $k_{on}$ and $k_{off}$ were...
NH$_4^+$-induced inactivation could be described by Scheme 1 (8).

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\begin{align*}
\text{O} & \xrightarrow{k_{\text{on}}} \text{I} \\
& \xleftarrow{k_{\text{off}}} 
\end{align*}
\]

Fig. 2C demonstrates that $k_{\text{on}}$ increased exponentially with hyperpolarization with an effective gating charge $\sim 0.4$. The rate of inactivation increased with elevated [NH$_4^+$] and saturated in $-100$ mM [NH$_4^+$], indicating that NH$_4^+$ binding is involved in the inactivation. Fig. 2D shows that the dose dependence of $k_{\text{on}}$ was similar at different $V_m$. Because NH$_4^+$ is itself a permanent ion through the Kir2.1 channel, the entering and leaving of the ion at both sides of the membrane complicates the analysis of $k_{\text{on}}$. However, the $V_m$ dependence of the $k_{\text{on}}$ is the same at both low and saturating [NH$_4^+$] levels, suggesting that the effective gating charge (0.4) is intrinsic to the on rate of the NH$_4^+$-induced inactivation. On the other hand, $k_{\text{off}}$ did not show $V_m$ dependence (Fig. 2E). Increasing symmetrical [NH$_4^+$] also accelerated $k_{\text{off}}$ to the same degree as it did to $k_{\text{on}}$ (Fig. 2F). Because steady-state open probability is equal to $k_{\text{on}}/(k_{\text{on}} + k_{\text{off}})$, the same [NH$_4^+$] dependence of $k_{\text{on}}$ and $k_{\text{off}}$ accounts for the same normalized I-$V_m$ relationships in various symmetrical [NH$_4^+$] shown in Fig. 2A. Fig. 2, D and F shows that the dependence of $k_{\text{on}}$ and $k_{\text{off}}$ on [NH$_4^+$] are about the same, and both are not $V_m$-dependent, suggesting again that the NH$_4^+$ binding site affecting inactivation is located outside the electrical field (possibility at the external pore mouth according to our previous study (8)).

In summary, our results show that the NH$_4^+$ binding site affecting inactivation is located outside of the electrical field yet the inactivating process is $V_m$-dependent. Also, $k_{\text{on}}$ and $k_{\text{off}}$ both depend on [NH$_4^+$]. Previously, we demonstrated that blocking rate would be too slow to account for NH$_4^+$ acting as a permanent blocker, and the inactivation is dependent on external rather than on internal NH$_4^+$ (8). Taken together our data suggest that the external NH$_4^+$-induced inactivation is because of the conformational changes of the Kir2.1 channels.

**NH$_4^+$-induced Inactivation Is Affected by Mutation of Amino Acids Ranging from the External to Internal Pore Mouth**—To test whether conformational changes are involved in the NH$_4^+$-induced inactivation, we first examined whether the NH$_4^+$-induced inactivation is affected by mutation of amino acids located at different parts of a Kir2.1 channel. We first examined whether the amino acids (Glu-125, Ile-137, and Thr-141) involved in Ba$^{2+}$ binding within the Kir2.1 channel (18, 19) may function in the NH$_4^+$-induced inactivation. Fig. 3 shows that the NH$_4^+$-induced inactivation in the E125N and I137L mutants is similar to the wild-type channels whereas it was greatly reduced in the T141V mutant. We also included our previous recording in the E145Y mutant (8), which shows little NH$_4^+$-induced inactivation.

To test whether the NH$_4^+$-induced inactivation involves amino acids located at different parts of a Kir2.1 channel, we next recorded currents through mutants whose mutation was located at the internal pore mouth. Both Asp-172 and Ghu-224 have been shown to be accessible to internal polyamines and Mg$^{2+}$ and thus are reckoned to be located at the internal pore mouth (3, 4, 20). Fig. 3 shows that the rate of the NH$_4^+$-induced inactivation in the D172N mutant was increased (see also Fig. 4C). Both the rate and degree of the NH$_4^+$-induced inactivation were reduced in the E224G mutant.

Fig. 4 summarizes the normalized I-$V_m$ relationship (Fig. 4A) and the $V_m$ dependence of the kinetic parameters (Fig. 4, B–D) obtained in the wild type and mutants. Except for the T141V mutant whose kinetics of macroscopic currents was not analyzed, $k_{\text{on}}$, of all mutants showed similar $V_m$ dependence as the wild-type channels. Fitting the $k_{\text{on}}$-$V_m$ relationship with a Boltzmann equation, we obtained $k_{\text{on}}$ and the effective gating charge ($z_{\text{on}}$), which are listed in Table I. The $k_{\text{on}}$ value at 0 mV, $k_{\text{on}}$ (0), was decreased about 2-fold in the E125N and E224G mutants. Values of $z_{\text{on}}$ ranged from 0.32 to 0.45 and do not seem to change dramatically although the change in the E125N mutant is statistically significant compared with the wild-type channels.

Table I also lists the parameters obtained from fitting the normalized I-$V_m$ relationships to Boltzmann distributions. The major findings shown in Fig. 4 and Table I are as follows. First, the degree of the NH$_4^+$-induced inactivation was dramatically reduced in the T141V mutant. Second, the rate of NH$_4^+$-induced inactivation was accelerated in the mutant D172N but was decreased in the E224G mutant. Third, the gating charge was significantly decreased in the E224G mutant. Fourth, $V_{0.5}$ was shifted toward negative $V_m$ in the E125N and E224G mutants.

Table I shows that the gating charge in the E224G mutant was reduced significantly. Recently, E224 has been shown to screen surface charge, thereby affecting ion conduction (21). We next examined whether surface-charge screening affects the gating properties of the NH$_4^+$-induced inactivation by comparing the inactivation in different ionic strengths. Fig. 5A shows the currents recorded from the wild types exposed to symmetrical 15 mM [NH$_4^+$] (the minimal [NH$_4$OH] added to keep the pH at 7.4 in the presence of 5 mM EDTA) and 200 mM sucrose. The normalized I-$V_m$ (Fig. 5B) relationship was identical to those exposed to solutions with larger ionic strength (Fig. 2A). These results suggest that the decrease in gating charge in the E224G mutant is not because of the reduced screening of surface charge. However, the rate of inactivation (Fig. 5C), $k_{\text{on}}$ (Fig. 5D), and $k_{\text{off}}$ (Fig. 5E) were all larger than those obtained in the wild types exposed to 10–50 mM [NH$_4^+$] plus 100 mM N-methyl-D-glucamine (Fig. 2), suggesting that reducing ionic strength (less surface-charge screening) increases the kinetics of the NH$_4^+$-induced inactivation. In summary,

calculated from Equations 1 and 2 and are plotted against $V_m$. The solid lines were the fit of the data to a Boltzmann equation, $k_{\text{on}} = k_{\text{on}}(0) \times \exp(-z_{\text{on}}FV_m/RT)$, where $k_{\text{on}}(0)$ is the on rate at 0 mV, $z_{\text{on}}$ is the gating charge, and $F$, $R$, and $T$ have their usual meanings. The $k_{\text{on}}$(0) and $z_{\text{on}}$ were as follows: 2.04 s$^{-1}$ and 0.39 in 10 mM [NH$_4$OH], 2.31 s$^{-1}$ and 0.45 in 30 mM [NH$_4$OH], 3.09 s$^{-1}$ and 0.43 in 50 mM, 5.3 s$^{-1}$ and 0.39 in 100 mM, and 4.7 s$^{-1}$ and 0.42 in 200 mM. D and F, dose dependence of $k_{\text{on}}$ and $k_{\text{off}}$. The solid lines in D were the fit of the data to a Hill equation in the form of $(v/v_0 + K_{CH}/[NH_4^+]$), $\beta$ and $K_c$ were as follows: 45.0 s$^{-1}$ and 32.4 at $V_m = -200$ mV (■), $10.1$ s$^{-1}$ and 22.3 at $V_m = -180$ mV (●), 77.2 s$^{-1}$ and 27.5 at $V_m = -160$ mV (□), 53.4 s$^{-1}$ and 26.0 at $V_m = -140$ mV (△), 39.5 s$^{-1}$ and 28.9 at $V_m = -120$ mV (▲). The solid lines in F were the fit of the data to a Hill equation in the form of $(\beta/\beta_0 + K_{CH}/[NH_4^+]$, $\beta$ and $K_c$ were as follows: 45.0 s$^{-1}$ and 32.4 at $V_m = -200$ mV, 39.5 s$^{-1}$ and 29.7 at $V_m = -180$ mV, 39.5 s$^{-1}$ and 29.7 at $V_m = -160$ mV, 36.7 s$^{-1}$ and 25.1 at $V_m = -140$ mV, 38.6 s$^{-1}$ and 24.3 at $V_m = -120$ mV.
Mutation of amino acids lining the channel pore but located at various portions of the Kir2.1 channel affects the gating for the NH\(_4^+\)/H11001-induced inactivation.

**Single-channel Kinetics of NH\(_4^+\)/H11001-induced Inactivation**—The NH\(_4^+\)/H11001-induced inactivation was reduced to a large degree in the T141V mutant. Because the degree of inactivation was very small the gating and kinetic values could not be determined reliably using Equations 1 and 2. To further determine how the kinetics of the NH\(_4^+\)/H11001-induced inactivation is affected in this mutant, we carried out single-channel recordings. Fig. 6 shows the single-channel traces and the corresponding histograms of open and closed dwell times of the wild type and T141V mutant recorded at \(-140\) mV in 100 mM symmetrical [NH\(_4^+\)]. The distributions of open and closed dwell times were well fitted to monoexponential functions. The values of \(k_{on}\) and \(k_{off}\) were then calculated as the reciprocals of fit time constants and are summarized in Table II. A 10-fold increase in \(k_{off}\) is the primary contributor to the almost abolished NH\(_4^+\)-induced inactivation.

**Fig. 3.** NH\(_4^+\) currents through the wild-type channels and various mutants. Current traces were obtained from inside-out patches exposed to 100 mM symmetrical [NH\(_4^+\)]. \(I_{inst}\) indicates the instantaneous current.

**Fig. 4.** Summaries of the kinetics of the NH\(_4^+\)-induced inactivation in the wild type and mutants. A and B, normalized I-\(V_m\) and rate-\(V_m\) relationships for the wild type and mutants. C and D, the \(k_{on}-V_m\) and \(k_{off}-V_m\) relationships for the wild type and the mutants. The solid lines were the fit of the data to a Boltzmann equation, \(k_{on} = k_{on}(0) \times \exp(z_{on}FV_m/RT)\). Both \(k_{on}\) and \(z_{on}\) are listed in Table I.
in the T141V mutant. These results suggest that several amino acids ranging from the external to the internal pore mouth of the Kir2.1 channel are involved in the NH4+/H11001-induced inactivation, which is thus likely because of global conformational changes of channel structure.

Chemical Modification of Substituted Cysteines in the Inner Pore Reduces Current and Alters the Kinetics of NH4+/H11001-induced Inactivation—To further confirm that the internal pore is involved in the NH4+/H11001-induced inactivation initiated by external NH4+ binding, we next examined whether chemical blocking in the internal pore changes the kinetics of the NH4+/H11001-induced inactivation. Lu et al. (22) have shown that the wild-type Kir2.1 channel is sensitive to modifications by MTS reagents. Thus, an MTS-insensitive channel, IRK1J (C54V, C76V, C89I, C101L, C149F, and C169V), was constructed (22). Several single-cysteine substitutions (constructed in IRK1J) in the M2 domain of a Kir2.1 channel are sensitive to internal MTSET modification in 140 mM [K+/H11001], indicating that the substituted residues are located at the internal pore mouth (22). Therefore, we carried out experiments in these M2 cysteine mutants, as well as in an E224C mutant. Fig. 7A shows that, in 100 mM symmetrical [NH4+/H11001] MTSET did not reduce the current through IRK1J nor did it affect the inactivation process. Except for the I171C mutant, the MTSET modification increased the steady-state open probability during the test pulse (−120 mV) in all the other cysteine mutants. Also, MTSET modification signifi-

| Mutant   | \( p_{\text{min}} \) (mM) | \( Z \) | \( V_{0.5} \) (mV) | \( k_{\text{on},0} \) (s\(^{-1}\)) | \( Z_{\text{on}} \) |
|----------|-----------------|-------|-----------------|----------------|---------|
| WT       | 0.22 ± 0.02     | 0.72  | −96 ± 2         | 6.13 ± 0.43     | 0.37 ± 0.01 |
| E125N    | 0.17 ± 0.01     | 0.70  | −105 ± 2\(^{a}\) | 2.72 ± 0.15\(^{a}\) | 0.45 ± 0.03 |
| I137L    | 0.22 ± 0.01     | 0.64  | −101 ± 5        | 4.44 ± 0.44\(^{a}\) | 0.37 ± 0.02 |
| T141V    | n.r.d.          | n.r.d.| n.r.d.          | n.r.d.          | n.r.d.   |
| D172N    | 0.18 ± 0.02     | 0.73  | −95 ± 1         | 10.78 ± 2.43\(^{a}\) | 0.35 ± 0.03 |
| E224G    | 0.29 ± 0.02     | 0.54  | −117 ± 1\(^{a}\) | 3.23 ± 0.37\(^{a}\) | 0.36 ± 0.02 |

\(^{a}\) \( p < 0.05.\)

\(^{b}\) \( p < 0.001.\)

\(^{c}\) \( p < 0.01.\)

FIG. 5. Effects of reduced ionic strength on the NH4+/H11001-induced inactivation. A, currents recorded in 15 mM [NH4+] and 200 mM sucrose with the voltage protocol shown in Fig. 1A. B and C, the \( V_m \) dependence of normalized I and rate of inactivation. The solid curve is the fit of the data to Boltzmann equation described in the legend for Fig. 2. D and E, the effects of \( V_m \) on \( k_{\text{on}} \) and \( k_{\text{off}} \). The solid line is the fit of data to a Boltzmann equation (\( n = 4 \)).
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Fig. 6. Effects of mutation on the single-channel currents. Each panel shows a sample sweep at −140 mV and the histograms for opening and closing time for the indicated channel. Histograms are plotted with square root (sqrt)-log ordinates. The distributions of the open and closed times were fitted to monoeponential functions (continuous curves). Mean open and closed times obtained from the fitted curve are given above the histograms (n = 4–6).

Effects of mutation on k_{on} and k_{off} for the NH_{4}^{+}-induced inactivation

|         | WT (cal) | T141V   | WT (cal) |
|---------|----------|---------|----------|
| k_{on} (s^{-1}) | 54.9 ± 6.4 | 72.1 ± 2.4\(^a\) | 50.3 ± 3.5 |
| k_{off} (s^{-1}) | 33.2 ± 1.5 | 360.7 ± 40.8\(^b\) | 34.8 ± 1.7 |

\(^a\) p < 0.05.
\(^b\) p < 0.001.

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pore mouth thereby changing the kinetics of the NH_{4}^{+} may alter the flexibility of the Kir2.1 channel at the internal pore mouth. Thus, modification at the internal pore mouth reduced induced inactivation. Together these results suggest that the global changes of channel structure may be involved in the NH_{4}^{+}-induced inactivation.

State Dependence of MTSET Modification of Substituted Cysteines in the Inner Pore—Fig. 7, B and C shows that the MTSET bound in the inner pore at 0 mV (at which there is no NH_{4}^{+}-induced inactivation) affects the kinetics of the NH_{4}^{+}-induced inactivation in some cysteine mutants. However, it remains to be determined whether the structure of the inner pore is changed during the inactivation such that the accessibility of the MTSET to the substituted cysteines is state-dependent. To further probe the structural changes of the inner pore during the NH_{4}^{+} inactivation, we next measured the state-dependent rates of MTSET modification in different conformational states of the Kir2.1 channel. Data shown in Fig. 7 were obtained by measuring MTSET modification mainly during an open state (at least 95% of time in the open state with a pulse frequency of 0.5 Hz and duration of 100 ms). Next the rates of MTSET modification during the inactivated state were estimated by holding the patches at 0 mV and stepping to −120 mV (100 ms) at 5 Hz. Using this protocol the channels were thus held at −120 mV for 50% of the total recording time. Shown in Fig. 8 are these experiments, which were carried out in the cysteine mutants whose kinetics of the NH_{4}^{+}-induced inactivation are greatly affected by MTSET modification (Fig. 7, B and C).

Fig. 8A shows the time courses of MTSET modification obtained with a pulse frequency of 0.5 and 5 Hz, respectively, in the Q164C mutant exposed to 100 mM symmetrical [NH_{4}^{+}]. The rate of MTSET modification was slightly higher with a pulse frequency of 5 Hz. Because MTSET is positively charged it is conceivable that its accessibility to the substituted cysteine located in the pore is affected by V_{m}. As a control, we also measured the MTSET modification in the cysteine mutants exposed to 100 mM symmetrical [K^{+}], in which all the cysteine mutants do not show inactivation during hyperpolarization (data not shown) (23). Fig. 8B illustrates the time courses of MTSET modification obtained with a pulse frequency of 0.5 and 5 Hz, respectively, in the Q164C mutant exposed to 100 mM symmetrical [K^{+}]. The rate of MTSET modification was slower with a pulse frequency of 5 Hz. Fig. 8C shows the averaged rates of MTSET modification obtained in the Q164C, D172C, and I176C mutants in 100 mM [NH_{4}^{+}]. An increase of pulse frequency from 0.5 to 5 Hz did not significantly accelerate the rates of MTSET modification in the Q164C mutant (p > 0.11) although the rate seemed to be slightly higher at 5 Hz. The rates of MTSET modification in the D172C and I176C mutants
were not significantly changed ($p > 0.45$) by an increase of pulse frequency. The rate of MTSET modification was greatly accelerated in the D172C mutant exposed to 100 mM $\text{NH}_4^+$ compared with 100 mM $\text{K}^+$. The effect is not related to the $\text{NH}_4^+$-induced inactivation and is currently under investigation in our laboratory. Fig. 8D shows the averaged rates of MTSET modification in 100 mM symmetrical $\text{K}^+$. An increase of pulse frequency significantly decreased the rates of MTSET modification in the Q164C, D172C, and I176C mutants. MTSET modification in all the cysteine mutants used in this study could not be reversed by washout in the control solutions (100 mM $\text{NH}_4^+$ and 100 mM $\text{K}^+$). Considering the following factors, the rates of MTSET modification during the $\text{NH}_4^+$-induced inactivation may be increased in the Q164C, D172C, and I176C mutants. First, hyperpolarization significantly decreases the accessibility of MTSET to the pore of the Kir2.1 channel (Fig. 8D). Second, the channels spend only 50% of the entire recording time at $-120$ mV where the channels inactivate. Third, not all of the channels are in the inactivated state at $-120$ mV (40% for Q164C, 60% for D172C, 70% for I176C). Together these results suggest that MTSET modification may be state-dependent to a certain degree in the Q164C, D172C, and I176C mutants.

**DISCUSSION**

Refinement of the Kinetic Scheme for $\text{NH}_4^+$-induced Inactivation—Scheme 1 is simplified from Scheme 2, where $K_o$ is the dissociation constant for $\text{NH}_4^+$ binding in the open state, $\alpha$ is the on rate, and $\beta$ is the off rate between the open and inactivated transition. The simplification was made by assuming that the O and O-$\text{NH}_4^+$ states have the same conductance and that the binding step is very rapid compared with any subsequent conformational change. $k_{\text{off}}$ is also dependent on $\text{NH}_4^+$, suggesting that an empty inactivated state may exist. Scheme 2 is therefore modified as shown in Scheme 3, where $K_i$ is the dissociation constant for $\text{NH}_4^+$ binding in the inactivated state. The fitting of the normalized I-$V_m$ relationships to Boltzmann distributions with non-zero offsets suggests that the system is not an equilibrium one. Previous studies have demonstrated theoretically (24) and experimentally (10) that a non-equilibrium distribution of conformational states is created, if there exists a coupling of ion translocation and confor-
An essential requirement for the coupling of ion translation and conformational transition is that transitions between the two states can take place both in the empty and occupied state of the binding site (24). In other words, it is essential to introduce a transition between the O and I state. Therefore, Scheme 3 is further modified as shown in Scheme 4. Scheme 4 predicts that the observed \( k_{on} \) and \( k_{off} \) have the following relationships with \( K_{o1} \) and \( K_{o2} \), respectively, as illustrated by Equations 3 and 4.

\[
\begin{align*}
k_{on} &= \frac{[\text{NH}_4^+]_o}{[\text{NH}_4^+]_o + [\text{K}^+]_o} \Rightarrow \frac{[\text{O} \cdot \text{NH}_4^+]_o}{[\text{O} \cdot \text{NH}_4^+]_o + [\text{I} \cdot \text{NH}_4^+]_o} \\
k_{off} &= \frac{[\text{K}^+]_i}{[\text{K}^+]_i + [\text{I} \cdot \text{NH}_4^+]_i} \Rightarrow \frac{[\text{O} \cdot \text{NH}_4^+]_i}{[\text{O} \cdot \text{NH}_4^+]_i + [\text{I} \cdot \text{NH}_4^+]_i}
\end{align*}
\]

Fitting data shown in Fig. 2, D and F to Equations 3 and 4, we obtained one \( \alpha \) and one \( \beta \) (data not shown). Because the gating is induced by \( \text{NH}_4^+ \) binding \( \alpha_2 \) should be much less than \( \alpha_1 \). Thus, \( \alpha_2 \) is equal to zero. On other hand, we propose that \( \beta_1 = \beta_2 \) to incorporate the coupling of ion translocation and state transitions in Scheme 4.

Fig. 2A shows that the gating charge is around 0.7, and the non-zero offset is about 0.2. Both values are similar to that of ClC0 channels (12). The effective gating charge is severalfold smaller than that for voltage-gated cation channels (25). Similar to previous discussion (12), we also suspect that the source of the gating charge is the \( \text{NH}_4^+ \) ion itself, moving inwards during the \( (\text{O} \rightarrow \text{NH}_4^+ \rightarrow \text{I} \rightarrow \text{NH}_4^+) \) transition rather than the movement of charge intrinsic to the protein. The reason for this hypothesis is 2-fold. First, there are few charged amino acids lining the pore of a Kir2.1 channel. So far, D172 and E224 are the only two charged residues that are known to be located in the electrical field of the Kir2.1 channel. Neutralization of Asp-172 to a non-polar residue did not affect the gating charge (Table I). The gating charge in the E224G mutant is decreased but not completely eliminated. Second, the gating depends on \( \text{NH}_4^+ \) ions, which are permeant ions and thus efficient gating-charge carriers. Thus, we propose that the conducting ions move through the Kir2.1 channel, entering and leaving on both sides, and keeping the cycle under consideration above out of equilibrium. Unlike the ClC0 channel, which shows a clear time-asymmetric single-channel record arising from the double-barreled nature of the channel (10), we could not actually observe a time-asymmetric single-channel record in the Kir2.1, which is a single-pore channel. However, we can reason that a channel may assume two states. One state has the binding site accessible only from the external side, and the other state has the binding site accessible only from the internal side. In this case, in the presence of a large electrochemical gradient, the channel exhibits a carrier-like behavior. An \( \text{NH}_4^+ \) moves from high to low electrochemical potential with each turn of the conformational cycle. Scheme 5 illustrates such an imaginary process.

\[ \text{NH}_4^+(o) \text{ and } \text{NH}_4^+(i) \text{ denote the } \text{NH}_4^+ \text{ at the external and internal sides, respectively.} \]
Conformational Changes in Kir2.1 Channels

Effects of E125N on the kinetics of the NH₄⁺-induced inactivation may be gated by NH₄⁺ located close to the K⁺ selectivity filter GYG, which, according to the structure of KcsA channel, constitutes to the narrowest part to the channel (26). Therefore, it is possible that Thr-141 may also be part of the narrow pore filter and thus stabilizes the inactivated state in the Kir2.1 channel. Furthermore, the single-channel current of the T141V mutant is larger than that of the wild type (Fig. 5). As shown in Scheme 5, the NH₄⁺-induced inactivation may be gated by NH₄⁺ itself. The conducting ions move through the Kir2.1 channel, entering and leaving on both sides, and keeping the cycle under consideration out of equilibrium. An increase in single-channel conductance may further drive the cycle out of equilibrium in the direction of prompting the exit of the T141V mutants from the inactivated state. In other words, \( k_{\text{off}} \) depends on the ion translocation of NH₄⁺.

Therefore, the increase in \( k_{\text{on}} \) may result from the increase of single-channel conductance in the T141V mutant.

Of the all the mutants tested, E224G is the only one that shows a significant decrease in the gating charge. Recently, Glu-224 has been shown to screen surface charge, thereby affecting ion conduction (21). However, Fig. 5 shows that the gating properties are the same for the wild-type channels exposed to 15 mM [NH₄⁺] and 200 mM sucrose, as well as for those exposed to 10–50 mM [NH₄⁺] plus 100 mM N-methyl-D-glucamine. Therefore, the effect of E224G mutant on gating charge cannot be attributed to a decrease in surface charge screening. It is possible that Glu-224 contributes directly to the gating charge in the NH₄⁺-induced inactivation. On the other hand, the permeability for K⁺ in the E224G mutant has been shown to decrease (20). Therefore, it is also possible that the effect of Glu-224 on the gating charge is because of the change in conductance for NH₄⁺, which may be the gating charge itself.

The mutation at Glu-224 (E224G) decreases both the degree and \( k_{\text{on}} \) but does not affect \( k_{\text{off}} \) of the NH₄⁺-induced inactivation. Fig. 5, D and E shows that a decrease in ionic strength increases both \( k_{\text{on}} \) and \( k_{\text{off}} \). Therefore, the decrease of \( k_{\text{on}} \) cannot be attributed to a reduction in the screening of surface charge in the E224G mutant. Because NH₄⁺ binding is located at the external pore, the effects of mutation at position 224 are likely to be because of a decrease in the transition rate from the open to inactivated state instead of a decrease in NH₄⁺ binding.

The second line of evidence for the conformational changes hypothesis is that the MTSET modification decreases \( k_{\text{on}} \) and \( k_{\text{off}} \) in several cysteine mutants whose mutation is constructed at the internal pore. Our results are consistent with the hypothesis stating that the NH₄⁺-induced inactivation is because of conformational changes of Kir2.1 channels. MTSET modification changes the flexibility of the Kir2.1 channels, which then close and reopen in a rate that is different from the unmodified channels. Is it possible that the changes of \( k_{\text{on}} \) and \( k_{\text{off}} \) are because of the interaction of MTSET and NH₄⁺ in the pore? For example, an effect of MTSET can be because of competition of the MTSET with the NH₄⁺ bound at the external site. However, we consider this an unseemly possibility for the following reasons. First, we have shown previously that the NH₄⁺-induced inactivation is inconsistent with the permeant ion block mechanism. Second, the NH₄⁺ binding site is located at the external pore mouth, yet the cysteine mutation is positioned within the internal pore. Thus, it is unlikely that MTSET would compete with NH₄⁺ within the pore to decrease \( k_{\text{on}} \). Third, in all the cysteine mutants where \( k_{\text{off}} \) are decreased, \( k_{\text{on}} \) values are also decreased. Thus, our results are inconsistent with a direct competition (\( k_{\text{off}} \) should not be affected) or knock-off (in that case, \( k_{\text{off}} \) should increased by MTSET modification).

According to Scheme 5, \( k_{\text{on}} \) can be because of variations in NH₄⁺ binding affinity or the on-rate for inactivation or both. However, the cysteine-replacement is at the internal pore mouth, and the NH₄⁺ binding site is at the external pore mouth, so the effect of \( k_{\text{on}} \) observed in the cysteine mutants seems to indicate changes of the on-rate for the NH₄⁺-induced channel closure.

State-dependent modification of ion channels by MTST agents has been used previously to probe the conformational changes of proteins in different states (27–29). We showed here that the MTSET modification may also be state-dependent to a certain degree in the cysteine mutants located in the inner pore of the Kir2.1 channel. However, the rates do not seem to be affected dramatically during the NH₄⁺-induced inactivation, indicating that the major structural changes of Kir2.1 channels during the NH₄⁺-induced inactivation may be located further...
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externally to site 164, e.g. close to the selectivity filter (7) and site 141. The effects then of MTSET modification during the open state (Fig. 7) on the NH4+-induced inactivation may be propagated to the narrow pore whose closure is restricted, because the wider inner vestibule is held in a fixed place.

conclusions
In this study, we performed further biophysical analyses of NH4+-induced inactivation. We find that the NH4+-induced inactivation is a non-equilibrium system. The gating properties are similar to those of the Cl- dependent activation for the ClC0 channel. Also, we provide further evidence that conformational changes are probably proceeding to the closure of the Kir2.1 channels during the NH4+-induced inactivation based on the following results. First, the mutation of several amino acids located at different parts of a Kir2.1 channel changes the gating of the NH4+-induced inactivation. Second, chemical modification at the internal pore mouth reduces the rate of the NH4+-induced inactivation, which is initiated by the binding of NH4+ at the external pore mouth.

Although we have provided additional evidence supporting the relationship between structural changes and gating mechanism, more conclusive evidence still awaits a direct probe of the conformational changes in the Kir2.1 channel during inactivation. Furthermore, it remains to be defined how the amino acids, which are involved in the NH4+-induced inactivation, move to effect changes of the kinetics of the inactivation.
