Direct inhibition of the cloned Kv1.5 channel by AG-1478, a tyrosine kinase inhibitor

BOK HEE CHOI, JIN-SUNG CHOI, DUCK-JOO RHIE, SHIN HEE YOON, DO SIK MIN, YANG-HYEOK JO, MYUNG-SUK KIM, AND SANG JUNE HAHN

Department of Physiology, College of Medicine, The Catholic University of Korea, Socho-gu, Seoul 137-701, Korea

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THE MODULATION OF ION CHANNELS by the activation of protein kinases linked to second-messenger systems has been studied extensively (16, 19). A number of protein kinase inhibitors have been widely used to investigate the role of protein kinases in the modulation pathways of ion channels. However, recent studies have indicated that protein kinase inhibitors have a direct action on voltage- or ligand-gated ion channels and ion transporters in a phosphorylation-independent manner. Of the protein kinase C (PKC) inhibitors, calphostin C and staurosporine have been shown to directly block L-type Ca\(^{2+}\) channels (12) and muscarinic K\(^+\) channels (21), respectively. H-89, a protein kinase A (PKA) inhibitor, also has nonspecific and direct inhibitory effects on sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (15). Although protein phosphorylation by serine/threonine kinases is a major mechanism for the modulation of ion channels, tyrosine kinases also play an important role in regulating the activity of several ion channels (9, 16, 23). Similarly, a protein tyrosine kinase (PTK) inhibitor, genistein, directly inhibits glycine receptors (14) and K\(^+\) currents (25, 33) and directly activates Cl\(^-\) channels (34). The possibility that genistein and tyrphostin 23 directly block Ca\(^{2+}\) channels has also been discussed (4, 36).

In previous studies (5, 8), we found that staurosporine and another PKC inhibitor, bisindolylmaleimide, directly block cloned Kv1.3 and Kv1.5, respectively, without mediation through PKC inhibition or any diffusible cytosolic molecules. Recently, we have also reported (7) that H-89 inhibits cloned Kv1.3 channels independently of PKA-involved pathways. Therefore, we designed the present study to investigate the direct action of tyrphostin AG-1478, a PTK inhibitor, on cloned rat Kv1.5 expressed in Chinese hamster ovary (CHO) cells by using the whole cell patch-clamp technique.

MATERIALS AND METHODS

Stable transfection and cell culture. Rat brain Kv1.5 channels stably expressed in CHO cells (5) were used in the present study. The method for establishing Kv1.5 expression in CHO cells is briefly described as follows. Kv1.5 cDNA (29) was transferred into the plasmid expression vector pCR3.1 (Invitrogen, San Diego, CA). CHO cells were stably transfected with Kv1.5 cDNA by using FuGENE 6 reagent (Boehringer Mannheim, Indianapolis, IN). The transfected cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM hypoxanthine, 0.01 mM thymidine, and 0.2 mg/ml G418 (Life Technologies) under a 95% humidified air-5% CO\(_2\) environment at 37°C. The cultures were passaged every 4–5 days by open-channel block.
using a brief trypsin-EDTA treatment. The trypsin-EDTA-treated cells were seeded onto glass coverslips (diameter 12 mm; Fisher Scientific, Pittsburgh, PA) in a petri dish. After 12–24 h, the cell-attached coverslips were transferred to a continually perfused recording chamber (RC-13; Warner Instrument, Hamden, CT) for electrophysiological recordings.

**Electrophysiological recordings.** At room temperature (22–24°C), whole cell currents of Kv1.5 were recorded and stored by using the patch-clamp technique (11) with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) and an IBM compatible computer equipped with a Digidata 1200A acquisition board (Axon Instruments). Currents were sampled at 5 kHz and filtered at 2 kHz (4-pole Bessel filter). Pulse generation and data acquisition were controlled with pCLAMP 6.05 software (Axon Instruments). Patch electrodes were fabricated by using PG10165-4 glass capillary tubing (World Precision Instruments, Sarasota, FL). Liquid junction potentials between external and pipette solutions were offset. Whole cell capacitive current compensation and 80% series resistances compensation were done without any leakage compensation. Whole cell currents of ~1–4 nA and series resistances of 2–3 MΩ were used for the analysis.

**Solutions and drugs.** The pipette solution contained (in mM) 140 NaCl, 1 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 EGTA and was adjusted to pH 7.3 with KOH. The bath solution contained (in mM) 140 NaCl, 5 KCl, 1.3 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 glucose and was adjusted to pH 7.3 with NaOH. Genistein (10 μM; Calbiochem, San Diego, CA) was added to the bath solution for genistein-pretreatment experiments. Genistein, lavendustin A (Calbiochem), and AG-1478 (Sigma Chemical, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical) to yield stock solutions of 50 mM. The concentration of DMSO in the final solution was <0.1% and had no effect on Kv1.5 currents.

**Data analysis.** Data were analyzed by using pCLAMP 6.05 (Axon Instruments) and Origin 6.1 software (Microcal Software, Northampton, MA). The concentration-dependent curve for current inhibition by AG-1478 was fitted to the Hill equation

\[
\% \text{Control} = 1/[1 + (IC_{50}/[D])^n_H] \tag{1}
\]

where \%control is represented as the current in the presence of drugs divided by the current under control conditions, multiplied by 100, at the various drug concentrations [D]. IC_{50} is the concentration at half-maximal inhibition, and \(n_H\) is the Hill coefficient. Interaction kinetics between drug and channel are described on the basis of a first-order blocking scheme, as previously described (28). The apparent rate constants for binding (\(k_1\)) and unbinding (\(k_{-1}\)) were calculated from the following equation

\[
\tau_D = 1/(k_1[D] + k_{-1}) \tag{2a}
\]

\[
K_d = k_{-1}/k_1 \tag{2b}
\]

in which \(\tau_D\) is the drug-induced time constant, which was calculated from single exponential fits to the inactivating current traces during depolarization to 50 mV. The steady-state activation curves were fitted with the Boltzmann equation

\[
y = 1/[1 + \exp(-(V - V_{1/2})/k)] \tag{3}
\]

where \(k\) represents the slope factor, \(V\) is the test potential, and \(V_{1/2}\) is the potential at which the conductance was half maximal. The steady-state inactivation curves under control conditions and in the presence of drugs were obtained by using a two-pulse protocol; currents were induced by a 250-ms depolarizing pulse of 50 mV with 20-s preconditioning pulses from −60 to 10 mV by increments of 10 mV. The experimental data were fitted to the following equation

\[
(I - I_a)/(I_{max} - I_a) = 1/[1 + \exp(V - V_{1/2}/k)] \tag{4}
\]

in which \(I_{max}\) represents the current measured at the most hyperpolarized preconditioning pulse, \(I_a\) represents a non-zero current that is not inactivated at the most depolarized 20-s preconditioning pulse, and \(V, V_{1/2}, k\) represent the precluding potential, half-inactivation point, and slope factor, respectively. We eliminated the non-zero residual current by subtracting it from the actual value. The dominant time constant of activation was calculated by fitting a single exponential to the latter 50% of activation (5, 27, 35). The deactivation kinetics were also determined by a single exponential fitting.

Results are expressed as means ± SE. Student’s t-test and analysis of variance (ANOVA) were used for statistical analysis. A value of \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Reversible and direct inhibition.** As shown in Fig. 1A, Kv1.5 currents were rapidly activated under control conditions, with a sigmoidal time course rising to a peak, and then slowly inactivated, as reported previously (5). The dominant time constant of activation...
In the presence of 10 mM 
with a 250-ms depolarizing pulse from 
ed by AG-1478. In the presence of AG-1478, the 
fi
under control conditions was 1.46 ± 0.23 ms (n = 5) 
A: control current recorded 
a 30-min preincubation with 10 μM genistein and the current 
measured after a further 3-min treatment with 10 μM AG-1478. For 
this experiment, a bath solution containing 10 μM genistein was 
used. B: control current, the current recorded after a 10-min exposure 
to 10 μM lavendustin A, and the current measured after a 
control value (n = 5). Moreover, pretreatment with 10 μM genistein had no effect on the activation or inactivation kinetics of Kv1.5 compared with control currents (Fig. 2A). The addition of 10 μM AG-1478 to a bath solution containing 10 μM genistein induced an acceleration of inactivation with little effect on peak current amplitude (Fig. 2, A and C). The steady-state current amplitude at the end of a 250-ms depolarizing pulse was decreased to 52.9 ± 2.7% of control values (n = 5). Figure 2, B and C, show the effects of lavendustin A on the inhibition of Kv1.5 by AG-1478. A 10-min exposure to 10 μM lavendustin A did not induce any inhibition of Kv1.5. Subsequent application of AG-1478 (10 μM) reduced the steady-state current of Kv1.5 measured at the end of a depolarizing pulse of 50 mV to 51.9 ± 1.2% of control values (n = 5). The values for the inhibition by AG-1478 after pretreatment with genistein and lavendustin A were not significantly different from values for inhibition by AG-1478 alone (Figs. 1 and 2C). Furthermore, pretreatment with genistein and lavendustin A did not affect the kinetics of activation and inactivation of AG-1478-induced Kv1.5 currents (Table 1). Because the concentrations of PTK inhibitors (genistein and lavendustin A) used in our experiments were high enough to inhibit PTK completely, these results indicate that the PTK pathway is not involved in AG-1478-induced inhibition of Kv1.5 and that AG-1478 inhibits Kv1.5 currents by direct interaction with Kv1.5.

under control conditions was 1.46 ± 0.23 ms (n = 5) 
with a 250-ms depolarizing pulse from −80 to 50 mV. 
In the presence of 10 μM AG-1478, the time constant of 
avtivation was 1.42 ± 0.21 ms (n = 6), which indicates 
that the activation kinetics were not significantly modified 
by AG-1478. In the presence of AG-1478, the 
current was initially activated as under control conditions 
without significantly affecting the peak current amplitude. However, the slow inactivation was markedly accelerated, resulting in an apparent decrease in the steady-state current amplitude at the end of a 250-ms depolarizing pulse. Thus AG-1478 (10 μM) inhibited the steady-state current of Kv1.5 at the end of a depolarizing pulse of 50 mV by 54.9 ± 1.7% of the control value (n = 6). As shown in Fig. 1B, repeated bath perfusions of 10 μM AG-1478 induced a rapid and reversible inhibition of Kv1.5 currents at the end of a 250-ms depolarizing pulse. Kv1.5 inhibition appeared within 20 s of the drug being applied and reached a steady state within 3 min. The effect of AG-1478 was largely reversed following a 2-min washout. Under control conditions without AG-1478, the peak amplitude of Kv1.5 currents did not display any noticeable change over a 20-min period (90.0 ± 2.5% of the control value, n = 5). Although the current was not completely restored after washout of the drug (86.1 ± 1.3% of control values, n = 8), the degree of inhibition was not affected by repeated treatment with the drug. A comparison plot of the time course of Kv1.5 currents for an individual experiment in the absence and presence of AG-1478 is shown in Fig. 1B.

Kv1.5 has multiple consensus sites for phosphorylation by PKC, PKA, and PTK (29, 32). Indeed, it has been reported (13) that the human Kv1.5 channel is downregulated by tyrosine phosphorylation in HEK cells. We tested whether the inhibition of Kv1.5 by AG-1478 is mediated through PTK inhibition by using other PTK inhibitors with markedly different structures (genistein, lavendustin A). Preincubation with 10 μM genistein had no effect on the activation or inactivation kinetics of Kv1.5 (Table 1). The values for the inhibition by AG-1478 after pretreatment with genistein and lavendustin A were not significantly different from values for inhibition by AG-1478 alone (Figs. 1 and 2C). Furthermore, pretreatment with genistein and lavendustin A did not affect the kinetics of activation and inactivation of AG-1478-induced Kv1.5 currents (Table 1). Because the concentrations of PTK inhibitors (genistein and lavendustin A) used in our experiments were high enough to inhibit PTK completely, these results indicate that the PTK pathway is not involved in AG-1478-induced inhibition of Kv1.5 and that AG-1478 inhibits Kv1.5 currents by direct interaction with Kv1.5.

Table 1. Time constants of activation and inactivation with AG-1478 alone and in the presence of genistein or lavendustin A

<table>
<thead>
<tr>
<th>Drug</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG-1478</td>
<td>1.42 ± 0.21</td>
<td>40.75 ± 2.18</td>
</tr>
<tr>
<td>AG-1478 + genistein</td>
<td>1.28 ± 0.29</td>
<td>38.68 ± 1.93</td>
</tr>
<tr>
<td>AG-1478 + lavendustin A</td>
<td>1.34 ± 0.23</td>
<td>36.88 ± 2.77</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. The time constants of activation and inactivation were calculated as described in text. For the concentration for each drug, see Fig. 2.
Concentration-dependent inhibition. Figure 3A shows superimposed Kv1.5 current traces produced by a 250-ms depolarizing pulse to 50 mV under control conditions and in the presence of various concentrations of AG-1478. AG-1478 induced a reduction in the steady-state current amplitude during the depolarizing pulse, with little effect on the peak amplitude. The current amplitude measured at the end of a 250-ms depolarizing pulse was used as an index of inhibition. A nonlinear least-squares fit of the Hill equation (Eq. 1) to the individual data points yielded an IC50 value of 9.82 ± 1.07 μM and a Hill coefficient of 1.45 ± 0.23 (n = 6). Furthermore, AG-1478 induced a concentration-dependent increase in the rate of current decay. The traces of current decay at each concentration (10, 30, and 100 μM) of AG-1478 were well fitted to a single exponential function, which yielded a time constant (τD) for Kv1.5 current inhibition. Because the Kv1.5 channel undergoes an intrinsic slow inactivation during the depolarizing pulse, we disregarded the time constant values at low concentrations (3 μM) of AG-1478 in calculating a good approximation of the time constant for the development of drug-induced inhibition. Plotting τD at 50 mV against each concentration and fitting it to a hyperbolic equation (Eq. 2a) yielded binding (k1) and unbinding rate constants (k−1) of 1.46 ± 0.06 μM−1 s−1 and 10.19 ± 2.79 s−1, respectively (Fig. 3C). The theoretical Kd value derived by k−1/k1 (Eq. 2b) was 6.98 μM. Although the derived Kd of 6.98 μM is independent of the IC50 of 9.82 μM calculated from the concentration-response curve (Fig. 3B), the two values are reasonably close, suggesting that AG-1478-induced inhibition of the Kv1.5 channel follows a simple one-to-one reaction.

Voltage dependence of inhibition. Figure 4A shows the current-voltage (I-V) relationships of Kv1.5 currents under control conditions and in the presence of 10 μM AG-1478. Under control conditions, the Kv1.5 current was activated at pulses greater than −30 mV. The I-V relationship shows a sigmoidal shape at potentials between −30 and 0 mV and is almost linear for depolarizing pulses >0 mV. In the presence of 10 μM AG-1478, an inhibition of steady-state currents was observed...
through the whole voltage range over which Kv1.5 was activated. When the relative current ($I_{AG-1478}/I_{control}$) was plotted against the membrane potential (Fig. 4B), a high degree of inhibition with strong voltage dependence was observed between −30 and 0 mV, which corresponds to the voltage range of channel opening. This suggests that AG-1478-induced inhibition of Kv1.5 currents occurs preferentially after channels open. However, the inhibition of Kv1.5 channels by AG-1478 in the range of voltages between 0 and 50 mV, where channels are fully activated, did not show voltage dependence. The linear curve fitting of the data at potentials >0 mV (Fig. 4B, solid line) yielded a voltage dependence of ~0 mV for the slope of the line: 51.3 ± 3.4% of the control value at 0 mV and 49.6 ± 2.2% of the control at 50 mV (n = 4, ANOVA, P > 0.05). The voltage dependence of the inactivation time constant (τ) is plotted in Fig. 4C. The inactivation time course under control conditions was not significantly altered (ANOVA, P > 0.05) at different voltages between 20 and 50 mV. The time constants of the decay phase of Kv1.5 were significantly reduced in the presence of 10 μM AG-1478 (111–137 ms for the control; 41–44 ms for AG-1478), but this effect did not vary over the voltage range examined (ANOVA, P > 0.05). Therefore, these results indicate that AG-1478-induced inhibition of Kv1.5 channels is voltage-independent over the voltage range in which channels are fully activated.

Voltage dependence of the steady-state activation and inactivation. We examined whether AG-1478 affected Kv1.5 by shifting the steady-state activation curve (Fig. 5A). A two-pulse protocol was used to estimate the voltage dependence of Kv1.5 by tail current (Fig. 5A, top) analysis in the absence and presence of AG-1478. As shown in Fig. 5A, the activation curve was unchanged in the presence of 10 μM AG-1478. The potential of half-maximum activation ($V_{1/2}$) was −11.08 ± 1.23 and −13.85 ± 0.92 mV before and after application of the drug (n = 4). Similarly, there was no significant shift in slope value (k) in the presence of AG-1478 (5.95 ± 0.67 mV for control; 7.39 ± 0.50 mV for AG-1478, n = 4). To further examine the possibility of inhibition in the inactivated state, we analyzed the effect of AG-1478 on the steady-state inactivation of Kv1.5. Figure 5B, top, shows control currents and those in the presence of 10 μM AG-1478. In the absence of drug, the midpotential ($V_{1/2}$) and slope value (k) of the steady-state inactivation curve measured −23.72 ± 0.32 and 6.19 ± 0.28 mV, respectively (Fig. 5B, bottom). After the addition of 10 μM AG-1478, the $V_{1/2}$ and k showed no change, measuring −25.86 ± 0.13 and 6.48 ± 0.11 mV, respectively.

Effects of AG-1478 on the deactivation kinetics of Kv1.5. To further investigate the voltage dependence of inhibition, the effects of AG-1478 on the Kv1.5 current deactivation kinetics were studied. Figure 6, A and B, shows the representative superimposed tail currents recorded with a 250-ms repolarizing pulse between −50 and −20 mV after a 250-ms depolarizing pulse of 50 mV from a holding potential of −80 mV under control conditions and in the presence of 10 μM AG-1478. These currents were well fitted to a single exponential function. Under control conditions, the tail current declined with a time constant of 22.19 ± 1.54 ms (n = 4) and was nearly completely deactivated.
during a 250-ms repolarizing pulse of ∼40 mV. In the presence of 10 μM AG-1478, the initial peak amplitude of the tail current was reduced and the subsequent decline of the current was slowed (57.16 ± 2.11 ms, n = 4, Student’s t-test, P < 0.05), which resulted in a crossover phenomenon (Fig. 6C). Figure 6D shows a summary of the time constants calculated for repolarizing pulses between −50 and −20 mV in the absence and presence of AG-1478. Although AG-1478 significantly increased the deactivation time constants of Kv1.5 over the voltage range tested, the differences between the time constants obtained from tail currents in the absence and presence of AG-1478 did not change (ANOVA, P > 0.05). These results provide further evidence of the voltage independence of inhibition of Kv1.5 by AG-1478.

Use-dependent inhibition. Original current traces, under control conditions and in the presence of 10 μM AG-1478, were produced by 15 repetitive applications of depolarizing pulses at two different frequencies, 1 and 2 Hz (Fig. 7A). Under control conditions, the peak amplitude of the Kv1.5 current decreased slightly by 9.0 ± 0.5% (n = 4) at a frequency of 1 Hz and by 16.1 ± 0.9% (n = 4) at a frequency of 2 Hz (Fig. 7B). In the presence of 10 μM AG-1478, the peak amplitude of Kv1.5 was not significantly reduced after the first pulse. The subsequent trace showed a progressive decrease in the peak amplitude of Kv1.5 to a steady level. The extent of the steady-state inhibition was 28.5 ± 0.8% (n = 4) and 42.7 ± 1.5% (n = 4) at 1 and 2 Hz, respectively. Thus AG-1478 exhibited use-dependent inhibition of Kv1.5 with little tonic block.

The membrane sidedness of AG-1478 action on Kv1.5. To study the possibility that AG-1478 acts at an intracellular site, AG-1478 (100 μM) was included in the pipette solution. After the whole cell configuration was established, the magnitude and kinetics of Kv1.5 were found to be unaltered compared with control experiments (92.0 ± 1.4% of control value, n = 4) over a 10-min period. Subsequent external application of 10 μM AG-1478 promptly inhibited Kv1.5 currents (51.3 ± 2.1% of control value, n = 4). These results suggest that the site of action of AG-1478 on this channel is accessible from outside the membrane.

DISCUSSION

The present study shows that AG-1478, a tyrosine kinase inhibitor, reversibly inhibits the steady-state current of Kv1.5 in a concentration-, time-, and use-dependent manner. These results suggest that this inhibitory action is not mediated via the inhibition of PTK but is probably due to a direct interaction of the drug with Kv1.5.

It is well known that the activity of K⁺ channels can be modulated by phosphorylation and dephosphorylation (9, 16, 19, 23). Consensus protein phosphorylation sites are prevalent in the deduced amino acid sequences of cloned Kv1.5 channel proteins (29, 32). Although tyrosine phosphorylation can play an important role in regulating long-term changes in cellular functions such as cell growth and differentiation, recent studies have shown that tyrosine phosphorylation can rapidly modulate native and cloned ion channels, including ligand-gated and voltage-gated channels (3, 9, 13, 23, 31).

AG-1478 is a potent inhibitor of PTK and has been used to investigate the physiological role of PTK in the regulation of various cellular functions (20). However, our results suggest that the effects of AG-1478 on Kv1.5 do not occur via phosphotyrosine-signaling pathways for the following five reasons.

First, Kv1.5 inhibition was detected rapidly within 20 s of the application of the drug and reached a steady state within 3 min. Its effect was also highly reversible within 2 min. The activation of receptor or nonreceptor PTKs, which results in the tyrosine phosphorylation of ion channels, modulates channel activity with a relatively slow time course of inhibition. The amplitude of Kv1.5 currents, for example, is modified by tyrosine phosphorylation with a half-time of ∼20 min, and the recovery of the suppressed current is much slower than the initial rate of decline (30). Furthermore, there is a delay of a few minutes before any changes in current amplitude are detected. Therefore, the slow time course of channel modulation by PTK inhibition in other studies (24, 30) does not parallel the time course of the inhibition of Kv1.5 currents in our study. We conclude, therefore, that the rapid time course taken to reach the steady-state inhibition of Kv1.5 by AG-1478...
and the rapid time course of the reverse reaction are not simply explicable by the inhibition of PTK activity.

Second, pretreatment with the structurally dissimilar PTK inhibitors genistein and lavendustin A had no effect on the AG-1478-induced inhibition of Kv1.5 and did not modify the AG-1478-induced current kinetics. Because genistein and lavendustin A are potent PTK inhibitors (2, 20) and are structurally different from AG-1478, these results suggest that AG-1478 directly interacts with Kv1.5 without the mediation of PTK inhibition. Furthermore, in the present study, AG-1478-induced inhibition of Kv1.5 was observed under nonphosphorylating conditions (no ATP in the pipette solution). Although AG-1478 is selective for tyrosine kinases, it is still possible that AG-1478 can also nonspecifically block other types of protein kinases, such as PKC (20). Although we cannot completely exclude the possibility that the inhibition of Kv1.5 results indirectly from the inhibition of PKC, our previous studies showed that PKC inhibition has no effect on Kv1.5 (5, 6).

Third, although the down- or upregulation of Kv1.5 by PTK is controversial (13, 22, 30), the observed pattern of Kv1.5 current suppression by tyrosine phosphorylation is completely different from the AG-1478-induced inhibition in the present case. Inhibition by tyrosine phosphorylation has been characterized by a slowing of the apparent rate of activation (13) and by a reduction in peak current amplitude, whereas inactivation acceleration has not been detected (13, 30). Furthermore, Kv1.3 and Kv3.1 channels have been shown to be inhibited by protein phosphorylation over a long time course with no changes in inactivation kinetics (1, 17). In the present study, time-dependent decay of the current was evident in the presence of AG-1478, reflecting different kinetics of inhibition for AG-1478.

Fourth, AG-1478 did not influence the kinetics of activation or the voltage dependence of the steadystate activation and inactivation curves of Kv1.5 currents. The major point made by the present work is that time-dependent inhibition of Kv1.5 occurred in the presence of AG-1478. Its inhibitory action is characterized by an acceleration of current inactivation. Moreover, AG-1478 decreases the rate of decay of the tail current and induces a crossover phenomenon. These results suggest that the inhibition of Kv1.5 by AG-1478 can be described in terms of an open-channel blocking mechanism (10). The effects of AG-1478 were also use dependent: the degree of current inhibition increased with repetitive depolarizations. Furthermore, in the presence of AG-1478, the peak amplitude of Kv1.5 currents was not significantly reduced after the first pulse, suggesting no tonic block by the drug. These phenomena are also cited as evidences of an openchannel blocking mechanism. Taken together, these results indicate that AG-1478 directly blocks the openstate Kv1.5 channel as a pore blocker and probably accesses its binding site from the extracellular face of the channel.

Fifth, a high degree of inhibition with strong voltage dependence was observed in the voltage range of channel opening. These results suggest that AG-1478 preferentially binds to the open state of the channel. The blocking effects of AG-1478 resemble those previously described for other positively-charged drugs (26). In contrast to the block produced by these drugs, however, AG-1478-induced inhibition of the Kv1.5 channel was voltage-independent over the voltage range at which channels are fully activated (Fig. 4, B and C). Further evidence of the voltage independence of Kv1.5 inhibition is the observation that AG-1478 had no effect on the voltage dependence of current deactivation kinetics as determined from tail currents (Fig. 6D). Because AG-1478 (pKₐ = 6.5) is mainly in the uncharged form at the intracellular pH of 7.3 (pH of the pipette solution), the interaction between the charged AG-1478 molecule and the Kv1.5 channel is not affected by the transmembrane electrical field. Therefore, the voltage independence could be explained in one of three ways. First, even if the drug did enter from the outside and bind deeply within the transmembrane electrical field, there could be little or no voltage dependence to its binding, given the uncharged nature of AG-1478. Another possibility is that the binding site for AG-1478 does not lie within the channel pore and therefore is exposed only slightly to the membrane electrical field. However, an alternative explanation for voltage independence could be that AG-1478 interacts with the Kv1.5 channel by a hydrophobic pathway, altering the inactivation kinetics by an allosteric mechanism. In general, the voltage dependence of an open-channel block produced is charged form of the drugs. Therefore, it is difficult to understand the nature of such a blocking mechanism. However, an open-channel block produced by the uncharged form of a drug has also been observed (5, 6, 18, 37).

We cannot completely rule out the possibility that the inhibition of Kv1.5 by AG-1478 results indirectly through the inhibition of unidentified signal transduction pathways. However, taking all these results together, we infer that the inhibition of Kv1.5 currents by AG-1478 does not occur via a phosphorylation-dependent signal transduction pathway but via a direct one-to-one interaction between the drug and the channel in the open state.

In conclusion, the present study shows that AG1478, a potent PTK inhibitor, is able to inhibit cloned rat brain Kv1.5 channels independently of PTK activity. On the basis of the present study and considering previous reports demonstrating the mechanisms of the direct actions of PKC, PKA, and PTK inhibitors on ion channels (4, 5, 7, 8, 14, 21, 25, 33, 34), we recommend caution in the use of these kinds of drugs in physiological experiments designed to determine the role of protein kinases in the modulation of ion channels. Alternatively, this study provides a pharmacological tool for the development of a specific ion-channel blocker.
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