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Block of Delayed-Rectifier Potassium Channels by Reduced Haloperidol and Related Compounds in Mouse Cortical Neurons

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ABSTRACT

Haloperidol is known as an antagonist of dopamine D2 receptors. However, it also blocks a variety of ion channels at concentrations above the therapeutic range. Reduced haloperidol (R-haloperidol), one of the main metabolites of haloperidol, has been reported to accumulate in certain tissues, particularly in brain cortex, and it may produce the pharmacological effects associated with haloperidol treatment. In this study, we assessed the effect of R-haloperidol and other related compounds on native delayed-rectifier potassium channels (K_{DR}) in mouse cortical neurons by using the whole-cell patch-clamp technique. Although R-haloperidol has much lower affinity to D2 receptors than haloperidol, the IC_{50} of R-haloperidol to block K_{DR} currents was 4.4 μM, similar to its parent compound. The binding site of R-haloperidol is on the cytoplasmic side of the channel because its quaternary derivative preferentially inhibited the currents from intracellular side. 4-Chlorophenyl-4-hydroxypiperidine (4C4HP) is the active fragment of haloperidol because other compounds containing this moiety, including L-741,626 (3-[4-(4-chlorophenyl)-4-hydroxypiperidin-L-yl]-methyl-1*H*-indole) and loperamide, also blocked K_{DR} channels. The potency of the 4C4HP fragment positively correlated with the hydrophobicity index (clogP) of the compounds tested. We conclude that R-haloperidol is a K_{DR} channel blocker, although it does not interfere with the normal channel function at a clinically relevant concentration.

Haloperidol is commonly prescribed in clinical practice to treat psychiatric and neurologic diseases and to control the symptoms of Tourette's syndrome. Its pharmacological effect is believed to be via the block of D2 dopamine receptors in the central nervous system (Seeman and Van Tol, 1994). However, effects of haloperidol other than D2 receptor antagonism have been observed. Up-regulation of A-type potassium channel on chronic haloperidol treatment has been proposed to suppress neuronal activity (Hahn et al., 2003). Conversely, acute application of haloperidol has been reported to block numerous types of ion channels, including ATP-sensitive potassium channel (Yang et al., 2004), G protein-activated inwardly rectifying potassium channel (Kobayashi et al., 2000), and human *ether-a-go-go*-related gene (HERG) potassium channels (Shuba et al., 2001). Drug-induced torsade de pointes, a lethal type of cardiac arrhythmia, has been correlated with blocking of the HERG channel by certain compounds, including haloperidol (Brown et al., 2004; Testai et al., 2004). Therefore, understanding the basic mechanism of drug-channel interaction may provide useful information for structure-based drug design (Fermini and Fossa, 2003).

Potassium channels have been found in all the known organisms and exhibit a high level of heterogeneity (Jan and Jan, 1997). Aberrant activity of potassium channels has been shown in pathophysiological conditions, such as neurologic disorders, cardiac arrhythmia, and diabetes (Shieh et al., 2000). Therefore, the therapeutic and toxicologic aspects of drug-induced alterations in potassium channel activity are particularly relevant (Fermini and Fossa, 2003; Testai et al., 2004). Slow opening and lack of a fast inactivation process characterize delayed-rectifier potassium channels (K_{DR}). Because of the nature of its kinetics, modification of K_{DR} channel activity affects the shape and duration of action potentials (Bekkers, 2000). Prolonged opening of the channels can lead to elevation of the local extracellular potassium concentration $[K^+]_o$. Under pathological conditions, even a moder-

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ABBREVIATIONS: HERG, human *ether-a-go-go*-related gene; K_{DR}, delayed-rectifier potassium channel(s); 4C4HP, 4-chlorophenyl-4-hydroxypiperidine; 3FBPA, 3-fluorobenzoyl propionic acid; R-haloperidol, reduced haloperidol; 4-AP, 4-aminopyridine; L-741,626, 3-[4-(4-chlorophenyl)-4-hydroxypiperidin-L-yl]methyl-1*H*-indole; *N*-Me-R-haloperidol, *N*-methyl-reduced haloperidol.

ate increase in $[K^+]_o$ drastically influences neuronal excitability and synaptic transmission (Moulder et al., 2004). On the other hand, $[K^+]_o$ also regulates potassium channel kinetics (Kuo, 1997), and interactions between drugs with ion channels can be modified at different $[K^+]_o$ (Kuo, 1998; Jo et al., 2000; Yang et al., 2004).

In humans, haloperidol is metabolized via three major pathways. One metabolic route is oxidative *N*-dealkylation by cytochrome P450 and generates two metabolites, 4-chloro-4-hydroxypiperidine (4C4HP) and 3-fluorobenzoyl propionic acid (3FBPA). About 20% of haloperidol is degraded via this pathway. Another 50 to 60% of haloperidol is glucuronidated at the hydroxyl group and excreted in the urine. Alternatively, about 20 to 30% of haloperidol is reduced at the carbonyl group by ketone reductase and converted to reduced haloperidol (R-haloperidol) (Froemming et al., 1989; Kudo and Ishizaki, 1999). R-Haloperidol is believed to have little clinical relevance because the binding affinity to D2 receptors is fairly low compared with haloperidol (Kirch et al., 1985). However, its prolonged half-life and bigger volume of distribution (V_d) than haloperidol produces a substantial buildup of R-haloperidol in certain tissues (Korpi et al., 1984). Because it can be oxidized back to haloperidol through the cytochrome P450 system, R-haloperidol has been proposed as an in vivo haloperidol reservoir (Kudo and Ishizaki, 1999).

In this study, we investigated the effect of R-haloperidol and other chemically related compounds on K_{DR} channels in mouse cortical neurons. We found that R-haloperidol is an open channel blocker for K_{DR} channels. The binding site of R-haloperidol is located at the intracellular side of the channel. Binding of R-haloperidol to the K_{DR} channel is modulated by $[K^+]_o$.

Materials and Methods

Primary Culture of Mouse Cortical Neurons. All the animal studies were conducted according to the National Institutes of Health's *Guidelines for Care and Use of Experimental Animals* and were approved by the Committee on Animal Care and Use of the local institution and state. Pregnant female C57/BL6 mice were killed by cervical dislocation, and embryos (E15–E17) were extracted from the uterus. Brain cortices were isolated from embryos and were dissociated by gentle trituration. Cells (5×10^6 ml^{-1}) were plated onto glass coverslips pretreated with laminin and poly-L-ornithine and cultured in chemically defined medium containing basal medium Eagle (Invitrogen, Carlsbad, CA) supplied with 2% B27 supplement (Invitrogen), 1% glucose, and 1% fetal bovine serum (Invitrogen). Cortical neurons were selected based on the pyramidal morphology and were used between 6 and 8 days in vitro.

Electrophysiology. Potassium currents were recorded from cultured cortical neurons using standard whole-cell patch-clamp configuration by an EPC10 amplifier (HEKA, Lambrecht/Pfalz, Germany). Data were acquired at 20 kHz with PULSE8.6 software (HEKA). Pipettes were pulled from 1.5-mm borosilicate glass capillaries (Harvard Apparatus Inc., Holliston, MA), and their tips were heat-polished using a microforge (MF830; Narishige, Tokyo, Japan). Pipette resistances were 3 to 5 MΩ in standard intracellular solution. Recordings with series resistance higher than 20 MΩ were excluded from this study. Unless otherwise indicated, the K_{DR} currents were elicited by 400-ms voltage steps from holding potential at −40 mV to +100 mV every 6 s. The capacitive transients were subtracted online by the P/4 method. All the experiments were done at room temperature (20–25°C).

Solutions and Chemicals. The extracellular solution contained 150 mM NaCl, 10 mM HEPES, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM 4-aminopyridine (4-AP) with pH adjusted to 7.2 (by NaOH). In certain experiments, equimolar KCl or RbCl was used to replace NaCl, and pH was adjusted by adding KOH or RbOH, respectively. The intracellular solution contained 150 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM Na_2ATP, and 5 mM EGTA, with pH adjusted to 7.2 (by KOH). Na_2ATP (5 mM) and EGTA (5 mM) were added to block ATP-sensitive potassium currents (I_{KATP}) and Ca^{2+}-activated potassium currents (I_{KCa}) (Speier et al., 2005). Contamination by voltage-activated sodium and calcium currents at voltage steps to +100 mV was negligible; therefore, no blockers were added to the extracellular solution. KCl (150 mM) in the intracellular solution was substituted with equimolar RbCl in some experiments. In these experiments, a minimum of 2 min was used for dialysis to diminish the contamination with residual intracellular potassium ions. The osmolarity of all the solutions was 300 ± 10 mOsm/l. The recording chamber had a volume of 2 ml, and chemicals were focally applied to the cell through a self-made manifold pipe and driven by gravity with a flow rate of 5 ml/min.

L-741,626, sulpiride, and loperamide were purchased from Tocris Cookson Inc. (Bristol, UK), and 4C4HP was obtained from Alfa Aesar (Ward Hill, MA). All the other chemicals were from Sigma-Aldrich Laborchemikalien (Seelze, Germany). Haloperidol, R-haloperidol, 4C4HP, 3FBPA, sulpiride, and L-741,626 were dissolved in dimethyl sulfoxide. The final concentrations of dimethyl sulfoxide were less than 0.1%, which did not affect the potassium currents tested.

N-Methyl-R-haloperidol (*N*-Me-R-haloperidol) was synthesized from haloperidol as follows. $NaBH_4$ (50 mg, 1.33 mmol) was added to a stirred solution of haloperidol (500 mg, 1.33 mmol) in MeOH (50 ml) at room temperature for 80 min; water (10 ml) then was added. After 15 min, the solvent was removed in vacuo, and the resulting crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 10:1) to provide R-haloperidol (450 mg, 90%) as a white powder.

A solution of R-haloperidol (100 mg, 265 μmol) in acetone (1 ml) was treated drop-wise with CH_3I (18 μl, 291 μmol) and heated under microwave irradiation conditions (Personal Chemistry SmithCreator microwave reactor; Biotage, Charlottesville, VA) at 60°C for 1 h. The solvent was then removed in vacuo, and the resulting crude product was recrystallized from CH_2Cl_2 to give the desired *N*-Me-R-haloperidol iodide (85 mg, 62%) as a pale yellow solid, which was subjected to ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. The obtained spectra matched the proposed structure of *N*-Me-R-haloperidol iodide.

Data Analysis. Curve fitting was done using PulseFit v8.65 (HEKA) and SigmaPlot v8.0 (SPSS Inc., Chicago, IL). Data are given as mean ± S.E.M., and *n* indicates the number of cells analyzed. Statistical significance of the difference between the samples was determined using Student's *t* test. The clogP value of each compound was predicted by using OSIRIS software (Actelion, South San Francisco, CA). The pK_a value of R-haloperidol was predicted by SciFinder database (Chemical Abstracts Service, Columbus, OH).

Results

Separation of Voltage-Gated Potassium Currents in Mouse Cortical Neurons. A large family of voltage-gated potassium channels are presented in mammalian neurons (Trimmer and Rhodes, 2004). We used the standard pharmacological method to isolate K_{DR} currents by including 4-AP into the extracellular solution. Figure 1A shows typical currents recorded from a cortical pyramidal neuron. The fast inactivating potassium currents (A-type K^+ currents) were suppressed by holding cells at −40 mV and adding 4-AP to the extracellular solutions further decreased the peak currents (Fig. 1B). The residual currents were insensitive to 4-AP and can be further blocked by tetraethylammonium extracellularly (data not shown), which resembles the typical K_{DR} currents (Bekkers, 2000). To reduce the contaminations of A-type potassium currents with minimum effects on K_{DR}

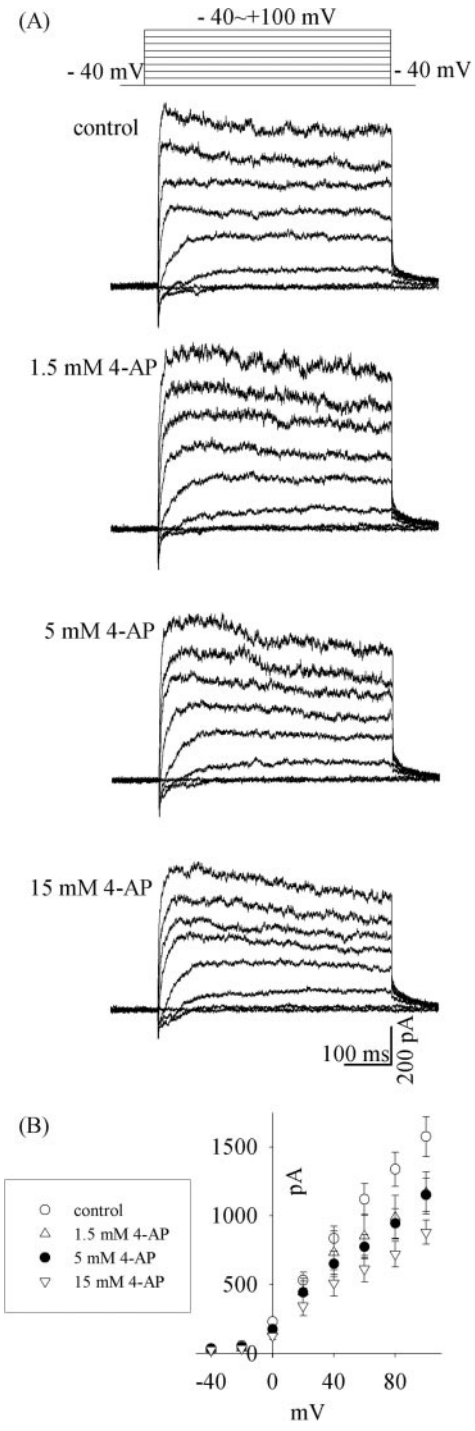

Fig. 1. Pharmacological separation of potassium currents in mouse cortical neurons. A, potassium currents were elicited by stepping from holding potential −40 mV to various voltages between −40 and +100 mV. Different concentrations of 4-AP were added as indicated. B, peak currents under different control and different 4-AP concentrations were plotted against the stepping voltages (n = 6–11 for each point).

currents, 5 mM 4-AP was added into the extracellular solutions.

Block of K_{DR} Currents by Haloperidol and Its Metabolites. We first examined the block of K_{DR} current by haloperidol and its metabolites listed in Fig. 2A. Haloperidol blocked the K_{DR} currents in a reversible manner (Fig. 2B). The concentration-dependence curve was constructed by fitting data to the Hill equation (Fig. 2C):

$$\frac{I_{drug}}{I_{control}} = \frac{1}{\left(1 + \left(\frac{[drug]}{IC_{50}}\right)^{n}\right)} \quad (1)$$

where I_{drug} and $I_{control}$ are the current amplitudes of the last 10 ms of the pulse (from 390 to 400 ms of the +100-mV voltage step) in the presence and absence of the drug, respectively, IC_{50} is the half-maximal inhibitory concentration of the drug applied, and n is the slope parameter (Hill coefficient).

The metabolites of haloperidol were also examined in a similar manner. 4C4HP (Fig. 2D) and 3FPBA (Fig. 2E), two metabolites generated by oxidative *N*-dealkylation, were much less potent than haloperidol. 3FPBA scarcely blocked the K_{DR}. On the other hand, R-haloperidol, a metabolite generated by carbonyl reduction, blocked the K_{DR} currents to a similar degree as the parent compound (Fig. 2F). Because R-haloperidol has higher concentrations in the human brain than haloperidol, we focused on this metabolite for the rest of the study.

Sidedness of R-Haloperidol Block. Potassium channels possess multiple drug binding sites, and some are only accessible from one side of the plasma membrane. R-haloperidol is a tertiary amine, and it can either directly bind to its binding site from the extracellular side or diffuse across the plasma membrane and dock to the binding site on the cytoplasmic side (Hille, 1977). To explore the sidedness of R-haloperidol binding, we synthesized *N*-Me-R-haloperidol, the quaternary amine derivative of R-haloperidol (Fig. 3A). Because it contains a permanent charge, it cannot cross the plasma membrane easily as its tertiary amine form when applied to either side of the membrane. Extracellular *N*-Me-R-haloperidol only partially blocks the K_{DR} current even at concentrations above 100 μM (Fig. 3, B and D), but 100 μM *N*-Me-R-haloperidol significantly blocked the K_{DR} currents when included in the intracellular solutions (Fig. 3, C and D). The diffusion of intracellular *N*-Me-R-haloperidol was so fast that the characteristic blocking kinetics could be observed in the first trace after the establishment of whole-cell configuration (Fig. 3C). Assuming *N*-Me-R-haloperidol binds to the same binding site as R-haloperidol, these experiments implied that R-haloperidol has to cross the membrane to access its binding site.

Voltage Dependence of R-Haloperidol Block. R-haloperidol has a predicted pK_a value of 8.5. In our standard extracellular solution, pH 7.2, about 95% of R-haloperidol was positively charged; thus, the membrane voltage can influence the binding of R-haloperidol if the binding site locates in the electric field (Garcia-Ferreiro et al., 2004). To assess the voltage-dependent block of R-haloperidol on K_{DR} currents, we used 400-ms voltage steps from −40 mV to potentials between +20 and +100 mV (Fig. 4A). R-Haloperidol (10 μM) blocked these currents to a similar extent (Fig. 4B). To quantify the voltage dependence of the R-haloperidol block, the normalized currents were plotted against the membrane potential and fitted by the Woodhull equation (Choi et al., 2001):

$$\frac{I_{drug}}{I_{control}} = \frac{[\text{R-halop}]}{([\text{R-halop}] + IC_{50}(0\text{ mV}) \times \exp(\delta FV/RT))} \quad (2)$$

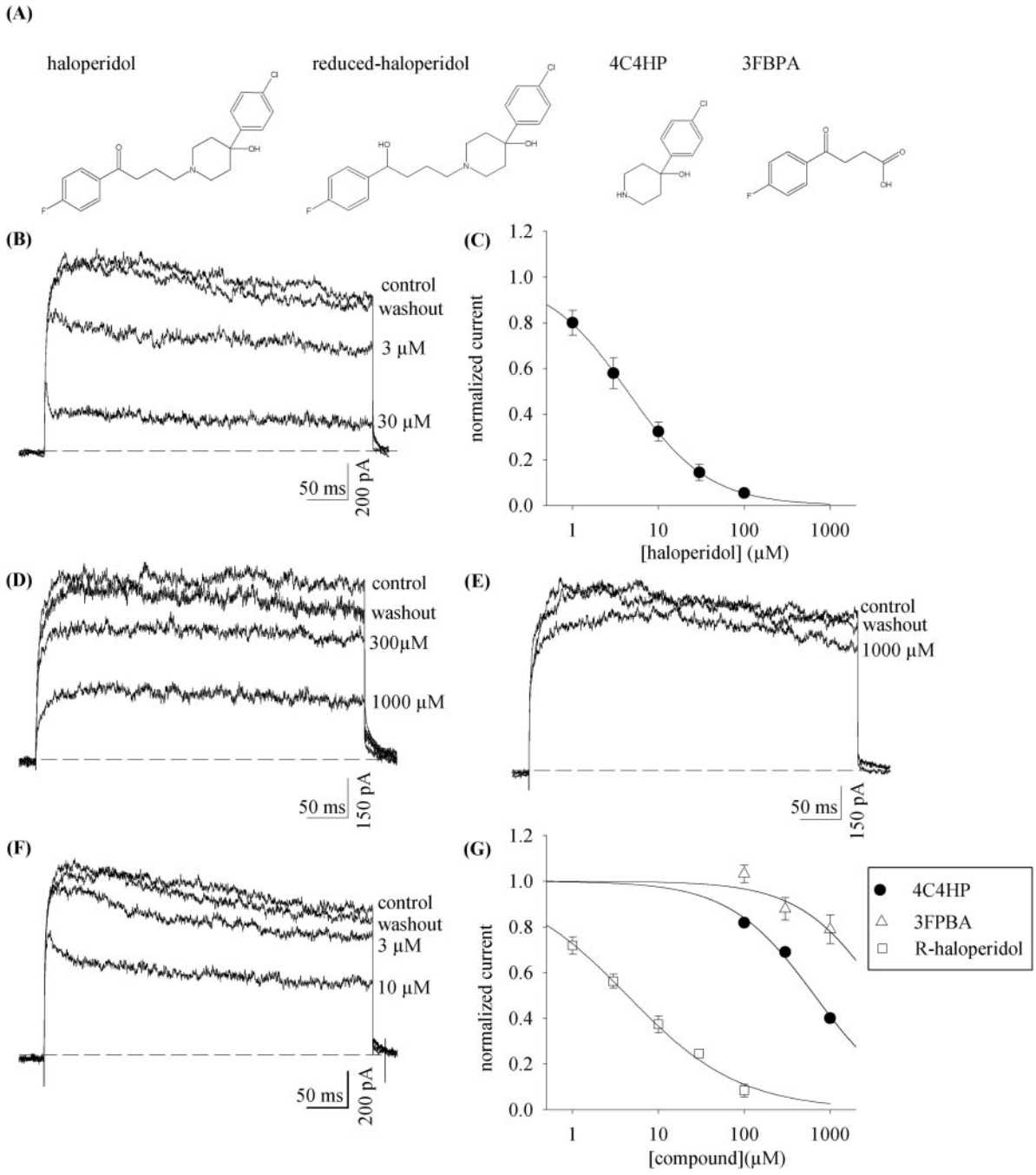

Fig. 2. Block of K_{DR} currents by haloperidol and its metabolites in mouse cortical neurons. A, chemical structures of haloperidol and its major metabolites. B, K_{DR} currents were blocked by haloperidol in a concentration-dependent manner. They were activated by a 400-ms voltage step to +100 mV from −40 mV. This step voltage was chosen to activate the channels at a maximum rate. The haloperidol block was reversible on the removal of haloperidol. C, concentration-dependent curve of haloperidol block of K_{DR} currents. The average current amplitude measured during the last 10 ms of the 400-ms voltage step at different haloperidol concentration was normalized to the currents in the control condition. The line was fitted by eq. 1 with IC_{50} = 4.4 μM and n = 0.9 (n = 5). The block of major haloperidol metabolites is shown in (D) 4C4HP, (E) 3FBPA, and (F) R-haloperidol. G, concentration-dependence curves of these metabolites. The IC_{50} values are 661, 4033, and 4.4 μM, and the Hill coefficients are 0.9, 0.9, and 0.7 for 4C4HP, 3FBPA, and R-haloperidol, respectively. The number of cells tested at each drug concentration was between four and eight.

where [R-halop] is the concentration of R-haloperidol used (10 μM), IC_{50}(0 mV) is the half-inhibition concentration of R-haloperidol at 0 mV, and δ is the fractional electrical distance from the external surface of the pore. F is the Faraday constant, V is the membrane potential, T is the absolute temperature, and R is the gas constant. A value of 25 was used for RT/F because the experiments were performed at room temperature (20°C). The calculated fractional electrical distance was 0.07 (Fig. 4C), indicating a weak voltage dependence of R-haloperidol block.

Elevation of $[K^+]_o$ Reduces the Affinity of R-Haloperidol to K_{DR} Channels. Binding of haloperidol and other compounds to the potassium channels is sensitive to the $[K^+]_o$ (Kuo, 1998; Boccaccio et al., 2004; Yang et al., 2004). The interaction of R-haloperidol and $[K^+]_o$ was tested by increasing the $[K^+]_o$ from 5 to 150 mM, and the degree of

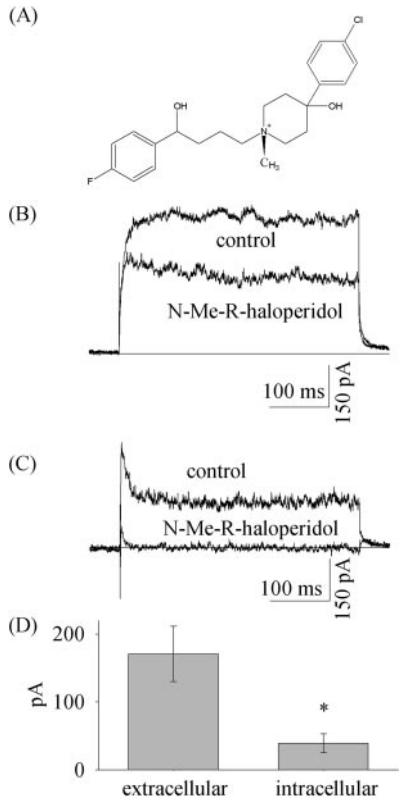

Fig. 3. Sidedness of *N*-Me-R-haloperidol block on K_{DR} currents. A, the chemical structure of *N*-Me-R-haloperidol. B, 100 μM *N*-Me-R-haloperidol was applied to the extracellular solution, and it only partially inhibited the K_{DR} currents. C, when 100 μM *N*-Me-R-haloperidol was included into the intracellular solution, it gradually blocked the K_{DR} currents on diffusion. First trace after the membrane rupture and a representative trace reaching steady state were shown here. D, comparison of 100 μM *N*-Me-R-haloperidol block from different sides of the membrane. K_{DR} currents were significantly smaller in the presence of intracellular *N*-Me-R-haloperidol (n = 8 and 6 for extracellular and intracellular *N*-Me-R-haloperidol, respectively; $p < 0.05$).

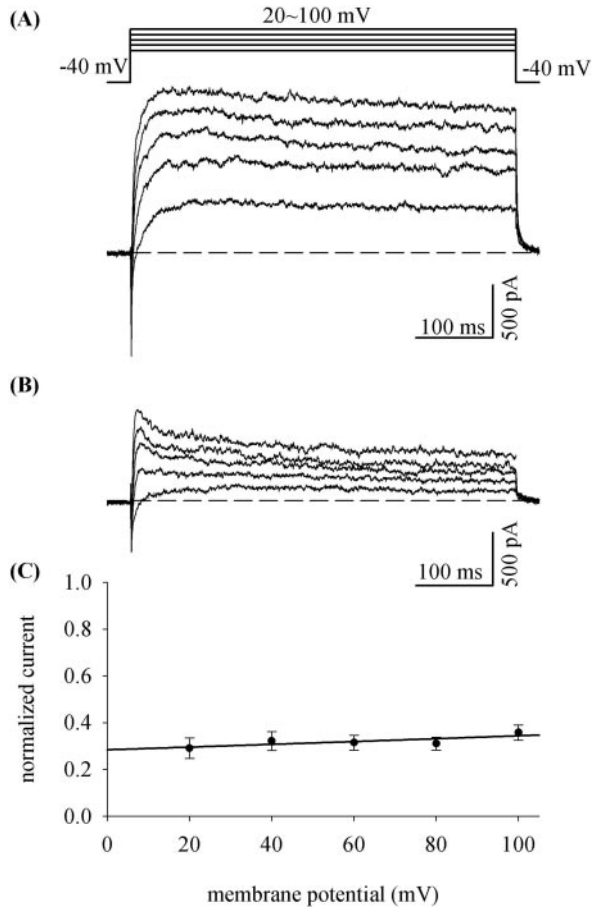

Fig. 4. Voltage dependence of R-haloperidol block of K_{DR} currents. Currents were obtained by applying 400-ms voltage steps from −40 mV to voltages between +20 and +100 mV with 20-mV increments every 6 s. The representative traces under control conditions are shown in (A), and the traces after adding 10 μM R-haloperidol are shown in (B). The initial inward currents are sodium and calcium, which are also partially blocked by 10 μM R-haloperidol. C, the fraction of block by 10 μM R-haloperidol was plotted against the test voltages. The line is drawn by eq. 2, which yielded $\delta = 0.07$ and $IC_{50}(0\text{ mV}) = 4.0$ μM (n = 8–9 at each voltage).

R-haloperidol block of K_{DR} currents was decreased at high $[K^+]_o$ (Fig. 5, A and B). The concentration-dependence curve was shifted toward the right, and the IC_{50} of R-haloperidol was elevated from 4.4 μM at 5 mM $[K^+]_o$ to 21 μM at 150 mM $[K^+]_o$ (Fig. 5E). However, when potassium ions on both sides of the membrane were substituted by rubidium, another potassium channel permeant ion, the Rb^+ currents were blocked by R-haloperidol to a similar extent, irrespective of $[Rb^+]_o$ (Fig. 5, C, D, and F).

R-Haloperidol not only decreased the current amplitude but also changed the kinetics by accelerating the current decay (Fig. 2F). This implied that R-haloperidol preferentially binds to an open state of the channels. We further analyzed the kinetics of the R-haloperidol binding and the effect of external ions on the binding kinetics. Current traces at different R-haloperidol concentrations were point-by-point divided by the trace recorded in control conditions, and these normalized traces were fitted by a single exponential function (Fig. 6, A–D):

$$\frac{I_{drug}}{I_{control}} = a + b\,\exp\left(\frac{-t}{\tau}\right) \qquad (3)$$

The block rate (τ^{-1}) is the inverted decay time constant of each normalized current trace and was increased at higher concentration of R-haloperidol (Fig. 6, E and F). This result suggests a bimolecular interaction between R-haloperidol and channels in the open state as shown in the following scheme:

$$C \rightarrow O \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} OD$$

where C and O represent the open and closed state of channels, and D denotes the R-haloperidol.

The block rate can be expressed as:

$$\tau^{-1} = k_{on} \times [\text{R-Halop}] + k_{off} \qquad (4)$$

k_{on} and k_{off} stand for the on-rate and off-rate of R-haloperidol to the potassium channels, and [R-halop] is the concentration of R-haloperidol.

The k_{on} values were comparable at either 5 or 150 mM $[K^+]_o$, but the k_{off} values were accelerated at 150 mM $[K^+]_o$ (Fig. 6E). In contrast, the binding and unbinding kinetics of R-haloperidol were insensitive to $[Rb^+]_o$ (Fig. 6F); the k_{on} was about twice as fast in rubidium solutions than in potas-

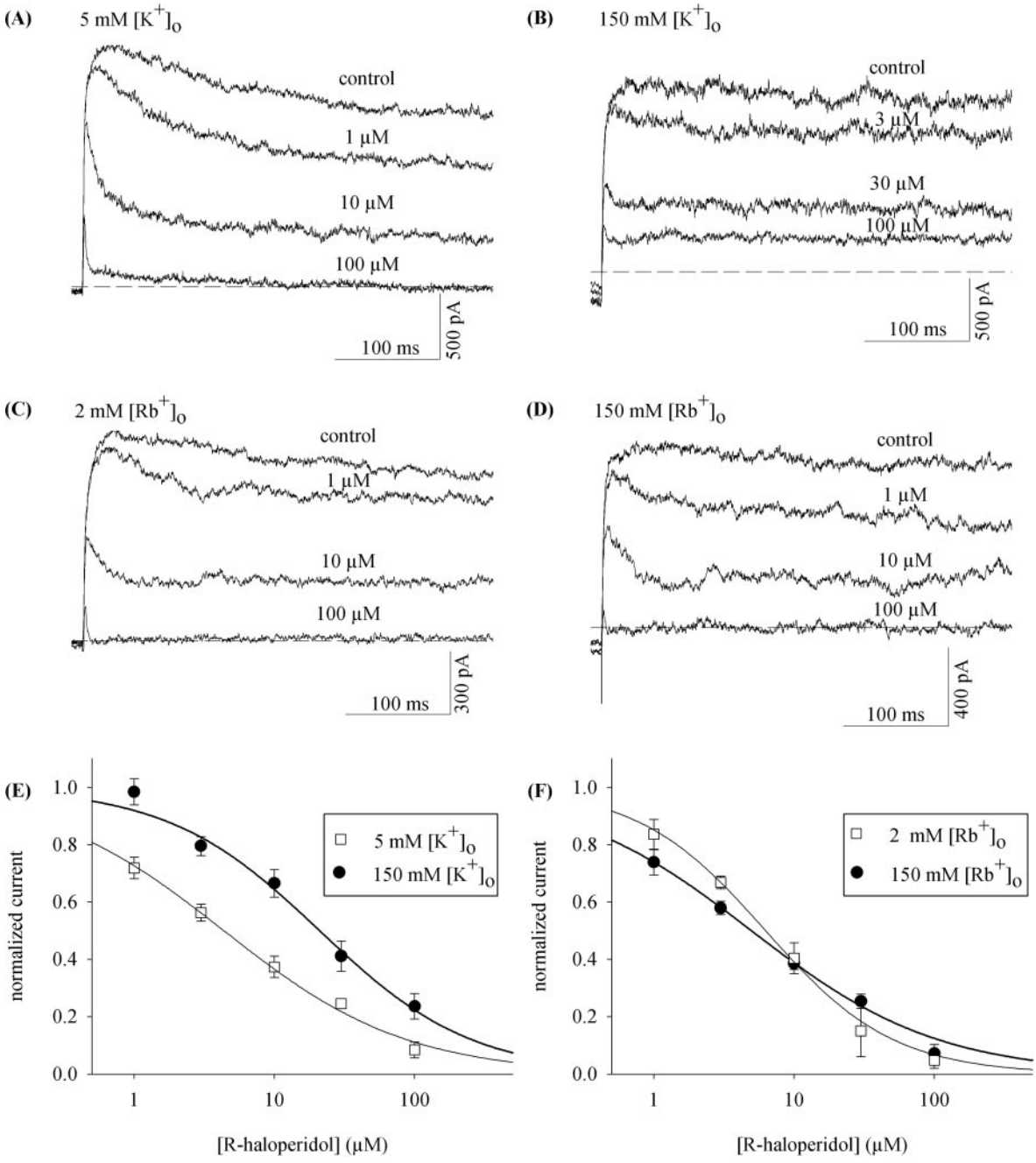

Fig. 5. Effect of permeant ions on the R-haloperidol block of K_{DR} channels. Currents were elicited by a 400-ms voltage step from −40 mV to +100 mV. Representative traces of potassium currents blocked by R-haloperidol at (A) 5 mM $[K^+]_o$ and (B) 150 mM $[K^+]_o$. When rubidium ions were used as charge carriers, the degree of R-haloperidol block was comparable at both (C) 2 mM $[Rb^+]_o$ and (D) 150 mM $[Rb^+]_o$. E, concentration-dependence inhibition curve of R-haloperidol at different $[K^+]_o$. The last 10 ms of current traces at different R-haloperidol concentration were normalized to the current at control condition. The data were fitted by eq. 1. For comparison, the data points of 5 mM $[K^+]_o$ are taken from Fig. 2G. The IC_{50} value was 21.0 μM with Hill coefficients of 0.7 at 150 mM $[K^+]_o$. F, concentration-dependence inhibition curves of R-haloperidol block of rubidium currents at different $[Rb^+]_o$. IC_{50} values were 6.3 and 5.0 μM, and the Hill coefficients were 0.9 and 0.7 for 2 mM $[Rb^+]_o$ and 150 mM $[Rb^+]_o$, respectively. For each data point in (E) and (F), 2 to 18 cells were used with a median cell number of seven.

sium solutions, and the k_{off} at both $[Rb^+]_o$ was comparable with the k_{off} at low $[K^+]_o$ (Fig. 6A).

The intercept of the normalized current traces on the *y*-axis represents the fraction of channels blocked before they opened (Fig. 6, A–D). Therefore, the affinity of R-haloperidol to the channels in the closed state can be estimated by plotting the *y*-intercept versus R-haloperidol concentration. The apparent binding affinities to the channel in the closed state were also decreased in the presence of extracellular potassium ions (Fig. 6G).

Block of K_{DR} Channels by Compounds Functionally or Structurally Related to Haloperidol. The backbone structure of R-haloperidol is similar to haloperidol, although the affinity to D2 receptor is reduced by about two orders of magnitude (Kirch et al., 1985; Froemming et al., 1989). Thus, binding of R-haloperidol to K_{DR} channels does not correlate

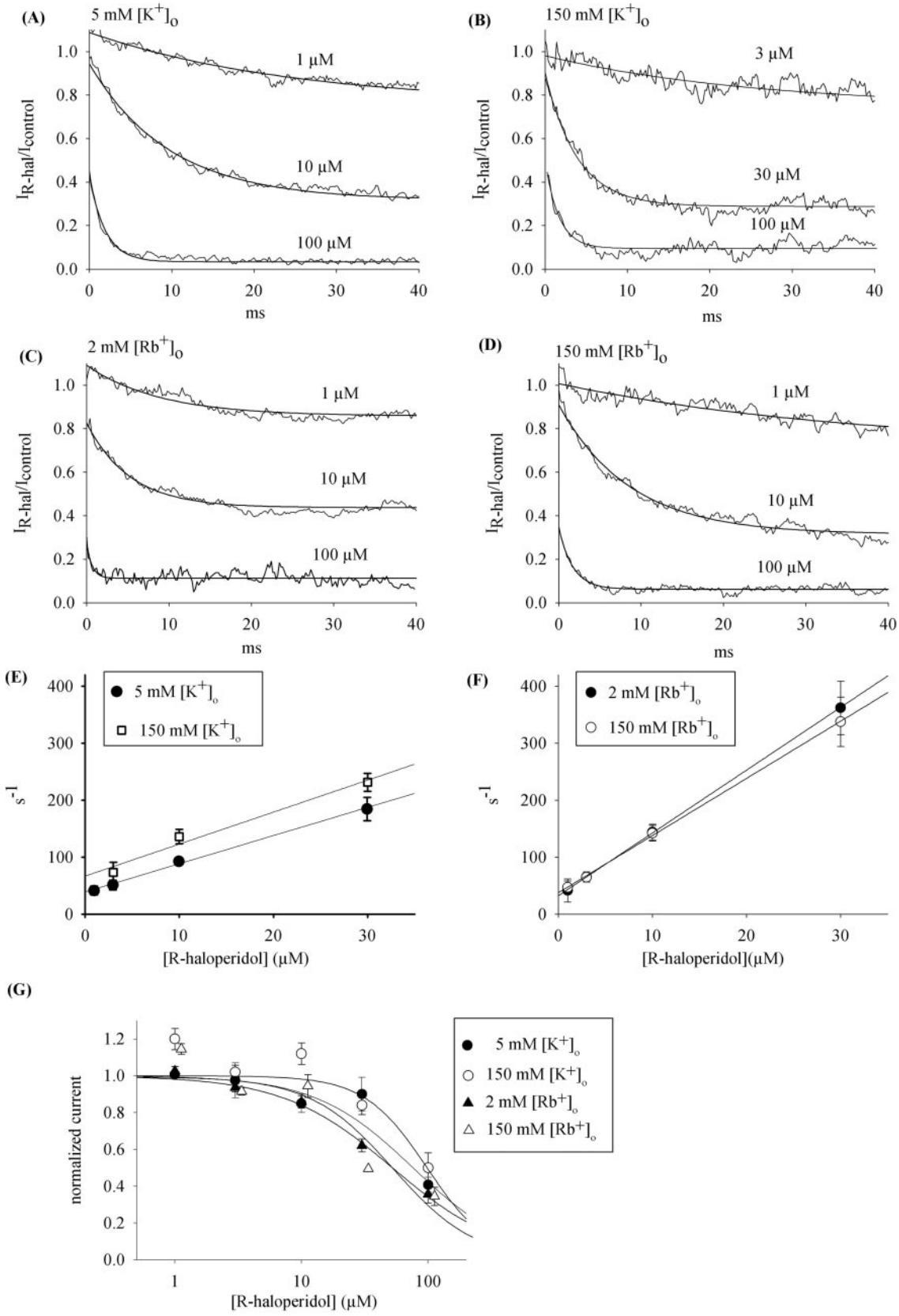

Fig. 6. Kinetics of R-haloperidol block at different ionic conditions. A–D, original current traces were recorded as in Fig. 4. The currents at the indicated extracellular cation concentration were normalized point by point to the control current trace without the addition of R-haloperidol and fitted with eq. 3. E, calculated block rates at different $[K^+]_o$ were plotted against the R-haloperidol concentration. According to eqs. 4 and 5, a straight line was used to fit the R-haloperidol binding kinetics. The slope and the *y*-intercept represent the on-rate (k_{on}) and the off-rate (k_{off}), respectively. The on-rates were comparable at different $[K^+]_o$, and the values were 4.9 μM·s and 5.6 μM·s for 5 and 150 mM $[K^+]_o$, respectively. The k_{off} was increased from 39/s to 66/s as the $[K^+]_o$ increased from 5 to 150 mM. In contrast, the R-haloperidol binding kinetics were almost indistinguishable in different

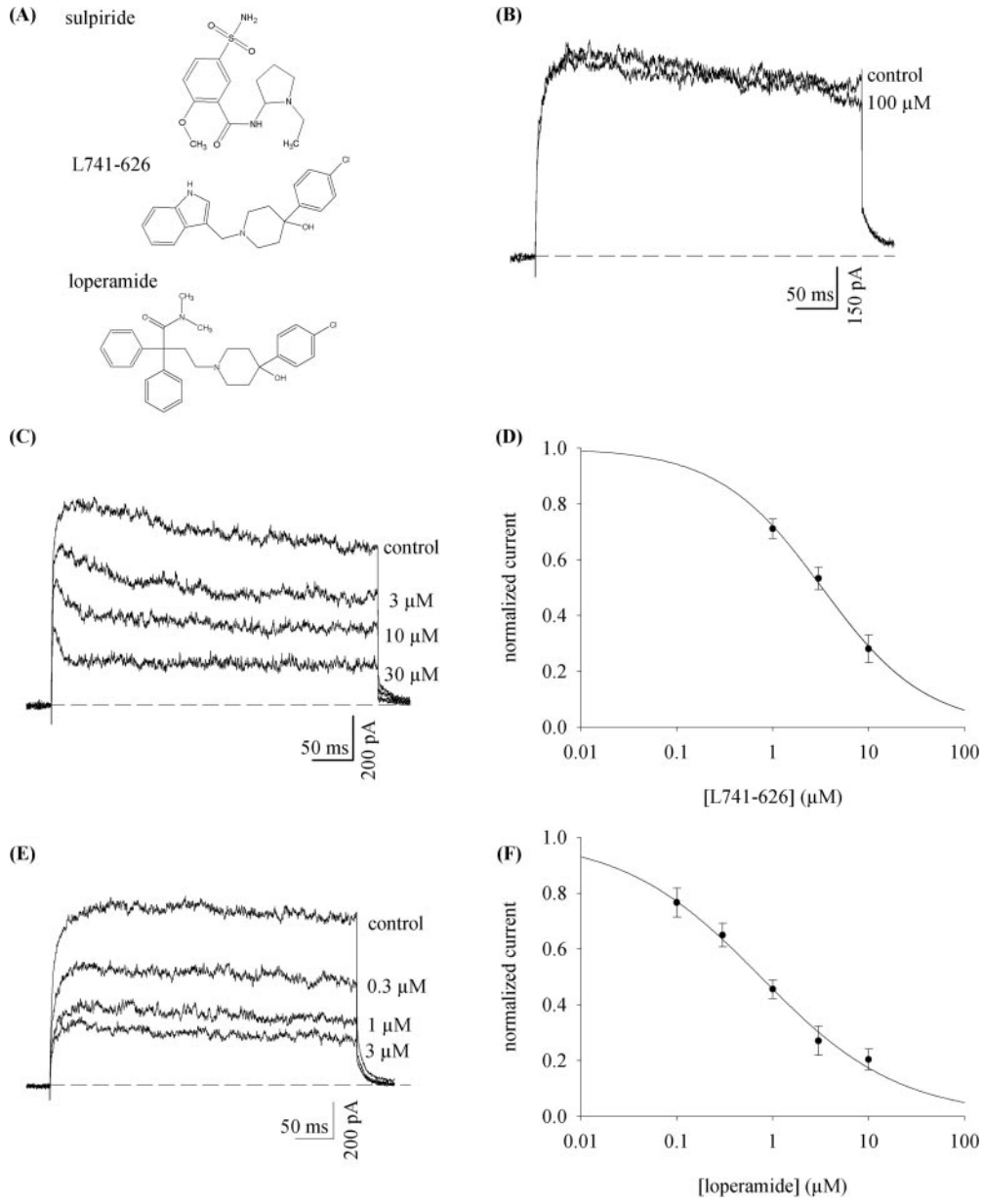

Fig. 7. K_{DR} currents blocked by other compounds with the 4C4HP moiety. A, chemical structures of compounds tested. B, representative current traces in the presence and absence of sulpiride. Only 2 ± 2% of currents were blocked by 100 μM sulpiride (n = 4). C, representative traces recorded at different concentrations of L-741,626. D, concentration-dependence curve of L-741,626 block on K_{DR} currents. The IC_{50} and Hill coefficient were 3.3 μM and 0.8, respectively (n = 5). E, representative traces recorded at different concentrations of loperamide. F, concentration-dependence curve of loperamide block on K_{DR} currents. The IC_{50} and Hill coefficient were 0.75 μM and 0.6, respectively (n = 5).

to the D2 receptor potency. To address the active chemical moiety, several compounds with either functional or structural similarity were tested (Fig. 7A). Sulpiride, another D2 receptor antagonist with similar potency as haloperidol but structurally unrelated, did not inhibit the potassium currents when applied extracellularly (Fig. 7B). In contrast, L-741,626, a D2 receptor antagonist with a similar chlorophenyl piperidine moiety, blocked the potassium currents to a similar extent as R-haloperidol (Fig. 7, C and D). If the chlorophenyl piperidine moiety determines the block ability, other compounds with this fragment should also block the channels. Loperamide, an antidiarrheal drug that activates opioid receptors, also contains this fragment. Therefore, we tested whether loperamide also blocked the K_{DR} currents. To avoid the activation of opioid receptor, 10 μM naloxone was continuously present in the extracellular solution before and

$[Rb^+]_o$. F, the k_{on} values were 11 μM·s and 10 μM·s for 2 and 150 mM $[Rb^+]_o$, respectively, which were about twice as fast as in the solutions containing potassium. The k_{off} values were 32/s and 38/s for 2 and 150 mM $[Rb^+]_o$, respectively. G, affinity of R-haloperidol to closed-state channels. The y-intercepts in (A–D) were the portion of channels blocked before the opening, and hence the affinity to the closed-state channels was estimated by fitting the concentration-dependence curves at various R-haloperidol concentrations. The lines were fitted by eq. 1. The IC_{50} values were 76 μM, 98 μM, 52 μM, and 47 μM for 5 mM $[K^+]_o$, 150 mM $[K^+]_o$, 2 mM $[Rb^+]_o$, and 150 mM $[Rb^+]_o$, respectively. The Hill coefficients were 1.1, 1.8, 1.0, and 1.3 for 5 mM $[K^+]_o$, 150 mM $[K^+]_o$, 2 mM $[Rb^+]_o$, and 150 mM $[Rb^+]_o$, respectively. The numbers of cells tested for each point were between 2 and 11 (median, 5).

during the application of loperamide. K_{DR} currents were not affected in the presence of 10 μM naloxone (data not shown). We found that loperamide was even more potent than other compounds tested, with an IC_{50} value of 0.75 μM (Fig. 7, E and F).

Discussion

In this study, we have shown that R-haloperidol, a metabolite of haloperidol, is an open channel blocker for K_{DR} channels in mouse cortical neurons. R-Haloperidol accelerates the current decay in a concentration-dependent manner. Elevating the $[K^+]_o$ antagonized the binding of R-haloperidol to K_{DR} channels. The binding site is shown to be at the intracellular side. The 4C4HP fragment determines the K_{DR} channel block ability, although the fragment itself only weakly blocks the channels.

Binding Site of R-Haloperidol on the K_{DR} Channels. According to the scheme shown in eq. 4, R-haloperidol binds to the K_{DR} channel in the open state. If the binding of R-haloperidol to the K_{DR} channels did not have the preferential state, a proportional reduction of current size should have been expected without altering the channel kinetics. There are two lines of evidence suggesting that R-haloperidol has a higher affinity to open K_{DR} channels. First, the current decayed faster at higher R-haloperidol concentration (Fig. 6). Equation 4 predicts a one-to-one relationship between R-haloperidol and the K_{DR} channel, and the binding rate should be linearly related to the ligand concentration. Second, the IC_{50} of R-haloperidol to the closed channels was estimated to be 76 μM, which was more than 10 times higher than 4.4 μM, the IC_{50} of the R-haloperidol block to the open channels (Figs. 2G and 5G). The voltage gate of potassium channels is located at the cytoplasmic end of the channel protein (Jiang et al., 2002). Therefore, we propose that the binding site of R-haloperidol is either formed or accessible when the channel is opened. The quaternary amine binding has been located in the central cavity of the channel (Zhou et al., 2001), and we also have shown that the *N*-Me-R-haloperidol, the quaternary amine derivative of R-haloperidol, preferentially blocked the K_{DR} currents from the intracellular side (Fig. 3). The fractional electrical distance of 0.07 indicated a weak voltage dependence of R-haloperidol block (Fig. 4). When the channel is in its open configuration, the voltage decreases significantly across the selective filter, and the central cavity is at the same membrane potential as the cytosolic solution (Jiang et al., 2002). If the R-haloperidol binding site is in the central cavity and it is only accessible when the channel is open, it is plausible that the R-haloperidol block is independent of membrane voltage. Nevertheless, we cannot exclude the possibility that R-haloperidol blocks the channel in its neutral form.

Interaction of R-Haloperidol and External Cations on the K_{DR} Channels. Raising of $[K^+]_o$ reduced the R-haloperidol binding affinity to its binding site, whereas an increase in $[Rb^+]_o$, another potassium channel permeant ion, does not produce a similar effect (Figs. 5 and 6). Interactions between permeant ions and blockers have been observed previously (Kuo, 1998; Boccaccio et al., 2004; Yang et al., 2004; Yang and Kuo, 2005). The main effect of external K^+ is accelerating the unbinding of R-haloperidol from the K_{DR} channel; therefore, it seems that only K^+, but not Rb^+, can knock off the R-haloperidol from its binding site. According to the structure of a potassium channel, the selective filter has different conformation when crystallized in solutions with different K^+ concentrations, but not Rb^+. Based on this scenario, the selective filter has a low energy barrier for K^+, and this ion can freely move across the filter, but it functions as a molecular hurdle for other permeant ions, for example, Rb^+ or Cs^+; even these ions can traverse through the channel as K^+ (Morais-Cabral et al., 2001; Zhou and MacKinnon, 2003). Increase of external K^+ but not Rb^+ boosts the ion flow through the permeant pathway and therefore pushes R-haloperidol out from the binding site. Recently, Lin et al. (2005) reported a similar phenomenon that K^+ but not Cs^+ regulates the binding of cisapride, a prokinetic compound, to HERG channels. Nevertheless, the possibility of allosteric modulation of a channel by K^+ cannot be completely ruled out.

Nature of the Binding Motif on R-Haloperidol. The ability of compounds to block the K_{DR} channels is dependent on the existence of the 4C4HP fragment but is not related to the pharmacological character of the compounds. We found a linear relationship between the $\log IC_{50}$ and calculated the hydrophobicity index (clogP) in compounds containing the 4C4HP fragment (Fig. 8). We proposed that the 4C4HP is the key fragment that binds to the K_{DR} channel, and the affinity of 4C4HP is enhanced by adding a hydrophobic motif at the nitrogen atom on the piperidine ring, which increases the accessibility of 4C4HP to its intracellular binding site. On the other hand, increase of hydrophobicity also facilitates the binding of the active fragment to the receptor, and the hydrophobic motif functioned as a "binder" of the active fragment. The binder concept was recently suggested in a fragment-based lead discovery, in which optimizing the binder part of the molecule augments the binding affinity (Rees et al., 2004). It has been shown that increase of the hydrophobicity of quaternary amines enhances the binding affinity to its binding site (Choi et al., 1993). The chemical nature of the binder can be unspecific because the key fragment determines the main biological effect. Therefore, it is worth checking the channel blocking ability of compounds with the 4C4HP motif at an early stage of drug development to avoid adverse effects caused by ion channel blocking (Fermini and Fossa, 2003).

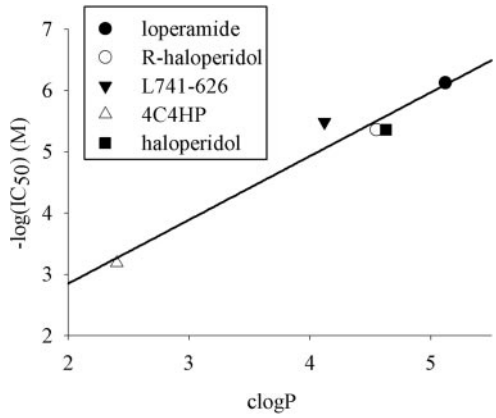

Fig. 8. Relationship between the potency and hydrophobicity of compounds studied. The binding affinities of each compound were plotted against the clogP values. A straight line with a slope of 1.0 with a *y*-intercept of −0.8 was used to describe the relationship.

The oxygen atom in the carbonyl group has been proposed to involve the binding of haloperidol to the central cavity of HERG channels in an in silico study (Testai et al., 2004). Most compounds listed in that study have already been reported as blockers for other potassium channels, including K_{DR} channels. R-Haloperidol, in contrast, has a hydroxyl group at this position. Nevertheless, the binding affinities to the K_{DR} channel of both R-haloperidol and haloperidol were comparable. It is likely the role of carbonyl oxygen was overestimated in the above-mentioned in silico study (Testai et al., 2004).

Therapeutic and Toxicological Relevance of R-Haloperidol Block on K_{DR} Channels. The plasma concentration of haloperidol and R-haloperidol is between 10 and 100 nM (Chang et al., 1989; Kudo and Ishizaki, 1999; Roh et al., 2001), which is about two orders of magnitude below the IC_{50} to block K_{DR} channels. Our results indicated the block of K_{DR} currents by haloperidol and/or R-haloperidol does not produce clinically relevant symptoms. However, in occasional cases, micromolar haloperidol and R-haloperidol have been detected in human cortex (Korpi et al., 1984; Kornhuber et al., 1999). A portion of potassium channels could be blocked under at these concentrations. The local concentration of haloperidol and R-haloperidol cannot be predicted from the plasma haloperidol concentration, and haloperidol metabolism is variable among different individuals (Chang et al., 1989; Lam et al., 1995; Roh et al., 2001). Adverse effects related to the R-haloperidol block of K_{DR} channels can be expected, particularly when other drugs capable of modifying metabolic enzymes are coprescribed (Kudo and Ishizaki, 1999).

The loperamide block of K_{DR} channel in cortical neurons should not be relevant because of the poor bioavailability and restricted blood-brain barrier penetration. The antidiarrheal effect of loperamide is suggested to result from a decrease in the local ganglia activity in the enteric nervous system (Awouters et al., 1983). Although the plasma concentration of loperamide is fairly low, in the nanomolar range, the local concentration in the gut may be much higher because of the low solubility and enterohepatic shunt (Awouters et al., 1983). Loperamide has been shown to decrease potassium efflux from epithelial cells, and direct block of potassium channels may be one explanation for this phenomenon (Epple et al., 2001). Interestingly, haloperidol has already been shown to reduce the fluid secretion in rabbit and human small intestine mucosa, a property that is necessary for antidiarrheal treatment (Smith and Field, 1980).

In conclusion, we have found that R-haloperidol, a main metabolite of haloperidol, is an open channel blocker for neuronal K_{DR} channels. The binding site is intracellular, as *N*-Me-R-haloperidol, a quaternary amine form of R-haloperidol, preferentially blocked the channel from intracellular side. Our findings also suggest the 4C4HP motif is the main fragment blocking the channel because increasing the hydrophobicity of compounds containing 4C4HP enhances the binding to the K_{DR} channels. Although the concentration of R-haloperidol needed to block the K_{DR} channels is above the clinical relevant range, it may produce some pharmacological or toxic effects because of the polymorphism of enzymes metabolizing haloperidol.

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