Differential Action of Riluzole on Tetrodotoxin-Sensitive and Tetrodotoxin-Resistant Sodium Channels

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ABSTRACT

The effects of riluzole, a neuroprotective drug, on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium channels in rat dorsal root ganglion neurons were studied using the whole-cell patch clamp technique. At the resting potential, riluzole preferentially blocked TTX-S sodium channels, whereas at more negative potentials, it blocked both types of sodium channels almost equally. The apparent dissociation constants for riluzole to block TTX-S and TTX-R sodium channels in their resting state were 90 and 143 μM, respectively. Riluzole shifted the voltage dependence of activation of TTX-R sodium channels in the depolarizing direction more than that of TTX-S sodium channels. The voltage dependence of the fast inactivation of both types of sodium channels was shifted in the hyperpolarizing direction in a dose-dependent manner, and the apparent dissociation constants for riluzole to block the inactivated channels were estimated to be 2 and 3 μM for the TTX-S and TTX-R sodium channels, respectively, indicating a much higher affinity for the inactivated channels than for the resting channels. Riluzole was equally effective in blocking both types of sodium channels in their slow inactivated state. Since more TTX-S channels are inactivated than TTX-R channels at the resting potential, riluzole blocks TTX-S sodium channels more potently than TTX-R sodium channels. It was concluded that one of the mechanisms by which riluzole exerts its neuroprotective action is to preferentially block the inactivated sodium channel of damaged or depolarized neurons under ischemic conditions, thereby suppressing excess stimulation of the glutamatergic receptors and massive influx of Ca++.
tion and inactivation and activate and inactivate at more positive potentials than TTX-S sodium channels.

Our study was undertaken to examine the differential effect of riluzole on TTX-S and TTX-R sodium channels using rat DRG neurons as a model. This preparation was chosen because the differential sensitivity of TTX-S and TTX-R sodium channels to riluzole provided us with an excellent model with which detailed mechanisms of action of riluzole could be elucidated. Riluzole has a much higher affinity for channels in the inactivated state than in the resting state of either TTX-S or TTX-R sodium channels. This difference in affinity accounts for the higher potency of riluzole to block TTX-S sodium channels than TTX-R sodium channels as TTX-S sodium channels are inactivated more at resting membrane potentials than TTX-R sodium channels.

Material and Methods

Cell preparations. DRG neurons were isolated as described previously (Roy and Narahashi, 1994; Tatebayashi and Narahashi, 1994). Rats (2–6 days postnatal, either sex) were anesthetized with methoxyflurane and the spinal column was removed and cut longitudinally. Ganglia were plucked from between the vertebræ of the spinal column, and incubated in phosphate-buffered saline solution (GIBCO BRL, Grand Island, NY) containing trypsin (2.5 mg/ml, type XI, Sigma Chemical Co., St. Louis, MO) at 37°C for 25 min. After enzyme treatment, ganglia were rinsed with Dulbecco’s modified Eagle medium (GIBCO BRL) supplemented with newborn calf serum (10%, v/v, GIBCO BRL) and gentamicin (80 μg/ml, Northwestern University Lurie Cancer Center). Single cells were mechanically dissociated with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips. Cells were incubated for 2 to 7 hr before patch clamp experiments.

TTX (0.2 μM) was used to separate TTX-R sodium currents from TTX-S sodium currents. For the study of TTX-S sodium channels, cells that expressed only TTX-S sodium currents were used. TTX-S sodium currents were completely inactivated at the end of a 5-msec depolarizing pulse to 0 mV, although TTX-R currents were still present. Thus, the difference in kinetics was used to identify the type of sodium current.

Electrophysiological recording. Currents were recorded using the whole-cell patch clamp technique (Hamill et al., 1981). Suction pipettes (borosilicate glass capillary tubes, 1.5–1.8 mm inner diameter, Kimble, Vineland, NJ) using a two-step vertical puller (Narishige, Tokyo, Japan). The pipette solution contained (in mM): CsF 135, NaCl 10 and HEPES 5. The pH was adjusted to 7.0 with CsOH and the osmolarity was 275 mOsmol. The membrane potentials than TTX-R sodium channels.

Results

Effects of riluzole on sodium channel currents. As has been reported previously, two types of sodium channels were found in rat DRG neurons (Kostyuk et al., 1981; Roy and Narahashi, 1992; Elliott and Elliott, 1993; Ogata and Tatebayashi, 1993). TTX-S sodium currents activated and inactivated quickly and were completely blocked by 200 nM TTX, whereas the TTX-R sodium currents activated and inactivated slowly and were not blocked by 200 nM TTX. When the membrane was held at −80 mV, which was near the resting membrane potential (Song and Narahashi, 1995), TTX-S sodium currents were more sensitive to the blocking action of riluzole than TTX-R sodium currents. At 3 μM, riluzole blocked 50% of the TTX-S sodium current (fig. 1Aa), whereas it took 30 μM for riluzole to produce a similar block of the TTX-R sodium currents (fig. 1Ab). Both types of sodium currents were blocked within 3 min after bath application of the drug and the currents recovered within a few minutes after washout with drug-free external solution. When the peak current amplitude in the presence of riluzole was normalized to the control value, the activation and inactivation kinetics were not changed by riluzole in TTX-S sodium current (fig. 1Ba), whereas the time course of inactivation of TTX-R sodium current was greatly accelerated by riluzole (fig. 1Bb).

The voltage dependence of the steady-state inactivation is
different between the two types of sodium channels (Roy and Narahashi, 1992; Tatemayashi and Narahashi, 1994). Because the steady-state inactivation for TTX-S sodium channels occurs at more negative potentials than that for TTX-R sodium channels, and because riluzole is known to have a higher affinity for the inactivated state than for the resting state of TTX-S sodium channels in other preparations (Benoit and Escande, 1991; Hebert et al., 1994), the apparent difference in the potency of riluzole block of TTX-S and TTX-R sodium channels in DRG neurons could be due to their different inactivation characteristics.

To examine this hypothesis, the membrane was held at large negative potentials, i.e., −120 mV for TTX-S and −100 mV for TTX-R channels. Under these conditions, riluzole blocked both types of sodium channels to almost the same degree (figs. 2A and 3A). Riluzole had no effect on the time course of TTX-S sodium currents evoked at 0 mV, as evidenced from superimposed currents in the presence and absence of drug (fig. 2Bb). These results suggest that riluzole does not alter either activation or inactivation of TTX-S sodium channel currents at 0 mV (fig. 2Bb). However, it slightly accelerated the inactivation time course of TTX-S sodium currents at −20 mV (fig. 2Ba). The time course of TTX-R sodium currents was greatly accelerated by riluzole at all membrane potentials tested; this effect was more pronounced at negative potentials than at positive potentials (fig. 3Ba, b and c). The small outward currents seen in riluzole (fig. 3a and b) may be due to an artifact of the P+P/4 procedure since the potassium channel was blocked by internal cesium.

**Effect of riluzole on the time constant of inactivation.** The decay of sodium currents was fitted to a single exponential function and the time constants are plotted as a function of membrane potential in figure 4. In TTX-S sodium channels, riluzole reduced the time constant of inactivation at potentials more negative than −25 mV but had little or no effect at potentials more positive than −25 mV (fig. 4A).

By contrast, the time constant of inactivation of TTX-R sodium channel currents was reduced by riluzole at potentials more negative than +20 mV, being more pronounced at more negative potentials (fig. 4B). The voltage dependence of inactivation time constant of TTX-R sodium channels appears to be shifted in the hyperpolarizing direction by riluzole. However, the difference between the effects on the two types of channels may arise from the differences in the inherent voltage dependence of the channels; as the rate of inactivation increases, the effectiveness of the drug diminishes.

**Effects of riluzole on the kinetics of sodium channel activation.** Effects of riluzole on the current-voltage relationship and the conductance-voltage curve are illustrated in figure 5 for TTX-S sodium channels and figure 6 for TTX-R sodium channels. As can be seen from the current-voltage curve, riluzole blocked TTX-S sodium currents to the same degree in the entire membrane potential range (fig. 5A). The membrane potential corresponding to half-maximum conductance (Vg0.5) was −27.9 ± 1.4 mV (n = 4) for TTX-S channels. Riluzole at 30 and 100 μM shifted Vg0.5 of TTX-R channels by 1.3 ± 1.5 and 5.0 ± 1.8 mV (n = 4), respectively, in the depolarizing direction (fig 5B; table 1). The slope factor (kg) for the conductance-voltage curve was increased by riluzole or the slope became less steep after application of riluzole (table 1).

Riluzole blocked TTX-R sodium currents more in the negative voltage range than in the positive voltage range (fig. 6A), resulting in a great shift in the conductance-voltage curve in the depolarizing direction (fig. 6B). The half-maximum activation of TTX-R sodium channels occurred at −16.6 ± 1.3 mV (n = 4). Riluzole at 30 and 100 μM shifted Vg0.5 by 8.7 ± 0.1 and 18.5 ± 0.1 mV (n = 4), respectively, in the depolarizing direction (fig. 6B, table 1). The slope factor (kg) for the conductance-voltage curve was also affected by riluzole, becoming larger (table 1).

**The apparent dissociation constant for riluzole block of sodium channels in the resting state.** To estimate the apparent dissociation constant for riluzole to block sodium channels in the resting state (Kd), the membrane was held at −120 mV for TTX-S sodium channels and −100 mV
for TTX-R sodium channels. At these potentials sodium channel inactivation was completely removed. To minimize errors due to the shift in conductance-voltage curve by riluzole, currents were elicited by depolarizing steps to +50 mV for both types of sodium channels to elicit the maximum conductance. The percentage of current inhibition is plotted as a function of riluzole concentration in figure 7. The dose-response data were fitted to the Hill equation with parameters of 90 and 143 μM, and Hill coefficients of 1.12 and 1.15, for the TTX-S and TTX-R sodium channels, respectively.

**Effects of riluzole on the time course of sodium channel inactivation.** The effects of riluzole on the time course of sodium channel inactivation are given in figure 8. Prepulses of 150-msec duration were evoked by 10-msec depolarizing steps to various levels from a holding potential of −120 mV. Test potentials ranged from −60 to +50 mV in 5-mV increments and were delivered at a frequency of 0.2 Hz. The conductance-voltage relationship for TTX-S sodium channels is shown in figure 8. Prepulses of 150-msec duration were evoked by 10-msec depolarizing steps to various levels from a holding potential of −120 mV. The effects of riluzole on the fast sodium channel inactivation are given in table 2.

### Effects of riluzole on Boltzmann parameters of sodium channel activation

<table>
<thead>
<tr>
<th>Riluzole (μM)</th>
<th>kg (mV)</th>
<th>Vg0.5 (mV)</th>
<th>ΔVg0.5 (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 ± 0.4</td>
<td>-27.9 ± 1.4</td>
<td>1.3 ± 1.5</td>
</tr>
<tr>
<td>30</td>
<td>7.0 ± 0.2</td>
<td>-26.6 ± 1.1</td>
<td>5.0 ± 1.8</td>
</tr>
<tr>
<td>100</td>
<td>6.0 ± 0.6</td>
<td>-16.6 ± 1.3</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>TTX-S</td>
<td>9.6 ± 0.8</td>
<td>-7.9 ± 1.4</td>
<td>18.5 ± 0.1</td>
</tr>
<tr>
<td>TTX-R</td>
<td>11.9 ± 0.5</td>
<td>1.9 ± 0.5</td>
<td>18.5 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (n = 4). kg is the slope factor (potential required for an e-fold change), Vg0.5 is the membrane potential for the half-maximum conductance and ΔVg0.5 is the shift in Vg0.5 relative to control value.

The apparent dissociation constant for riluzole block of sodium channels in the inactivated state. The apparent dissociation constant for riluzole to block sodium channels in the inactivated state, Kf, was estimated from Kd and the shift in Vh0.5. As shown in figure 9, the shift in Vh0.5 is plotted as a function of riluzole concentration. The data are plotted along with three lines with different parameters ac-
Riluzole on TTX-S/TTX-R Na⁺ Channels

**Fig. 6.** A. The current-voltage relationship for TTX-R sodium channels in the absence (●) and presence (▲) of 100 μM riluzole, and after washout with riluzole-free solution (○). Currents were evoked by 40-msec depolarizing steps to various levels from a holding potential of −100 mV. Test potentials ranged from −40 to +50 mV in 5 mV increments and were delivered at a frequency of 0.2 Hz. B. The conductance-voltage relationship for TTX-R sodium channels in the absence (●) and presence of 30 μM (■) and 100 μM (▲) riluzole. The method to determine the sodium channel conductance is the same as that for figure 5 (n = 4).

**Fig. 7.** Dose-response relationships for the riluzole block of both TTX-S (●) and TTX-R (○) sodium channel currents in the resting state. Currents were evoked by depolarizing steps (10 msec for TTX-S and 40 msec for TTX-R) to +50 mV from large negative holding potentials (−120 mV for TTX-S and −100 mV for TTX-R). The percentage of the block is plotted as a function of the riluzole concentration. The dose-response data were fitted to the Hill equation, %inhibition = 100/[1 + ([RZ]/[KR])^n], where [RZ] and [KR] represent the concentration of riluzole and apparent dissociation constant for riluzole block of sodium channels in the resting state, respectively, and h represents the Hill coefficient (n = 4).

and TTX-R channels, respectively, than those of fast inactivation using 150 msec prepulse. V_{h0.5} was shifted by −8.5 ± 1.0 mV (n = 6) by 3 μM riluzole in TTX-S channels and 15.6 ± 0.7 mV by 30 μM riluzole in TTX-R channels (fig. 10; table 2). Both shifts were comparable to those obtained for fast inactivation curve.

**Discussion**

Our study demonstrated that riluzole at low concentrations preferentially blocked both TTX-S and TTX-R sodium channels of rat DRG neurons in their inactivated state although it had much less effect on the channels in the resting state. Riluzole blocked both TTX-S and TTX-R sodium channels nearly the same extent under the experimental conditions where the inactivation of two types of channels was minimal.

The apparent dissociation constants for riluzole to block sodium channels in their resting states were estimated to be 90, and 143 μM for the TTX-S and TTX-R sodium channels, respectively. These values are in the same order of magnitude as those previously reported. In frog nodes of Ranvier and rat brain IIA sodium channel ɑ-subunits expressed in *Xenopus* oocytes, Kₚ values were estimated to be 90, and 30 μM, respectively (Benoit and Escande, 1991; Hebert et al., 1994).

Riluzole shifted the steady-state inactivation curves to the same extent with either 150 msec or 20 sec prepulse. Therefore, riluzole does not distinguish between the fast and slow inactivated states of sodium channels, and blocks both inactivated states almost equally. The apparent dissociation constants for riluzole to block the sodium channels in the fast inactivated state were estimated from the concentration-dependent shift in the steady-state inactivation curve. The K_f values were 2 and 3 μM for the TTX-S and TTX-R sodium channels, respectively. These values are considerably smaller than those for blocking the resting channels. However, our estimates of the K_f values are almost 10 times larger than those previously reported in other preparations (Benoit and Escande, 1991; Hebert et al., 1994).

Inasmuch as riluzole does not exhibit much difference in blocking TTX-S and TTX-R sodium channels at either resting states.
or inactivated state, how can one explain the differential block of TTX-S and TTX-R channels by riluzole near the resting membrane potential? When the membrane is held at −80 mV, 61% of TTX-S channels and 95% of TTX-R channels are available for activation (fig. 10). More TTX-S channels are in the inactivated state than the TTX-R channels. Thus, riluzole preferentially blocks the TTX-S sodium channels. Because the resting membrane potential of DRG neurons with either type of sodium channels is around −80 mV (Song and Narahashi, 1995), it is expected that TTX-S sodium channels experience more block than TTX-R sodium channels by the same concentration of riluzole. The percentage of riluzole block of sodium channels at a given membrane potential can be estimated from the following equation (Hebert et al., 1994):

\[
1 - \frac{I_{RZ}}{I_{\text{control}}} \times 100
\]

\[
= 100/\left(1 + (h_c \times [RZ]/K_p) + [(1 - h_c) \times (RZ)/K_i]\right)
\]

where \(I_{RZ}\) is the current amplitude in the presence of riluzole, \(I_{\text{control}}\) is the control current amplitude, \(h_c\) is the availability of sodium channels for activation at a given membrane potential, and \([RZ]\) is the concentration of riluzole. At the resting membrane potential of −80 mV, riluzole at 10 and 30 μM blocks 67 and 86% of TTX-S sodium channels, respectively, although the same concentrations of riluzole block only 19 and 41% of TTX-R sodium channels, respectively.

Riluzole greatly accelerated the time course of inactivation of sodium channels in rat DRG neurons especially in TTX-R sodium channels. This is a unique phenomenon because it is not observed in the TTX-S sodium channels in DRG neurons, frog nodes of Ranvier, or rat brain IIA sodium channel α subunit expressed in Xenopus oocytes (Benoit and Escande, 1991; Hebert et al., 1994). The voltage dependence of inactivation time constant of TTX-R sodium channels appears to be shifted in the hyperpolarizing direction by riluzole. However, the difference between the effects on the two types of channels may arise from the differences in the inherent voltage dependence of the channels.

Another difference between two types of sodium channels was found with respect to the riluzole effect on the sodium
channel activation. Riluzole shifted the conductance-voltage curves for both TTX-S and TTX-R sodium channels in the depolarizing direction. However, the degree of shift was far greater for TTX-R sodium channels than for TTX-S sodium channels. Also the conductance curves became less steep after riluzole treatment and the effect was more pronounced in TTX-R sodium channels. The effects of riluzole on the sodium channel activation kinetics were not observed in other preparations (Benoit and Escande, 1991; Hebert et al., 1994).

Ischemic conditions will cause gradual depolarization of neuronal membranes evoking repetitive discharges and glutamate release from nerve terminals that in turn stimulate the NMDA receptors. Massive calcium influx will ensue through the open NMDA receptor channels causing cell death. Because riluzole blocks the sodium channels much more potently in the inactivated state than in the resting state, it will effectively suppress the sodium channel activity and action potentials in the ischemic conditions, preventing cell death. Riluzole block of high voltage-gated N-type and P/Q-type calcium channels (Huang et al., 1996, submitted for publication) and the NMDA receptor (Debono et al., 1993) also contributes to neuroprotective activity. It should be noted that the plasma concentration of riluzole in healthy human volunteers is estimated to be 1.62 μM 1 hr after administration of 100 mg riluzole, a usual dose (Bryson et al., 1996). This plasma concentration is in the same order of magnitude as the apparent dissociation constants of riluzole to block the TTX-S and TTX-R sodium channels in their inactivated state that are estimated to be 2 and 3 μM, respectively.

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References


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