Evidence for Chloride Ions as Intracellular Messenger Substances in Astrocytes

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Bekar, Lane K. and Wolfgang Walz. Evidence for chloride ions as intracellular messenger substances in astrocytes. J. Neurophysiol. 82: 248–254, 1999. Cultured rat hippocampal astrocytes were used to investigate the mechanism underlying the suppression of Ba²⁺-sensitive K⁺ currents by GABA_A receptor activation. Muscimol application had two effects on whole cell currents: opening of the well-known Cl⁻ channel of the GABA_A receptor and a secondary longer-lasting blockade of outward K⁺ currents displaying both peak and plateau phases. This blockade was independent of both Na⁺ (inside and outside) and ATP in the pipette. It also seemed to be independent of muscimol binding to the receptor because picrotoxin application showed no effect on the K⁺ conductance. The effect is blocked when anion efflux is prevented by replacing Cl⁻ with gluconate (both inside and out) and is enhanced with more permeant anions such as Br⁻ and I⁻. Moreover, the effect is reproduced in the absence of muscimol by promoting Cl⁻ efflux via lowering of extracellular Cl⁻ levels. These results, along with the requirement for Cl⁻ efflux in muscimol experiments, show a strong dependency of the secondary blockade on Cl⁻ efflux through the Cl⁻ channel of the GABA_A receptor. We therefore conclude that changes in the intracellular Cl⁻ concentration alter the outward K⁺ conductances of astrocytes. Such a Cl⁻-mediated modulation of an astrocytic K⁺ conductance will have important consequences for the progression of spreading depression through brain tissue and for astrocytic swelling in pathological situations.

INTRODUCTION

Although activation of GABA_A receptors in both astrocytes and neurons results in Cl⁻ currents, the consequences of this activation are different. In neurons the E_Cl is usually somewhat more negative than the membrane potential and therefore a net Cl⁻ influx is evoked, leading to hyperpolarization (Alvarez-Leefmans and Russel 1990). In astrocytes, the E_Cl is far more positive than the membrane potential, and GABA_A receptor activation therefore will result in net Cl⁻ efflux and depolarization (Fraser et al. 1995). One exception is the situation in neonatal animals, where neurons can have an E_Cl more positive than the resting membrane potential, and GABA_A receptor activation results in depolarization (Cherubini et al. 1991). There is no corresponding difference in the E_Cl of astrocytes between neonatal and adult animals (Bekar et al. 1999).

GABA_A-receptor-mediated Cl⁻ current is not the only effect of GABA_A receptor activation: in cultured astrocytes and neonatal and adult astrocytes in situ and in one adult neuronal preparation (cerebellar granule cells), a longer-lasting blockade of K⁺ outward currents occurs concomitant with the Cl⁻ current (Bekar et al. 1999; Labrakakis et al. 1997; Muller et al. 1994; Pastor et al. 1995). The mechanism of this blockade and its link to the GABA_A receptor is unknown. However, an analogous situation exists with the astrocytic kainate receptor, where cation currents are followed by K⁺ channel blockade. In this case, it was found that Na⁺ net influx through the kainate receptor, with an increase in internal Na⁺ concentration, is responsible for the secondary K⁺ channel blockade (Robert and Magistretti 1997). Na⁺ increases alone were able to reduce outward K⁺ currents.

In the present project, we investigate the mechanism that underlies the K⁺ channel blockade after GABA_A-receptor-mediated Cl⁻ currents in astrocytes. We used cultured rat hippocampal astrocytes because we previously verified that the GABA_A receptors of this preparation have properties similar to those of “complex” hippocampal astrocytes in situ (Bekar et al. 1999).

METHODS

Cell culture

Primary cultures of hippocampal astrocytes were prepared from 1-day-old Wistar rat pups. Animals were anesthetized with methoxyflurane and decapitated. Hippocampi were removed aseptically, and cells were mechanically disaggregated using an 80-μm Nitex filter. The cells were seeded (10⁵ cells/35 mm dish) onto round coverslips in DMEM Gibco BRL containing 20% low endotoxin horse sera (HyClone) and incubated at 37°C in 5% CO₂, 95% humidified air. After 3 days, the culture medium was replaced by DMEM containing 10% low endotoxin horse sera. Cells then were fed twice a week in this manner. Cells were used for whole cell patch recordings when cells formed a confluent monolayer (~9–15 days). Cultures were checked periodically for purity: >85% of the cells are glial fibrillary acidic protein (GFAP) positive with the major other cell type being microglial cells.

Electrophysiological setup and protocols

For electrophysiological recordings, coverslips were placed in a perfusion chamber. The chamber was perfused continuously with normal Cl⁻ containing solution (see following text). Cells were analyzed under optical control using the conventional whole cell patch-clamp technique. Membrane currents were recorded in voltage-clamp mode using the EPC-7 patch-clamp amplifier (List Electronics), filtered at 3 kHz, and connected to a computer system serving as a stimulus generator. Recording pipettes were made of borosilicate capillaries (Hilgenberg, Germany) with resistances of 4–8 MΩ, and the reference electrode was a chlorided silver wire. The voltage-clamp holding potential was ~−80 mV and cell membranes were measured in current-clamp mode immediately after formation of whole cell patch. Cell membrane capacitance and membrane input resistance were calculated from the average of 10 10-mV depolarizing voltage steps.
from the holding potential. Current-voltage (I-V) relationships were
calculated from data obtained in the first 10 ms of the 100-ms voltage
steps ranging from −160 to +80 mV in 20-mV increments. These
voltage step protocols were applied every 5 s throughout the experi-
ments.

All currents (pA) obtained using the whole cell patch-clamp tech-
nique were normalized to cell size. To normalize values, all currents
were divided by the cell capacitance (pF) to give current densities.
Cell membrane capacitance is directly proportional to the size of the
were incubated in normal Cl−-containing solution and normal internal solution was the pipette
equilibrium potential is 0 mV due to symmetrical
demonstrating that the effects are not a culture artifact (Bekar
and K+ conductance was calculated using an
80 mV driving force (driving force for all the anions at
80 mV, where presumably there is very little voltage-
gated channel activity. Receptor conductance was calculated using an
80-mV driving force (driving force for all the anions at −80 mV
because the equilibrium potential is at 0 mV due to symmetrical
distribution). To calculate the conductance at +80 mV, the current at
−80 mV first was subtracted from the total current at +80 mV
(receptor current + voltage-gated currents), leaving solely voltage-
gated channel current. Receptor currents at −80 and +80 mV are
assumed to be equal because the anion equilibrium potential is 0 mV
(equal driving forces). Conductance then could be calculated using a
driving force of 170 mV (driving force for K+ at +80 mV; −90 mV
equilibrium potential).

When comparing muscimol receptor mediated effects on K+ channel
conductance, all values were normalized to muscimol receptor
current densities opposed to just cell capacitance. The rationale for
normalizing in this manner is due to the fact that K+ conductance
changes are linked directly to the size of the muscimol receptor
current. Furthermore by normalizing to muscimol current density, one
can rule out any variations in experimental conditions such as perfusion/diffusion speed, which would affect current size and desensit-
ization rate.

Solutions

The composition of the different external solutions used is listed in
Table 1, and the internal pipette solutions are listed in Table 2. Unless
otherwise mentioned, the cells always were incubated in normal Cl−
containing solution and normal internal solution was the pipette
electrolyte. Kainic acid was obtained from Sigma-Aldrich and picro-
toxin and muscimol were purchased from Tocris Cookson.

Statistical methods

All values obtained are expressed as means ± SE. All statistical
comparisons were performed using Excel software on an IBM com-
patible computer system. An unpaired two-tailed t-test, assuming
equal variance, was performed on all series of data. Values of P <
0.05 were considered significant.

RESULTS

Two effects of receptor activation

When cultured astrocytes in normal Cl−-containing solution
were exposed to the GABA<sub>A</sub> receptor agonist muscimol (200
μM), we consistently saw an increase in receptor inward
conductance with a concomitant longer lasting outward K<sup>+</sup>
conductance decrease (Fig. 1). The peak conductance decrease
always occurred with the peak receptor inward conductance.
The peak conductance blockade averaged 35%, whereas the
plateau conductance decrease averaged 15% (Table 3; Fig. 1).
The two effects of GABA<sub>A</sub> receptor activation have been
shown in astrocytes of neonatal and adult hippocampal slices,
demonstrating that the effects are not a culture artifact (Bekar
et al. 1999). Figure 2 illustrates both kainate and muscimol
effects on cultured hippocampal glial cells in normal Cl−
-containing external and Na<sup>+</sup>-free external solutions. In normal
Cl−-containing external solution (Fig. 3A), application of 200
μM kainate (10 ± 1.5 pA/pF, n = 10) or 200 μM muscimol
(56 ± 15 pA/pF, n = 33) results in a receptor current and a
secondary blockade of outward currents.

### TABLE 1. External salt solutions

<table>
<thead>
<tr>
<th></th>
<th>Na&lt;sup&gt;+&lt;/sup&gt;</th>
<th>K&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Cl&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Glucose</th>
<th>HEPES</th>
<th>Gluconate</th>
<th>Ba&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Br</th>
<th>I&lt;sup&gt;-&lt;/sup&gt;</th>
<th>NMDG</th>
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<td>HEPES</td>
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<td>130</td>
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<td>10</td>
<td>5</td>
<td>—</td>
<td>22</td>
<td>—</td>
<td>—</td>
<td>150</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-free HEPES</td>
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<td>3.5</td>
<td>130</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>5</td>
<td>—</td>
<td>22</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Low Cl&lt;sup&gt;-&lt;/sup&gt; HEPES</td>
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<td>10</td>
<td>5</td>
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<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;-containing HEPES</td>
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<td>22</td>
<td>—</td>
<td>130</td>
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<tr>
<td>Gluconate</td>
<td>150</td>
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<td>2</td>
<td>2</td>
<td>10</td>
<td>5</td>
<td>152</td>
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</table>

All solutions (in mM) buffered to pH 7.3. NMDG, N-methyl-D-glucamine.
TABLE 2. Internal pipette solutions

<table>
<thead>
<tr>
<th></th>
<th>K⁺</th>
<th>Cl⁻</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>EGTA</th>
<th>Na₂ATP</th>
<th>HEPES</th>
<th>Br⁻</th>
<th>I⁻</th>
<th>Gluconate</th>
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<td>130</td>
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<td>5</td>
<td>3</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Na₂ATP free</td>
<td>130</td>
<td>—</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
<td>—</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bromide</td>
<td>130</td>
<td>—</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>130</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Iodide</td>
<td>130</td>
<td>—</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>130</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gluconate</td>
<td>130</td>
<td>—</td>
<td>0.5</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>130</td>
<td>—</td>
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</tr>
</tbody>
</table>

All solutions (in mM) buffered to pH 7.3.

**Role of Na⁺**

To test the involvement of Na⁺ in the secondary blockade of the two agonists, we tested the responses in Na⁺-free external solution: the two responses to kainate are abolished completely, whereas the responses to muscimol are not significantly different (Table 3). These results suggest that Na⁺ is not a major factor involved in the secondary blockade of outward currents linked to GABA_A receptor activation (Fig. 2B).

**Need for Cl⁻ pore opening**

Figure 3, A and B, shows two ways in which we isolated binding of muscimol to the GABA_A receptor. First (Fig. 3A), we applied 200 μM muscimol in the presence of 200 μM of the noncompetitive GABA_A receptor antagonist picrotoxin. Picrotoxin does not affect binding of muscimol to the receptor but rather inserts itself into the receptor channel pore, effectively blocking the Cl⁻ conductance. Picrotoxin effectively blocks both effects of muscimol, demonstrating dependence on Cl⁻ conductance through the channel for the secondary blockade. The second way we isolated muscimol binding to the receptor (Fig. 3B) was by replacing Cl⁻ with gluconate in both the pipette filling solution and the external superfusion solution, which is impermeable for the GABA receptor channel. Muscimol will bind under these circumstances and open the channel, but no current carrier is available to generate a current flux as the result of the conductance change. Again, muscimol binding under these conditions induces neither an anion flux nor a decrease in K⁺ current, reinforcing the hypothesis that the secondary blockade is linked directly or indirectly to Cl⁻ net flux through the receptor channel pore out of the cell.

**[ATP]ᵢ dependency**

That this action is not due to modulation of a second-messenger effect of muscimol is supported by the observation that removal of ATP from the pipette solution had no effect on the secondary blockade (Fig. 3C). Therefore not only is the blockade of outward current not dependent, solely, on muscimol binding to the receptor, but it is also not dependent on phosphorylating mechanisms.

**Effect of changing external Cl⁻ concentrations**

Figure 4A demonstrates that the secondary blockade of outward current involves inhibition of a barium-sensitive K⁺ current. Application of 10 mm barium causes a decrease of the membrane conductance from 0.67 ± 0.07 to 0.21 ± 0.05 nS/pF (n = 5). Muscimol application in the presence of barium results in an inward receptor current (73 ± 20 nA/pF, n = 5) that is not different from controls without barium (56 ± 15 nA/pF, n = 33). However, the secondary blockade is abolished in barium-containing solution (Fig. 4A). Experiments involving the reduction of the Cl⁻ concentration from 130 to 30 mM in the external solution displayed interesting results. This reduction mimicked the action of barium, decreasing the outward current at depolarizing potentials as shown by the I-V relationship in Fig. 4B. The membrane conductance was reduced from 0.73 ± 0.09 to 0.29 ± 0.04 nS/pF (n = 5). Figure 4C illustrates how the secondary reduction of outward current associated with increased efflux of Cl⁻ is occluded by barium. These experiments suggest that decreasing efflux of Cl⁻ results in the inhibition of a barium-sensitive K⁺ outward current and that a Cl⁻ conductance does not play a role under these circumstances.

**Effect of replacing Cl⁻ with halide anions**

If the secondary blockade of outward K⁺ channels is indeed a direct result of a decrease in the Cl⁻ concentration, then replacement of Cl⁻ (both in the internal pipette solution and in the external perfusion solution) with other halide anions (different sizes) might show differing results. Furthermore the results should agree with the anion selectivity isotherms proposed by Wright and Diamond (1977). Figure 5 shows I-V relationships of cultured hippocampal astrocytes in three different salt solution. All three I-V relationships were obtained in

**TABLE 3. Effects of ions on the secondary blockade in hippocampal cultures**

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>33</td>
<td>56 ± 15</td>
<td>35 ± 2.7</td>
<td>15 ± 1.6</td>
<td>1.0 ± 0.14</td>
<td>0.45 ± 0.08</td>
<td>0.22 ± 0.034</td>
</tr>
<tr>
<td>Na⁺-free Cl⁻</td>
<td>14</td>
<td>33 ± 4.4</td>
<td>35 ± 5.4</td>
<td>16 ± 3.7</td>
<td>1.3 ± 0.19</td>
<td>0.54 ± 0.13</td>
<td>0.2 ± 0.037</td>
</tr>
<tr>
<td>ATP⁺-free Cl⁻</td>
<td>7</td>
<td>31 ± 6.8</td>
<td>36 ± 8.3</td>
<td>12 ± 3.0</td>
<td>1.2 ± 0.16</td>
<td>0.38 ± 0.07</td>
<td>0.23 ± 0.097</td>
</tr>
<tr>
<td>Bromide</td>
<td>8</td>
<td>70 ± 15</td>
<td>70 ± 4.9</td>
<td>35 ± 3.6</td>
<td>1.2 ± 0.16</td>
<td>0.66 ± 0.14</td>
<td>0.40 ± 0.11</td>
</tr>
<tr>
<td>Iodide</td>
<td>10</td>
<td>67 ± 11</td>
<td>43 ± 7.3</td>
<td>18 ± 4.6</td>
<td>0.72 ± 0.13</td>
<td>0.3 ± 0.075</td>
<td>0.38 ± 0.061</td>
</tr>
</tbody>
</table>

Values given are means ± SE. Values are shown as percent change peak/plateau blockade, as well as percent change peak/plateau blockade normalized to receptor current density (see METHODS). * Values are significantly different than results in chloride-containing salt solution (P < 0.05).
internal and external salt solutions with symmetrical distribution of the major halide anion. The solutions contained Cl\(^{-}\) (chloride), I\(^{-}\) (iodide), or Br\(^{-}\) (bromide) as the major anion. The I-V relationship obtained in the Br\(^{-}\) containing salt solution (Fig. 5) was not significantly different from that in the Cl\(^{-}\) containing solution. The I-V relationship in the I\(^{-}\) salt solution, however, was only significantly different at 180 mV compared with the Cl\(^{-}\) solution. Results of the secondary conductance blockade in response to muscimol application in the different anion containing solutions are summarized in Table 3. When comparing the percentage of peak conductance blockade normalized to receptor current density (see METHODS), Br\(^{-}\) (0.40) and I\(^{-}\) (0.38) exhibited significantly larger blockade on the membrane outward K\(^{+}\) conductance as compared with Cl\(^{-}\) ions (0.22).

**DISCUSSION**

We investigated a long-lasting blockade of voltage-gated outward K\(^{+}\) currents in cultured hippocampal rat astrocytes after muscimol exposure in normal Cl\(^{-}\)-containing external solution. This blockade is identical to the one seen in adult and neonatal glial cells in situ in external solutions buffered with bicarbonate (Bekar et al. 1999; Muller et al. 1994; Pastor et al. 1995). It also is seen in cerebellar granule cells (Labrakakis et al. 1997).

The mechanism that connects this blockade with agonist binding to the GABA\(_A\) receptor has, to this date, remained unknown. An analogous blockade associated with the ionotropic AMPA/kainate receptors in glial cells is much better elaborated. These AMPA/kainate receptors mediate a mixed Na\(^{+}/K^+\) current (Seifert and Steinhauser 1995), resulting in increased intracellular Na\(^{+}\) concentrations. Na\(^{+}\) itself has been found to be responsible for the blockade of the outward K\(^{+}\) currents (Borges and Kettenmann 1995; Robert and Magistretti 1997). Our astrocytes exhibited such a AMPA/kainate-evoked, Na\(^{+}\)-dependent blockade of the outward K\(^{+}\) channels, but the muscimol-induced blockade was unaffected (Table 3) when Na\(^{+}\) was removed from both internal and external solutions. Uptake of the agonist cannot play a role because muscimol is not transported into the cell (Kanner 1997).
In symmetrical 130 mM Cl\textsuperscript{−} conditions (\(E_{\text{Cl}} = 0\) mV), the opening of the GABA receptor channel would cause a Cl\textsuperscript{−} efflux (driving force of 80 mV). Cl\textsuperscript{−} efflux is necessary for blockade, suggesting that the blockade is dependent on a decrease in the level of intracellular Cl\textsuperscript{−}. Muscimol binding alone without such a current is insufficient. Replacement of Cl\textsuperscript{−} inside and outside the cell with gluconate abolished both the receptor current and secondary blockade. Direct blockade of Cl\textsuperscript{−} efflux by picrotoxin (Fraser et al. 1995), prevented the secondary blockade and induction of efflux without affecting muscimol binding to the GAB\textsubscript{A} receptor. Induction of Cl\textsuperscript{−} efflux without activating GABA\textsubscript{A} receptors, through reduction of extracellular Cl\textsuperscript{−} mimicked the blockade. The blockade is also independent of the presence of ATP in the pipette. Thus the involvement of G proteins (unlikely in any event to play a role in GAB\textsubscript{A} receptor activation) in the secondary blockade of outward K\textsuperscript{+} currents is unlikely.

Evidence supporting the involvement of Cl\textsuperscript{−} ions in the secondary blockade comes from experiments involving perfusion of low [Cl\textsuperscript{−}]\textsubscript{o} to the cell, mimicking Cl\textsuperscript{−} efflux changes that occur in muscimol-receptor binding. Under these conditions, we saw a reduction of outward currents without any
effect on inward currents, similar to the secondary response of muscimol. Furthermore reducing the Cl\(^-\) concentration in the presence of the broad spectrum K\(^+\) channel blocker barium, no significant effect on the membrane conductance was seen. This indicates that Cl\(^-\) efflux is inhibiting voltage-gated outward K\(^+\) currents. Indeed the secondary blockade caused by muscimol is occluded by barium, a blocker of a number of K\(^+\) channels. The indication that a reduction of the external Cl\(^-\) concentration is causing a significant Cl\(^-\) efflux points to a large Cl\(^-\) conductance of the resting membrane, a large carrier mediated efflux or both. Because we found no significant change in the membrane conductance when the Cl\(^-\) was reduced in the presence of barium, we have to exclude a significant Cl\(^-\) conductance, at least under the circumstances tested. Another possibility is the efflux via the Na-K-Cl cotransporter, which is expressed by astrocytes and whose mode of action depends on the combined driving forces of the participating ions (Walz 1995) and which would be reversed by the reduction of external Cl\(^-\).

There is evidence for Cl\(^-\)-dependent modulation of other physiological processes: Adams and Oxford (1983) showed that inorganic anions reduce and slow delayed rectifying K\(^+\) channel conductance in the squid giant axon. They found that F\(^-\), Br\(^-\), and Cl\(^-\) all reversibly reduced outward K\(^+\) conductance (Adams and Oxford 1983). However, this effect of Cl\(^-\) also might be explained by the formation of a junction potential when changing Cl\(^-\)-containing solutions. Changing from 130 to 30 mM Cl\(^-\) always shifted the membrane potential more negative, as obtained in current-clamp mode on the EPC-7. As a result, the voltage jump protocol may not depolarize the membrane to the same extent, resulting in smaller outward currents relative to the control situation. A strong argument against this hypothesis is the activation kinetics (normally around ~40 mV) of the resulting I-V relationship would appear more positive than control, which was never the case. Furthermore the reversal potential of the I-V relationship does not shift more positively, demonstrating that the effect is solely on outward currents at depolarized potentials more positive than 0 mV. If it was a result of a junction potential, the I-V relationships would look identical but shifted more positively. Similar studies in the squid giant axon also showed that reduced or slowed K\(^+\) conductance by F\(^-\) or Br\(^-\) did not shift I-V relationships more positively (Adams and Oxford 1993).

Although there was no significant difference in resting membrane currents, the secondary conductance decrease in response to muscimol is significantly larger when both intra- and extracellular Cl\(^-\) is replaced by either Br\(^-\) or I\(^-\). Although studies have shown anions to effect G-protein-mediated responses (Higashijima et al. 1987; Lenz et al. 1997), Br\(^-\) and I\(^-\) in these studies always showed a lesser ability to affect G proteins than Cl\(^-\) (Lenz et al. 1997). In contrast, the secondary response in our study shows an increase in size during replacement of Cl\(^-\) with Br\(^-\) or I\(^-\), suggesting, again, that the secondary response is not dependent on G-protein-mediated effects. The effects of the different halides in our glial cell culture system display activity dependent characteristics (Br\(^-\) = I\(^-\) > Cl\(^-\); conductance decrease) consistent with sequences 1 and 2 of the selectivity isotherms proposed by Wright and Diamond (1977), indicating interaction of the halide anions with a selective site, likely, on voltage gated outward K\(^+\) channels.

**Physiological significance**

Our results suggest that the intracellular Cl\(^-\) concentration is modulating the conductance of outward K\(^+\) channels. In cultured astrocytes with symmetrical Cl\(^-\) distribution (130 mM inside and outside), muscimol causes a decrease in the internal Cl\(^-\) concentration through Cl\(^-\) outflux. This decrease is large enough to cause a 35% blockade of K\(^+\) outward conductances. Because the same phenomenon is seen in astrocytes in situ (Bekar et al. 1999), it is probably not a cell culture artifact. However, it is not clear under what conditions this mechanism is playing a physiological role.

The normal physiological Cl\(^-\) concentration of an astrocyte in situ and in vitro is probably between 30 and 40 mM (Walz 1995). At this Cl\(^-\) concentration level, it is not clear how large the modulatory effect will be because the outward driving force for Cl\(^-\) will be diminished.

It might well turn out that the largest changes seen in the intracellular astrocytic Cl\(^-\) concentration are not due to GABA binding but involve Cl\(^-\) concentration increases due to pathological swelling and spreading depression waves when massive anion channel opening occurs (Phillips and Nicholson 1979). Whatever the circumstances, we established that intracellular changes in the Cl\(^-\) concentration per se have to be considered as influencing important physiological processes. Because this secondary blockade also is seen in granular cerebellar neurons, this mechanism might not be unique to astrocytes.

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