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Retinal glial cell glutamate transporter is coupled to an anionic conductance

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ABSTRACT **Application of L-glutamate to retinal glial (Müller) cells results in an inwardly rectifying current due to the net influx of one positive charge per molecule of glutamate transported into the cell. However, at positive potentials an outward current can be elicited by glutamate. This outward current is eliminated by removal of external chloride ions. Substitution of external chloride with the anions thiocyanate, perchlorate, nitrate, and iodide, which are known to be more permeant at other chloride channels, results in a considerably larger glutamate-elicited outward current at positive potentials. The large outward current in external nitrate has the same ionic dependence, apparent affinity for L-glutamate, and pharmacology as the glutamate transporter previously reported to exist in these cells. Varying the concentration of external nitrate shifts the reversal potential in a manner consistent with a conductance permeable to nitrate. Together, these results suggest that the glutamate transporter in retinal glial cells is associated with an anionic conductance. This anionic conductance may be important for preventing a reduction in the rate of transport due the depolarization that would otherwise occur as a result of electrogenic glutamate uptake.**

High-affinity excitatory amino acid transporters are present in both glial cells and neurons, and play an important role in removing the neurotransmitter glutamate from the extracellular space (1–3). Because glutamate is excitotoxic, these transporters are thought to play a vital role in reducing the possibility of cell death following extended excitation. From studies in both synaptosomes and glial cells, it appears that the transport of glutamate against its electrochemical gradient is coupled to the cotransport of two sodium ions, and the countertransport of one potassium and one hydroxyl ion, down their electrochemical gradients. The net result of this stoichiometry is the influx of one electrical charge per molecule of glutamate transported into the cell.

However, more recent studies of glutamate transporters cloned from the human motor cortex and cerebellum and expressed in *Xenopus* oocytes indicate that the transport of excitatory amino acids for all four known subtypes is also associated with an anionic conductance (4, 5), which is stoichiometrically uncoupled (5). The presence of this conductance results in a glutamate-elicited current that is inward at negative potentials but becomes outward at positive potentials. Such an outward current is unexpected for a classical model of a transporter, which predicts that the glutamate-elicited current should be inward at all potentials.

It is not certain, however, whether such a conductance is an artifact associated with expression in oocytes, or whether it is also a general property of native glutamate transporters. Although it has been suggested that retinal cones, rods, and bipolar cells all have glutamate transporters with an associated chloride conductance (6–8), the existence of glutamate transporters in these cells has been somewhat controversial. We have therefore decided to examine whether this conductance exists in retinal glial cells, because glutamate transport in these cells has been studied extensively (9, 10). To date, there is little evidence of a chloride conductance associated with glutamate transport in retinal glial cells. Although a glutamate-elicited outward current at positive potentials has been reported in one study (9), in another study (10), no outward current was seen at any potential. Both papers reported that removing chloride from either side of the membrane has no effect on transporter currents.

In this paper, we demonstrate that glutamate activates an outward current at positive potentials and that this outward current is the result of an anionic conductance associated with glutamate transporters in retinal glial cells. The existence of such an anionic conductance may be important for preventing a large depolarization associated with electrogenic uptake of glutamate. By remaining hyperpolarized, the glial cell can remove extracellular glutamate more rapidly, thus reducing the excitotoxic effects of glutamate.

MATERIALS AND METHODS

Cell Preparation. Retinas were isolated from aquatic phase tiger salamanders (*ambystoma tigrinum*) and placed in a divalent free Ringer's solution containing 8 units of papain (Worthington) and 1 mg of cysteine per ml. The retinas were incubated twice for 15 min at 30°C, rinsed once, quenched in bovine serum albumin at 1 mg/ml for 10 min, and rinsed twice more. The retina was then chopped into pieces roughly 500 μm in diameter and stored at 4°C until needed. Just prior to an experiment, a small portion of chopped retina was gently triturated with a fire-polished Pasteur pipette and the resulting cell suspension was placed in a recording chamber with a clean glass bottom. Retinal glial cells, or Müller cells, were clearly identified by their unique morphology (10). Often, recordings were from cells missing the endfoot, where most of the potassium channels are located (11).

Recording Solutions. Control external solution contained 115 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, 15 mM glucose, and 0.1 mM $CdCl_2$ (pH 7.4). For chloride-free solutions (see Fig. 2), gluconate salts were substituted. In solutions containing other anions (SCN^-, ClO_4^-, NO_3^-, and I^-), NaCl was replaced by the appropriate sodium salt. For experiments with varying concentrations of $NaNO_3$ (see Fig. 5), extracellular solution consisted of combinations of the $NaNO_3$ and gluconate solutions. In sodium-free extracellular solution, $NaNO_3$ was replaced by Tris nitrate and the pH was adjusted by the addition of tris base. Cells were continuously perfused via flow pipes (i.d., 400 μm), positioned within 250 μm of the cell.

The high-chloride pipette solution contained 100 mM CsCl, 5 mM NaCl, 1 mM $CaCl_2$, 5 mM EGTA, and 5 mM Hepes. The low-chloride pipette solution contained 110 mM cesium gluconate, 5 mM sodium gluconate, 1 mM $CaCl_2$, 5 mM EGTA, and 5 mM Hepes. In experiments where external nitrate was

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Abbreviations: THA, *threo*-3-hydoxyaspartate; DHKA, dihydrokainate.
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present (see Figs. 4 and 5), the pipette consisted of 85 mM cesium gluconate, 25 mM $CsNO_3$, 5 mM sodium gluconate, 1 mM $CaCl_2$, 5 mM EGTA, and 5 mM Hepes. The 20 mM bis(2-aminophenoxy)ethane-*N,N',N'*-tetraacetate (BAPTA) pipette solution contained 20 mM tetracesium BAPTA, 40 mM cesium gluconate, 25 mM $CsNO_3$, 5 mM sodium gluconate, and 5 mM Hepes. For all internal solutions, 4 mM MgATP and <1 mM CsF were added fresh daily and stored on ice. The final pH was set to 7.0 by the addition of CsOH.

Recording and Analysis. Whole-cell patch clamp recordings were measured using a List EPC-7 (Medical Systems, Greenvale, NY). Currents were filtered at 2 kHz, and the data were sampled at 5 kHz. Liquid junction potentials were measured directly (12) and subtracted from the command and holding potentials.

Borosilicate patch pipettes had resistances of 1–3 MΩ, and whole-cell series resistances of 2–4 MΩ. Membrane resistances ranged from 150 to 350 MΩ and membrane capacitances from 125 to 250 pF.

Cells were held at −60 mV, and stepped at −100 to +80 mV in 10-mV intervals for 125 ms, in the presence or absence of various transporter substrates (Fig. 1*A*). To avoid contamination by time-varying, substrate-independent currents occasionally seen at extreme potentials, steady-state currents were measured as the average current during the last 15 ms of the voltage step. The glutamate-elicited current was measured as the difference between the steady-state currents in the absence and presence of substrate. The difference current is shown in Fig. 1*B*, and the steady-state current is shown in Fig. 1*C*. The differences were taken by using the control currents both before and after the application of substrate, and the two resulting current–voltage curves were averaged together. Cells not showing complete reversibility were excluded from analysis.

When cross-cell comparisons were necessary (see Figs. 3 and 5), variations in transporter number between cells were taken into account as follows. In each cell, glutamate was applied in both the control (chloride) solution and test extracellular solutions; the control current–voltage relations were scaled to have the same magnitude over the range of −100 to 0 mV. These scale factors were then applied to the current–voltage relations in the test solutions, which are plotted in dimensionless units relative to the average response of glutamate in the control solution at −60 mV. Similar results were obtained when the currents were scaled by membrane capacitance (10).

RESULTS

In contrast with the classical model of a transporter, previous work with glutamate transporters in glial cells (9) and in three of the four subtypes of glutamate transporters cloned from human (4, 5) showed that glutamate can elicit an outward current at positive potentials. As shown in Fig. 2 (□), in high-chloride extracellular solution and high-chloride intracellular solution, 100 μM L-glutamate in Müller cells evoked an inwardly rectifying current that has a significant outward current at potentials more positive than the chloride equilibrium potential (E_{Cl} = 0 mV). The average reversal potential was +10.6 ± 7.5 mV (mean ± SD, n = 6).

In the human transporter clones, the outward current is carried predominantly by the influx of external chloride ions (4, 5). In the present experiments, substitution of external chloride with the relatively large anion gluconate eliminated the outward current in all 5 cells studied (Fig. 2, ○). In contrast, the inward current was unaffected by the removal of external chloride ions. A similar result occurred when the intracellular solution consisted of low chloride (n = 10).

Voltage and ligand-gated chloride channels allow the flux of a variety of small anions, including SCN^-, ClO_4^-, I^-, NO_3^-, F^-, and Cl^- (13). Similarly, an influx of the external anions NO_3^-,

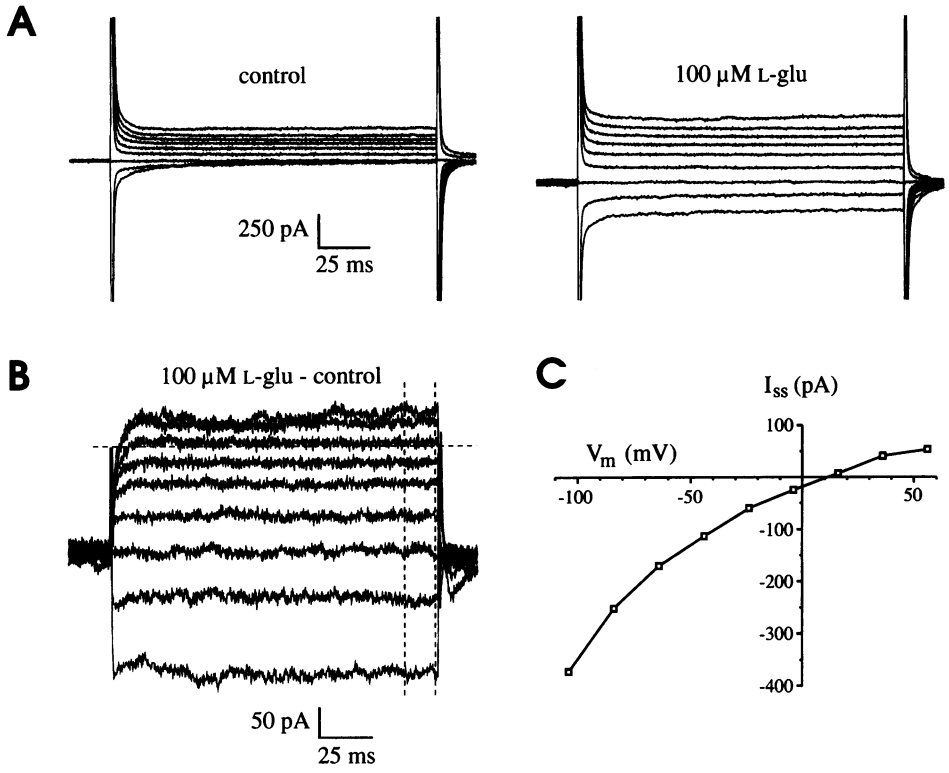

FIG. 1. Determination of steady-state current–voltage relations. (*A*) Currents measured in response to voltage steps ranging from −100 to +60 mV from a holding potential of −60 mV, in high-chloride (control) extracellular solution (*Left*), and in the presence of 100 μM L-glutamate (*Right*). (*B*) Glutamate-elicited current, calculated by subtracting the currents measured in control solution from the currents measured in glutamate, as shown in *A*. The horizontal dashed line indicates the zero-current level. The vertical dashed lines indicate the time range over which the current was averaged for producing the current–voltage relation in *C*. (*C*) Steady-state current–voltage relation. I_{ss}, steady-state current; V_m, membrane potential.

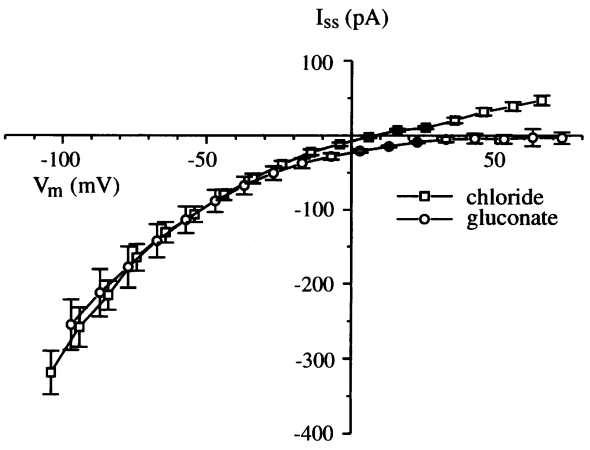

FIG. 2. Removal of external chloride eliminates the glutamate-elicited outward current. The averaged steady-state current–voltage curves were elicited by 100 μM L-glutamate in high chloride (□) and in zero chloride (gluconate; ○) extracellular solutions (n = 6). The intracellular solution was high chloride. Error bars represent standard error. Similar results were obtained with low chloride intracellular solutions (n = 10).

I^-, Br^-, Cl^- and F^-, is associated with the transport of D-aspartate through the cloned glutamate transporters from human cortex (5). We have examined the effect of replacing external chloride with NO_3^-, I^-, SCN^-, and ClO_4^- on the glutamate-elicited current (Fig. 3). The intracellular solution contained 2 mM chloride, so that the majority of current through the anionic conductance will be outward, due to the influx of external permeant anions. Each current-voltage relation is plotted relative to the current elicited by glutamate in a normal chloride extracellular solution at −60 mV, to allow cross-cell comparison (see *Materials and Methods*). All four anionic substitutions produced glutamate-elicited currents that reversed direction. The outward currents at positive potentials were considerably larger than when the external anion was chloride. The slope conductance of the outward current (at potentials more positive than 0 mV where the contribution by the electrogenic transport of glutamate is smallest) for the anions SCN^-, ClO_4^-, NO_3^-, I^-, and Cl^- has a relative magnitude of 38.1:11.4:12.3:7.96:1.00, respectively.

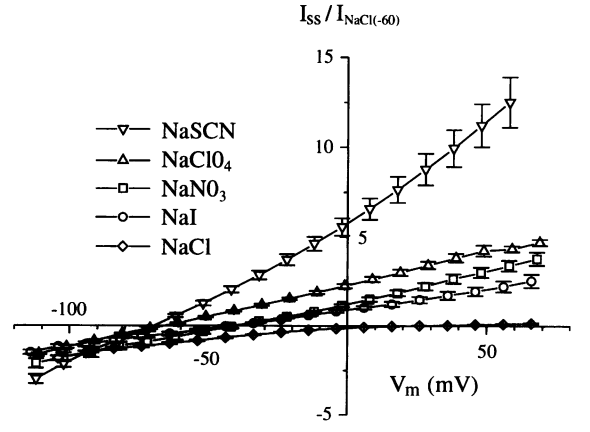

FIG. 3. The magnitude of the glutamate-elicited outward current depends on the external anion. Steady-state current–voltage curves were elicited by 100 μM L-glutamate in the presence of external 115 mM NaSCN (n = 6), $NaClO_4$ (n = 5), $NaNO_3$ (n = 7), NaI (n = 5), or NaCl (23), each replacing 115 mM NaCl. Each curve is plotted relative to the response to glutamate in normal chloride extracellular solution at −60 mV. Response in NaCl is the averaged test response from all other cells.

One interpretation of these results is that the anionic current is due to a glutamate-elicited, calcium-activated anionic current, perhaps activated by a metabotropic glutamate receptor. This seems unlikely, based on the following experiment. Five cells were dialyzed for 20 min with 20 mM bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate in the recording pipette. Under these conditions, 100 μM L-glutamate still produced both an inward and outward current in the presence of extracellular nitrate (external $CdCl_2$ was increased to 250 μM, to completely block calcium channels; data not shown). The voltage dependence was similar to what was seen immediately upon breaking into the cells.

A more likely alternative is that the glutamate-elicited current is the result of an anionic conductance associated with a glutamate transporter, as has been reported elsewhere (4, 5). Several experiments were performed to test whether the large glutamate-elicited outward current present at positive potentials in external nitrate is due to the same glutamate transporter that has been described (9, 10, 14). First, glutamate transport in Müller cells requires the presence of extracellular sodium (9, 10, 14). As shown in Fig. 4*A*, replacement of external $NaNO_3$ by Tris·NO_3 eliminated the glutamate-elicited current at all potentials (n = 5). When external sodium was restored, the current recovered to control levels. Thus, both the inward and outward currents in nitrate require the presence of external sodium.

Second, previous work has shown that the EC_{50} of the Müller cell glutamate transporter for L-glutamate is approximately 20 μM over the range of −100 to 0 mV (10, 14). We examined the effects of various concentrations of glutamate in the same seven cells when nitrate was present (Fig. 4*B*). The shape of the current-voltage relation and the reversal potential in nitrate were independent of glutamate concentration. Furthermore, the EC_{50} for L-glutamate at −100 mV (14.7 ± 4.8 μM) and at +50 mV (9.68 ± 2.28 μM) are approximately the same and are similar to the EC_{50} published previously. Thus, it seems likely that the inward and outward currents elicited by glutamate in extracellular nitrate are due to the same mechanism.

Third, THA has been reported to be a substrate inhibitor of glutamate uptake in Müller cells (10). That is, THA is transported with a higher affinity but lower rate of transport than L-glutamate and thus competitively inhibits glutamate transport. Consistent with these findings, we find that THA elicits a current in the presence of nitrate that closely mimics the response to L-glutamate. When measured over the entire voltage range, the response to 100 μM THA is 44.4 ± 14.6% (n = 7) of the response to 100 μM L-glutamate (in the same cells) and the response to 100 μM L-glutamate in the presence of 100 μM THA was 57.8 ± 6.7% of the response to L-glutamate alone (Fig. 4*C*). Note that the inward and outward currents are both reduced to a similar extent, and the reversal potentials do not change, again suggesting that both the inward and outward currents in nitrate are elicited by a similar mechanism.

Finally, DHKA has been reported previously to be a relatively weak blocker of glutamate transport in Müller cells (10). We also found that DHKA was an incomplete blocker of glutamate. With low intracellular chloride and high extracellular chloride we found that 500 μM DHKA, reduced the response to 30 μM L-glutamate to 39.0 ± 24.9% (n = 6) of control. In the presence of nitrate, a similar effect was seen; 500 μM DHKA reduced the response to 30 μM L-glutamate to 44.6 ± 5.7% (n = 5) of control (Fig. 4*D*). Both the inward and outward currents in nitrate were reduced to a similar degree by DHKA, and the reversal potential did not change.

Varying the anionic concentration gradient across the membrane should shift the reversal potential of a current produced by an anionic conductance. We measured the response to 100 μM L-glutamate with 25 mM nitrate in the pipette, and various concentrations of nitrate outside the cell. The steady-state current–voltage curves are shown in Fig. 5*A*, plotted relative to

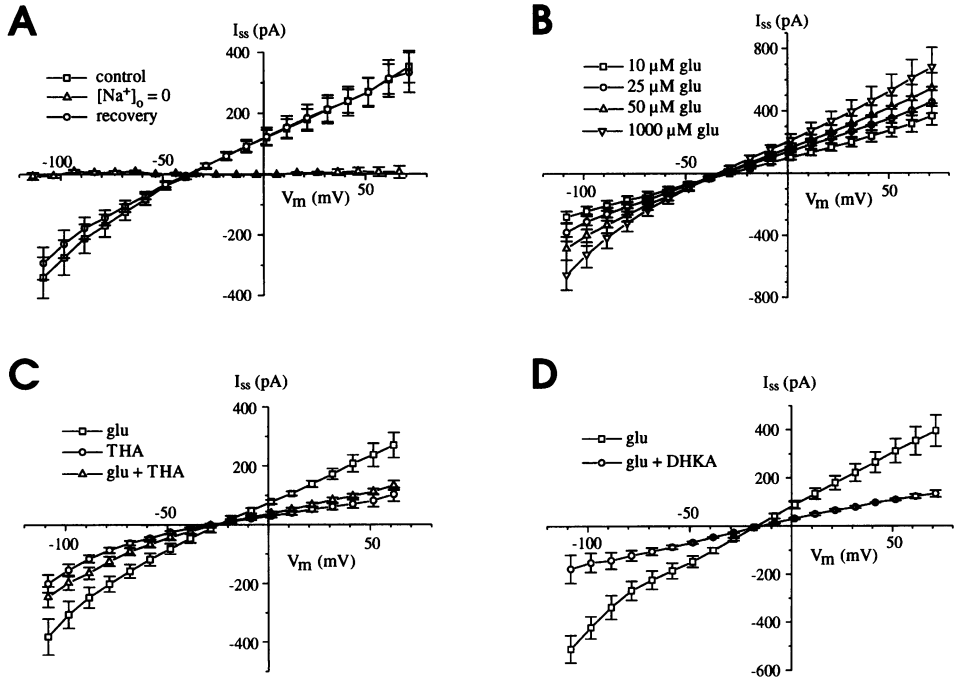

FIG. 4. Both the inward and outward glutamate currents in nitrate result from a glutamate transporter. In each experiment (*A–D*), the same cells were used for every condition. (*A*) Currents evoked by 100 mM L-glutamate (control) are reversibly blocked when extracellular sodium is completely replaced by the larger cation Tris (n = 5). (*B*) Currents evoked by 10, 25, 50, or 1000 μM L-glutamate (n = 7). (*C*) Currents evoked by either 100 μM L-glutamate alone, 100 μM *threo*-3-hydroxyaspartate (THA) alone, or 100 μM L-glutamate in combination with 100 μM THA (n = 5). (*D*) Currents evoked by 30 μM L-glutamate alone or in the presence of 500 μM dihydrokainate (DHKA; n = 4).

the response to glutamate in control Ringer's at −60 mV. The reversal potentials are plotted as a function of external nitrate concentration in Fig. 5*B*. The measured reversal potentials deviate from the nitrate equilibrium potential (Nernst equation; dashed line), especially at more negative potentials. A fit to the measured reversal potentials yields a slope of 44.9 mV per decade concentration of nitrate. The deviation from the Nernst equation suggests that we have not isolated the anionic current from other contaminating currents, principally the transport current (see *Discussion*).

DISCUSSION

The transport of one molecule of glutamate leads to the net influx of one positive charge, resulting in an inward current which is greatest at negative potentials (1). At positive potentials our results show that in external chloride, a small outward current is present in retinal glial cells. This observation is consistent with previous work (9), which attributed the outward current to a block of an uncoupled influx of sodium through the transporter that occurs in the absence of glutamate. Our results also suggest a conductance associated with the glial glutamate transporter, but we find that the outward current can be blocked by replacing chloride with gluconate, suggesting that this current is due to a glutamate-elicited influx of chloride. These findings are consistent with studies of the human glutamate transporter clones expressed in oocytes (4, 5), which concluded that the transport of glutamate does not require the presence of chloride, but rather a flux of chloride occurs simultaneously with glutamate transport. However, our experiments do not rule out the possible existence of additional uncoupled currents associated with glutamate transport.

In contrast to our findings, previous papers have stated that replacing extracellular chloride with gluconate had no effect

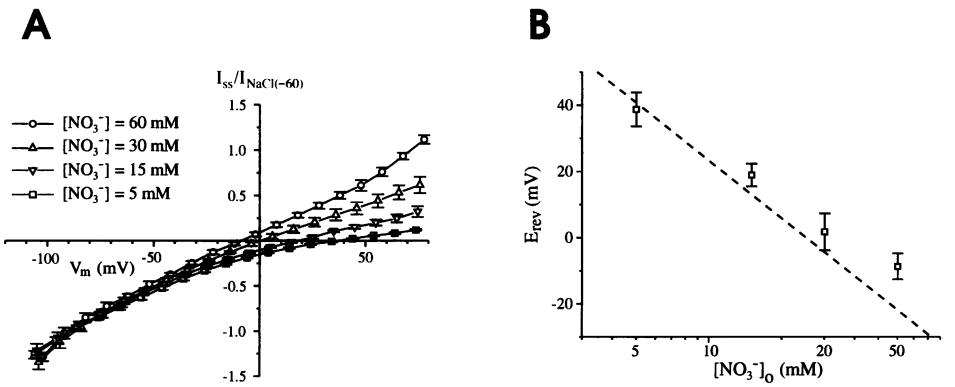

FIG. 5. Varying the concentration of external nitrate shifts the glutamate-elicited reversal potential. Internal pipette solution contained 25 mM nitrate. (*A*) Steady-state current–voltage curves elicited by application of 100 μM L-glutamate in the presence of 60 (n = 5), 30 (n = 5), 15 (n = 5), and 5 (n = 7) mM $NaNO_3$. Each curve is plotted relative to the response to glutamate in normal chloride extracellular solution at −60 mV. (*B*) Plot of the reversal potential as a function of external concentration of NO_3^- from the same cells as in *A*. Dashed line represents the Nernst equilibrium potential for nitrate. Error bars represent standard deviation.

on the glial glutamate transport current (9, 10). However, neither paper sufficiently described the effect of replacing chloride with gluconate over the positive potential range, where the effect would be the largest. It is possible that both papers overlooked the small gluconate-blocked outward current seen at these potentials.

The outward current elicited by glutamate application at positive potentials can be substantially enhanced by substituting different anions for chloride. By measuring the slope of the current-voltage curve at positive potentials, we find that the conductance follows the sequence: $SCN^- > NO_3^- \approx ClO_4^- > I^- > Cl^-$. This work extends previous studies performed on the human glutamate transporter EAAT1, which had a relative conductance of $NO_3^- > I^- > Br^- > Cl^- > F^-$ (5). A similar conductance sequence has been described in some chloride channels (15, 16), but not others (17, 18).

In the presence of external nitrate, both the inward and outward currents have an identical ionic dependence, apparent affinity, and pharmacology as has been reported previously for glutamate transport in these cells. Thus, it seems likely that the total current elicited by glutamate at all potentials is associated with glutamate transport.

In addition to these tests, the reversal potential was measured as a function of external nitrate concentration to determine if the outward current is caused by a coupled anionic conductance. The Nernst equation states that lowering the external anionic concentration will shift the reversal potential of a channel permeable only to anions in the depolarizing direction. Using four different concentrations of extracellular nitrate, we see that this relationship indeed holds. However, the Nernst equation also predicts that the reversal potential should shift by approximately 58 mV for a 10-fold change in nitrate concentration, instead of 44.9 mV as observed. This result is not surprising, however, because we are not examining the anionic conductance in isolation.

One major source of error is a contamination from the inwardly rectifying, transporter current. The presence of the electrogenic transport current will result in a more positive measured reversal potential, and also will reduce the overall shift due to a change in anionic concentration; as the concentration of the permeant anion (in this case nitrate) is increased, the negative shift in the reversal potential of the anionic conductance will be countered by an increase in the transport current at these more negative potentials. The greatest deviation occurs at the most negative potentials (the highest concentrations of external nitrate) where the transporter current is the largest. Thus, the transport current will result in a decreased slope of reversal potential as a function of extracellular nitrate concentration. Additional uncoupled fluxes associated with glutamate transport could also make the relationship between reversal potential and nitrate concentration deviate from the Nernst equation.

Our studies indicate that the glial cell glutamate transporter has anionic conductance properties that are quite similar to glutamate transporters reported previously in retinal cones (6), bipolar cells (7), and rods (8). Glutamate elicits a current in cones whose reversal potential closely follows the chloride equilibrium potential (19). Based on studies of the pharmacology (6, 20), ionic dependence (6, 19–21), and noise characteristics in the presence of transporter substrates (20, 21), it has been suggested that there exists a tight association between the glutamate transporter and a chloride channel, perhaps both part of a single molecule (6, 21). More recent work with glutamate transporter clones (4, 5) is consistent with this idea. We cannot, however, rule out the possibility that the glutamate transporter is tightly coupled to a separate chloride channel molecule.

The existence of an anionic conductance associated with the glial cell glutamate transporter will require a reexamination of studies indicating a thermodynamically coupled counterexchange of glutamate with pH-changing anions (22). In these studies, substitution of internal chloride with thiocyanate, perchlorate, and nitrate all produced larger inward currents elicited by glutamate, suggesting that these anions speed uptake of glutamate by binding more favorably to an intracellular site on the glutamate transporter. An alternative explanation for the enhanced inward current, based on our studies, is that the efflux of these more permeable anions through the uncoupled, associated anionic conductance is greater than for chloride. It is possible, however, that the anionic conductance can modulate the transport of glutamate. Our data do not address whether there is a coupled, counterexchange of an anion, as suggested previously (22).

In the presence of internal and external chloride, the gluconate-blocked outward current is small, suggesting that at depolarized potentials, the total current due to the uncoupled chloride flux is small. However, the chloride conductance in the human glutamate transporter EAAT1 is inwardly rectifying (5) and so at negative potentials, the inward chloride current may represent a significant fraction of the total glutamate-elicited current. What role could such a chloride conductance play? Depending on the chloride equilibrium potential relative to the normal membrane potential, the chloride conductance could provide a hyperpolarizing influence on the cell membrane that counters the depolarizing effect of the inward current caused by the electrogenic uptake of glutamate.

In the absence of such a conductance, glutamate uptake will result in a depolarization, which would decrease uptake and, in the case of neurons, may increase calcium-dependent glutamate release. This depolarization is prevented, in part, by the large potassium conductance present in retinal glial cells (11); however, this conductance is reduced by the presence of a metabotropic glutamate receptor (23). Furthermore, the current elicited by glutamate in Müller cells is quite large, and the chloride conductance may therefore be necessary to prevent a depolarization-induced decrease in glutamate uptake.

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