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Anion Conductance Behavior of the Glutamate Uptake Carrier in Salamander Retinal Glial Cells

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Glutamate uptake is driven by the cotransport of Na^+ ions, the countertransport of K^+ ions, and either the countertransport of OH^- or the cotransport of H^+ ions. In addition, activating glutamate uptake carriers has been shown to lead to activation of an anion conductance present in the carrier structure. Here we characterize the ion selectivity and gating of this anion conductance. The conductance is small with Cl^- as the permeant anion, but it is large with NO_3^- or ClO_4^- present, undermining the earlier use of NO_3^- and ClO_4^- to suggest that OH^- countertransport rather than H^+ cotransport helps drive uptake. Activation of the anion conductance can be evoked by extra- or intracellular glutamate and can occur even when glutamate transport is inhibited. By running the carrier backward and detecting glutamate release with AMPA receptors in neurons placed near the glial cells, we show that anion flux is not coupled thermodynamically to glutamate movement, but OH^-/H^+ transport is. The possibility that cell excitability is modulated by the anion conductance associated with glutamate uptake suggests a target for therapeutic drugs to reduce glutamate release in conditions like epilepsy.

Key words: glutamate; transporter; anion conductance; uptake; pH; glial cell

The extent to which glutamate uptake can lower the extracellular glutamate concentration in the CNS is determined by the ionic stoichiometry of the uptake process (Attwell et al., 1993). Entry of each glutamate ion into the cell is thought to be accompanied by the cotransport of two Na^+ ions (Baetge et al., 1979; Stallcup et al., 1979; Erecinska et al., 1983) and the countertransport of one K^+ ion (Kanner and Sharon, 1978; Barbour et al., 1988; Amato et al., 1994). In addition, glutamate uptake carriers generate pH changes, acid inside the cells and alkaline outside (Erecinska et al., 1983; Bouvier et al., 1992). Bouvier and colleagues (1992) suggested that, for the glutamate transporter in salamander retinal glia, power is obtained from the transmembrane pH gradient by the transport of an OH^- ion out of the cell rather than the (thermodynamically equivalent) transport of an H^+ ion into the cell. This was based on the observation that, when certain anions (NO_3^-, ClO_4^-, and SCN^-) were inside the cell, the inward current evoked by external glutamate was larger, but the pH change generated was unaffected or reduced, and that anion-sensitive electrodes detected the efflux of ClO_4^- from cells containing ClO_4^-. It was suggested that NO_3^-, ClO_4^-, and SCN^- competed for transport on a carrier site that normally transports OH^-.

The cloned mammalian glutamate transporters (Fairman et al., 1995; Wadiche et al., 1995) and the transporters in salamander photoreceptors and glia and in fish bipolar cells (Sarantis et al., 1988; Eliasof and Werblin, 1993; Grant and Dowling, 1995; Picaud et al., 1995; Eliasof and Jahr, 1996) activate an anion conductance when they bind external glutamate and Na^+. However, it is unclear how the binding of substrate to the carrier gates the anion conductance. Can the anion conductance component of the transporter be activated by intracellular glutamate during reversed uptake as well as by external glutamate during forward uptake? Is it even necessary for glutamate transport to occur for the anion conductance to open, or is glutamate binding sufficient? The presence of an anion conductance in the transporter structure also raises the possibility that the effects of NO_3^-, ClO_4^-, and SCN^- on the salamander glial carrier were exerted not through the postulated OH^--binding site but through the anion conductance component of the transporter molecule (Eliasof and Jahr, 1996). It further brings into question whether the observed pH changes are a result of substrate transport or are attributable to pH-changing anions passing through the anion conductance.

Here we investigate the gating of the anion conductance in the salamander glial glutamate transporter. We show that the anion conductance can be activated by glutamate binding to either side of the membrane and can occur independently of whether net glutamate transport occurs, suggesting that it is activated by a conformation change, which is allowed when the carrier is in a particular state of its uptake cycle. Transport of OH^- (or H^+) is shown to be coupled to glutamate transport and shown not to occur through the anion conductance.

MATERIALS AND METHODS

All experiments were done at room temperature, 25°C.

Salamander retinal glial cells. Glial (Müller) cells were isolated from tiger salamander retinae by using papain, as described previously (Barbour et al., 1991), and whole-cell-clamped with pipettes of series resistance (in whole-cell mode) ~3 MΩ, which leads to negligible series resistance voltage errors (<2 mV). Large pipettes are essential for dialyzing the cell adequately in experiments removing intracellular potassium from the cell (Szatkowski et al., 1991). When currents were compared in different cells, they were normalized by cell capacitance to compensate for variations in cell size (Barbour et al., 1991).

Solutions. Unless otherwise stated, the extracellular solution contained (in mM): NaCl 105, KCl 2.5, $CaCl_2$ 3, $MgCl_2$ 0.5, glucose 15, HEPES 5, and $BaCl_2$ 6 (to block the inward rectifier potassium channels of the cells), pH-adjusted to 7.3 with NaOH. A 1 M NaCl agar bridge was used as the bath electrode to reduce (to <0.4 mV) junction potential changes when changing the anion in the external solution. Unless otherwise stated, the standard pipette solution for uptake experiments contained (in

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mM): KCl 95, NaCl 5, HEPES 5, $MgCl_2$ 7, Na_2ATP 5, $CaCl_2$ 1, and K_2EGTA 5, pH-adjusted to 7.0 with 14 mM KOH. The pipette solution for studying the effect of $[K^+]_i$ on the anion conductance (see Fig. 3*C,D*) was as above [but pH-adjusted with *N*-methyl-D-glucamine (NMDG) and with $(NMDG)_2EGTA$ instead of K_2EGTA] or with KCl replaced by choline-Cl; for these experiments, the external solution was as above, but with 0.1 mM ouabain added, KCl omitted (Barbour et al., 1988), and with 105 mM NaCl replaced by 25 mM NaCl and 30 mM choline-Cl plus 50 mM of either NaCl or $NaNO_3$ (pH was adjusted with NMDG). The pipette solution for studying uptake with strong pH buffering contained (in mM): KCl 50, HEPES 71, NMDG 26, NaCl 5, $(NMDG)_2EGTA$ 5, $CaCl_2$ 1, $MgCl_2$ 7, and Na_2ATP 5, pH 7.0; that for reversed uptake usually contained (in mM): Na-glutamate 10, choline-Cl 40, HEPES 71, NMDG 25, $(NMDG)_2EGTA$ 5, $CaCl_2$ 1, $MgCl_2$ 7, and Na_2ATP 5, pH set to 7.0 with NMDG. When we studied the effects of internal anions on the anion conductance activated during reversed uptake (see Fig. 4*B*), the pipette solution contained (in mM): Na-glu 10, choline-Cl 85 (for Cl^-) or 35 (ClO_4^-), choline-ClO_4^- 0 (Cl^-) or 50 (ClO_4^-), NaCl 5, HEPES 5, $(NMDG)_2EGTA$ 5, Na_2ATP 5, $CaCl_2$ 1, and $MgCl_2$ 7, pH set to 7.0 with NMDG. When we studied activation of the anion conductance with net glutamate transport inhibited (see Fig. 5*A*), the pipette solution contained (in mM): Na-glu 100, MgATP 5, HEPES 5, $CaCl_2$ 1, $(NMDG)_2$-EGTA 5, and $MgCl_2$ 2, pH set to 7.0 with NMDG; the external solution contained (in mM): NaCl 100, choline-Cl 10, $MgCl_2$ 0.5, $CaCl_2$ 3, HEPES 5, glucose 15, $BaCl_2$ 6, and ouabain 0.1. When we studied the effect of intracellular $[Na^+]$ and $[glu^-]$ on the anion conductance with net glutamate transport inhibited (see Fig. 5*B*), the pipette solution contained blockers of the Krebs' cycle, glutamate transaminase, and glutamine synthetase to allow better control of $[glu^-]_i$ and comprised (in mM): NaCl 0 or 10, NMDG-glutamate 0 or 10, choline-Cl 100 (for 0 Na^+/0 glu^-), 90 (for 10 Na^+/0 glu^- or 0 Na^+/10 glu^-), or 80 (for 10 Na^+/10 glu^-), $CaCl_2$ 1, $(NMDG)_2EGTA$ 5, MgATP 5, HEPES 5, $MgCl_2$ 2, malonic acid 0.2, amino-oxyacetic acid 5, and L-methionine sulfoximine 2, pH set to 7.0 with NMDG; the external solution was as just described, but with choline-Cl replaced by NaCl. Electrode junction potentials were compensated (Fenwick et al., 1982).

I–V plots. These were derived from the steady-state current measured at the end of 150 msec voltage steps from a holding potential of −50 mV. Glutamate-evoked currents were obtained from the *I–V* data in glutamate by subtracting the average of control *I–V* data obtained before and after glutamate.

Sensing glutamate release with isolated Purkinje cells. This was performed as described by Billups and Attwell (1996). Cerebellar Purkinje cells were isolated from 200-μm-thick slices of cerebellum from 12-d-old rats by incubation in papain, as for salamander glia (Barbour et al., 1991), except that the tonicity of the incubation and washing solution was increased to that for rat cells by raising the NaCl concentration by 20 mM. After isolation and plating of the cells into the recording chamber, the solution outside the cells was altered to standard solution of the tonicity for salamander cells: this did not seem to damage the cells. Purkinje cells were recognized by their large cell bodies and stumps of dendrites and axon. They were whole-cell-clamped with a pipette solution containing (in mM): CsCl 110, HEPES 10, $MgCl_2$ 2, $CaCl_2$ 0.5, $(NMDG)_2EGTA$ 5, and Na_2ATP 5, pH set to 7.0 with NMDG. Desensitization of Purkinje cell non-NMDA receptors was reduced with 1 mM trichlormethiazide, and the Purkinje cell then generated a non-NMDA current related to the glutamate concentration by a Hill equation with an EC_{50} of 23 μM and a Hill coefficient of 1.2 (Billups and Attwell, 1996).

Measurement of intracellular pH. This was done as described previously with the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)carboxyfluorescein (BCECF; 100 μM) loaded into the cell in the standard whole-cell pipette solution but buffered with only 0.5 mM HEPES (Bouvier et al., 1992). Calibration of the pH was obtained from the response to a weak acid and two concentrations of a weak base (Bouvier et al., 1992).

RESULTS

Glutamate uptake into salamander retinal glia activates an anion conductance

Earlier experiments (Brew and Attwell, 1987) found that the glutamate-evoked current in salamander retinal glia is inward and smaller at positive potentials (see Fig. 2*B,D*; data for 0 mM $[NO_3^-]_o$ and $[ClO_4^-]_o$), as expected from activation of a carrier that transports two Na^+ ions into the cell with each glu^- anion while countertransporting a K^+ and an OH^- ion (Bouvier et al., 1992). If, as has been shown for the cloned mammalian glutamate transporters, activation of the retinal glial carrier also activates an anion conductance, then altering the extracellular chloride concentration should influence the glutamate-evoked current. When external Cl^- was lowered from 126.5 to 19 mM, the inward current evoked by 200 μM glutamate was increased, consistent with an increase in Cl^- efflux through an anion conductance (Fig. 1*A,B*). In four cells at −100 mV, lowering $[Cl^-]$ increased the current by 15 ± 6% SEM. Interestingly, although one might expect the current increase produced by chloride removal to be larger at positive potentials (where the driving force for Cl^- influx would be greatest), a larger change was seen at negative voltages (Fig. 1*B*; in 4 cells the current change at 0 mV was 34 ± 13% of that at −100 mV). This might be attributable to more activation of the anion conductance occurring at more negative potentials when the carrier is cycling more often.

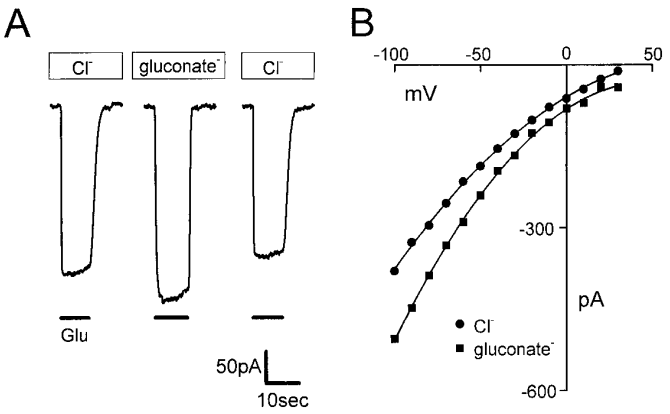

Figure 1. Chloride dependence of the glutamate-evoked current in salamander retinal glia. *A*, Lowering external $[Cl^-]$ from 126.5 to 19 mM (replaced with $gluconate^-$) increased the current evoked by 200 μM glutamate (*Glu*, *black bar*) at −60 mV. *B*, Voltage dependence of the glutamate-evoked current in the presence and absence of external Cl^-. Data typical of nine cells; pipette and external solution as discussed in Materials and Methods.

Consistent with the effect of removing external Cl^-, we found that the glutamate-evoked current was smaller in cells clamped with a pipette solution with reduced $[Cl^-]$. In four and six cells clamped with a pipette solution containing 116 or 21 mM Cl^-, respectively, the current evoked by 200 μM glutamate at −100 mV (normalized by cell capacitance) was 2.7 ± 0.2 and 2.0 ± 0.2 pA/pF. In the six cells studied with lowered $[Cl^-]_i$, lowering external $[Cl^-]$ increased the glutamate-evoked inward current, as in Figure 1.

The anion conductance is more permeable to NO_3^- and ClO_4^- than to Cl^-

Intracellular NO_3^-, SCN^-, and ClO_4^- (replacing Cl^-) increase the inward current generated by the uptake carrier when glutamate is applied extracellularly (Bouvier et al., 1992). With ClO_4^- or NO_3^- present extracellularly but not intracellularly, the glutamate-evoked current was outward at depolarized potentials (Fig. 2*A,B,D*). An outward current is not expected from external glutamate activating a carrier that transports two Na^+ ions in with each glu^- and transports a K^+ and an OH^- out of the cell, as proposed earlier (Bouvier et al., 1992), but it could be explained by ClO_4^- entering the cell through an anion conductance linked to the carrier (Eliasof and Jahr, 1996).

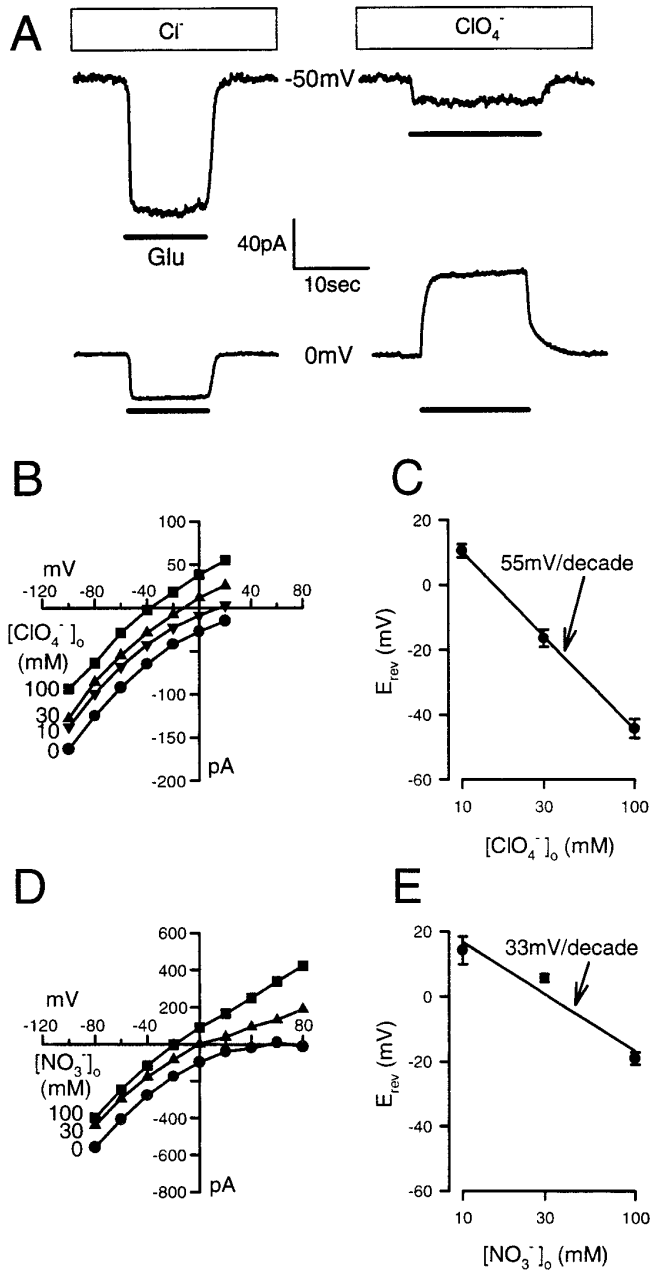

Figure 2. With ClO_4^- or NO_3^- present outside the cell, glutamate produces an outward current at positive potentials. *A*, Specimen currents evoked by 200 μM glutamate (*Glu*) at −50 and 0 mV with Cl^- as the main external anion (*left*; external solution as in Materials and Methods) or with 100 mM Cl^- replaced by ClO_4^- (*right*). *B*, *I–V* data for the glutamate-evoked current show that, with Cl^- outside, the current is inward at all potentials but decreases toward zero at positive potentials, whereas with ClO_4^- outside, the current reverses at depolarized potentials. In four cells the mean ± SEM outward current at +20 mV in 100 mM ClO_4^- was 60 ± 9 pA. *C*, Dependence on $[ClO_4^-]_o$ (log scale) of the reversal potential for currents studied as in *B* (mean ± SEM; $n = 4$ cells for 10 mM, 5 for 30 mM, 6 for 100 mM). *D*, Experiments are as in *B* but with NO_3^- replacing external Cl^-. *E*, Dependence on $[NO_3^-]_o$ of the reversal potential for currents studied as in *D* ($n = 5$ cells for 10 mM, 7 for 30 mM, 17 for 100 mM).

The glutamate-evoked currents recorded with different external $[ClO_4^-]$ showed *I–V* relations characteristic of an anion conductance with a relatively high permeability to ClO_4^- (Fig. 2*B*). Best-fitting a straight line to the dependence on $\log([ClO_4^-]_o)$ of the reversal potential of the *I–V* relations gave an average shift of 55 mV per 10-fold change of $[ClO_4^-]_o$ (Fig. 2*C*). Similar results were obtained with NO_3^- as the anion replacing Cl^-, except that less outward current was generated at positive potentials (relative to the inward current at negative potentials) with NO_3^- present outside (Fig. 2*D,E*); on average, the reversal potential shifted by 33 mV for a 10-fold change of $[NO_3^-]_o$. To test whether the anion conductance is permeable to HCO_3^-, we changed the external solution to one buffered to pH 7.3 with 5% CO_2/26 mM HCO_3^- (using a highly buffered pipette solution to minimize changes of intracellular pH; see Materials and Methods). This made the glutamate-evoked current less inward at negative potentials (by 24 ± 8% SEM at −40 mV in 4 cells), consistent with some HCO_3^- entering through the anion conductance, but it did not result in the current becoming net outward at positive potentials (+50 mV) unlike with 30 mM NO_3^- or ClO_4^-.

We interpret these results, similar to Wadiche et al. (1995), in terms of the glutamate-evoked current having two components: a current generated by the glutamate-transporting part of the molecule, which is always inward and decreases at more positive potentials, and a current generated by an anion conductance, which is highly permeable to ClO_4^- (giving a reversal potential that depends in an almost Nernstian manner on $[ClO_4^-]$), less permeable to NO_3^- (producing a less-than-Nernstian dependence of reversal potential on $[NO_3^-]$), and even less permeable to Cl^- and HCO_3^-. With Cl^- as the main intra- and extracellular anion, the glutamate-evoked current at positive potentials is dominated by the transporter part of the molecule (as judged by the lack of a net outward current seen at positive potentials). With external ClO_4^- or NO_3^- present, an outward current through the anion conductance is seen at positive potentials. Because glutamate transport is greatly reduced at positive potentials, this suggests that, for the anion conductance to be activated, it may not be necessary for net glutamate transport to occur. Experiments described below will confirm this and show that ion movements through the anion conductance are not coupled to the flux of glutamate.

Activation of the anion conductance by external glutamate is dependent on external Na^+ and internal K^+

Replacing external sodium with choline abolished the outward current evoked by glutamate with NO_3^- or ClO_4^- present outside the cell (Fig. 3*A,B*) (see also Eliasof and Jahr, 1996) as well as the inward current at negative potentials that may (with Cl^- as the main intracellular anion) be generated primarily by glutamate transport. Thus, activation of the anion conductance by external glutamate, like activation of the uptake process, requires external sodium.

Removing intracellular potassium greatly reduces the inward glutamate-evoked current at negative potentials (Barbour et al., 1988, 1991) and abolishes glutamate uptake (Kanner and Sharon, 1978). It also reduces the outward current seen at positive potentials with NO_3^- present outside the cell (Fig. 3*C,D*). With K^+ in the whole-cell pipette, at +40 mV no glutamate-evoked current is produced with Cl^- as the external anion, because transport of glutamate with a net positive charge into the cell is inhibited. With 50 mM NO_3^- outside the cell, however, an outward current of ~0.43 pA/pF of cell capacitance is seen because of NO_3^- influx through the anion conductance (Fig. 3*C*). When the same experiment was done without K^+ in the pipette (Fig. 3*D*), no glutamate-evoked current was seen at any potential with Cl^- as the external

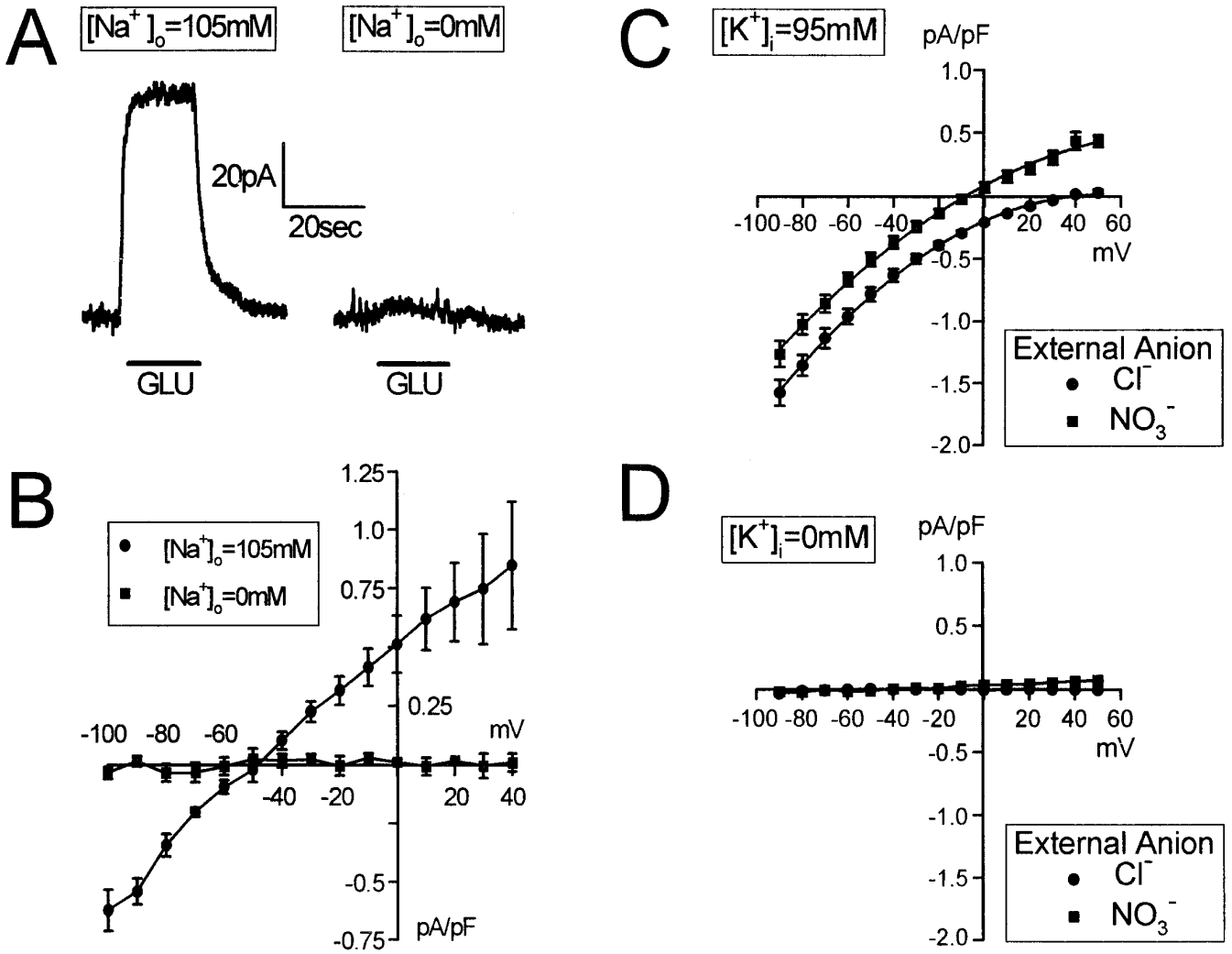

Figure 3. $[Na^+]_o$ and $[K^+]_i$ dependence of anion conductance activation evoked by 200 μM glutamate. *A*, Glutamate-evoked currents at 0 mV with ClO_4^- as the external anion (solutions as in Fig. 2) and with external sodium present (*left*) or absent (*right*; replaced by choline, conducted on 5 cells). *B*, Average *I–V* data from experiments as in *A* on three cells. Sodium removal abolishes both the outward current at positive potentials (produced by ClO_4^- entry through the anion conductance of the carriers) and the inward current at negative potentials (primarily current produced by the cotransport into the cell of a net positive charge with each glutamate anion). Similar results were obtained in four cells when NO_3^- was the external anion. *C, D*, Mean *I–V* relations (normalized by cell capacitance) for currents evoked by 200 μM glutamate, with K^+ either present (*C*, 5 cells) or omitted (*D*, 5 cells) from the pipette and with 50 mM NO_3^- either present in (*curves* labeled NO_3^-) or absent from (*curves* labeled Cl^-) the external solution. Pipette and external solutions are described in Materials and Methods.

anion (because glutamate uptake is absolutely dependent on the countertransport of K^+), and the outward current produced at +40 mV with NO_3^- present was reduced by 86 ± 3% (5 cells studied with and 5 without K^+ in the pipette). Thus, activation of the anion conductance shows a similar ionic dependence to that for activation of glutamate transport.

The anion conductance can be activated by intracellular glutamate

With sodium and glutamate present inside the cell, raising the external potassium concentration evokes an outward membrane current, which is attributed to reversed operation of the uptake carrier, transporting glutamate and net positive charge out of the cell (Szatkowski et al., 1990). Experiments described below (Fig. 6; Billups and Attwell, 1996) confirm that glutamate is released from the cell by reversed uptake in this situation.

When the external Cl^- was replaced by NO_3^- or ClO_4^-, the outward current evoked by a rise of $[K^+]_o$ was increased (Fig. 4*A*,*C*), the mean increase being by a factor of 1.58 ± 0.06 (SEM; 6 cells) for NO_3^- and by 3.34 ± 0.41 (5 cells) for ClO_4^-. Experiments described below (Fig. 6) show that there is no increase in the glutamate release by reversed uptake when Cl^- is replaced in this way. Furthermore, when sodium and glutamate were omitted from the pipette (replaced with choline-Cl), raising $[K^+]_o$ evoked no current in cells superfused with NO_3^- solution (mean current/capacitance was 0.49 ± 0.08 pA/pF in 5 cells with Na-glu inside and −0.05 ± 0.03 pA/pF in 5 cells with Na-glu omitted), as found by Szatkowski et al. (1990) with Cl^- outside, indicating that the extra K^+-evoked outward current seen during Cl^- substitution is generated by the glutamate transporter. We therefore attribute the extra outward current to an influx of NO_3^- or ClO_4^- through the anion conductance of the carrier, with the anion conductance being activated when reversed operation of the carrier is evoked by the simultaneous presence of intracellular glu^- and Na^+ and extracellular K^+. When activated by reversed uptake, the selec-

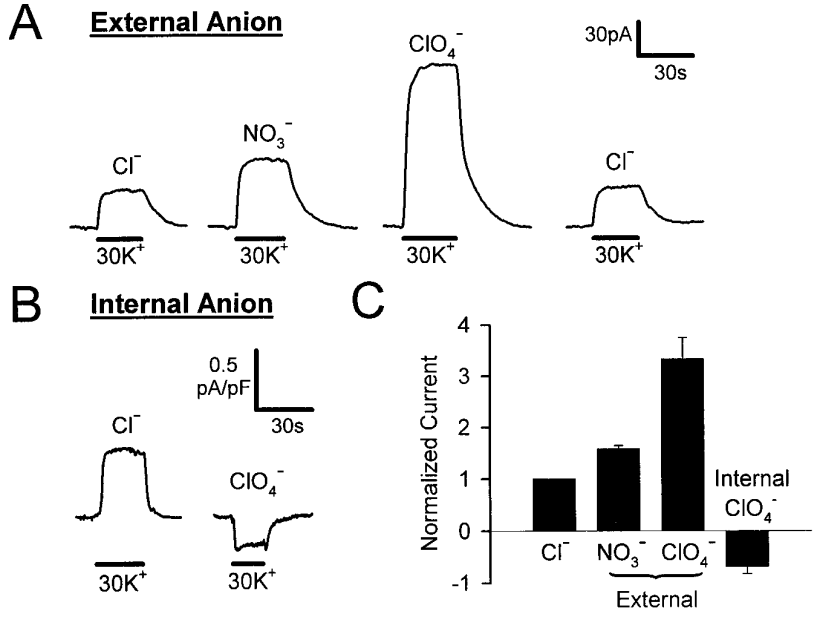

Figure 4. Activation of the anion conductance during reversed uptake evoked by raising $[K^+]_o$ from 0 to 30 mM (*black bars*) around cells whole-cell-clamped with solutions containing 10 mM Na-glu. *A*, K^+-evoked currents at 0 mV in a Müller cell bathed sequentially in external solutions containing Cl^- as the main anion, including 50 mM NO_3^- or ClO_4^-, and then with just Cl^- again. External solution as in Figure 3*C* and with 30 mM choline-Cl replaced by KCl when $[K^+]$ was raised. Pipette solution for reversed uptake as in Materials and Methods. *B*, K^+-evoked currents in two different cells (normalized by cell capacitance) clamped with pipette solutions (see Materials and Methods) containing either Cl^- or ClO_4^- as the main anion. External solution as in *A* for Cl^-. *C*, Mean (±SEM) K^+-evoked currents measured as in *A* and *B* for six cells with external NO_3^-, for five with external ClO_4^-, and for five cells clamped with internal ClO_4^- (normalized to 3 cells clamped with internal Cl^-).

tivity sequence of the anion conductance for different anions is $ClO_4^- > NO_3^- > Cl^-$, as was found above for activation of the conductance by transport of glutamate into the cell.

Conversely, when the $[K^+]_o$ was raised around cells containing 50 mM intracellular ClO_4^- (replacing Cl^-), instead of an outward current being evoked, an inward current shift occurred (Fig. 4*B*,*C*), presumably because activation of the anion conductance leads to an efflux of ClO_4^-, generating an inward current that is larger than the outward transport current produced by glutamate efflux.

These data show that the anion conductance can be activated by extracellular or intracellular glutamate, provided that the transport part of the molecule is allowed to cycle by provision of Na^+ and K^+ on appropriate sides of the membrane. A kinetic scheme consistent with these observations is presented in Discussion (Fig. 7). The following section provides evidence that the anion conductance can also be activated when the transport activity of the carrier molecule is greatly reduced.

Anion conductance activation in the absence of net glutamate transport

In the absence of intra- and extracellular K^+, forward and reversed transport of glutamate are inhibited (Kanner and Sharon, 1978; Barbour et al., 1988; Szatkowski et al., 1990; Billups and Attwell, 1996) (homoexchange of glutamate can still occur: Kanner and Bendahan, 1982). Figure 3*D* shows that external glutamate does not evoke a detectable current when Cl^- is the main intra- and extracellular anion present. However we found that, with net glutamate transport abolished in this way, if glutamate and sodium were present inside the cell (via the whole-cell pipette), addition of extracellular glutamate did evoke a current. This current was inward at negative and outward at positive potentials and showed the pharmacology of the uptake carrier (Fig. 5*A*). Activation of this current was dependent on the presence of both glutamate and sodium inside the cell (Fig. 5*B*). Relative values of glutamate-evoked current (normalized to cell capacitance) at +20 mV with 10 mM Na-glu, 10 mM glu^- but no Na^+, 10 mM Na^+ but no glu^-, or no Na^+ and no glu^- in the pipette, were, respectively, 1.0, 0.014 ± 0.024, −0.041 ± 0.031, and 0.0 ± 0.0 (6 or 7 cells for each pipette solution).

Changing the external chloride concentration revealed that the current was produced by activation of an anion conductance (Fig. 5*C*,*D*), although the absolute value of the reversal potential for the current and its less-than-Nernstian dependence on $[Cl^-]_o$ indicated that this conductance was not very specific for Cl^-. Interestingly, Vandenberg and colleagues (1995) have shown the presence of a Cl^--dependent cation leak through a cloned human glutamate transporter, and, if present in the salamander transporter, this might explain the lack of a Nernstian dependence on $[Cl^-]_o$ in Figure 5*D*. With ClO_4^- instead of Cl^- as the major anion present inside and outside the cell (Fig. 5*E*), the glutamate-evoked conductance was greatly increased (in 4 cells the currents evoked at −80 and +40 mV were increased by factors of 30.1 ± 6.5 and 9.7 ± 1.8, respectively: the fact that the reversal potential was near −10 mV instead of 0 mV with 50 mM ClO_4^- in the pipette and outside the cell may reflect incomplete dialysis of the cell with ClO_4^-). A similar, but smaller increase in the outward current at positive potentials was seen with NO_3^- outside the cell (data not shown). The increase in current when Cl^- was replaced by NO_3^- or ClO_4^- is consistent with the selectivity sequence described above for the anion conductance evoked during forward or reversed transport of glutamate.

From these data, it seems that the anion conductance can be activated even when the carrier molecule is not producing net transport of glutamate across the membrane. We took advantage of this to investigate the external glutamate and sodium dependence of anion conductance activation without contamination from current generated by glutamate transport. With 100 mM NO_3^- outside the cell, the anion conductance (assessed as the outward current at +20 mV) showed a Michaelis–Menten dependence on external glutamate concentration, with a K_m (mean value 15.1 ± 0.3 μM in 5 cells) similar to that found for the glutamate transport current (Barbour et al., 1991; Eliasof and Jahr, 1996). Varying the external sodium concentration (replaced with choline; 5 cells) revealed a sigmoid dependence on $[Na^+]_o$ at low $[Na^+]_o$ (rising as $[Na^+]_o^{2.5}$ for $[Na^+]_o \leq 15$ mM at −40 mV), similar to that for the current associated with glutamate transport (Barbour et al., 1991). These data are consistent with one glutamate anion and two Na^+ ions having to bind to activate the anion

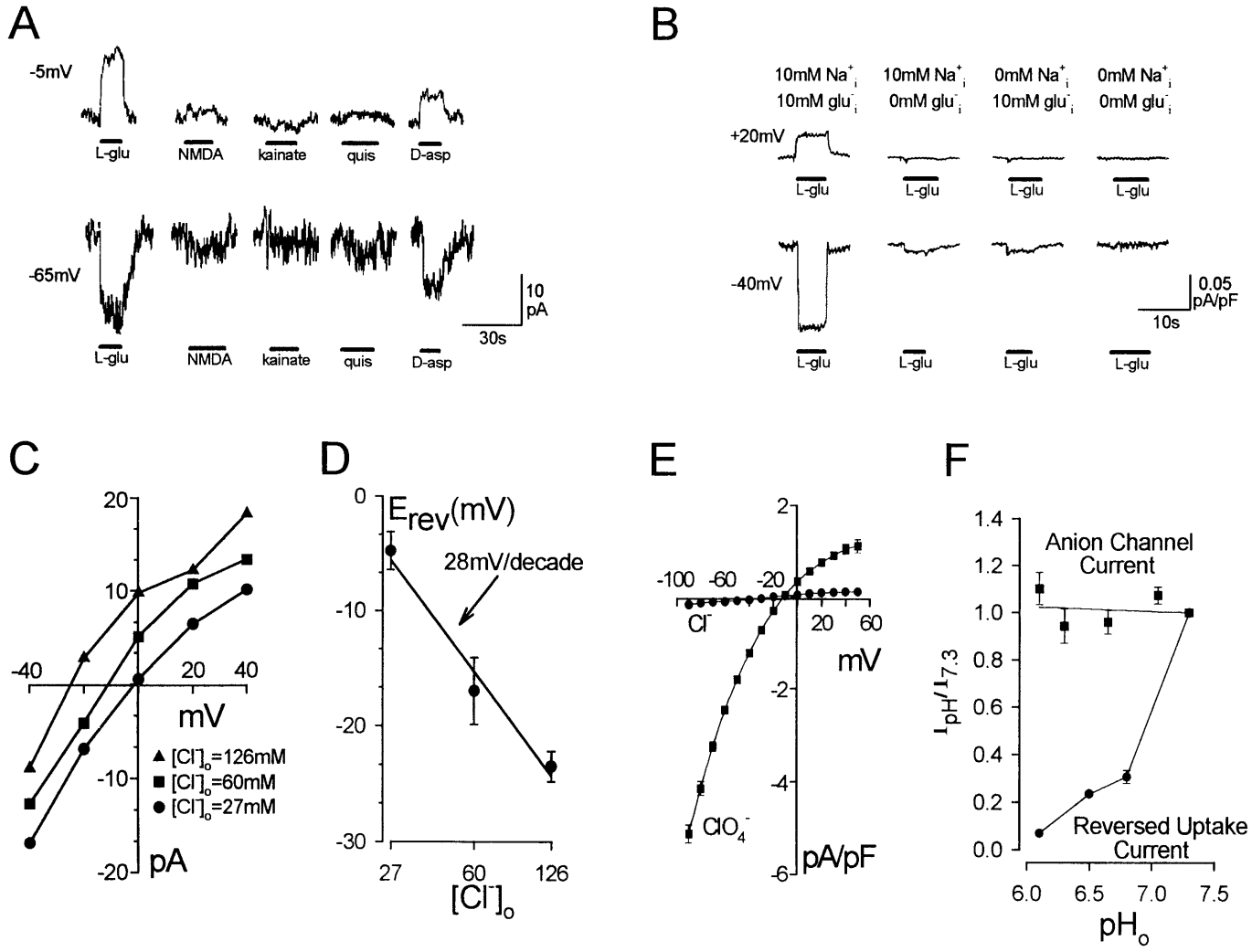

Figure 5. Activation of the anion conductance with glutamate transport inhibited by the absence of extra- and intracellular K^+ and with Na^+ and glu^- added intracellularly. *A*, Currents evoked at −5 and −65 mV by 100 μM glutamate and analogs. Pipette and external solution are described in Materials and Methods. Analogs that activate non-NMDA, NMDA, and metabotropic receptors produced no current change. Glutamate and D-aspartate (also transported on uptake carriers) evoked a conductance increase. *B*, Specimen glutamate-evoked currents (normalized by cell capacitance) as in *A* but with varying $[Na^+]$ and $[glu^-]$ in the pipette. *C*, Specimen *I–V* data from one cell as in *A* with varying external $[Cl^-]$. External solution as in *A*, but with no ouabain, with choline-Cl replaced by 7 mM NaCl, and Cl^- replaced by gluconate as needed. Internal solution as in Figure 4*A*. *D*, The reversal potential of data obtained as in *C* (data from 5–8 cells/point) changes by 28 mV per 10-fold change of $[Cl^-]_o$. *E*, *I–V* data (normalized by cell capacitance) obtained as in *A* from five cells with Cl^- and five cells with ClO_4^- as the main internal and external anion. With ClO_4^- the mean ± SEM outward current at +20 mV was 122 ± 14 pA. External solutions as Figure 3*C*, but with ClO_4^- replacing NO_3^-; pipette solution as Figure 4*B*. *F*, Dependence on external pH (pH_o) of the anion conductance current for five cells studied as in *A* (current evoked by 1 mM glutamate at −40 mV, *squares*). Pipette solution as Figure 4*A*; external solution as for Cl^- in Figure 3*C*. For comparison, we show the pH_o dependence of the reversed uptake current (at 0 mV, *circles*) produced by raising $[K^+]$ from 0 to 30 mM with 10 mM glu^- and 20 mM Na^+ in the pipette (Billups and Attwell, 1996).

conductance—a result that is incorporated into a kinetic scheme proposed in Discussion.

With Na^+ and glu^- inside the cell, the reversed uptake current produced by raising $[K^+]_o$ is greatly reduced when the extracellular pH is made acid (Billups and Attwell, 1996), presumably because with an acid pH_o there is not enough OH^- present for countertransport into the cell (or because the carrier cannot lose H^+ cotransported out of the cell). An acid extracellular pH had no effect, however, on the anion conductance activated by adding external glutamate with glu^- and Na^+ inside the cell (and no K^+ inside or outside; Fig. 5*F*), again suggesting that it is possible to dissociate the anion conductance function of the molecule from its glutamate-transporting activity. A kinetic model consistent with this observation is presented in Discussion.

Lack of coupling of anion movements to glutamate transport

To determine whether alteration of the ion flux through the anion conductance part of the transporter molecule has any effect on the rate of glutamate transport, we evoked reversed uptake in salamander retinal glial cells while monitoring glutamate release with non-NMDA receptor channels in isolated rat Purkinje cells placed just outside the glial cells (Billups and Attwell, 1996).

Glutamate release by reversed uptake was evoked with 10 mM Na-glu in the glial cell and 30 mM K^+ in the extracellular solution by depolarizing the cell from −60 mV (at which potential glutamate release by reversed uptake is small; Szatkowski et al., 1990; Billups and Attwell, 1996) to +20 mV. This procedure produced a current in the adjacent Purkinje cell consistent with glutamate

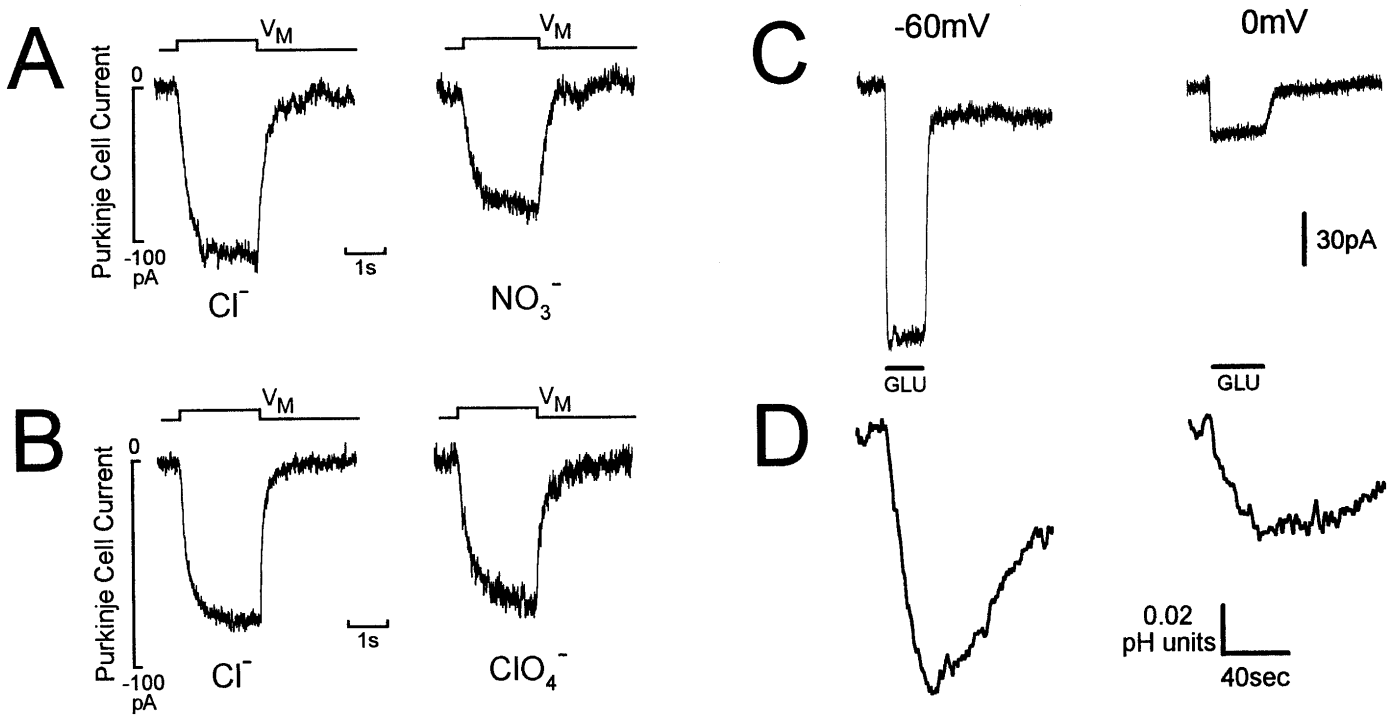

Figure 6. Investigation of coupling of anion movements to glutamate transport. *A, B*, Test of whether increasing current flow through the anion conductance increases glutamate transport. *A*, Currents evoked in a Purkinje cell (clamped to −60 mV) by glutamate released from an adjacent Müller cell by reversed uptake in external solution containing Cl^- (*left*) or NO_3^- (*right*) as the main anion. Reversed uptake was evoked by bathing the cells in 30 mM K^+ solution (as in Fig. 3*C* with KCl replacing choline-Cl) and stepping the Müller cell voltage from −60 to +20 mV (*top trace*). The decrease in the Purkinje cell current response in NO_3^- is produced by a decrease in glutamate sensitivity of the non-NMDA receptors of the Purkinje cell and not by a decrease of glutamate release from the Müller cell (see text). Purkinje cell pipette solution as described in Materials and Methods; Müller cell pipette solution for reversed uptake as described in Materials and Methods. *B*, Same experiment as in *A* but testing the effect of external ClO_4^-. The glutamate sensitivity of the Purkinje cell is more than doubled by ClO_4^- (see text), so the small decrease of response seen here implies a large decrease of glutamate release from the Müller cell. *C, D*, Test of whether H^+/OH^- movements on the uptake carrier are through the anion conductance and are passive or are coupled to glutamate transport. *C*, Membrane currents evoked at −60 and 0 mV by 200 μM glutamate in a Müller cell clamped with standard internal solution, pH 7.0, but containing only 0.5 mM HEPES and also 100 μM BCECF. Standard external solution was used but with its pH adjusted to 7.7. *D*, Glutamate-evoked changes in intracellular pH, measured at the same time as the current records in *C*, are acid both below and above the reversal potential for H^+/OH^-, implying coupling of the movement of H^+/OH^- to glutamate transport.

release by reversed uptake activating non-NMDA channels (Billups and Attwell, 1996). Replacing external Cl^- with NO_3^- or ClO_4^- increased the outward current shift evoked in the glial cell by a rise of $[K^+]_o$ at 0 mV (Fig. 4*A*), because NO_3^- and ClO_4^- can enter the cell through the anion conductance better than Cl^- can but had little effect on the change of membrane current evoked in the Purkinje cell by glutamate release from the glial cell (Fig. 6*A,B*). On average, the responses with 50 mM external NO_3^- or ClO_4^- present were 0.69 ± 0.04 (3 cells) and 0.98 ± 0.10 (6 cells) of those in external Cl^-. Control experiments, in which the sensitivity of three Purkinje cells to 3 μM glutamate was tested, showed that NO_3^- reduced the current evoked in the Purkinje cell to 0.76 ± 0.18 of its value in Cl^-, whereas ClO_4^- increased it to 2.33 ± 0.29 (data not shown). The increased current in ClO_4^- is consistent with the fact that chaotropic ions like ClO_4^- increase the affinity of AMPA receptors (Honore and Drejer, 1988). Combining these alterations of glutamate sensitivity with the data in Figure 6, *A* and *B*, suggests that in the presence of NO_3^- (which increases the glial cell current evoked by a rise of $[K^+]_o$ by a factor of 1.58; Fig. 4) glutamate release is essentially unaffected (0.91 ± 0.22 of its value in Cl^-), whereas in ClO_4^- (which increases the K^+-evoked current in the glial cell 3.34-fold) glutamate release is actually reduced to 0.42 ± 0.06 of its value in Cl^-. Clearly, the amount of glutamate transported is not proportional to the movement of charge through the anion conductance part of the carrier molecule, as was suggested also by the experiments above in which anion conductance activation was still seen in the absence of K^+ and at positive potentials when transport is inhibited.

Coupling of movement of pH-changing ions to glutamate transport

Changes of pH produced by glutamate uptake carriers have been attributed previously to a cotransport of H^+ ions with glutamate or to a countertransport of OH^- ions (Erecinska et al., 1983; Bouvier et al., 1992)—interpretations that imply that glutamate accumulation is powered partly by the transmembrane pH gradient. However, if glutamate transporters contain an anion conductance, an obvious possibility is that the pH changes are generated by passive movement of OH^- ions through the anion conductance, not coupled to the transport of glutamate. To investigate this possibility, we measured changes of intracellular pH evoked by external glutamate in Müller cells clamped to different potentials. For this experiment the intra- and extracellular solutions had pH values of 7.0 and 7.7, respectively, giving a reversal potential for OH^- of −41 mV. Thus, at potentials more positive than −41 mV, if OH^- were moving passively through the glutamate-evoked anion conductance, it would move into the cell, making the cell more alkaline, whereas if movement of OH^-/H^+ were coupled

thermodynamically to glutamate entry, then glutamate should make the cell go acid at all potentials. Experimentally, the latter was found to be the case (Fig. 6*C,D*). Indeed, at 0 mV the ratio of the rate of acidification to the glutamate-evoked current was similar to that at −60 mV (0.27 ± 0.04 and 0.23 ± 0.05 pH units/sec per nA at 0 mV and −60 mV, respectively, in 6 cells: the slightly, although not significantly, smaller value at −60 mV might be expected because the inward glutamate-evoked current, but not the pH change, is increased by chloride efflux through the anion conductance). Thus, the movement of pH-changing ions is coupled to glutamate transport, rather than occurring through the anion conductance.

DISCUSSION

The salamander glial cell glutamate transporter has an anion conductance

Data presented here show that the glutamate transporter in salamander retinal glial cells activates an anion conductance (see also Eliasof and Jahr, 1996). Removing external chloride increases, and lowering internal chloride decreases, the glutamate-evoked inward current, consistent with the results of Wadiche et al. (1995) on cloned mammalian transporters. With Cl^- as the main anion inside and outside the cell, the glutamate-evoked current remains inward at positive potentials (Fig. 1), presumably because it is dominated by the current associated with glutamate transport rather than that generated by the anion conductance. The contribution of the anion conductance can be greatly enhanced by replacing Cl^- with more permeant anions, resulting in the glutamate-evoked current becoming outward at positive potentials (Fig. 2). Inspection of the data in Figures 2, *C* and *E*, and 4 of this paper and in Bouvier et al. (1992), suggests a selectivity sequence $SCN^- > ClO_4^- > NO_3^- > Cl^- \simeq Br^- \simeq I^-$ for the anion conductance. This is similar to the theoretical sequence 1 of Wright and Diamond (1977) but differs in that, for sequence 1, $ClO_4^- > SCN^-$ and $I^- > Br^- > Cl^-$. The apparent position of ClO_4^- in our selectivity sequence could, however, be altered by the fact that ClO_4^-, in addition to permeating the anion conductance, seems to slow carrier cycling (see Lack of Coupling of Anion Movements to Glutamate Transport) and so may reduce opening of the anion conductance. Conceivably, the selectivity sequence of the anion conductance would be identical to sequence 1 of Wright and Diamond (1977) if currents through the open anion conductance could be investigated independently of changes in conductance activation. In earlier work, Barbour and colleagues (1991) observed a small (12%, but statistically insignificant) decrease of glutamate-evoked current on removing internal chloride, as we report here, but did not see the effect of removing external chloride shown in Figure 1. This may be attributable to the use of a nonsaturating glutamate dose or to the presence of acetate in the internal solution in the experiments of Barbour et al. (1991); we are performing experiments to examine these possibilities.

Different modes of gating of the anion conductance

The glutamate carrier anion conductance can be activated by the simultaneous presence of extracellular glutamate and sodium and intracellular potassium when the carrier operates in forward uptake mode (Figs. 1–3). However, it is also activated by the simultaneous presence of intracellular glutamate and sodium and extracellular potassium when the carrier transports glutamate out of the cell (Fig. 4). These data suggest that activation of the anion conductance may occur when a particular state of the carrier cycle is reached, independent of whether that state is reached by the carrier cycling in the forward or the reversed direction. A possible example of such a scheme is shown in Figure 7.

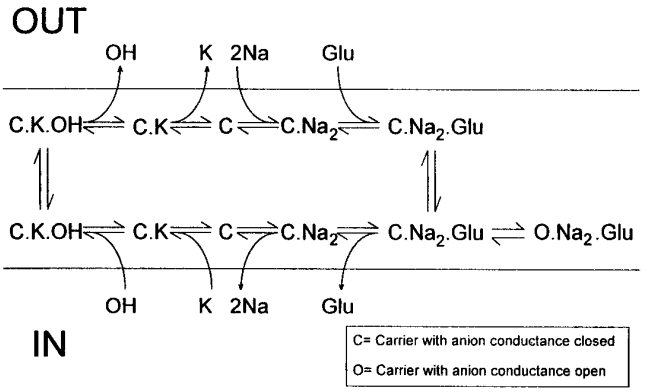

Figure 7. A possible kinetic scheme for the glutamate transporter and associated anion conductance. *C* denotes the carrier in conformations for which the anion conductance is not activated (closed). In these conformations the carrier can bind extracellular glu^- and two Na^+ ions, transport them to the inner face of the membrane, then bind K^+ and OH^- at the inner membrane surface and transport them to the outside of the cell, shifting one net positive charge into the cell during the carrier cycle. (Note that the order in which K^+ and OH^- bind is unknown. Furthermore, although we show OH^- countertransport out of the cell, as discussed in the text the carrier might get energy from the transmembrane pH gradient by cotransporting H^+ with glu^-). *O* denotes a conformation of the transporter in which the anion conductance is open. Here we postulate simply that the open state can be accessed from the closed conformation that has Na^+ and glu^- bound at the inner face of the membrane. Our data would also be consistent with an *O* state being accessed from the closed conformation with Na^+ and glu^- bound at the outer membrane surface (or from both of the *C.Na₂.Glu* conformations).

If the anion conductance only opens once each carrier cycle (during forward uptake), its open probability could be larger at negative potentials when the carrier cycles more rapidly. This might explain why the change in glutamate-evoked current produced by removing external Cl^- is larger at more negative potentials (Fig. 1) rather than at positive potentials when the driving force for Cl^- entry is greatest; similarly, it would explain why the outward shift of glutamate-evoked current produced by external ClO_4^- or NO_3^- is only slightly larger at positive potentials (Fig. 2).

One constraint on which state of the carrier cycle allows activation of anion conductance is provided by the observation that the anion conductance can be activated (Fig. 5) when net glutamate transport is inhibited by the absence of intra- and extracellular K^+. This implies that the anion conductance is activated by a state of the carrier cycle at which the carrier does not have K^+ bound. If, as suggested by Kanner and Bendahan (1982) and as shown in Figure 7, the K^+ translocating part of the carrier cycle is distinct from the Na^+ and glu^- translocating part of the cycle, then activation of the anion conductance must occur from one of the states of the glu^-/Na^+ transporting limb of the carrier cycle. The observation (see Anion Conductance Activation in the Absence of Net Glutamate Transport) that anion conductance activation shows a first-order dependence on external glutamate concentration and a sigmoid dependence on $[Na^+]_o$ implies it occurs from a state with one glu^- and two Na^+ ions bound (Fig. 7). When intra- and extracellular K^+ are absent, the anion conductance is activated by external glutamate only if glutamate and sodium are present intracellularly (Fig. 5*B*); this could be explained by binding of the internal glutamate and sodium resulting

in the carrier spending more time in the state with glu^- and two Na^+ ions bound that is suggested in Figure 7 to lead to anion conductance activation. (Similarly, external glutamate is needed to prevent the carrier accumulating in the state at the external surface with Na^+ bound but with no glutamate bound.) Figure 7 proposes that the OH^-/H^+ transporting part of the carrier cycle is associated with the K^+ transporting limb of the cycle rather than with the glu^-/Na^+ transporting limb. This would explain the fact that an acid pH (equivalent to lack of transported substrate OH^- in Fig. 7) does not affect the activation of the anion conductance seen in the absence of intra- and extracellular K^+ (Fig. 5*F*), although it does block reversed uptake of glutamate (Billups and Attwell, 1996).

The data in Figure 6, *A* and *B*, suggest that there is no energetic coupling between the anion flux through the conformation of the carrier denoted O in Figure 7 and the cycle of reactions that transport glutamate.

Reinterpretation of the effects of intracellular ClO_4^- and NO_3^-

Bouvier and colleagues (1992) found that intracellular ClO_4^- and NO_3^- increased the inward current evoked by external glutamate, that ClO_4^- came out of the cell when glutamate was applied, and that the presence of these ions intracellularly reduced the ratio of the pH change generated by the carrier to the current that it generated. Those results were interpreted as showing that the glutamate-evoked pH changes were generated by the transport of OH^- ions out of the cell and that ClO_4^- and NO_3^- could compete for transport at the OH^- site. It is now clear that the effects of ClO_4^- and NO_3^- were produced by these ions leaving the cell (at a much higher rate than Cl^-) through the anion conductance associated with the uptake carrier, generating an extra inward current. This invalidates the earlier conclusion that the pH changes generated by the carrier are produced by the transport of OH^- out of the cell rather than the (thermodynamically equivalent) transport of H^+ into the cell: our data reopen the possibility that H^+ is cotransported with glutamate.

Figure 6*D* shows that, irrespective of whether OH^- or H^+ is transported, movement of the pH-changing ion is coupled to glutamate transport. Thus, glutamate uptake does derive energy from the transmembrane pH gradient, consistent with the observation that, in the kidney, a pH gradient alone can drive uptake (Nelson et al., 1983).

Therapeutic possibilities offered by the existence of the anion conductance

Our demonstration that the anion conductance part of the transporter molecule can be activated even when glutamate transport is inhibited (at positive potentials in Fig. 2 and in the absence of K^+ in Fig. 5) suggests some independence between these two functions of the molecule and, hence, that they may be capable of being modulated separately by pharmacological agents. This suggests a possible strategy for developing drugs to treat conditions in which excessive glutamate is released, such as epilepsy. If, for glutamate transporters in presynaptic terminals, the anion conductance activation could be greatly enhanced, then whenever glutamate was released, activation of the anion conductance during glutamate re-uptake would tend to clamp the presynaptic terminal at a negative potential, reducing further exocytotic release (by making it harder for action potentials to invade the synaptic terminal) and potentiating the (voltage-dependent) reuptake. Interestingly, in the retina at least, the glutamate transporter in cone synaptic terminals expresses a particularly large anion conductance (Sarantis et al., 1988, Eliasof and Werblin, 1993), like the human EAAT4 carrier (Fairman et al., 1995), suggesting that evolution already might have arrived at this strategy for controlling glutamate release. Recently Rothstein and colleagues (1996) have shown that preventing the expression of neuronal EAAC-1 carriers leads to epileptic behavior of neurons (whereas preventing expression of glial uptake carriers leads to a rise of extracellular glutamate concentration but no epilepsy). It is not yet known whether the antiepileptic properties of EAAC-1 transporters derive solely from their ability to take up glutamate or whether their contribution to the anion conductance of neurons is also involved.

During ischemia the glutamate concentration in glial cells rises (Storm-Mathisen et al., 1992). Because activation of the anion conductance in glial uptake carriers can be potentiated by intracellular glutamate (Fig. 5*B*), it is possible that the uptake carrier might contribute to the glial cell chloride conductance, which, by allowing Cl^- influx, could facilitate glial cell swelling in ischemia (Walz et al., 1993).

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