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# Amino Acid Current through Anion Channels in Cultured Human Glial Cells

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**Abstract.** During volume regulation in hypotonic media, glial cells release a large portion of their amino acids. These amino acid losses appear to be mediated by a diffusion type of transport and a swelling-activated chloride channel seems to be involved. The objective of this project was to provide direct evidence that amino acids could diffuse through a $Cl^-$ channel. Using a human glial cell line, $Cl^-$ currents activated in hypotonic media were measured in whole-cell patch clamp. To measure the currents produced by amino acids, it was necessary to increase the pH of external solutions to basic values reaching 9.6 and 10.0 to raise the concentration of the anionic form of these amino acids. Introducing external hypotonic media containing high concentrations of amino acids, like glycine, taurine, glutamine and glutamate, it was possible to measure their respective current-voltage curves with NMDG-Cl-filled pipettes. From the reversal potentials, their permeability ratios with respect to chloride were determined. It was found that the low molecular weight amino acids, like glycine, were most permeant, while the larger ones, like glutamine, had a lower permeability with respect to chloride. The amino acids with two carboxyl groups, like glutamate, had a much lower permeability ratio. The reversal potentials for some metabolites, like lactate and malate were also measured for comparison. These results demonstrate that amino acids can diffuse through anion channels and that activation of these channels in pathological conditions could be at least partly responsible for the observed increase in external amino acids.

**Key words:** Amino acids — Chloride channels — Glial cells — Volume regulation — Glutamate — Neurotransmitters

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## Introduction

Osmolyte losses after swelling of glial cells in hypotonic media have been studied recently and it was found that these cells were releasing large amounts of their internal amino acids, particularly glutamate, aspartate, taurine, glycine, glutamine and alanine (Kimelberg et al., 1990*a;* Pasantes-Morales et al., 1993). It was shown that the permeability of these amino acids increased in hypotonic conditions but it was not clear what transport mechanism was activated by the swelling of these cells. Some evidence has been provided demonstrating that the amino acid transport activated by astrocyte swelling was not Na-dependent and that the fluxes were linearly dependent on their external amino acid concentration (Kimelberg et al., 1990*a,* Sanchez-Olea et al., 1991). These results suggested that a diffusional type of transport was involved. Additional evidence was provided by similar studies performed on other cell types. With the MDCK cell line, Roy and Malo (1992) have shown that the swelling-induced fluxes of amino acids, like taurine, glycine, alanine, aspartate, glutamate and even phenylalanine, did not depend on the presence of $Na^+$ and were linearly related to their external concentration. Also Kirk et al. (1992) and Haynes and Goldstein (1993) using erythrocytes have shown that the taurine fluxes activated by cell swelling had the same properties. To explain their results, Roy and Malo (1992) suggested that the transport pathway for amino acids activated by cell swelling was provided by an anion channel of low selectivity. More direct evidence for this possibility was obtained by Banderali and Roy (1992) who measured amino acid currents for aspartate, glutamate and taurine through single channels in MDCK cells. The channel that was involved was the volume-sensitive $Cl^-$ outward rectifier which has a low selectivity for anions, but excludes cations in MDCK cells. Additional support for this hypothesis was provided by the use of anion channel blockers like DIDS and NPPB (Kimelberg et al., 1990*a;*

Sanchez-Olea et al., 1991; Kirk et al., 1992; Haynes and Goldstein, 1993). Recent results with a glial cell line (rat C6 glioma) were also obtained by Jackson and Strange (1993) who have clearly demonstrated that cell swelling activated anion channels permeable to taurine and inositol. They could measure a taurine and chloride current with whole-cell patch clamp and determine the permeability ratio of taurine relative to chloride.

All this evidence indicates that anion channels of low selectivity may be responsible for the amino acid losses occurring during swelling of glial cells, but current measurements for all these amino acids through anion channels are still lacking. Therefore whole-cell patch clamp experiments were performed to measure those currents in hypotonic conditions using a human glial cell line. Chloride currents activated in hypotonic media were measured first and afterwards the currents produced by many different amino acids were determined. In the case of neutral amino acids, the pH of the solutions were increased to provide a sufficient concentration of the anionic form of the amino acids. The permeability ratio of each amino acid with respect to chloride was obtained. It was found that amino acids could have high, intermediate or low permeabilities with respect to $Cl^-$, depending on their molecular weight and their negative charge.

## Materials and Methods

### Cell Cultures

The U-138MG cell line was obtained from the American type Culture Collection starting at the 180th serial passage. Cells were used for patch clamp experiments up to the 200th serial passage. Cells were seeded at medium density and grown until confluence in plastic bottles (Falcon 3023) with DMEM (Gibco). The medium contained 10% fetal bovine serum (Gibco) with Gentamycin as an antibiotic and the medium was changed every two days. Cultures were maintained at 37°C in a humid air atmosphere in closed Falcon bottles. Subculture was performed by detaching the cells from the bottles using a trypsin-EDTA solution containing 0.05% trypsin and 0.5 mM EDTA-Na. For patch clamp experiments, cells were detached mechanically from a culture bottle by blowing the Earle medium on the cell layer with a pipette. The cells were maintained in suspension and a sample was introduced into the perfusion chamber. After a few minutes the cells settled on the bottom of the chamber and were ready for patch clamp experiments.

### Solutions

The standard bathing solution was an Earle's medium containing (in mM): 121 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 6.0 $NaHCO_3$, 1.0 $NaH_2PO_4$, 5.5 glucose, 25.0 HEPES, 10.0 NaOH (pH: 7.3, osmolarity: 290 mosm/Kg). All the other experimental media were hypotonic at 200 mosm. The normal NMDG-Cl solution contained (in mM): 100 NMDG-Cl, 10 HEPES, 1 Mg-$SO_4$; the pH was adjusted at 7.3 with 4 NMDG base. The NMDG-Cl solution at pH 9.6 contained 60 NMDG base and 70 NMDG-$Cl^-$. In the low Cl solutions (40, 25, 10 and 1 mM), NMDG-Cl was partly replaced by raffinose to keep the same osmolarity. The amino acid media contained only 1 NMDG-Cl, 1 Mg-$SO_4$ and no HEPES. For the glutamate and aspartate solutions, 100 NMDG-Cl was replaced by 100 NMDG-Glut or NMDG-Asp at pH 7.3. For the other amino acid solutions, 100 NMDG-Cl was replaced by different concentrations of amino acids, depending on the pH, which was adjusted at different values with NMDG base. The pH, amino acid and NMDG concentrations of these solutions are given in Table 1.

### Patch Clamp Technique

Whole cell patch clamp experiments were performed with pipettes containing the hypotonic (200 mosm) NMDG-Cl pH 7.3 solution with in addition 1 mM EGTA and 1 mM Na-ATP. The patch clamp amplifier was a PC-501, Warner Instrument (Hamden CT). The pipettes were made from pyrex glass capillaries and pulled using a David Kopf programmable puller (model 750). The pipette resistance ranged from 9 to 12 Megohm and the seal resistance was usually between 8–10 Gigaohm. The whole-cell current was initially recorded on a strip chart using a 30 mV pulse (+ and −) to monitor the current increase. When a stable current was reached after a few minutes, the current voltage (*I-V*) curve was measured, using a X-Y tracer and a voltage ramp command. A positive voltage produced a flow of anions into the cells and corresponded to the positive current. The perfusion chamber had a small volume and the solutions could be changed in 15 seconds. All experiments were performed at room temperature (22–24°C).

When the external $Cl^-$ was reduced to determine the selectivity of the whole-cell current, important deviations from the calculated reversal potentials appeared for the low external $Cl^-$ solution (1 mM). Usually, that reversal potential was between 80–90 mV, while the calculated value is 110 mV. This result is expected when the seal leakage is taken into account. In symmetrical 100 mM $Cl^-$ solutions, the $Cl^-$ conductance is usually about 5–10 nS and the seal leak (0.1 nS) is negligible. As the $Cl^-$ concentration is reduced in the external medium to measure the reversal potential, the conductance near that potential decreases. The reversal potential is determined by the sum of the $Cl^-$ current and the leak current. As the external $Cl^-$ concentration becomes lower, the $Cl^-$ current near the reversal potential becomes lower and the seal leak current becomes relatively more important. At potentials between 80–90 mV, they become approximately equal, giving an apparent reversal potential. In some experiments, the reversal potentials for 10 and 1 mM $Cl^-$ were lower than those shown on Fig. 2. These results were considered to be caused by an increased seal leakage current appearing after the whole-cell configuration was reached. Therefore, it was important to test if the seal resistance did not increase during cell swelling and during solution exchanges. Control solutions with 1 mM $Cl^-$ were always used to verify that the reversal potential was greater than 80 mV at the beginning and at the end of the experiments.

### Calculation of Permeability Ratios

From the *I-V* curves, reversal potentials were measured and their average values calculated. With the concentrations of anions in the solutions, given in Table 1, the permeability ratios were calculated with the Goldman-Hodgkin-Katz (GHK) equation. In the case of the basic pH solutions, the anion concentrations were calculated on the basis of the pK, the pH and the total concentration of each amino acid. When a divalent ion (malate) was used in the outside solution, the GHK equation is no longer valid. It is necessary to calculate the ionic current for $Cl^-$ and $Mal^{2-}$ with the Goldman constant field equation.

With the concentration and the valence of each anion and the reversal potential $E_r$, the permeability ratio is given by,

$$P_m/P_{cl} = \frac{([Cl_i]/4[Mal_o])(1 - \exp(E_r/25)\ \exp(2E_r/25)}{(1 - \exp(2E_r/25)}$$

## Results

A very common method to study swelling induced currents in whole-cell recordings is to use pipettes filled with an isotonic solution and change the external medium from isotonic to hypotonic. In such conditions the cell swells, but because the pipette solution is isotonic and has a large volume, it continually draws water and the cell volume keeps increasing, eventually bursting the cells or breaking the seal (Kimelberg et al., 1990*b*, Ross et al., 1994). In performing such experiments, it was also found that the cells frequently swelled continuously until the seal broke or the whole-cell current became so large that it was limited by the electrode current. It was desirable to avoid a large volume increase, because it could damage the membrane and also increase the seal leakage current, thereby introducing errors in estimating the reversal potentials for various external media. Therefore, it appeared necessary to modify this method and use instead an approach that produced cell swelling but leading to a stable volume. This is possible if the pipette is filled with a hypotonic rather than isotonic solution. After the whole-cell configuration is obtained, the external isotonic medium is immediately switched to the hypotonic medium. This method produces a volume increase only if the external medium is changed rapidly (within 30 sec) after reaching the whole-cell configuration. It is necessary that cell swelling occurs before the pipette has dialized the internal cell medium. If the external medium is switched to hypotonic 3 to 4 min after reaching the whole-cell configuration, there is no increase in the whole-cell current, presumably because the pipette had sufficient time to change the internal medium from isotonic to hypotonic. If the external medium is changed to hypotonic rapidly after reaching the whole-cell configuration, the cell swells immediately and later on it is dialized by the pipette solution containing the same hypotonic NMDG-Cl medium as the external medium. In these conditions, there is no regulatory volume decrease (RVD) because the cellular $K^+$ is replaced by an impermeant cation, $NMDG^+$. Since the cell is exposed to two identical hypotonic media, it reaches an internal osmolarity and composition equal to that of these media and remains stable at an increased cell volume. Unfortunately, it was not possible to measure reliable diameter increases under the microscope; that diameter increase would be at most 13% with the 200 mosm hypotonic solution and such variations are within the error range of microscopic measurements. But the fact that large diameter increases could not be observed means that the cell volume did not increase beyond the value expected. With pipettes containing an isotonic solution and with a hypotonic solution in the external medium, large diameter increases were observed in whole-cell configuration.

Normally, immediately after breaking in whole cell, the baseline current remained the same, indicating that the seal resistance had not changed and that the whole-cell current was negligible in isotonic conditions. When the external hypotonic solution was introduced, the conductance increased slowly and reached a stable value after a few minutes, as shown on Fig. 1. Normally, currents between 100–200 pA for a 30 mV pulse were reached and remained stable for long periods; they were tested for 10 min and have shown no sign of increase or decrease. If the isotonic Earle's medium was restored during or at the end of the current increase, the latter started decreasing and returned to nearly its initial value with a similar time delay (Fig. 1).

### Chloride Currents

A typical current-voltage (*I-V*) curve with symmetrical $Cl^-$ concentrations is shown on Fig. 2. It has a small outward rectification for potentials between −80 to +50 mV. For larger positive potentials, the current flattens and even decreases. If the *I-V* curve is measured rapidly (1 sec) this flattening does not appear, but it is much pronounced when the voltage ramp is slow (10 sec), indicating that some slow inactivation takes place for potentials above +50 mV. As the potential returns to values less than +50 mV, the current recovers its initial value with a few seconds delay. The rectification observed with a fast ramp (1 sec) usually gives a current 50% larger for positive potentials compared to that for negative potentials. This rectification and inactivation of the volume-sensitive $Cl^-$ current is well known and has been observed previously (Kubo & Okada, 1992; Chan et al., 1993; Nilius et al., 1994).

To determine if the measured whole-cell current depended only on Cl ions, external solutions with varying NMDG-$Cl^-$ concentrations were used; raffinose replaced NMDG-Cl to keep the osmolarity at 200 mosm. Reversal potentials of 22, 33 and 53 mV (±1 mV, n = 3) for external Cl concentrations of 40, 25 and 10 mM respectively were obtained with 100 mM $Cl^-$ in the pipette, as shown on Fig. 2. Using activity ratios, the calculated Nernst potentials are very close to these values. Important deviations from the calculated values appeared for the low $Cl^-$ solution (1 mM). Usually, that reversal potential was between 80–90 mV, while the calculated value is 110 mV. This result is expected when the seal leakage is taken into account (*see* Materials and Methods).

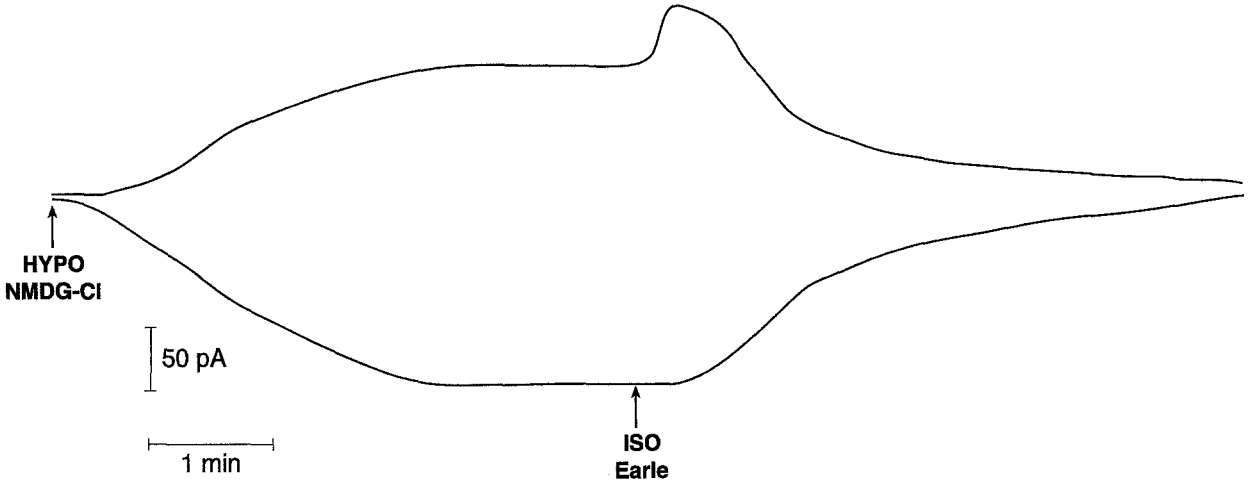


**Fig. 1.** Whole cell current increase after perfusing glial cells with the NMDG-Cl hypotonic solution (200 mosm) and current decrease upon restoring the isotonic Earle's solution. The pipette was filled with the hypotonic NMDG-Cl solution. The potential was clamped and alternated between + and −30 mV. Only the envelope of the current steps is shown. The maximum current could remain stable for periods much longer than shown on the tracing.

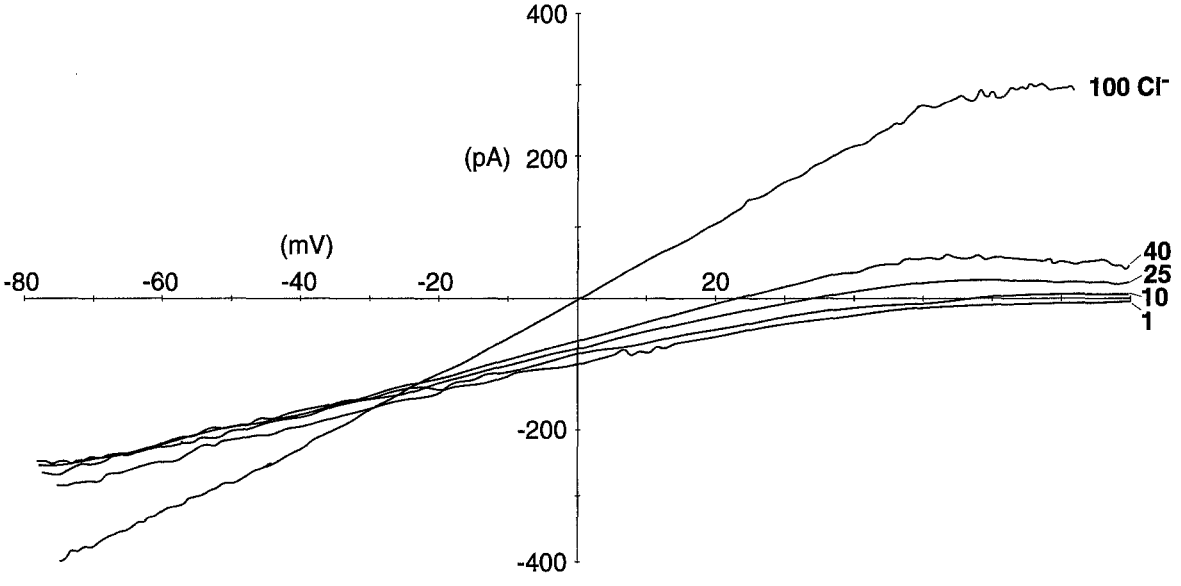


**Fig. 2.** Current-voltage curves measured in whole cell with varying concentrations of hypotonic (200 mosm) NMDG-Cl in the external medium (100, 40, 25, 10, 1mM). The pipette contained the hypotonic 100 mM NMDG-Cl solution and the command potential was a ramp varying between + and −80mV. Reversal potentials were obtained directly from each *I-V* curve tracing.

### Effect of Basic pH

To measure currents for neutral amino acids, it was necessary to increase the pH of the external medium such that a sufficient concentration of the negatively charged form of these amino acids was present in the solution. For taurine, having a pK of 8.8, the pH was increased to 8.2 and 9.0. For glycine, the pK is much higher at 9.6 and the pH was increased to 9.0 and 9.4. For GABA, the pK is even higher at 10.6. In this case the pH was raised to 9.5 and 10.0. The total amino acid concentration used in each solution is given in Table 1; it varies because the pK is different for each amino acid and because NMDG was used to titrate the amino acids at the desired pH. Since NMDG is not a strong base (pK = 9.7), more is required as the pH increases. To determine if such basic pH values were tolerated by the cells, *I-V* curves were measured with NMDG-Cl solutions at pH 7.3 and 9.6. Since the pH 9.6 solution had a lower $Cl^-$ concentration (70 mM), required to reach pH 9.6, the effect of the pH on the currents were more clearly evaluated at −75 mV, where the currents depended almost exclusively on the

**Table 1.** Solute concentrations and pH for experimental media. Measured reversal potentials and calculated permeability ratios

| | $X_t$ mM | $X^-$ mM | NMDG mM | pH | $pK_2$ | $E_r$ mV | $P_x/P_{cl}$ | MW | n |
|---|---|---|---|---|---|---|---|---|---|
| Gly1 | 162 | 32 | 34 | 9.0 | 9.6 | 45 ± 3 | 0.52 ± 0.06 | 75 | 5 |
| Gly2 | 130 | 50 | 66 | 9.4 | 9.6 | 32 ± 2 | 0.56 ± 0.05 | 75 | 5 |
| β-Ala | 150 | 30 | 48 | 9.6 | 10.2 | 45 ± 2 | 0.55 ± 0.04 | 89 | 3 |
| GABA1 | 170 | 16 | 27 | 9.5 | 10.6 | 52 ± 2 | 0.78 ± 0.06 | 103 | 5 |
| GABA2 | 125 | 25 | 70 | 10.0 | 10.6 | 43 ± 2 | 0.72 ± 0.05 | 103 | 3 |
| Tau1 | 173 | 35 | 27 | 8.2 | 8.8 | 52 ± 2 | 0.36 ± 0.03 | 125 | 5 |
| Tau2 | 120 | 73 | 80 | 9.0 | 8.8 | 37 ± 3 | 0.31 ± 0.03 | 125 | 5 |
| Leu | 110 | 55 | 90 | 9.7 | 9.6 | 44 ± 3 | 0.31 ± 0.03 | 131 | 6 |
| Asp | 100 | 100 | 100 | 7.3 | | 64 ± 2 | 0.077 ± 0.006 | 133 | 5 |
| Glut | 100 | 100 | 100 | 7.3 | | 65 ± 2 | 0.074 ± 0.006 | 147 | 6 |
| Gln | 135 | 59 | 80 | 9.1 | 9.1 | 48 ± 3 | 0.25 ± 0.03 | 147 | 5 |
| Lac | 100 | 100 | 100 | 7.3 | | 37 ± 3 | 0.23 ± 0.03 | 90 | 4 |
| Mal | 71 | 71 | 142 | 7.3 | | 51 ± 2 | 0.052 ± 0.006 | 134 | 5 |
| Gluc | 100 | 100 | 100 | 7.3 | | 65 ± 2 | 0.074 ± 0.006 | 190 | 4 |

The anion concentrations ($X^-$) in the basic solutions were calculated from the total concentrations ($X_t$), using the pH and the $pK_2$ values. The amino acid solutions at two different pH are indicated by 1 and 2. The reversal potentials $E_r$ are averages from n results. The permeability ratios $P_x/P_{cl}$ were calculated from the averaged reversal potentials and anion concentrations using the GHK equation, except for the malate permeability ratio which was calculated from the equation shown in methods. MW represents the molecular weight.

$Cl^-$ concentration in the pipette. It was found that the current at pH 9.6 was 20% lower at this potential. Also, to verify that the basic pH solutions did not change the reversal potential in the low Cl solutions, *I-V* curves were measured using the 1 mM NMDG-Cl and raffinose solution at pH 7.3 and 9.6. The high reversal potentials (80–90 mV) were the same for both pH solutions, while the current, measured at −75 mV, was also decreased by 20% with the basic pH solution. Upon restoring the pH 7.3 solutions, the *I-V* curves were the same as those observed initially.

### Amino Acid Currents

The *I-V* curves for glycine, GABA and taurine, measured at three pH values for each amino acid, are given in Fig. 3, Fig. 4 and Fig. 5, respectively. The control *I-V* curves measured with the amino acid solutions at pH 7.3 gave a large reversal potential (80–90 mV), demonstrating that there was a negligible concentration of anionic amino acids available. As the pH of each amino acid solution was increased, more anions were present in the solutions and the reversal potentials decreased. The *I-V* curves measured with the basic pH solutions clearly demonstrate the presence of an inward amino acid current. Similar experiments were performed with a glutamate solution at pH 7.3 and a glutamine solution at pH 7.3 and 9.1. As shown on Fig. 6, the reversal potential for glutamate (65 mV) is higher than the one for glutamine at pH 9.1 (45 mV), despite the fact that the anion concentration of glutamine at pH 9.1 is lower than that of glutamate (Table 1). It means that glutamine is more permeant than glutamate, although both have the same molecular weight. Current-voltage curves were also obtained with an aspartate solution at pH 7.3 and they gave an average reversal potential nearly equal to that for glutamate, as shown in Table 1, indicating that both amino acids had similar permeability ratios relative to chloride. Additional measurements were performed with a β-alanine and a leucine solution at pH 9.6 and 9.7 respectively. From their *I-V* curves, reversal potentials were obtained and they are given in Table 1. The reversal potential for leucine (44 mV) is much lower than that of aspartate even if these amino acids have almost the same molecular weights.

Current-voltage curves were also measured with pipettes containing the hypotonic NMDG-aspartate solution. With the hypotonic NMDG-Cl solution as the external medium, reversal potentials were measured and they were found to be the same, with the opposite polarity, as those obtained with the aspartate solution in the external medium and NMDG-Cl in the pipettes. Similar experiments were also performed with the taurine solution at pH 8.2 in the pipettes. Unfortunately, it was not possible to obtain reliable reversal potential measurements in those conditions, because of a large increase of the leakage current. This leakage current was found when the external NMDG-Cl solution was changed to the raffinose solution. It is possible that a pH 8.2 inside the cell is not tolerated and is responsible for this increased leakage.

### Metabolite Currents

When comparing the chemical composition of glutamate and glutamine (Table 2), the only difference is the re-

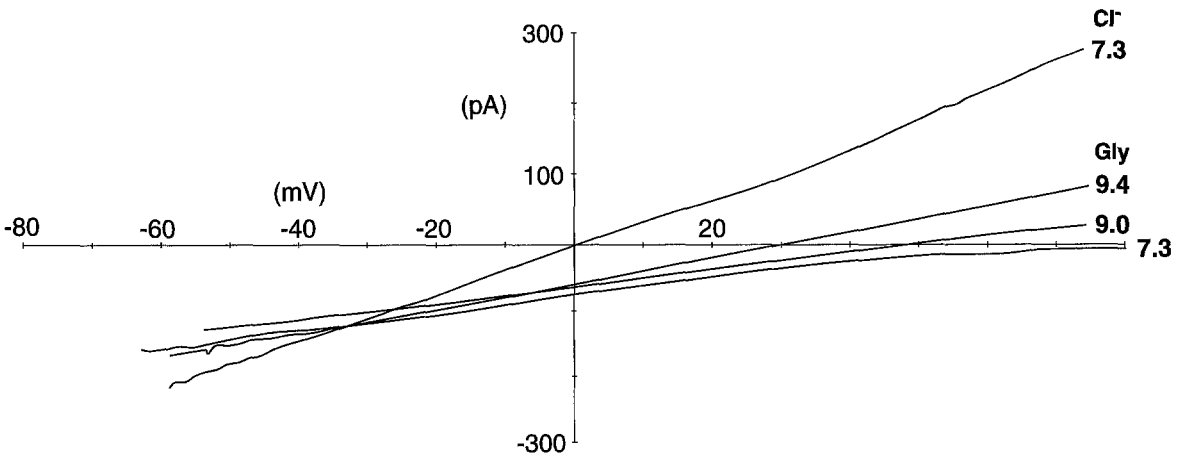


**Fig. 3.** Current-voltage curves measured in whole cell with the 100 mM NMDG-Cl and the glycine external hypotonic (200 mosm) media. The pH of the glycine (Gly) solutions was changed from 7.3 to 9.0 and 9.4 by increasing NMDG concentrations (Table 1). The pipette contained the hypotonic 100 mM NMDG-Cl solution at pH 7.3 and the command potential was a ramp varying between + and −80mV. Reversal potentials were obtained directly from each *I-V* curve tracing.

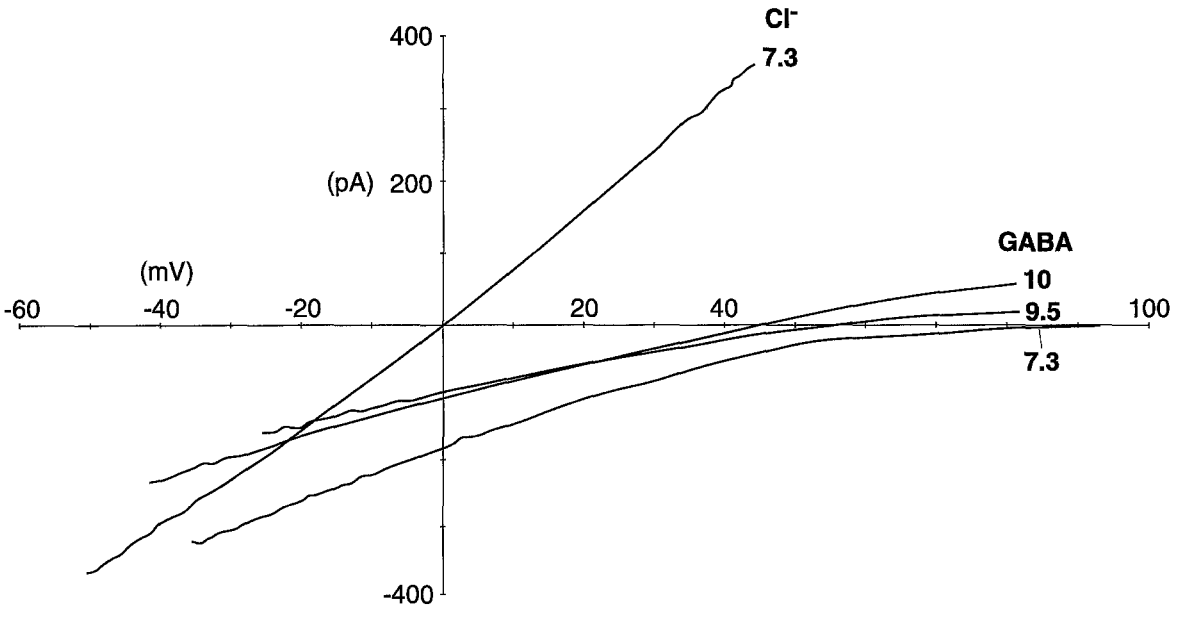


**Fig. 4.** Current-voltage curves measured in whole-cell with the 100 mM NMDG-Cl and the GABA external hypotonic (200 mosm) media. The pH of the GABA solutions was changed from 7.3 to 9.5 and 10.0 by increasing NMDG concentrations (Table 1). The pipette contained the hypotonic NMDG-Cl solution at pH 7.3 and the command potential was a ramp varying between + and −80mV. Reversal potentials were obtained directly from each *I-V* curve tracing.

placement of a hydroxyl by an amine group, thereby removing a negative charge from glutamate. To determine if the presence of two negative charges could be an important factor in reducing the permeability, *I-V* curves were measured with malic acid, which has the same molecular weight and configuration as aspartate, with two negative charges, but without the positively charged amino group which is replaced by a hydroxyl (Table 2). The *I-V* curves for malate gave an average reversal potential of 51 mV, which is lower than that of aspartate. This result does not mean malate has a permeability ratio higher than aspartate, because malate is a divalent anion. Another metabolic acid, lactate, was studied to compare its permeability ratios with amino acids having similar molecular weights. Lactate has some similarity with glycine but does not have an amino group. Current-voltage curves were measured with a lactate solution at pH 7.3 and the average reversal potential was 37 mV, as shown on Table 1. It was also interesting to determine the reversal potential of another organic acid having a molecular weight larger than that of glutamine and glutamate. Glucuronate has such a large molecular weight (190)

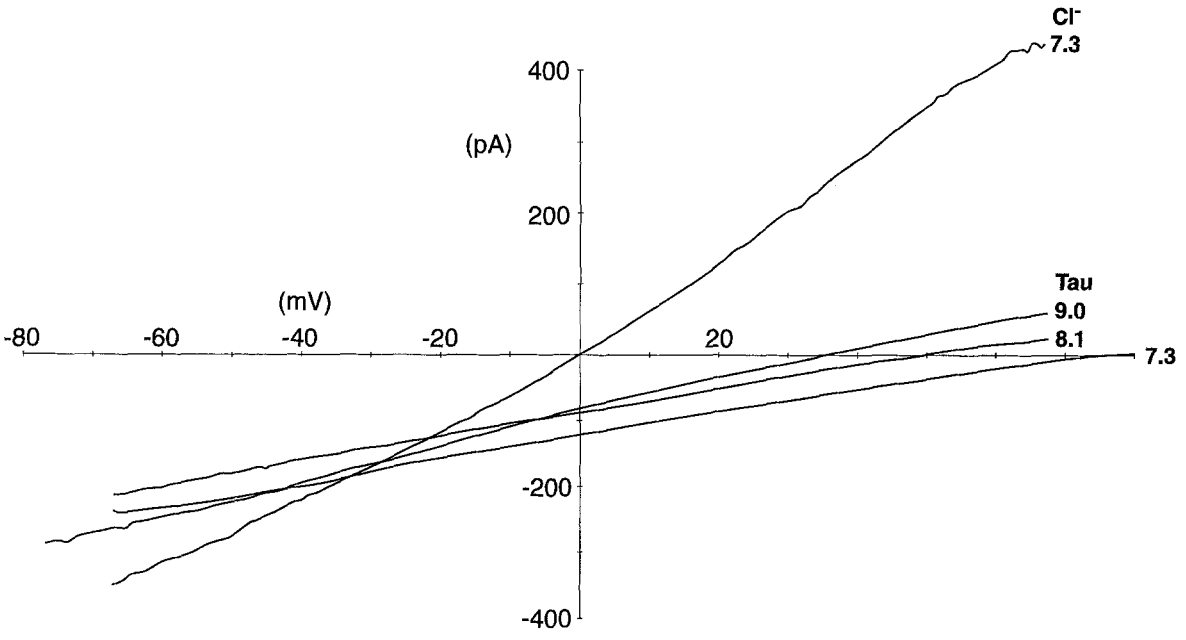


**Fig. 5.** Current-voltage curves measured in whole cell with the 100 mM NMDG-Cl and the taurine external hypotonic (200 mosm) media. The pH of the taurine solutions was changed from 7.3 to 8.2 and 9.0 by increasing NMDG concentrations (Table 1). The pipette contained the hypotonic NMDG-Cl solution at pH 7.3 and the command potential was a ramp varying between + and −80mV. Reversal potentials were obtained directly from each *I-V* curve tracing.

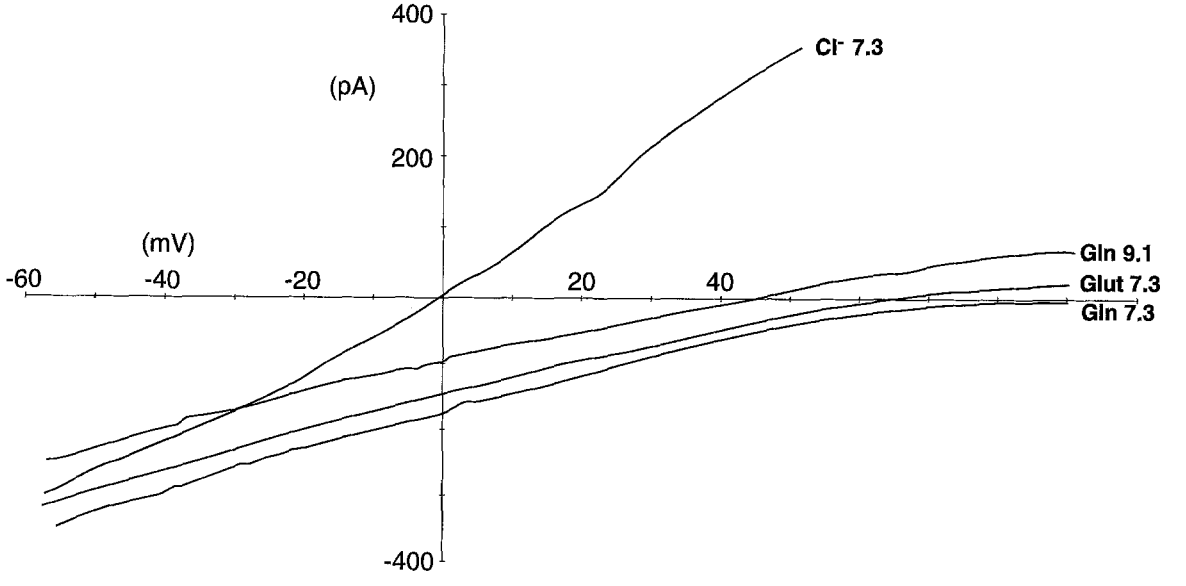


**Fig. 6.** Current-voltage curves measured in whole cell with the 100 mM NMDG-Cl, the glutamate (pH 7.3) and the glutamine (pH 7.3 and 9.1) external hypotonic (200 mosm) media (Table 1). The pipette contained the hypotonic NMDG-Cl solution at pH 7.3 and the command potential was a ramp varying between + and −80mV. Reversal potentials were obtained directly from each *I-V* curve tracing.

and a composition similar to glucose and inositol, two neutral metabolites which could diffuse through anion channels. Current-voltage curves were measured with a glucuronate solution at pH 7.3 and the average reversal potential was 65 mV, as shown on Table 1. This value is equal to the reversal potential for glutamate. In one experiment, *I-V* curves for both glutamate and glucuronate were measured successively on the same cell, and both reversal potentials were the same.

### Organic Cation Currents

These results demonstrate that the volume-sensitive chloride channel is permeable the many different anions

**Table 2.** Chemical composition of amino acids and metabolites

| | | | |
|---|---|---|---|
| $H_2N\text{-}CH_2\text{-}CO_2H$<br>Glycine | $H_2N\text{-}CH_2\text{-}CH_2\text{-}CO_2H$<br>β-Alanine | $H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO_2H$<br>GABA | $H_2N\text{-}CH_2\text{-}CH_2\text{-}SO_3H$<br>Taurine |
| $CH_3$<br>$CH\text{-}CH_2\text{-}CH\text{-}CO_2H$<br>$CH_3$ $NH_2$<br>Leucine | $HO_2C\text{-}CH_2\text{-}CH\text{-}CO_2H$<br>$NH_2$<br>Aspartate | $HO_2C\text{-}CH_2\text{-}CH_2\text{-}CH\text{-}CO_2H$<br>$NH_2$<br>Glutamate | $H_2NOC\text{-}CH_2\text{-}CH_2\text{-}CH\text{-}CO_2H$<br>$NH_2$<br>Glutamine |
| $H_3C\text{-}COH_2\text{-}CO_2H$<br>Lactate | $H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2OH$<br>Aminopropanol | $HO_2C\text{-}CH_2\text{-}COH\text{-}CO_2H$<br>Malate | $H_2OC\text{-}COH_2\text{-}COH_2\text{-}COH_2\text{-}COH\text{-}CO_2H$<br>Glucuronate |

and particularly to anionic amino acids and metabolites. But it was not established if organic cations of low molecular weights similar to glycine could not be permeant. Although the channel appears to be very selective against large cations like $NMDG^+$, as shown on Fig. 2, it is possible that smaller cations are permeant. To test for this possibility, *I-V* curves were measured with a hypotonic solution containing a low molecular weight amine, 3-amino-1-propanol, replacing $NMDG^+$. This amine has a composition similar to glycine and β-alanine, but it does not have the carboxyl group which is replaced by a hydroxyl group (Table 2); its molecular weight is 75. It was found that the *I-V* curves were the same as those obtained with the NMDG-Cl solution and no reversal potential was produced when the amino-propanol-Cl solution replaced the NMDG-Cl solution. If amino-propanol was permeant, a positive reversal potential should have been observed. To determine if a basic pH could induce a permeability to such small organic cations, *I-V* curves were measured with the amino-propanol-Cl solution at pH 9.6. Again, the *I-V* curves were the same as those obtained with the NMDG-Cl solution and no reversal potential was produced.

## Discussion

The objective of this study was to demonstrate that amino acids could diffuse through volume-sensitive $Cl^-$ channels by measuring their current through these channels. At first, a new method was introduced to obtain a stable volume increase in hypotonic media during whole-cell recordings. The pipette medium was hypotonic instead of isotonic, to maintain the cell in osmotic equilibrium with the pipette and the external medium during measurements in hypotonic conditions. This approach appears more appropriate than the usual protocol where the pipette is isotonic and the external medium hypotonic. When the external medium was changed from isotonic to hypotonic, the cell initially swelled, triggering a slow current increase that reached a stable state. The whole-cell current was produced by Cl ions and had an outward rectifying *I-V* curve, with inactivation appearing for potential larger than +50 mV. It is now well established in many cell types that swelling in hypotonic media activates similar outward rectifying $Cl^-$ channels with inactivation (Kubo & Okada, 1992; Chan et al., 1993; Nilius et al., 1994).

Since most amino acids do not have a net charge at pH 7, their currents through the $Cl^-$ channel had to be measured at different external basic pH values, depending on the pK of the amino acids. Increasing the external pH to 9.6 and 10.0 could have effects on the properties of the channels, even though the internal pH was maintained at 7.3. But it seems that the channel characteristics were not dramatically modified by such high pH values, at least for a short time, because the chloride current was decreased by only 20% at pH 9.6 and the initial current could be recovered when the pH was restored to 7.3. The current reduction at pH 9.6 could be related to an increase of the negative surface charges on the external side of the membrane. A similar current reduction at basic pH values was observed by Halm and Frizzell (1992) on a $Cl^-$ channel of T84 cells. This effect of the basic pH on the $Cl^-$ currents should similarly reduce the amino acid currents and should not affect the reversal potentials.

The permeability ratios given in Table 1 clearly demonstrate that many amino acids are permeant through the volume-sensitive anion channel and therefore this channel provides a pathway to explain the large losses of amino acids produced by swelling in many types of cells, including glial cells (Kimelberg et al., 1990*a*, Pasantes-Morales et al., 1993). The permeability ratios for glycine, GABA and taurine were calculated from reversal potentials measured at two different basic pH values. The two values are not significantly different for each of these amino acids, providing evidence that the amino acid permeabilities are not affected by the basic pH. The permeabilities with respect to $Cl^-$ are very large for the low molecular weight amino acids, like glycine, β-alanine and GABA, and decrease for those with a higher molecular weight, like taurine, leucine and glutamine. But molecular weight is clearly not the only fac-

tor involved, because the lowest permeability ratios were obtained for aspartate and glutamate, which have molecular weights equal to those of leucine and glutamine respectively. It seems that the charge configuration is also important. Glutamate and aspartate both have two carboxyl and one amino group, while glutamine and leucine have only one carboxyl and one amino group (Table 2). The charge distribution of these amino acids is quite different. Aspartate and glutamate have two negative and one positive charges, while glutamine and leucine have only one negative charge, their amino group being neutral at basic pH values. To determine if a second negatively charged carboxyl group was important for the permeability, *I-V* curves and reversal potentials were measured for malate. This metabolic acid has two carboxyl groups as aspartate, but no amino group. As shown in Table 1, the permeability ratio obtained for malate is even lower than that of aspartate and glutamate. This indicates that the presence of a second negative charge on aspartate and glutamate could be responsible for their lower permeability ratios compared to those of leucine and glutamine. It was also found by Stoddard et al. (1993) that the divalent ion sulfate had a much lower permeability ratio than the monovalent ion nitrate in a volume sensitive Cl channel. It appears therefore that an anion with two negative charges has a much lower permeability than one with a single negative charge. This is supported by the much larger permeability ratio of lactate compared to malate. But it is also possible that the absence of a positive charge on the amino group of glutamine and leucine gives them a higher permeability. The permeability ratios of glycine and β-alanine are about twice that of lactate, although these molecules have similar molecular weights and comparable compositions. It is possible that the presence of a neutral amino group instead of a hydroxyl is responsible for these differences. Also the permeability ratios for these amino acids could be different at neutral pH, when both a positive and a negative charge are present on them.

It was also demonstrated that this anion channel is not permeable to organic cations with molecular weights similar to that of glycine. Molecules with a positive charge are not permeant through this channel, indicating that some positively charged groups are present in the channel selectivity filter. But these positive charges do not seem to prevent the diffusion of amphoteric amino acids, bearing both a negatively and a positively charged group. It is possible that the binding of the carboxyl on the positive charges shields the amino group.

The permeability ratio for lactate is similar to that obtained by Halm and Frizzell (1992) on T84 cells. The values for glutamate and aspartate are about half of those obtained by Banderali and Roy (1992) on MDCK cells and by Jackson et al. (1994) on C6 glioma cells. The permeability ratio for taurine is similar to that observed by Jackson and Strange (1993) on C6 glioma cells, but much lower than the value measured in single channels by Banderali and Roy (1992) on MDCK cells. It appears that the taurine permeability relative to chloride is larger for epithelial cells than for glial cells. The permeability ratio obtained for glucuronate indicates that this large molecule is permeant through the channel and provides additional evidence that inositol and glucose are also permeant through this anion channel, as demonstrated by Jackson and Strange (1993) and Kirk et al. (1992). The permeability ratio for glucuronate or gluconate were measured in other volume-sensitive anion channels and it was found to be at the limit of measurable permeability ratios obtained from reversal potentials (Stoddard et al., 1993; Nilius et al., 1994). By making a CPK model of large molecules, like gluconate and glucuronate, it is possible to determine the minimum size of the channel; it would have a diameter of about 0.6 nm (Halm & Frizzell, 1993; Jackson & Strange, 1993).

The amino acid permeability sequence obtained in this study can be compared to the results of Pasantes-Morales et al. (1994) who have shown that replacing NaCl (60 mM) in the external medium by an amino acid (120 mM) could block volume regulation. The amino acid influx is about equal to the $K^+$ and $Cl^-$ efflux and it replaces these ions in the cells. From these results, it appears that taurine and β-alanine are the most permeant, followed closely by glycine, GABA, aspartate and glutamate, with glutamine having a lower permeability. Although the external amino acid concentration (120 mM) sufficient to block RVD in many of those experiments is larger than that of cellular $Cl^-$, it indicates that these amino acid permeabilities were only a little lower than that of $Cl^-$. This compares favorably with the permeability ratios given in Table 1, where glycine, β-alanine, GABA and taurine have about half the permeability of $Cl^-$. But the permeability ratios for glutamate and aspartate are lower than that of glutamine in Table 1, contrary to the results of Pasantes-Morales et al. (1994). It should be realized that the permeability ratios given in Table 1 were obtained from currents produced by the anionic form of amino acids. At basic pH values, these anionic amino acids lose their positive charge and their permeabilities through the channel could be modified. The permeabilities of the neutral form of amino acids at a physiological pH could be different from that of the anionic form in the channel. Nevertheless, the results of both these studies compare favorably and support the hypothesis that amino acids, neutral or anionic, diffuse through an anion channel activated by cell swelling. To provide further evidence that these channels are permeable to neutral amino acids in hypotonic media at a physiological pH, experiments will be performed with anion channel blockers to demonstrate that both the whole-cell anion channel current and the amino acid release can be inhibited similarly with the same blocker.

Glial cells play a vital role in brain functions and they are particularly known for their ability to reabsorb the amino acid neurotransmitters released by neurons in the external medium. But in pathological conditions, like ischemia, astrocytes swell (Kimelberg & Randsom, 1986; Waltz et al., 1993) and could loose some of their osmolytes among which there are amino acids. It seems reasonable to suppose that a channel permeable to organic anions in glial cells is activated in pathological conditions, like ischemia, and that some of the cellular amino acids are released through this channel. Although many different processes could contribute to amino acid losses during ischemia, it is possible that the activation of a volume-sensitive anion channel is partly responsible. Future experiments will attempt to provide additional support for such an hypothesis.

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