Regulation of Glutamate Transport into Synaptic Vesicles by Chloride and Proton Gradient*

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Glutamate uptake into synaptic vesicles is driven by an electrochemical proton gradient formed across the membrane by a vacuolar H⁺-ATPase. Chloride has a bi- phasic effect on glutamate transport, which it activates at low concentrations (2–8 mM) and inhibits at high concentrations (>20 mM). Stimulation with 4 mM chloride was due to an increase in the Vmax of transport, whereas inhibition by high chloride concentrations was related to an increase in Km to glutamate. Both stimulation and inhibition by Cl⁻ were observed in the presence of A23187 or (NH₄)₂SO₄, two substances that dissipate the proton gradient (ΔpH). With the use of these agents, we show that the transmembrane potential regulates the apparent affinity for glutamate, whereas the ΔpH antagonizes the effect of high chloride concentrations and is important for retaining glutamate inside the vesicles. Selective dissipation of ΔpH in the presence of chloride led to a significant glutamate efflux from the vesicles and promoted a decrease in the velocity of glutamate uptake. The H⁺-ATPase activity was stimulated when the ΔpH component was dissipated. Glutamate efflux induced by chloride was saturable, and half-maximal effect was attained in the presence of 30 mM Cl⁻. The results indicate that: (i) both transmembrane potential and ΔpH modulate the glutamate uptake at different levels and (ii) chloride affects glutamate transport by two different mechanisms. One is related to a change of the proportions between the transmembrane potential and the ΔpH components of the electrochemical proton gradient, and the other involves a direct interaction of the anion with the glutamate transporter.

Glutamate is the major excitatory neurotransmitter found in the mammalian central nervous system and is released into the synaptic cleft by synaptic vesicles exocytosis (Jahn and Sudhof, 1994). Re-uptake of the released glutamate is mediated by two transport systems. One is a high affinity, Na⁺-dependent carrier located in the plasma membrane, and the other is a low affinity, Na⁺-independent transport system located in the synaptic vesicles (Kanner, 1983; Maycox et al., 1990). Glutamate uptake into synaptic vesicles is driven by a ΔμH⁺ formed across the vesicle membrane by a bafilomycin A₁-sensitive vacuolar H⁺-ATPase (Disbrow et al., 1982; Naito and Ueda, 1983, 1985; Maycox et al., 1988; Cidon and Sihra, 1989; Floor et al., 1990). As H⁺ is pumped into the vesicle lumen, a ΔpH, acidic inside, and a ΔΨ, positive inside, are built across the membrane. The relative proportions of ΔpH and ΔΨ vary greatly. In the absence of a permeating anion, the proton charge is not counterbalanced, and thus ΔΨ predominates over ΔpH. When the concentration of the physiological permeating anion chloride is increased there is a progressive fall of the ΔΨ, and a significant ΔpH is formed across the membrane (Van Dyke, 1988). There is no consensus in the literature on whether glutamate uptake into synaptic vesicles is driven solely by ΔΨ (Maycox et al., 1988; Cidon and Sihra, 1989; Hartinger and Jahn 1993; Moriyma and Yamamoto, 1995) or by both the ΔΨ and ΔpH components of the ΔμH⁺ (Naito and Ueda, 1985; Shiomi and Ueda, 1990; Tabb et al., 1992). Low concentrations (2–8 mM) of chloride stimulate glutamate uptake, and high Cl⁻ concentrations (>20 mM) inhibit it. Tabb et al. (1992) proposed that low chloride stimulates glutamate uptake because it increases the vesicle ΔpH, and the inhibition by high Cl⁻ would be related to the dissipation of ΔΨ (Maycox et al., 1988). Recently, Hartinger and Jahn (1993) found that high concentrations of chloride prevented inhibition of glutamate transport promoted by DIDS, an anion transporter blocker. These authors proposed that the vesicular glutamate transporter possesses a DIDS-sensitive chloride binding site. In the present study we examined the effects of chloride on steady state glutamate uptake. Different ΔpH dissipation agents were used to alter the balance between the ΔpH and ΔΨ contribution to the ΔμH⁺ formed across the synaptic vesicles membrane. It was found that ΔΨ controls the apparent affinity for glutamate, whereas ΔpH is important for antagonizing the effect of high chloride concentrations.

EXPERIMENTAL PROCEDURES

Vesicle Preparation—Synaptic vesicles were isolated from rat brains as described in detail by Hell et al. (1988). The vesicles were used omitting the chromatography on controlled pore glass beads, as described by Hell et al. (1990). The glutamate uptake and purity of this preparation have been previously determined (Hell et al., 1990; Hartinger and Jahn, 1993). The vesicles were stored under liquid nitrogen until use. Protein concentration was determined by the method of Lowry et al. (1951). All the experiments were performed at least three times with different vesicle preparations.

Neurotransmitter Uptake—The vesicles were incubated with 1-[³⁵S]glutamate at 35 °C in different media as described in the figure legends. The Cl⁻ concentration was varied using different proportions of potassium gluconate and KCl and maintaining H⁺ concentration fixed at 140 mM. Glutamate uptake was started by the addition of 4 mM MgATP, and the reaction was stopped by filtration of the assay medium.
The Dual Effect of Cl⁻ on the Kinetics of Glutamate Transport—Previous reports have shown that the transport of glutamate is stimulated by 2–8 mM Cl⁻. This effect is not observed with glutamate, a nonpermeating anion (Naito and Ueda, 1985; Maycox et al., 1988; Cidon and Sihra, 1989). The present study shows that the effect of Cl⁻ varies depending on the glutamate concentration in the medium and on whether the initial velocity or the steady state level of glutamate uptake is measured (Fig. 1). The steady state is achieved when the rate of glutamate efflux equals the rate of glutamate influx. The initial rate of glutamate uptake was measured after 1 min of incubation, and the steady state level of glutamate uptake was measured after 10–20 min of incubation (Fig. 1). Cl⁻ (4 mM) stimulated the initial rate of uptake regardless of the glutamate concentration used (Fig. 1). Measurement of the initial rate of uptake in the presence of different glutamate concentrations indicated that the activation promoted by Cl⁻ is related to an increase in the $V_{\text{max}}$ of glutamate transport (Fig. 2A and Table I). The activation was abolished, and inhibition of glutamate uptake was observed when the Cl⁻ concentration was raised from 4 to 80 mM (Figs. 1 and 2). The effect of high Cl⁻ concentrations seems to be related to an increase of the apparent $K_m$ of the transport system for glutamate; the inhibition by Cl⁻ was more pronounced in the presence of low glutamate concentrations and decreased progressively as the concentration of glutamate was raised to 4 mM (Figs. 1B and 2A and Table I). Therefore, although Cl⁻ modified the initial rate of glutamate uptake in the presence of high glutamate concentrations (Fig. 2A), it did not alter the maximal filling capacity of the vesicles observed after the steady state was reached (Figs. 1B and 2B). The values shown in Table I for the $K_m$ and $V_{\text{max}}$ of glutamate uptake in the absence of Cl⁻ were determined by double-reciprocal plots. The effect of A23187 at each Cl⁻ concentration was analyzed by a paired $t$ test, and the results are shown in parentheses under each pair of values. The effects of Cl⁻ within each column were compared by one-way ANOVA, followed by Duncan’s multiple range test. The values are means ± S.E. of 4 experiments with three vesicle preparations. Other conditions are the same as described in the legend to Fig. 1.

### Table I

| $V_{\text{max}}$ | $K_m$ |
|------------------|-------|
| (nmol/min/mg)    | (mM)  |
| Control A23187   | Control A23187 |
| No Cl⁻           | 3.6 ± 0.5 | 3.7 ± 0.5 |
| (N.S.)           | 0.8 ± 0.1 | 0.5 ± 0.1 |
| 4 mM Cl⁻         | 8.4 ± 0.9b | 6.1 ± 0.8b |
| (p = 0.01)       | 1.1 ± 0.1 | 0.6 ± 0.1 |
| 80 mM Cl⁻        | 4.2 ± 0.9ab | 1.9 ± 0.3ab |
| (p = 0.003)      | 2.7 ± 0.2ab | 2.6 ± 0.3ab |
|                  | (N.S.)   | (N.S.)   |

- Value that is statistically different from that obtained without Cl⁻ in the same column at $p < 0.01$.
- Value that is statistically different from that obtained with 4 mM Cl⁻ in the same column at $p < 0.01$.
- Value that is statistically different from that obtained without Cl⁻ in the same column at $p < 0.05$.

Other research groups favor a different explanation for the effect of Cl⁻ on glutamate transport (Maycox and Ueda, 1992). However, our results indicate that the activation of glutamate transport by Cl⁻ is not the only mechanism involved in the regulation of glutamate uptake.
et al., 1988; Hartinger and Jahn, 1993; Moriyama and Yamamoto, 1995). In the following experiments, different combinations of ΔpH dissipating agents and Cl− and glutamate concentrations were used. The aim was to estimate the relative contributions of ΔΨ and ΔpH to the kinetics of glutamate transport.

Alteration of ΔH+ Components by (NH₄)₂SO₄ and A23187—The electrochemical proton gradient formed across the vesicle membrane varies with the permeating anion concentration used (Van Dyke, 1988). Without added Cl−, a large ΔΨ and a small ΔpH are formed across the membrane. Increasing Cl− concentration promotes a progressive decrease of the ΔΨ, and this is associated with an increase of ΔpH (Figs. 3 and 4). The effect of chloride can be modified with the use of ammonium sulfate and A23187 (Figs. 3 and 4). These two substances dissipate ΔpH and increase the ΔΨ (Johnson and Scarpa, 1976). Note that these effects were observed over a wide range of Cl− concentrations (Fig. 4). During H+ pumping, the uncharged species NH₃ derived from (NH₄)₂SO₄ diffuse into the lumen of the vesicles and associate with H+ forming NH₄⁺. A23187 is a dicarboxylic ionophore capable of transporting 2H⁺ in exchange for either one Mg²⁺ or one Ca²⁺ ion (Johnson and Scarpa, 1976; Pressman, 1976; Romani and Scarpa, 1992). Under the conditions of the present study, the collapse of the ΔH+ by A23187 is promoted by the exchange between H⁺ and Mg²⁺. Removal of contaminating Ca²⁺ from the medium with EGTA did not modify the effect of A23187 on the ΔH+ (data not shown). In the subsequent experiments, we show that the effect of A23187 and (NH₄)₂SO₄ on the steady state level of glutamate uptake varies with the Cl− concentrations used.

The Effect of A23187 and (NH₄)₂SO₄ in the Presence of Low Cl− Concentrations—Both A23187 (Fig. 5) and (NH₄)₂SO₄ (Fig. 6) stimulated 2-fold the uptake when the latter was measured with glutamate concentrations far below the Kₘ value and either in the absence or in the presence of 4 mM Cl− (Figs. 5, A and B, and 6A). Under these conditions, these agents not only increased the rate of uptake but also enhanced the amount of glutamate stored by the vesicles after the steady state was reached. This could be best seen if A23187 was added to the medium after the steady state uptake was reached (Fig. 7A). The enhancement of the filling capacity of the vesicles was only observed with the use of low glutamate concentrations. A23187 had no effect on steady state glutamate uptake when saturating glutamate concentrations were used (Fig. 7B). The different effects observed with the use of low and high glutamate concentrations can be ascribed to the 2-fold decrease of the Kₘ for glutamate promoted by A23187 (Table I). The experiments illustrated in Figs. 3 and 4 show that A23187 dissipates the preexisting ΔpH and increases the ΔΨ component of the gradient. This indicates that ΔΨ alone can drive glutamate uptake and that the apparent affinity of the vesicles for glutamate is determined by the magnitude of ΔΨ.

The Combined Effects of High Cl− and ΔpH on Glutamate Efflux—High Cl− concentrations seem to activate the efflux of glutamate by interacting directly with the glutamate transporter of the membrane, but this is only observed after the ΔpH is collapsed with A23187. Thus, the ΔpH seems to be important to antagonize the effect of high Cl− and for the retention of glutamate inside the vesicles. The data that led to these conclusions are the following: (i) In the absence of Cl−, A23187 had no effect on the Vmax of glutamate transport. In the presence of 4 mM Cl−, A23187 promoted a small decrease of Vmax but this decrease become more pronounced when the Cl− concentration...
The significant stimulation of the bafilomycin A1-sensitive ATPase was raised to 80 mM (Table I). The amount of glutamate retained by the vesicles depends on both the velocity of uptake and the rate of glutamate efflux from the vesicles. The effect of Cl\(^{-}\) and A23187 can be analyzed better after steady state is reached, a condition in which the rate of glutamate uptake equals the rate of glutamate efflux. Using saturating concentrations of glutamate and in the absence of Cl\(^{-}\) (Fig. 7B), there was no change in the level of glutamate retained by the vesicles when A23187 was added to the medium, indicating that dissipation of the pre-existing ΔpH did not lead to a change in either the rate of glutamate uptake or the rate of glutamate efflux. However, when 20 mM Cl\(^{-}\) was present in the medium, the addition of A23187 led to an increase in the efflux rate (Fig. 7, C and D), and although the H\(^{+}\)-ATPase was still pumping protons and the driving the transport of glutamate (Fig. 8), the rate of efflux was faster than the rate of uptake. As a result, there was a net decrease in the amount of glutamate retained by the vesicles until a new steady state was reached (Fig. 8A). A comparison of Fig. 7 (D and F) indicates that glutamate efflux rate increased with Cl\(^{-}\) concentration in the medium. This was observed in the presence of both a low and a high glutamate concentration (Fig. 7, E and F) regardless of the order of A23187 and Cl\(^{-}\) addition to the medium. (iii) In the presence of Cl\(^{-}\), dissipation of ΔpH by A23187 promoted a significant stimulation of the bafilomycin A1-sensitive ATPase activity (Fig. 8B). This suggests that similar to other H\(^{+}\)-transport ATPases (Dufour et al., 1982), the vesicular H\(^{+}\)-ATPase is also back-inhibited by the accumulation of protons inside the vesicles (ΔpH) and that the dissipation of the ΔpH by A23187 uncouples the glutamate uptake from the ATPase activity (Fig. 8). (iv) The effect of Cl\(^{-}\) on glutamate efflux revealed a saturation kinetics, with the half-maximal effect being attained with 30 mM Cl\(^{-}\) (Fig. 9). Notice in Fig. 9 that gluconate did not induce glutamate efflux from the vesicles, thus indicating that the effect is specific for the chloride anion and is not related to a possible osmotic imbalance of the system.

The Critical Cl\(^{-}\) and Glutamate Concentration Ranges—

**Fig. 5.** Dual effect of chloride at various glutamate concentrations and in the presence of A23187. The reaction was carried out at 35°C in medium containing 10 mM Mops-Tris (pH 7.0), 4 mM MgATP, 1 mg of synaptic vesicle protein/ml, a mixture of potassium gluconate and KCl to achieve the desired chloride concentrations, maintaining total K\(^{+}\) at 140 mM, either in the absence (●) or in the presence of 10 μM A23187 (○). Glutamate concentrations were 10 μM (A), 50 μM (B), or 1 mM (C). The reaction was stopped after 20 min of incubation at 35°C. The values represent the average of three different experiments with two different vesicle preparations. glu, glutamate.

**Fig. 6.** Dual effect of chloride at various glutamate concentrations and in the presence of NH\(_4\)\(^{+}\). The conditions were the same as described in the legend to Fig. 5. Glutamate concentrations were 50 μM (A) or 1 mM (B) in the presence of either 10 mM K\(_2\)SO\(_4\) (●) or 10 mM (NH\(_4\))\(_2\)SO\(_4\) (○). The reaction was stopped after 10 min of incubation at 35°C. The values represent the average of three different experiments with three different vesicle preparations. glu, glutamate.

**Fig. 7.** Effect of dissipation of ΔpH (A, B, C, and D) and chloride addition (E and F) at steady state glutamate uptake. A, B, C, and D, glutamate uptake was measured in the presence of 10 mM Mops-Tris (pH 7.0), 4 mM MgATP, 120 mM potassium gluconate, 1 mg of synaptic vesicles/ml, with 50 μM (●) or 4 mM L-[\(^{3}\)H]glutamate (△, ▲), either in the absence (A and B) or in the presence of 20 mM KCl (C and D). After steady state was reached (10 min, arrow) A23187 was added (●, ▲) to a final concentration of 10 μM, or no A23187 was added (○, △). E and F, glutamate uptake was measured in the presence of 10 mM Mops-Tris (pH 7.0), 4 mM MgATP, 60 mM potassium gluconate, 1 mg of synaptic vesicles/ml, 10 μM A23187 with 50 μM (●), or 4 mM L-[\(^{3}\)H]glutamate (△, ▲). After steady state was reached (10 min, arrow) KCl was added (●, ▲) to a final concentration of 80 mM, or no KCl was added (○, △). The values are means of three experiments with three different vesicle preparations.
As suggested by the present results under these conditions, small changes of intraneuronal chloride concentrations may lead to the release of glutamate accumulated by the vesicles. This mechanism may contribute to the inhibitory action of neuron blockers in neurotransmission.

Under optimal conditions, the \( \Delta \psi \) across synaptic vesicles was found to be of one unit (Tabb et al., 1992). The \( K_H \) of the \( \gamma \)-carboxylic group of glutamate is 4.25 and is far from the \( \Delta \psi \) range of this study. After decreasing the \( \Delta \psi \) from 7.0 to 6.0, the concentration of the negative forms of glutamate decreases from 99.8 to 98\%, whereas a neutral species concentration increases from 0.2 to 2\% of the total. Under the conditions of our study, only 1-5\% of the glutamate was taken up by the vesicles. Thus, if the neutral species of glutamate will be less permeable than the negative forms, the possibility exists that glutamate will be progressively trapped inside the vesicles.
Dual Effect of Chloride on Glutamate Uptake

because a continuous H⁺ flux is provided by the vacuolar H⁺-ATPase. Another possibility is a direct effect of internal ΔpH on the glutamate transporter protein as suggested by Tabb et al. (1992).

Hartinger and Jahn (1993) found that high chloride concentration prevents inhibition of glutamate uptake by DIDS, indicating that the glutamate carrier has a chloride binding site on the cytoplasmic side. In this view, a second mechanism for the action of chloride implies a direct interaction with the glutamate transporter protein, and in the present study this is supported by the following data: (i) Both activation and inhibition of glutamate uptake by chloride can be observed at subsaturating glutamate concentrations after the ΔpH is abol-

ished with either A23187 or (NH₄)₂SO₄ (Figs. 5, A and B, and 6A). Thus, stimulation with 4 mM chloride is not essentially due to the formation of a ΔpH as previously suggested by Tabb et al. (1992); (ii) Chloride significantly increased the Kᵣₑₐ₅ for glutamate and altered the Vₑ₅ₐₓ in the presence of A23187 (Table I); (iii) In the absence of a ΔpH, the addition of chloride led to glutamate efflux, an effect that exhibited saturation kinetics (Fig. 9). A possible explanation for the two effects of chloride is that chloride may bind to the glutamate carrier and act as a counter anion to glutamate (Fig. 11, B and C, reactions 1–4). Glutamate influx during glutamate accumulation may be coupled to chloride movement in the opposite direction (Fig. 11B). For the influx of glutamate, Maycox et al. (1990) have already proposed that chloride may act as a counter anion. These authors, however, did not observe an effect of high chloride on efflux. We now raise the possibility that release of glutamate will also be coupled to chloride influx. The occurrence of this reaction is inversely related to the magnitude of the ΔpH component. In this view, the affinity of the glutamate carrier for chloride would vary depending on the side of the membrane to which glutamate binds. When glutamate binds to the external surface of the membrane, the carrier would bind chloride with high affinity to the part of the carrier that faces the vesicle lumen (Fig. 11B). Conversely, during efflux, the binding of glutamate in the vesicles lumen would be coupled with the binding of chloride to a low affinity site located in the part of the carrier facing the external surface of the membrane.

In the first situation, the binding of chloride will facilitate the uptake and in the second the release of glutamate. It is not clear whether binding of chloride and glutamate to the carrier will be simultaneous (Fig. 11B) or follow a sequential pattern (Fig. 11C, reactions 1–4). Although a chloride-glutamate counter transport has still to be directly demonstrated, it would provide charge balance and thus explain the previous finding that the membrane potential remains largely intact during glutamate accumulation (Maycox et al., 1988).

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