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The role of chloride ions on the transport of glycine in plasma membrane vesicles from glial cells

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The high-affinity transport system for glycine in plasma membrane vesicles from C6 glioma cells is dependent on Na^+ and also on the presence of Cl^- in the incubation medium. This anion requirement is relatively specific for Cl^-, since other anions are also capable of stimulating the glycine transport in the following order of decreasing efficacy: $Cl^- > Br^- > SCN^- \simeq I^- > NO_3^- > F^-$. Chloride ions raise the V_{max} for transport and, to a lesser extent, act on the K_m. The data provided by direct measurements of the coupling of sodium and chloride to the transport of glycine by using a kinetic approach suggest a stoichiometry for the translocation cycle catalyzed by the glycine transporter of two sodium ions and one chloride ion per glycine zwitterion.

Introduction

During the past years, studies have provided growing evidence suggesting that glial cells of the central nervous system play an important role in the modulation of neuronal excitability via the control of the levels of neuroactive substances in the extracellular milieu of neurons [1–3]. The reuptake of neurotransmitter substances into presynaptic nerve endings or glial cells provides one way of clearing the extracellular space of potentially neuroactive substances and so constitutes an efficient mechanism by which postsynaptic action can be terminated.

Na^+-dependent transport systems located in mammalian plasma membranes are generally accepted to function by cotransport of substrate and Na^+, and it has become clear that the electrochemical potential created by a Na^+ gradient serves as a direct driving force for the process [4–6]. A growing number of Na^+-dependent transport systems also require Cl^- for activity, suggesting that Cl^- might also be cotransported [7–9]. Recently, we have reported the existence of two efficient uptake systems for glycine in plasma membrane vesicles derived from glioma cells; one is sodium- and chloride-dependent with high affinity for the substrate, whereas the other has been shown to be Na^+-dependent and Cl^--independent with low affinity for glycine. The former shows kinetic features and energetic requirements similar to those found in nerve terminals [10,11].

Considerable evidence now indicate that glycine, besides its role in numerous metabolic functions [12], acts as an inhibitory neurotransmitter in the mammalian CNS, mainly in the spinal cord [13], and probably in some localized areas of the brain, such as substantia nigra [14]. On the other hand, membrane vesicles isolated from various bacterial and mammalian cells, including neurons [15,16], and specifically from the glial plasma membrane [10], have been shown to be extremely useful in studying the amino-acid transport mechanisms, entailing a well-defined ion environment and energy sources and avoiding metabolic and compartmentation interferences.

The present paper extends previous data from our laboratory on glycine transport in glial cells in order to obtain a more detailed understanding about the mode of action of Cl^- in the Na^+-coupled transporting systems for amino-acid neurotransmitters.

Materials and Methods

Materials

[U-^{14}C]Glycine (113 mCi/mmol) was obtained from Amersham International, U.K., DMEM and fetal calf serum were obtained from Gibco, Paisley, U.K. Dishes

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid; DMEM, Dulbecco's modified Eagle's medium.

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for tissue culture were purchased from Costar, The Netherlands. Valinomycin and furosemide were purchased from Sigma, U.S.A. SITS was purchased from BDH, U.K. Ficoll was provided by Pharmacia, Sweden and was exhaustively dialysed against water before use. All other reagents used were of the highest purity available.

Cell culture

The cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cultures were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The growth medium was changed at 3-day intervals, the medium containing 10% fetal calf serum. Only cell cultures at the stationary growth phase were used for cell membrane isolation.

Preparation of membrane vesicles

Membrane vesicles were prepared essentially as described previously [10]. Cells from about 20 confluent roller bottles were used for each membrane preparation. Bottles were rinsed twice with 0.3 M mannitol/1 mM EDTA/10 mM Tris-HCl (pH 7.4) (solution A) and then cells were harvested by scraping with a rubber blade. Subsequent steps were carried out at 4°C. Cells were centrifuged at $3000 \times g$ for 10 min, resuspended in 60 ml of solution A and broken down with ten strokes of an all-glass Potter-Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged for 10 min at $3000 \times g$ in a Sorval SS34 rotor and the pellet was washed by resuspension in 30 ml of solution A and centrifugation at $3000 \times g$ for 10 min. Both supernatants were combined (S1) and centrifuged for 20 min at $27000 \times g$, to obtain a crude mitochondrial pellet (PII). PII was resuspended in about 3 ml of solution A and layered over two discontinuous gradients, each consisting of 5 layers of 20, 16, 12, 8 and 2% Ficoll in solution A, respectively. The gradients were then centrifuged in a Beckman SW28 rotor at 20000 rpm for 90 min. The 2–8% and 8–12% interfaces (F_1 and F_2, respectively) were collected, combined and diluted with approx. 4 vols. of solution A. F_1 and F_2 were centrifuged at $27000 \times g$ for 20 min. The pellet was subjected to osmotic disruption in 20 ml of 5 mM Tris-HCl/1 mM EDTA (pH 7.4). After stirring for 45 min, the suspension was centrifuged again (20 min at $27000 \times g$) and the pellet resuspended in 10 ml of a 300 mosM medium (pH 7.4) with ionic composition depending on each particular experiment. Finally, the suspension was centrifuged at $27000 \times g$ for 15 min and the pellet was resuspended in the former medium to a protein concentration of 5 mg/ml. Portions were frozen in liquid nitrogen, and stored at −70°C. Under these conditions, membrane vesicles were functional for at least 1 month.

Transport assays

Aliquots of 10–20 μl of the suspension of membrane vesicles (about 100 μg of protein) were preincubated for 1 min at 25°C. The uptake was started by adding 100 μl of a solution containing [U-^{14}C]glycine (10 μM final concentration) in 150 mM NaCl/1 mM $MgSO_4$/5 mM Hepes-Tris, (pH 7.4) (NaCl medium), or 150 mM KCl/1 mM $MgSO_4$/5 mM Hepes-Tris (pH 7.4) (KCl medium), unless otherwise stated in the figure legends. The experiments were terminated by diluting with 5 ml of ice-cold 0.8 M NaCl and immediately filtering through a moistened Millipore filter HAWP (0.45 μm pore size) attached to a vacuum assembly. The filters were washed once with the ice-cold medium. The dilution, filtration and washing procedures were performed within 15 s. The filters were dried at 60°C, placed in microvials and their radioactivity was measured in a liquid scintillation counter (LKB 1219 Rackbeta). Results were corrected by a control before adding the radioactive substrate solution. When ionophores were used, they were preincubated with the membrane suspension for 1 min. All solutions used in the preparation of the membrane vesicles and in the uptake experiments were prepared with distilled deionized water and filtered through Millipore filters (0.45 μm) to avoid possible bacterial contamination. The osmolarity of all solutions was kept constant during the uptake experiments, unless otherwise indicated. The pH of the external and internal medium was 7.4 throughout the experiments. The ionic composition of the internal and external medium of the membrane vesicles was modified to investigate the effect of different ions.

All incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations. For estimating statistical differences, the data were compared using Student's *t*-test; differences at the 0.05 level were considered to be statistically significant.

Protein determination

Membrane protein was determined by the method of Resch et al. [17].

Computer optimization

The equation for each kinetic model (those of Garay and Garrahan [18] and Hill [19]), was fitted to the experimental data for membrane vesicles contents (calculated from the label contents) by means of a computer program with a weighted least-squares iterative algorithm.

Results

The high affinity transport system for glycine in plasma membrane vesicles derived from C6 astrocytoma cells shows a typical dependence for Na^+. In addition,

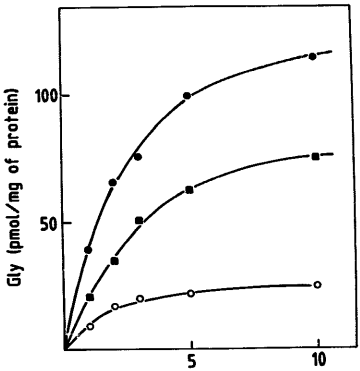

Fig. 1. Ion dependence of glycine transport. Vesicles were preloaded with KCl medium (150 mM KCl/1 mM $MgSO_4$/5 mM Hepes-Tris) (pH 7.4) (●), or sodium gluconate medium (150 mM sodium gluconate/1 mM $MgSO_4$/5 mM Hepes-Tris) (pH 7.4) (■), or potassium gluconate medium (150 mM potassium gluconate/1 mM $MgSO_4$/5 mM Hepes-Tris) (pH 7.4) (○) and incubated as described in Materials and Methods in the presence of 10 μM [^{14}C]glycine in NaCl medium (150 mM NaCl/1 mM $MgSO_4$/5 mM Hepes-Tris) (pH 7.4) (●, ■) or sodium gluconate medium (○).

as can be observed in Fig. 1, there is an influence of the anion associated with Na^+ in the incubation medium. Thus, chloride anions are also requires for the uptake process, because no accumulation above the non-gradient level was observed when an Na^+ gradient (out > in) was imposed in the absence of chloride. Previous data from our laboratory demonstrated that an Na^+ gradient (out > in) is absolutely necessary for maximal glycine uptake and that a Cl^- gradient can drive transport of glycine into the vesicles only if Na^+ is present. Thus, maximal glycine uptake takes place when both ion gradients are present.

We compared the efficacy with which certain anions stimulate glycine transport in the presence of the same external Na^+ concentration (150 mM). The specificity of the anion requirement for glycine uptake is documented in detail in Fig. 2. Chloride is by far the most effective anion tested, but several others are able to mimic its stimulatory effect on glycine uptake ($Cl^- > Br^- > SCN^- \simeq I^- > NO_3^- > F^-$). By contrast, propionate, acetate, sulphate and gluconate are not able to substitute for chloride. Taking into account the hydration radius of all anions tested [20], it can be concluded that, with minimal exceptions, these anions are classed according to increased size. Therefore, there is a relationship between the capacity of an anion to stimulate glycine transport and its size: the smaller the anion, the greater the efficacy.

Note that the effect of the anions tested in this experiment can not be ascribed to electrical effects (diffusion potentials) since the tests were carried out under voltage-clamp conditions in the presence of 5 μM valinomycin.

The effect of Cl^- on the high-affinity glycine transport was studied for substrate concentrations ranging from 10 to 200 μM, using vesicles preloaded with potassium gluconate and then incubated in media with different sodium and chloride concentrations. Fig. 3 shows that the high-affinity transport for glycine is a saturable function either at 30 or 150 μM of chloride in the external medium. The analysis of the kinetic data for the high-affinity system of glycine transport obtained by the Eadie-Hofstee method suggested that V_{max} but not K_m of glycine uptake depends on the external Cl^- concentration (Fig. 4). When Cl^- is partially replaced by gluconate, the lower the amino-acid concentration, the greater the inhibition of glycine transport. This substitution has an unequal effect on the two kinetic constants of the transport of the amino acid: there is an important decrease in the V_{max} (from 385 to 175 pmol/mg of protein), whereas it has no significant effect on the K_m. On the other hand, when Na^+ concentration in the external medium was lowered from 150 to 50 mM (maintaining the Cl^- concentration con-

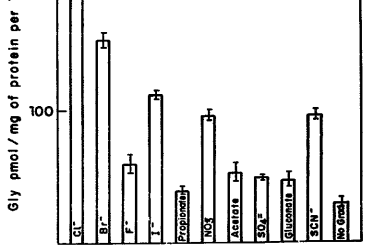

Fig. 2. Specificity of the effect of anions on the glycine transport. Membrane vesicles were preloaded with potassium gluconate medium and incubated as described in Materials and Methods for 10 min in the presence of 10 μM [^{14}C]glycine and 5 μM valinomycin in NaCl medium or in ionic media in which Cl^- was isoosmotically substituted by the indicated anions. The no-gradient conditions were obtained with potassium gluconate as external medium. The composition of the media are given in the legend to Fig. 1.

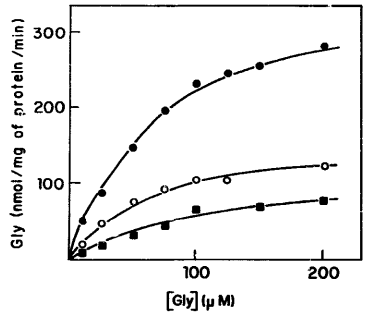

Fig. 3. Effect of Na^+ and Cl^- on the kinetics of high-affinity glycine transport. Vesicles were preloaded with potassium gluconate medium and incubated for 1 min as described in Materials and Methods in the NaCl medium (●), or in medium 30 mM NaCl/120 mM sodium gluconate/1 mM $MgSO_4$/5 mM Hepes-Tris, (pH 7.4) (○), or in medium 50 mM NaCl/100 mM LiCl/1 mM $MgSO_4$/5 mM Hepes-Tris (pH 7.4) (■), and radioactive glycine at the indicated concentrations. Data were corrected by subsracting both the diffusion and the low-affinity components from the total transport. The composition of the media are given in the legend to Fig. 1.

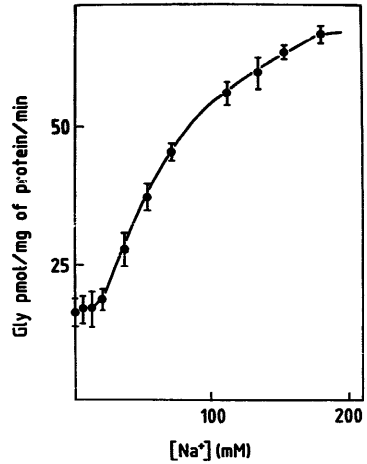

Fig. 5. Effect of increasing Na^+ concentrations on the transport of glycine. Membrane vesicles were preloaded with 200 mM KCl/1 mM $MgSO_4$/5 mM Hepes-Tris (pH 7.4) and incubated for 1 min as described in Materials and Methods in the presence of 10 μM [^{14}C]glycine and ionic media comprising 1 mM $MgSO_4$ 5 mM Hepes-Tris (pH 7.4), 5 μM valinomycin and increasing concentrations of NaCl. Osmolarity was maintained with LiCl.

stant), V_{max} decreased from 385 to 145 pmol/mg of protein min with a slight change in the K_m (from 75 to 170 μM). If chloride is absent in the external medium, the high-affinity component of the transport system disappeared, whereas low-affinity transport was not affected (data not shown).

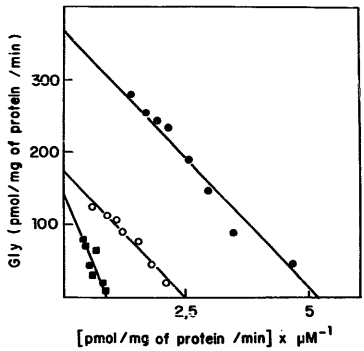

Fig. 4. Eadie-Hofstee plot of initial specific high-affinity uptake of glycine by membrane vesicles of C6 cells. The experimental conditions were as described in the legend to Fig. 3.

The following step is the description of the effects of Na^+ and Cl^- on glycine transport under well-defined experimental conditions and the results of the direct measurements of the coupling of Na^+ and Cl^- to the transport of glycine with the aim of obtaining ion/solute stoichiometries. We used a kinetic approach involving direct measurements on initial rates of glycine upon imposition of an ion gradient [21]. The results of such experiments are shown in Figs. 5 and 6, presented as initial rates of glycine uptake versus different Na^+ and Cl^- concentrations across the membrane.

The sigmoid relationship between the initial rate of glycine uptake and the increase in Na^+ concentration (iso-osmolarities maintained with lithium chloride) observed in Fig. 5, suggest that more than 1 Na^+ is associated with the process. On the other hand, as shown in Fig. 6, an increasing Cl^- concentration in the extravesicular medium (iso-osmolarity maintained with sodium gluconate) produces a hyperbolic stimulation in the rate of glycine transport. The kinetic characteristics

of the interaction of Na^+ and Cl^- with the glycine carrier were analyzed by using two models designed to describe the kinetics of multiple substrate/activator reactions. Similar results were obtained for Cl^- with either the Garay and Garrahan model and/or the Hill model, the best fit being attained when the number of Cl^- molecules involved in the process is 1. In both cases (Garay-Garrahan and Hill models), a Michaelis-Menten type of dependence on chloride concentration is obtained. This suggests that the chloride sites are non-interacting and could mean that 1 Cl^- interacts with the carrier for each glycine molecule. However, the optimum fit for sodium is achieved when the experimental data are analyzed by the Hill equation, corresponding to an *n* (Hill index) value of 2.

The chloride-dependence of glycine transport suggested the possibility that drugs known to affect various mechanisms of Cl^- movement in glial cells might alter glycine uptake. Furosemide, an inhibitor of active chloride transport in glial cells [22], or SITS, an inhibitor of the anion exchange system for Cl^- and HCO_3^- in glial cells [23], had no effect on the glycine transport in plasma membrane vesicles from C6. The lack of effect of furosemide or SITS on glycine transport (Table I) renders unlikely any model in which glycine transport directly depends on the function of other transport systems, namely, K^+/Cl^- cotransport system or Cl^-/HCO_3^- exchange system.

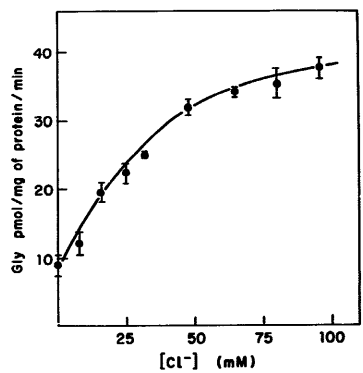

Fig. 6. Effect of increasing Cl^- concentrations on the transport of glycine. Membrane vesicles were preloaded with potassium gluconate medium and incubated for 30 s (in order to avoid dissipation of the Cl^- gradient) in the presence of 10 μM [^{14}C]glycine and ionic media containing 1 mM $MgSO_4$, 5 mM Hepes-Tris (pH 7.4), 5 mM valinomycin and increasing concentrations of NaCl. Osmolarity was maintained with sodium gluconate.

TABLE I

Effect of Cl^- transport inhibitors on the glycine transport

Vesicles were preloaded with sodium gluconate medium and incubated as described in Materials and Methods for either 1 min (initial rate) or 15 min (steady-state) in the presence of 10 μM [^{14}C]glycine in NaCl medium and the indicated additions. Each value ± S.E. is the mean of four experiments.

Addition	Glycine transport	
	initial rate (pmol·min^{-1}·mg^{-1})	steady-state (pmol·mg^{-1})
None	20±3	103±10
Furosemide (1 mM)	23±2	95±15
SITS (1 mM)	18±3	98±17

Discussion

The results presented herein and those previously reported by our laboratory [10] demonstrated that the glycine transport in plasma membrane vesicles derived from C6 glioma cells can be driven by either an Na^+ gradient or by a Cl^- gradient when the other essential ion is present. The effect of external Cl^- on the Cl^--gradient-dependent transport of amino acids such as GABA, glycine and β-alanine [15,16,24] has been often described in nerve terminals, and it is noteworthy that all of them are inhibitory neurotransmitters. On the other hand, a chloride-dependent transport of L-glutamic acid has been described in glioma cells in culture [25].

Several possibilities have been proposed in order to explain the mechanism by which Cl^- stimulates these transports. One mechanism that has been discussed is the independent crossing of the membrane by chloride ions in order to maintain electrical neutrality because positively charged substrate molecules are being transported. Our results present evidence which argues against a strictly electrical requirement for Cl^-. It has been demonstrated in our laboratory (data not shown) that the accumulation of tetraphenylphosphonium ion, a lipophilic cation which is taken up in a $\Delta\psi$-dependent fashion, is what could be expected according to anion permeability; $SCN^- > Cl^- > SO_4^{2-}$ > gluconate. So an anion such as SCN^- which is more permeant than Cl^- should stimulate transport better than Cl^-. Instead, it does not even fully replace Cl^-. Moreover, drugs that affect Cl^- movement by different mechanisms, do not affect glycine transport.

Another explanation for the Cl^- requirement is that it may facilitate the interaction of the amino acid with the carrier. The main effect of Cl^- on the kinetic constants of glycine transport is an increase in V_{max} of the transported molecule, whereas it has no significant

effect on the K_m for the substrate. According to enzyme kinetics, V_{max} is an indicator of total enzyme activity. Thus, in the case of uptake kinetics, V_{max} should be proportional to the number of transport sites. Thus, chloride may enhance the turnover rate of the solute-loaded carrier sites or enhance the number of carrier sites available at the outer surface of the membrane. Our data are insufficiently precise to determine accurately the coupling of the Cl^- in the traslocation of glycine, but they suggest, however, that chloride ions are cotransported with glycine; the fact that a chloride gradient (out > in) can serve as a driving force for glycine accumulation is consistent with this possibility. Other anions (Br^-, SCN^-, etc.,) can substitute Cl^- only partially in this effect.

The dependence of glycine uptake by plasma membrane of glioma cells on $[Cl^-]$ is hyperbolic as evidenced by our results, which are consistent with a Cl^- : glycine stoichiometry of 1 : 1, as was demonstrated in plasma membrane vesicles from nerve terminals [26]. On the other hand, the sigmoidal dependence of the glycine influx on $[Na^+]$ indicates the involvement of multiple sodium ions in the glycine transport event. The fact that the process is electrogenic [10] (positive charge moving inward) imposes restrictions on the possibilities for the stoichiometry of the process. Assuming that glycine is transported in its predominant form at neutral pH, the zwitterion, a relationship for the carrier such as $2Na^+ : Cl^- : Gly$, is suggested by our results, and is adequate to explain the electrogenity of the process and the kinetic characteristics of the interaction of Na^+ and Cl^- with the glycine transporter. It is interesting to speculate on the potential importance of coupling multiple Na^+ ions to the transport of any substrate. Coupling coefficients greater than 1 imply a thermodynamic advantage for uphill transport; the potential energy in the Na^+ electrochemical gradient is a power function based on the coupling coefficient [27].

Acknowledgements

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