Glycine transport into plasma-membrane vesicles derived from rat brain synaptosomes

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1. Transport of glycine has been demonstrated in membrane vesicles isolated from rat brain, using artificially imposed ion gradients as the sole energy source. 2. The uptake of glycine is strictly dependent on the presence of Na^+ and Cl^- in the medium, and the process can be driven either by an Na^+ gradient (out > in) or by a Cl^- gradient (out > in) when the other essential ion is present. 3. The uptake of glycine is stimulated by a membrane potential (interior negative), as demonstrated by the effects of the ionophores valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone and anions of different permeabilities. 4. The kinetic analysis shows that glycine is accumulated by two systems with different affinities. 5. The presence of ouabain, an inhibitor of $(\text{Na}^+ + \text{K}^+)$ -activated ATPase, does not affect glycine transport. 6. The existence of a high-affinity, Na^+ -dependent glycine-uptake system in membrane vesicles derived from rat brain suggests that this amino acid may have a transmitter role in some areas of the rat brain.

The existence in the nervous tissue of specific transport systems for neurotransmitter amino acids, neurotransmitter precursors and other amino acids has been established in tissue slices (Lajtha & Sershen, 1975; Sershen & Lajtha, 1979) and synaptosomes (Logan & Snyder, 1972; Bennett *et al.*, 1973; Kuhar & Zarbin, 1978).

During the past years, evidence has been accumulated about ion dependence for transport and it has become clear that the electrochemical potential created by an Na^+ gradient serves as a direct driving force for the process (Schultz & Curran, 1970; West, 1980). Preparations of membrane vesicles obtained from other different mammalian cells (Sacktor, 1977; Garcia *et al.*, 1980*a,b*; Giménez-Gallego *et al.*, 1980), and specifically from the synaptosomal fraction of rat brain (Kanner, 1978; Kanner & Sharon, 1978, 1980), have been demonstrated to be extremely useful for the study of amino acid-transport mechanisms, allowing the use of a well-defined ion environment and energy sources and avoiding metabolic and compartmentation interferences.

Glycine, besides its important role in numerous metabolic functions (Meister, 1975), has been reported to be an inhibitory neurotransmitter in the mammalian central nervous system, mainly in the

spinal cord (for review see Aprison & Daly, 1978), and probably in some localized areas of the brain, such as substantia nigra (James & Starr, 1979; Kerwin & Pycoc, 1979).

The presence of high-affinity and low-affinity transport systems have been demonstrated for several amino acids including glycine in different areas of the mammalian central nervous system (Johnston & Iversen, 1971; Logan & Snyder, 1972; Bennett *et al.*, 1973; Peterson & Raghupathy, 1973; Balcar *et al.*, 1977; Sershen & Lajtha, 1979). High-affinity Na^+ -dependent systems are thought to be implicated in the termination of transmitter action (Iversen, 1971), whereas low-affinity systems should play a 'metabolic' role, maintaining appropriate concentrations of the amino acids in the neural cells (Hedqvist & Stjarne, 1969; Johnston & Iversen, 1971).

In view of these observations, it is of interest to study glycine transport into membrane vesicles derived from rat brain synaptosomes. In the present paper, the general properties, ion requirements, kinetics and bioenergetics of glycine uptake have been investigated.

Experimental

Materials

[U- ^{14}C]Glycine was obtained from The Radio-

Abbreviation used: $\Delta\psi$, membrane potential across the plasma membrane.

chemical Centre, Amersham, Bucks., U.K. Ouabain, valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone were purchased from Sigma. Ficoll was provided by Pharmacia and was exhaustively dialysed against water before use. Nigericin was a gift from Lilly Laboratories, Indianapolis, IN, U.S.A. [^3H]Triphenylmethylphosphonium bromide was a gift from Dr. H. R. Kaback of Roche Institute, Nutley, NJ, U.S.A. All other reagents used were of the highest purity available.

Methods

Preparation of membrane vesicles. Adult male rats of the Wistar strain, weighing 150–200 g, were used. Membrane vesicles were isolated from rat brain essentially as previously described (Kanner, 1978). After the osmotic disruption of synaptosomes, the suspension was centrifuged at 27 000 *g* for 20 min, and the pellet was resuspended in 10 ml of a medium with osmolarity 284 mosm, pH 7.4, with the ionic composition depending on each particular experiment. Finally, the suspension was centrifuged at 27 000 *g* for 15 min, and the pellet was resuspended in the former medium up to a protein concentration of 15–25 mg/ml. Portions were frozen in liquid N_2 , and stored at -70°C . When required, portions were quickly thawed at 37°C . Under these conditions, membrane vesicles were functional for at least 1 month.

As noted by Kanner & Sharon (1980), the specific uptake of the membranes can vary with the preparation.

Transport assays. Portions (20 μl) of the suspension of membrane vesicles derived from rat brain (about 0.15 mg of protein), preloaded with 120 mM-KCl/22 mM-potassium phosphate buffer, pH 7.4 (KCl medium), unless otherwise stated, were pre-incubated for 1 min at 25°C . The uptake was started by adding 100 μl of a solution containing [^{14}C]glycine (18 μM final concentration, sp. radioactivity 118 Ci/mol) and the desired ionic composition. The experiment was terminated by diluting with 5 ml of ice-cold 0.8 M-NaCl, and immediately filtering through a moistened Millipore filter RAWP 02500 (1.2 μm pore size) attached to a vacuum assembly. The filters were rinsed twice 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 radioactive content was measured in a liquid-scintillation counter (Beckman LS-350). When the [^3H]triphenylmethylphosphonium ion uptake was measured, Millipore EHWP 02500 filters (1.0 μm pore size) were used to avoid binding of triphenylmethylphosphonium ion to the filter (Schuldiner & Kaback, 1975). All the experiments were corrected for a control obtained by diluting the

membrane suspension before adding the radioactive substrate solution. When ionophores were used, the membrane suspension was pre-incubated with them for 1 min before the experiments. All solutions used in the preparation of the membrane vesicles and in the uptake experiments were prepared with distilled de-ionized water and previously filtered through Millipore filters (0.45 μm) to avoid possible bacterial contamination. The osmolarity of all the 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 effects of different ions.

Protein determination. Membrane protein was determined by the method of Resch *et al.* (1971).

Results

The time course of [^{14}C]glycine uptake by membrane vesicles derived from rat brain is shown in Fig. 1. Vesicles were prepared in KCl medium and when an Na^+ gradient (out > in) was imposed artificially, by diluting at time zero the membrane vesicles 6-fold into a NaCl medium containing [^{14}C]glycine, the accumulation of glycine was five to ten times higher than in control conditions (non-gradients). Similar gradients of other univalent

Fig. 1. Time course of glycine uptake by membrane vesicles derived from rat brain

The vesicles were preloaded with KCl medium (120 mM-KCl/22 mM-potassium phosphate buffer, pH 7.4), and incubated as described in the Experimental section in the presence of 18 μM -[^{14}C]glycine in the NaCl medium (120 mM-NaCl/22 mM-sodium phosphate buffer, pH 7.4) (●) or the KCl medium (○).

cations (Tris^+ , K^+ , Li^+) were ineffective (results not shown).

Fig. 2 shows that chloride anions are also required for the uptake process, because no accumulation above the non-gradient level is observed when an Na^+ gradient (out > in) is imposed in the absence of chloride. Chloride cannot be replaced by other anions, such as sulphate or phosphate. However, a chloride gradient (out > in) in the absence of Na^+ is not able to promote the accumulation of $[\text{U-}^{14}\text{C}]$ glycine. As is shown in Fig. 3, a Cl^- gradient can produce uptake of $[\text{U-}^{14}\text{C}]$ glycine into the vesicles only when Na^+ is present. In the same way, an Na^+ gradient (out > in) can only drive the accumulation of glycine if Cl^- is in the medium. Maximal glycine uptake takes place when both ion gradients are present.

The requirement of internal cations seems to be less stringent; thus internal K^+ can be replaced by other cations, such as Tris^+ . A gradient of K^+ (in > out) cannot itself drive the transport of glycine.

Fig. 2. Ion dependence of glycine transport

The vesicles were preloaded with K_2SO_4 medium (60 mM- K_2SO_4 /60 mM-sucrose/22 mM-potassium phosphate buffer, pH 7.4) and incubated as described in the Experimental section in the presence of $18\text{ }\mu\text{M}$ - $[\text{U-}^{14}\text{C}]$ glycine in the following media: NaCl medium (Na^+ and Cl^- gradients), $\bullet$; KCl medium (Cl^- gradient in the absence of Na^+), Δ ; Na_2SO_4 medium (60 mM- Na_2SO_4 /60 mM-sucrose/22 mM-sodium phosphate buffer, pH 7.4) (Na^+ gradient in the absence of Cl^-), $\square$; K_2SO_4 medium (no gradients), $\circ$. The composition of the NaCl and the KCl media is described in the legend to Fig. 1.

To demonstrate that glycine is accumulated in an intravesicular space and is not bound to sites on the membrane surface, the equilibrium uptake was measured at different osmolarities of the incubation medium. When the osmolarity of the external medium was increased, the amount of glycine accumulated was proportionally less (Fig. 4). By plotting these uptake data against the reciprocal of osmolarity of the incubation medium, and extrapolating to infinite osmolarity (intravesicular volume zero), the value of the $[\text{U-}^{14}\text{C}]$ glycine uptake was zero, both in the presence and absence of an Na^+ gradient (Fig. 4, inset). Additional data, consistent with the suggestion that glycine is accumulated in an intravesicular space, are given by the fact that an efflux of labelled glycine can be observed in vesicles preloaded with $[\text{U-}^{14}\text{C}]$ glycine in a NaCl medium, when diluted with KCl medium (Fig. 5).

Kinetics of glycine uptake. To determine the kinetic characteristics of the system, the initial glycine uptake rates were examined as a function of its concentration (Fig. 6). In the presence of an Na^+

Fig. 3. Ion dependence of glycine transport

The vesicles were preloaded with K_2SO_4 medium ($\bullet$, Δ , $\square$), KCl medium ($\circ$) or Na_2SO_4 medium ($\blacktriangle$), and incubated as described in the Experimental section in the presence of $18\text{ }\mu\text{M}$ - $[\text{U-}^{14}\text{C}]$ glycine in the following media: NaCl medium (Na^+ and Cl^- gradients), $\bullet$; KCl medium (Cl^- gradient in the absence of Na^+), Δ ; NaCl medium (Na^+ gradient in the presence of Cl^-), $\circ$; NaCl medium (Cl^- gradient in the presence of Na^+), $\blacktriangle$; K_2SO_4 medium (no gradients), $\square$. The composition of the NaCl, KCl, Na_2SO_4 and K_2SO_4 media is described in the legends to Figs. 1 and 2.

Fig. 4. Effect of external osmolarity on the extent of glycine uptake

The membrane vesicles were preloaded with KCl medium and incubated for 15 min in the presence of labelled $18\mu\text{M}$ -glycine in the NaCl medium (●) or the KCl medium (○) and sucrose to reach the indicated osmolarity. The composition of the media is given in the legend to Fig. 1.

Fig. 5. Reversibility of glycine transport. The vesicles were preloaded with KCl medium and incubated for 15 min in the presence of $60\mu\text{M}$ - $[\text{U-}^{14}\text{C}]$ glycine in the NaCl medium (●) or the KCl medium (○). The vesicles were diluted 5-fold with KCl medium and incubated for different times as described in the Experimental section. The amount of $[\text{U-}^{14}\text{C}]$ glycine accumulated at the end of the pre-incubation period was taken to be 100% of retention. The composition of the media is given in the legend to Fig. 1.

Fig. 6. (a) Kinetics of glycine uptake and (b) Eadie-Hofstee plot of initial (30 s) specific Na^+ -dependent uptake of glycine by membrane vesicles derived from rat brain

In (a) the membrane vesicles, preloaded with KCl medium, were incubated for 30 s, as described in the Experimental section, in the NaCl medium (●) or in the KCl medium (○) and radioactive glycine [S] at the indicated concentrations. The specific Na^+ -dependent glycine uptake (□) was obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium at each concentration of glycine. The inset details the kinetics of glycine uptake in the concentration range 0–100 μM . Each point represents the mean $\pm$ S.E.M. of three experiments. The composition of the NaCl and the KCl media is described in the legend to Fig. 1. (b) Experimental conditions were as for (a).

gradient, the system approaches saturation kinetics, whereas in non-gradient conditions the glycine uptake rates increase linearly with substrate concentration. The saturability is clearer when the uptake rates, in the presence of an Na⁺ gradient, are corrected for the rates under non-gradient conditions (i.e. KCl medium). The analysis of the kinetic data for this Na⁺-specifically-dependent uptake system from Lineweaver-Burk plots and by the Eadie-Hofstee method (Fig. 6, inset) suggest the existence of two transport systems with different affinities. *K_m* values of 1500 μM and 50 μM, and *V_{max}* values of 460 pmol/30 s per mg of protein and 70 pmol/30 s per mg of protein have been obtained for the low- and high-affinity systems respectively.

Inhibitors. Table 1 shows the effect of several compounds on the initial rate of glycine uptake by membrane vesicles derived from rat brain. The uptake system seems to be specific for glycine, since other related amino acids do not inhibit glycine transport significantly. A decrease in the rate of [U-¹⁴C]glycine uptake is only observed when unlabelled glycine is added. Ouabain, an inhibitor of (Na⁺ + K⁺)-activated ATPase, has no effect on glycine uptake, demonstrating that this enzyme does not play any role in the transport process in our experimental conditions.

Table 1. Inhibition of glycine uptake by different compounds

The vesicles were preloaded with KCl medium and incubated for 20 s as described in the Experimental section in the presence of 18 μM-[U-¹⁴C]glycine and the indicated additions in the NaCl medium or the KCl medium. The specific Na⁺-dependent glycine uptake was obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium, taking the value of control without addition to be 100%. All added compounds were at a concentration of 0.5 mM except ouabain (1 mM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (10 μM). The values were calculated from the mean of at least three experiments. The composition of the media is described in Fig. 1.

Addition	Initial rate of specific Na ⁺ -dependent [U- ¹⁴ C]glycine uptake (% of control)
None	100
Glycine	45
L-Alanine	106
β-Alanine	100
γ-Aminobutyrate	94
Taurine	98
Proline	85
Aspartate	96
Ouabain	97
CCCP	68

Effect of specific ionophores. Nigericin, which is able to collapse Na⁺ gradients across the membranes electroneutrally through an Na⁺/K⁺ or an Na⁺/H⁺ antiport mechanism, completely inhibits the Na⁺-dependent glycine uptake (Fig. 7a). To investigate if glycine uptake is an electroneutral or electrogenic process, the effects of Δψ changes on glycine uptake were tested.

The ionophore valinomycin specifically increases the membrane permeability to K⁺, producing a transient increase in the membrane electrical potential (negative inside) when a K⁺ gradient (vesicle > medium) is present, as demonstrated in Fig. 8 by the higher accumulation of triphenylmethylphosphonium ion, a lipophilic cation that is taken up in a Δψ-dependent fashion (Ramos & Kaback, 1977). Glycine uptake is slightly stimulated by valinomycin, suggesting that glycine is accumulated electrogenically (Fig. 7b). The effect of the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, which collapses the membrane potential allowing the influx of protons, in decreasing the specific Na⁺-dependent uptake of glycine, also

Fig. 7. Effect of ionophores on the glycine transport (a) Effect of nigericin. The membrane vesicles were preloaded with KCl medium and incubated as described in the Experimental section in the presence of 18 μM-[U-¹⁴C]glycine in the NaCl medium (●, ○) or the KCl medium (△) with the following additions: ●, △, none; ○, nigericin (5 μg/mg of membrane protein). (b) Effect of valinomycin. The membrane vesicles were preloaded with KCl medium and incubated in the presence of 18 μM-[U-¹⁴C]glycine in the NaCl medium, with the following additions: ●, none; ▲, valinomycin (10 μg/mg of membrane protein). The composition of the media is described in the legend to Fig. 1.

Fig. 8. Effect of the ionophore valinomycin on $[^3\text{H}]$ -triphenylmethylphosphonium ion (TPMP $^+$) uptake. The membrane vesicles were preloaded with KCl medium and incubated as described in the Experimental section in the presence of $5\text{ }\mu\text{M}$ - $[^3\text{H}]$ -triphenylmethylphosphonium ion in the NaCl medium, with the following additions: ●, none; ▲, valinomycin ($5.55\text{ }\mu\text{g}/\text{mg}$ of membrane protein). The composition of the NaCl medium is described in the legend to Fig. 1.

Table 2. Effect of external anions on glycine uptake

The vesicles were preloaded with KCl medium and incubated for 5 min as described in the Experimental section in the presence of $18\text{ }\mu\text{M}$ - $[\text{U-}^{14}\text{C}]$ glycine in the indicated external media.

External medium	Uptake (% of control)
NaCl (control)	100
NaSCN	87
Na_2SO_4	57
KCl	19

suggests the existence of an electrogenic component for glycine transport.

Another way to modify $\Delta\psi$ is by using anions of different permeabilities. The accumulation of triphenylmethylphosphonium ion under these conditions is what would be expected on the basis of anion permeability: $\text{SCN}^- > \text{Cl}^- > \text{SO}_4^{2-}$ (results not shown). When Cl^- is replaced by SO_4^{2-} or SCN^- in the external medium a smaller glycine accumulation is found (Table 2). This result can be explained by the stringent requirement for Cl^- shown by the uptake system.

Discussion

The results reported herein clearly show that glycine is accumulated in membrane vesicles derived from rat brain synaptosomes, using artificially imposed ion-gradients as the sole energy source.

The uptake of glycine is strictly dependent on the presence in the medium of both Na^+ and Cl^- . The Na^+ dependence of glycine transport has been established in tissue slices and synaptosomes from spinal cord, substantia nigra and cerebral cortex (Lajtha & Sershen, 1975; Bennett *et al.*, 1973; James & Starr, 1979), whereas a Cl^- requirement for glycine transport in spinal-cord synaptosomes has been reported by Kuhar & Zarkin (1978).

In addition, the accumulation of glycine into membrane vesicles can be driven either by an Na^+ gradient or by a Cl^- gradient, when the other essential ion is present. The observations on Cl^- requirement and Cl^- -gradient-dependent uptake of glycine are similar to those on γ -aminobutyrate transport by the same preparation (Kanner, 1978). It is noteworthy that both substances are inhibitory neurotransmitters. With the present data, it is not possible to determine if the role of Cl^- in the process is to create a membrane potential (interior negative), to activate the carrier, or, if Cl^- is co-transported across the membrane together with Na^+ and the substrate.

Two lines of evidence suggest that uptake represents transport as opposed to binding. First, the extent of the accumulation of glycine decreases when the osmolarity of the medium is increased. Secondly, an efflux of glycine can be observed from membrane vesicles preloaded with $[\text{U-}^{14}\text{C}]$ glycine, demonstrating that the system is reversible.

The kinetic analysis of the transport of glycine into membrane vesicles shows a break in the Eadie-Hofstee plot, indicating that glycine is accumulated by both low-affinity and higher-affinity systems. The existence of both systems has been described in the spinal cord (Logan & Snyder, 1971; Balcar & Johnston, 1973). Although the presence in the brain of only a low-affinity system has been postulated, Peterson & Raghupathy (1973) have reported the existence of a high-affinity Na^+ -dependent system for glycine uptake in cerebral cortex. High-affinity uptake systems for glycine have been established more recently in rat substantia nigra (James & Starr, 1979; Kerwin & Pycoc, 1979). The high-affinity system could play an important role in the inactivation of glycine in the synaptic environment, whereas the low-affinity high-capacity system would serve general metabolic functions (Iversen, 1971; Hedqvist & Stjarne, 1969).

The importance of an Na^+ gradient as a direct driving force for the process of accumulation into membrane vesicles is clearly demonstrated by the

effect of the ionophore nigericin (able to collapse transmembranous Na^+ gradients) that completely inhibits glycine uptake. This is in accordance with the hypothesis that Na^+ gradients created by the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase system are the driving forces for active solute accumulation across mammalian plasma membranes (Crane, 1965; Holtz & Coyle, 1974). The fact that ouabain, an inhibitor of the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase and of glycine transport in synaptosomes from cerebral cortex and spinal cord (Aprison & McBride, 1973), does not affect glycine uptake into membrane vesicles clearly indicates that $(\text{Na}^+ + \text{K}^+)$ -activated ATPase as well as ATP are not directly involved in active transport, although they are essential for the generation of ion gradients.

On the other hand, the effects of valinomycin, carbonyl cyanide *m*-chlorophenylhydrazide and anions with different permeabilities indicate that a membrane potential (interior negative) contributes to energize transport. As has been previously suggested (Kanner & Sharon, 1980), it is noteworthy that intact membrane vesicles derived from rat brain synaptosomes are highly permeable to K^+ ions, as suggested by the accumulation of the lipophilic cation triphenylmethylphosphonium ion even in the absence of valinomycin.

Since the existence of a high-affinity, Na^+ -dependent uptake system is considered as a common feature for the neurotransmitter compounds, the results reported in the present work suggest that glycine, a well known inhibitory neurotransmitter in spinal cord, may have a neurotransmitter role in some areas of the rat brain.

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