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Efflux and exchange of glycine by synaptic plasma membrane vesicles derived from rat brain

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The influx and exchange of glycine were studied in synaptic plasma membrane vesicles isolated from rat brain. The vesicles were loaded with [U-^{14}C]glycine by active transport driven by an Na^+ as well as a Cl^- gradient (out > in). Dilution-induced efflux requires the simultaneous presence of internal Na^+ and Cl^-. As the efflux of glycine has been demonstrated to be strictly dependent on the presence of both ions (Mayor, F., Jr., Marvizón, J.G., Aragón, M.C., Giménez, C. and Valdivieso, F. (1981) Biochem. J. 198, 535–541), it can be concluded that the efflux of glycine is, in many aspects, symmetrical with its influx. Glycine efflux from the membrane vesicles is stimulated by external glycine, this exchange being partially dependent on external sodium and not on external chloride. The parallelism observed in influx and efflux processes shows that the release of glycine (by efflux and homoexchange) occurs via the carrier system. These results suggest that glycine is translocated in both directions across the membrane, probably by interacting with the carrier. Both sodium and chloride have to be present on the same side as glycine.

Introduction

Nerve terminals isolated from rat brain are able to accumulate neurotransmitter amino acids by high-affinity, sodium-dependent transport systems. These systems are directly involved with the termination of neurotransmitter action. These reuptake systems for neurotransmitters would ensure the maintainance of constant levels of neurotransmitters in the nerve terminal and a low concentration in the synaptic cleft [1,2]. Under depolarizing conditions these systems may also contribute to the release of the amino acid neurotransmitters into the synaptic cleft by a carrier-mediated efflux [3,4]. This fact is consistent with the site of origin of the released neuroactive amino acids, which has been shown to be largely cytoplasmic [4–6]. Both proposed functions confer to these transport systems a central position in the presynaptic events implicated with neurotransmission.

Sodium-dependent neurotransmitter transport has been studied mainly in brain slices and synaptosomes [7–11]. In the past few years, preparations of membrane vesicles from various mammalian cells [12,13] and specifically from the synaptosomal fraction of rat brain have been useful in the study of amino acid transport [14–21]; they allow the use of a well-defined ionic environment and energy sources, thus avoiding complications due to metabolism and compartmentation.

Glycine has been postulated to be an inhibitory neurotransmitter in the central nervous system of vertebrates, mainly in the spinal cord and in some

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

areas of the brain [22,25]. We have previously reported [17] that the transport of glycine in membrane vesicles derived from rat brain synaptosomes is an electrogenic process strictly dependent on the presence of Na^+ in the medium. In addition, the influx process of glycine, as well as a number of other inhibitory neurotransmitter amino acids (e.g., GABA and β-alanine), is absolutely dependent on the simultaneous presence of Cl^- [14,20,26].

The measurement of efflux, in addition to that of influx, can provide a better knowledge of the role of these ions in the transport process. Moreover, the ability of a solute to trans-stimulate its efflux through the membrane shows evidence that the same transport system can move the solute in both directions in a reversible way and that the direction of movement across the membrane may be related to the bioenergetics and mechanisms of uptake.

In this article we report the effects of membrane potential, Na^+ and Cl^-, and exchange with several amino acids across the membrane on the efflux of glycine from synaptic plasma membrane vesicles.

Materials and Methods

Materials

[U-^{14}C]Glycine (specific radioactivity 118 mCi/mmol) was obtained from Amersham, Bucks., U.K. Ficoll 400 was obtained from Pharmacia and was exhaustively dialysed against water before use. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was provided by Sigma and nigericin was a gift from Lilly Laboratories, Indianapolis, IN. All other reagents used were of the highest purity available.

Methods

Membrane vesicle preparation. Adult male rats of the Wistar strain, weighing 150–200 g, were used. The membrane vesicles were isolated from rat brain essentially as previously described [14,16]. After osmotic disruption of synaptosomes, the suspension was centrifuged at $27000 \times g$ for 20 min, and the pellet was resuspended in 10 ml of a 284 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 15–25 mg/ml. Portions were frozen in liquid nitrogen and stored at −70°C. When required, aliquots were quickly thawed out at 37°C. Under these conditions, membrane vesicles were functional for at least 1 month.

Efflux from actively loaded vesicles. Aliquots of 5 μl (about 45 μg protein, unless otherwise indicated) were preincubated for 1 min at 25°C. Uptake was started by adding 20 μl of a solution containing [U-^{14}C]glycine (10 μM final concentration) in 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) (influx solution). After the indicated times, the incubation mixtures were diluted 20-fold with the specified efflux solutions. The experiment was terminated by dilution with 5 ml of ice-cold 0.8 M NaCl, and immediate filtration 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 in less than 15 s. The filters were dried at 60°C, placed in microvials and their radioactivity was measured in a liquid scintillation counter (Beckman LS-350). The zero-time value was obtained by adding the cold stop solution prior to the efflux solution. In order to avoid possible bacterial contaminations, all solutions used in the experiments were prepared with distilled-deionized water and filtered through Millipore filters (0.45 μm). The osmolarity of all solutions was kept constant during the uptake experiments. All incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations. Closely agreeing results were obtained; thus only typical experiments are reported.

Protein determination. Membrane proteins were determined according to the method of Resch et al. [27].

Results

Fig. 1 shows the effect of external glycine and proton ionophore CCCP on glycine efflux from plasma membrane vesicles actively pre-loaded with [U-^{14}C]glycine. CCCP, which is able to collapse

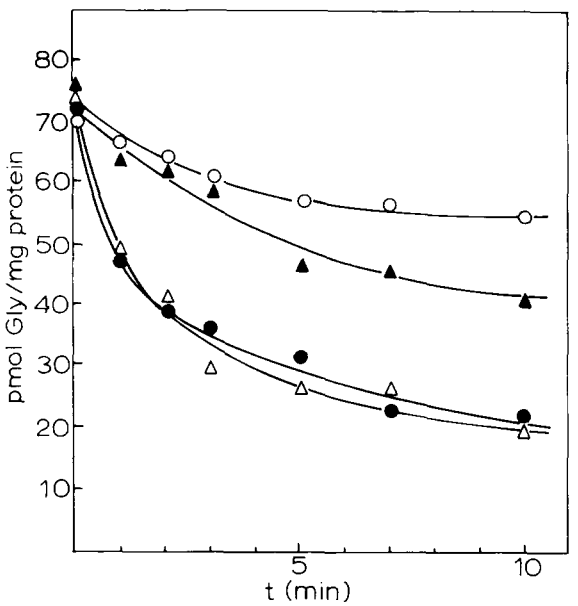

Fig. 1. Effect of external glycine and CCCP on the efflux of glycine. Membrane vesicles were preloaded with K_2SO_4 medium (60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$, pH 7.4). The vesicles were actively loaded (during 5 min) and the dilution induced efflux was measured. The efflux medium contained 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) and the following additions: none (○); 10 μM CCCP (▲); 400 μM glycine (△); 10 μM CCCP + 400 μM glycine (●).

$\Delta\psi$ by allowing the influx of protons, enhanced the efflux under these experimental conditions.

An interesting observation is the effect of unlabelled glycine on the efflux of [U-^{14}C]glycine from the vesicles (Fig. 1); this actually represents a comparison between net efflux and exchange. The addition of unlabelled glycine at concentrations far above its K_m value strongly stimulated the efflux of labelled glycine from the vesicles. The lack of effect of CCCP under exchange conditions could be explained by the fact that this process is not electrogenic.

Fig. 2A and B shows the effect of Na^+ and Cl^- on the efflux and exchange of glycine. Because the membrane vesicles present a relative permeability to Na^+ and Cl^- [26,28] it is possible to impose gradients of these ions (i.e. $[Na^+]$ in > $[Na^+]$ out, $[Cl^-]$ in > $[Cl^-]$ out) by diluting (20-fold) the actively preloaded vesicles with labelled glycine in the absence of either external Na^+ (A) or Cl^- (B). As shown in this experiment, the efflux rate of glycine is faster in the absence of Na^+ and Cl^- than when both ions are present in the external medium. Fig. 2 (A and B) also shows that the exchange is faster when Na^+ and Cl^- are present in the external medium. However, if either chloride or sodium are replaced by sulphate or lithium, respectively, the dilution-induced efflux proceeds very also actively. These observations suggest that glycine exchange shows a low stringent requirement for external Na^+ and Cl^-. The experiment in Fig. 3 shows the effect of the internal chloride on glycine efflux. The vesicles were loaded with a different ionic composition and the influx period was shorter than in previous experiments to limit the entrance of chloride, necessary at this stage. Glycine efflux from vesicles loaded with potassium chloride is more active than the efflux from vesicles loaded with potassium sulphate. The ionic composition of the efflux medium was the same in both cases. It included nigericin (sodium ionophore) and CCCP (proton ionophore) to avoid the limitation of sodium in the internal medium and to prevent the buildup of a membrane potential, respectively.

The dependence of glycine efflux on internal sodium is shown in the experiment described in Fig. 4. It uses nigericin, which is able to exchange Na^+/K^+ or Na^+/H^+ electroneutrally across the membranes. In vesicles preloaded with either potassium or lithium sulphate, the addition of nigericin to the Na^+-containing efflux medium produces an increase in the internal Na^+ concentration, which results in a dramatically enhanced glycine efflux. This indicates that the effect of nigericin is not the removal of the internal potassium but the increase in internal sodium concentration. The same experiment also shows that in the presence of nigericin, no efflux stimulation is observed when Na^+ is replaced by Li^+.

The data shown in Fig. 5 show the effect of internal Na^+ and Cl^- on the glycine efflux under homoexchange conditions. When vesicles preloaded with different ionic species are diluted into an NaCl medium, addition of nigericin causes a stimulation of efflux by increasing the internal Na^+ concentration. It is worth noting that when vesicles are preloaded with Na_2SO_4 (Fig. 5B) a small effect of nigericin on glycine efflux is ex-

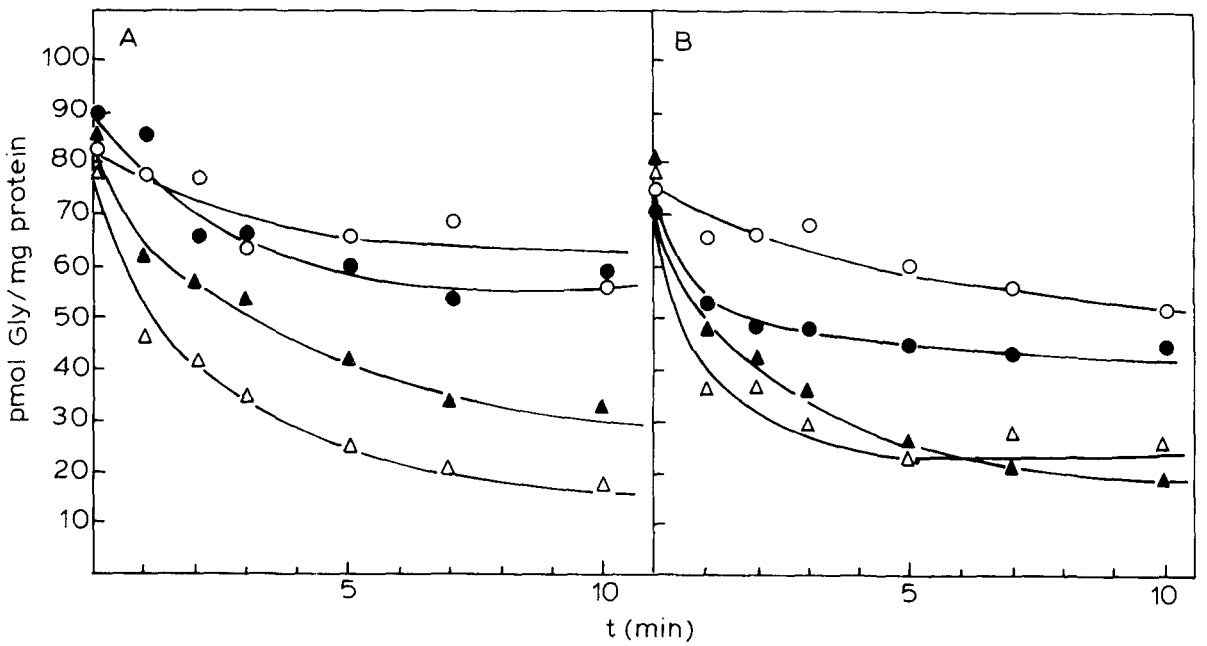

Fig. 2. Effect of Na^+ and Cl^- on the efflux and exchange of glycine. Influx (5 min) was performed as described in the legend of Fig. 1. Composition of the efflux media: (A) 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) (○); the same plus 400 μM glycine (△); 120 mM LiCl, 22 mM lithium phosphate, 1 mM $MgSO_4$ (pH 7.4) (●); the same plus 400 μM glycine (▲). (B) 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) (○); the same plus 400 μM glycine (△); 60 mM Na_2SO_4, 60 mM sucrose, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) (●); the same plus 400 μM glycine (▲).

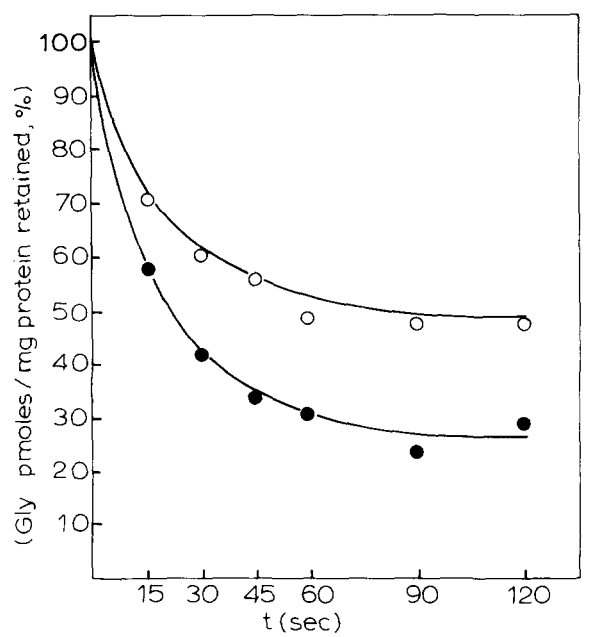

Fig. 3. Effect of internal Cl^- on glycine efflux. Membrane vesicles were preloaded with (○) 60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4) or (●) 120 mM KCl, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4). After 1 min of influx (chloride-loaded vesicles) or 0.5 min (sulphate-loaded vesicles) the efflux was initiated by adding 60 mM Na_2SO_4, 60 mM sucrose, 22 mM sodium phosphate, 1 mM $MgSO_4$ and 5 μM nigericin + 10 μM CCCP in both cases. ○, K_2SO_4-loaded vesicles, 108.8 pmol of glycine/mg protein after active loading; ●, KCl-loaded vesicles, 84.8 pmol glycine/mg protein after active loading.

erted, because enough internal Na^+ is already present. In contrast, with KCl-preloaded vesicles (Fig. 5C), a stronger stimulation of the glycine efflux in the presence of nigericin is observed. In these conditions, although both nigericin and external glycine alone stimulate efflux, the simultaneous presence of nigericin and external glycine does not further stimulate the glycine efflux. The latter effect is attained, however, in vesicles loaded with K_2SO_4 (Fig. 5A), when Cl^- are not present inside the vesicles. These results (in addition to those shown in Fig. 2) suggest that the efflux process is dependent on internal Na^+ and Cl^-, and do not rule out a partial dependency on internal and external Na^+ for the glycine exchange.

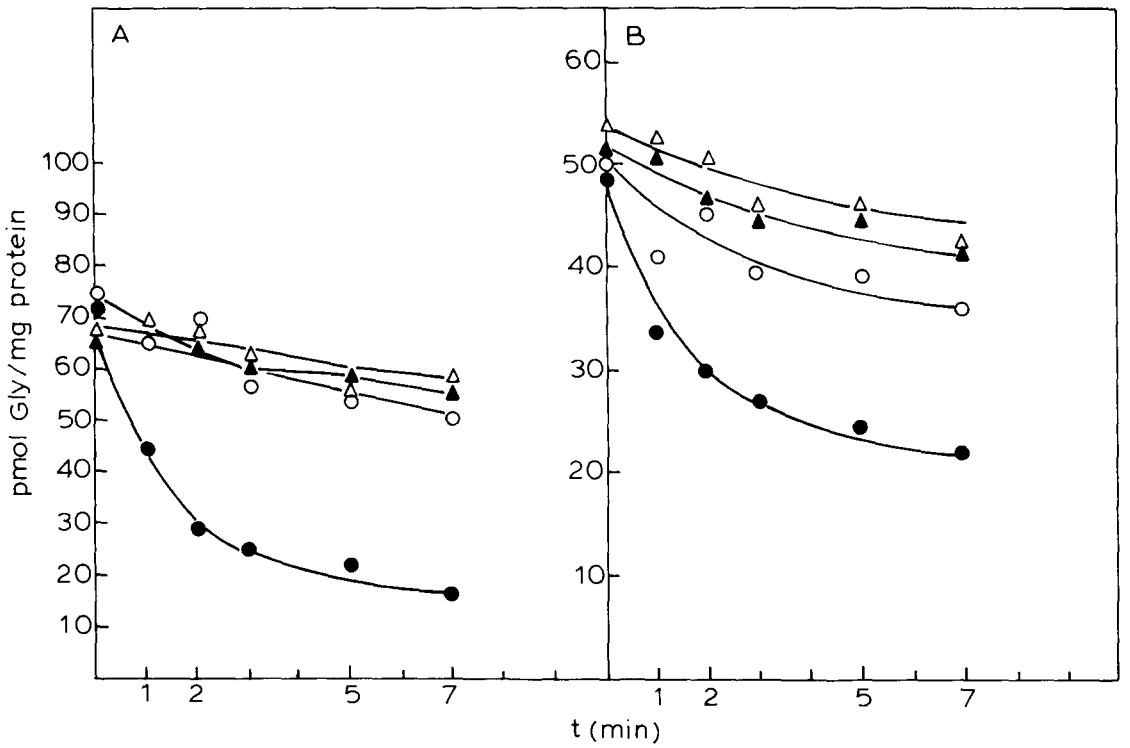

Fig. 4. Effect of nigericin on the efflux of glycine. Vesicles were loaded with: (A) 60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4); (B) 60 mM Li_2SO_4, 60 mM sucrose, 22 mM lithium phosphate, 1 mM $MgSO_4$ (pH 7.4). After 5 min of influx, the efflux was induced by dilution with the following media: 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) (○); the same plus 5 μM nigericin (●); 120 mM LiCl, 22 mM lithium phosphate, 1 mM $MgSO_4$ (pH 7.4) (△); the same plus 5 μM nigericin (▲).

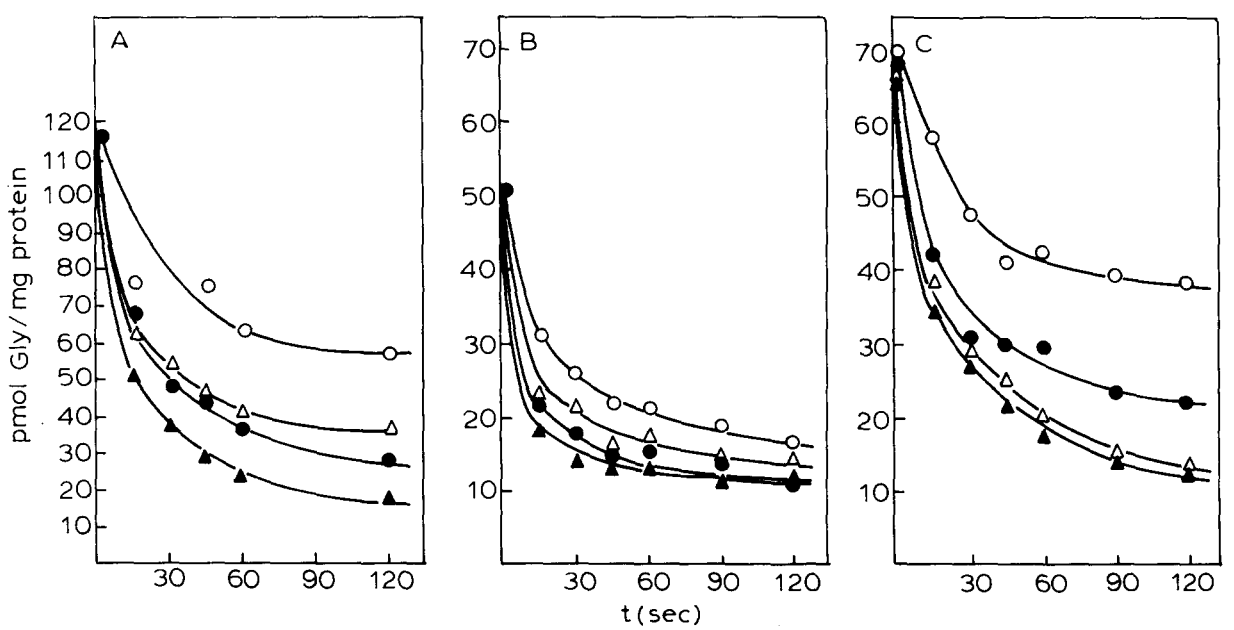

Fig. 5. Effect of nigericin and external glycine on efflux of glycine. Vesicles were loaded with: (A) 60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4); (B) 60 mM Na_2SO_4, 60 mM sucrose, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) or (C) 120 mM KCl, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4). After 2 min of influx, the efflux was initiated by adding 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) and either of the following additions: none (○); 400 μM glycine (●); 5 μM nigericin (△); 400 μM glycine plus 5 μM nigericin (▲). 90 μg protein were used per assay point.

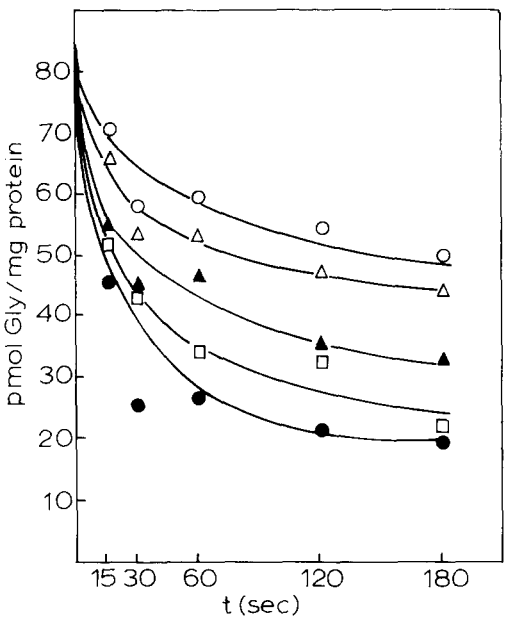

Fig. 6. Influence of external glycine concentration on glycine efflux. Influx (2 min) was performed by using 95 μg of protein. Membrane vesicles were preloaded with K_2SO_4 medium. The composition of the efflux medium was (○) 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) and the same plus (△) 10 μM, (▲) 50 μM, (□) 100 μM and (●) 500 μM glycine.

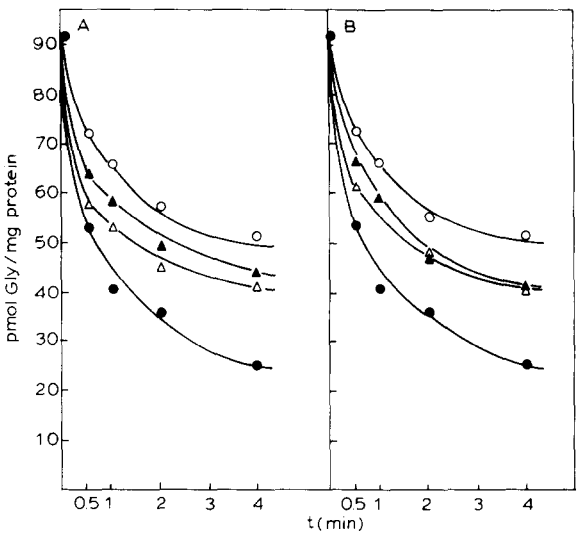

Fig. 7. Effect of several amino acids on the efflux of glycine. Membrane vesicles preloaded with 60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$ (pH 7.4) were actively loaded during 5 min. The efflux was initiated by adding 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$ (pH 7.4) with the following additions: (A) none (○); 500 μM glycine (●); 500 μM γ-aminobutyric acid (△); 500 μM L-proline (▲). (B) none (○); 500 μM glycine (●); 500 μM β-alanine (△); 500 μM taurine (▲).

Results shown in Fig. 6 indicate that the exchange proceeds via the transporter. Because the process is very rapid, an accurate estimation of the initial rate is very difficult to make. That fact imposes an important restriction on the determination of the K_m for external glycine in its ability to promote exchange. Nevertheless, some evidence can be found from results shown in Fig. 6. The stimulation of efflux by external glycine is concentration-dependent and its half-maximal effect is obtained with 50 μM concentrations, a value which is in agreement with the K_m for glycine influx [17].

To compare further the properties of the efflux and influx systems, the effect of several related amino acids on the efflux of glycine was also examined (Fig. 7). The efflux system seems to be specific for glycine, since other related ligands do not stimulate glycine efflux significantly. An activation in the [U-^{14}C]glycine release from vesicles is only observed when unlabelled glycine is added. This is consistent with the fact that γ-aminobutyric acid, proline, taurine and β-alanine show a lack of effect on glycine influx [17].

Discussion

The results reported herein clearly show that the Na^+-coupled transport system in membrane vesicles derived from rat brain synaptosomes mediates the exit, as well as the entry, of glycine.

The glycine efflux appears to require internal Na^+ and Cl^-, just as the glycine uptake was demonstrated to be strictly dependent on the simultaneous presence of both ions in the external medium [17].

As glycine transport in membrane vesicles has been demonstrated to be an electrogenic process [17], the slow efflux observed in Fig. 1 might be due to a lack of charge compensation, showing that efflux can be limited by the buildup of a membrane potential. The enhanced rate of efflux observed when CCCP is in the medium provides additional data in favour of the glycine efflux in membrane vesicles having an electrogenic compo-

nent. This also explains, at least in part, why exchange, which is electroneutral, is much faster than efflux, even in the presence of CCCP (Fig. 1). From a mechanistic point of view, efflux (in the absence of external glycine) and exchange (with glycine present in the external medium) differ by one step which occurs under net efflux conditions, namely, the return across the membrane of the unloaded carrier. The results (Fig. 1) are thus consistent with the idea that this step could be rate-limiting. Similar implications have also been made for the lactose carrier of *Escherichia coli* [29], the serotonin carrier of the platelet plasma membrane [30] and the γ-aminobutyric acid and glutamate carriers from the synaptic plasma membrane of rat rain [26,31].

Another possible reason for the slow efflux rate observed is that Na^+ and Cl^- inside vesicles limit the process. Results from Figs. 2, 3 and 4 show that efflux of glycine requires both internal sodium and chloride. An example of this is illustrated by the strong stimulation observed in the efflux rate of radioactive glycine in the presence of nigericin (which increases internal sodium) (Fig. 4).

As we reported before [17], the accumulation of glycine in plasma membrane vesicles is an electrogenic process, which can be driven by either an Na^+ or a Cl^- gradient (out > in) when the other essential ion is present. These results, together with the observation that in such membrane vesicles there is a considerable membrane potential (negative inside) even in the absence of chloride [28], suggest that chloride seems to be involved in the transport process by interacting with the carrier.

It is worth noting that similar results have been obtained by Kanner and Kifer for γ-aminobutyric acid [26], another inhibitory neurotransmitter. Moreover, Imler and Vidaver [32] have studied the glycine transporter from erythrocytes, which displays a strict requirement for Cl^-.

In addition to the spontaneous efflux there is another mode of exit of glycine from the vesicles. This is the exchange in the presence of external glycine, which appears to be independent of external Cl^- and only partially dependent on external sodium (Figs. 2 and 5). It is unclear whether the efflux stimulation by glycine in either LiCl (Fig. 2A) or Na_2SO_4 (Fig. 2B) media is due to carry-over of the NaCl-containing influx medium, or whether the interaction of external glycine with the carrier under exchange conditions is different from the interaction during net influx, as has been suggested by Kanner and co-workers for the γ-aminobutyric acid and glutamate carriers [26,31].

Additional evidence in favour of the fact that external glycine exerts its effects via the carrier system is given by the effect of the external glycine concentration on the stimulation of efflux (Fig. 6). The half-maximal effect coincides with the K_m value proposed for glycine influx. Moreover (Fig. 7), several amino acids, such as γ-aminobutyric acid, β-alanine, L-proline and taurine which are not substrates for the glycine transporter [17] do not induce much release of [U-^{14}C]glycine.

In conclusion, the parallelism observed in influx and efflux processes demonstrates that the release of glycine (by efflux and homoexchange) occurs via the carrier system, the glycine being translocated in both directions across the membrane with Na^+ as well as with Cl^-. For this reason, this transport system could have a dual role in physiological (depolarization) conditions, although this does not rule out the contributions of other mechanisms in the releasing process.

Acknowledgments

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