Biochimica et Biophysica Acta, 941 (1988) 209–216
Elsevier

BBA 74041

Depolarization-induced release of glycine and β-alanine from plasma membrane vesicles derived from rat brain synaptosomes

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(Received 13 January 1988)

Key words: Amino acid transport; Glycine; β-Alanine; Membrane potential; Depolarization; Membrane vesicle; (Rat brain)

Glycine and β-alanine actively loaded into brain synaptic plasma membrane vesicles were released into the external medium by using the classical depolarization agents high K^+ and veratridine. This release occurs via a Ca^{2+}-independent process. Measurements of membrane depolarization using tetraphenylphosphonium uptake show a close correlation between changes in the membrane potential and stimulation of the efflux process. Results shown herein and previously reported by our group (Aragón, M.C. and Giménez, C. (1986) Biochim. Biophys. Acta 855, 257–264; Agulló, L., Jiménez, B., Aragón, M.C. and Giménez, C. (1986) Eur. J. Biochem. 159, 611–617), suggest that the glycine and β-alanine transport systems in synaptic plasma membranes are susceptible of modulation by changes in ionic fluxes and hence in the membrane potential, similar to those occurring during depolarization and repolarization.

Introduction

High-affinity, sodium-dependent transport systems for neurotransmitter amino acids in nerve endings are directly involved in the termination of the transmitter action on postsynaptic receptors [1]. Thus, the inactivation step in the synaptic transmission by amino acids appears to be established. In contrast, their release mechanism is still not well known. Although an exocytosis-like process by means of the release of amino acid neurotransmitters has been suggested, arguments for and against this as the only mechanism, have been presented by many authors (for a review see Fagg and Lane [2]). An additional mechanism may involve a bidirectional carrier-mediated membrane transport process mediating either release or uptake, depending on the thermodynamic conditions across the membrane [3–11].

Since the amino acid neurotransmitters are localized in the cytoplasm rather than in synaptic vesicles [12–14] and the high affinity transport systems of these amino acids are dependent on the plasma membrane potential [8,15–21], these systems possibly operate in the efflux direction under depolarization conditions, releasing neurotransmitters from the nerve endings, whereas during repolarization and resting periods the neurotransmitters would be subject to reuptake.

Classical in vitro depolarization experiments mimicking the effect of an action potential on the release of amino acid neurotransmitters have given contradictory results regarding the calcium de-

Abbreviations: TPP^+, tetraphenylphosphonium ion; $\Delta\psi$, membrane potential across the plasma membrane; $\Delta\bar{\mu}_{Na^+}$, sodium electrochemical potential; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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pendency of te process [2]. Some show a partial dependency, which is interpreted as consistent with the existence of two pools of neurotransmitters [22], a Ca^{2+}-dependent, vesicular pool whose release would involve exocytosis, and a Ca^{2+}-independent cytoplasmic pool whose release could involve membrane transport processes. In support of this, the evidence for at least two neurotransmitter pools in certain systems [5,7,13] may be cited. A vesicular uptake of glutamate, driven by an electrochemical H^+-gradient, has been shown in rat brain synaptosomes [23] and more recently, the group of Nicholls [24–26] have demonstrated a rapid Ca^{2+}-dependent exocytotic release of this amino acid.

Both glycine [27,28] and β-alanine [29–32] have been postulated to be inhibitory neurotransmitters in the central nervous system of vertebrates, glycine being particularly involved in the spinal cord. Previous studies from our laboratory [17,20,33,34] have demonstrated that influx, efflux and homoexchange of these amino acids across synaptic plasma membranes are mediated by specific high-affinity transport systems, the influx and efflux processes being strictly dependent on the Na^+ and Cl^- ions on either side of the membrane and influenced by the membrane potential.

In the present study we have examined the characteristics of the glycine and β-alanine efflux in synaptic plasma membrane vesicles from rat brain, under different depolarization conditions, in order to investigate the possible involvement of the high-affinity transport systems in the release process in synaptic transmission.

Materials and Methods

Materials. β-[2,3-^{3}H]Alanine (34 Ci/mmol), [U-^{14}C]glycine (118 mCi/mmol), [^{3}H]tetraphenylphosphonium (TPP^+) (24 Ci/mmol) as the Br^- salt and $^{45}CaCl_2$ (25 mCi/mg Ca) were obtained from Amersham International, Amersham, Bucks., U.K. Ficoll 400 was provided by Pharmacia, Uppsala, Sweden and was exhaustively dialysed against water before use. Veratridine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and A23187 was provided by Calbiochem GmbH, Frankfurt, F.R.G. Veratridine and A23187 were dissolved in ethanol which was inactive. All other reagents were of the highest purity available.

Preparation of membrane vesicles. 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 [15,35]. After osmotic disruption of the synaptosomes, the suspension was centrifuged at $27\,000 \times g$ for 20 min and the pellet was resuspended in 10 ml of a 284 mosM medium (pH 7.4); the ionic composition varied according to the particular experiment. Finally the suspension was centrifuged at $27\,000 \times g$ for 15 min and the pellet resuspended in the former medium to a protein concentration of 15–25 mg/ml. Aliquots were frozen in liquid nitrogen and stored at −70 °C, and quickly thawed out at 37 °C when required. Membrane vesicles remained functional for at least 1 month at −70 °C.

Influx and efflux experiments. Samples of the membrane suspension (about 90 μg of protein) were preincubated for 1 min at 25 °C. The composition of the loading solution was 60 mM K_2SO_4, 60 mM sucrose, 22 mM potassium phosphate, 1 mM $MgSO_4$, pH 7.4 (K_2SO_4 medium), unless stated otherwise in the figure legends. The vesicles were actively loaded with glycine or β-alanine during 2 min at 25 °C in a external solution consisting of 120 mM NaCl, 22 mM sodium phosphate, 1 mM $MgSO_4$, pH 7.4 (NaCl medium) and 10 μM [U-^{14}C]glycine or 10 μM β-[^{3}H]alanine, unless stated otherwise in the figure legends. The incubation mixtures were diluted 10-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. When the TPP^+ uptake was measured, Millipore EHWP 02500 filters (1.0 μm pore size) were used to avoid the binding of the TPP^+ to the filter [36]. The washed filters were dried and the radioactivity measured by liquid scintillation spectrometry. The zero-time value was obtained by adding the cold stop solution before the efflux solution. In order to avoid possible bacterial contamination, all solu-

tions 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 experiments. All incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations.

Measurement of membrane potential. The membrane potential ($\Delta\psi$) was measured indirectly by noting the distribution of the lipophilic cation TPP^+ across the membrane using the method of Lichtshtein et al. [37]. A volume of 4.5 μl/mg of protein was taken as the intravesicular volume in order to calculate the internal TPP^+ concentration. The membrane potential was calculated by using

$$\Delta\psi = -(RT/F)\ln([TPP^+]_{in}/[TPP^+]_{out})$$

Protein determination. Membrane proteins were determined according to the method of Resch et al. [38].

Analysis of the data. Data are expressed as the mean ± S.E. of the percentage of the glycine and β-alanine retained. This was determined by dividing the retained radioactivity by the total radioactivity taken up before inducing the efflux. All comparisons for significance were made by using the Student's *t*-test.

Results

Fig. 1 shows the efflux of glycine and β-alanine from plasma membrane vesicles under depolarization induced by an increase in extravesicular K^+ ($[K^+]_{out}$) concentration. Previously the vesicles were actively preloaded with [U-^{14}C]glycine and β-[2,3-^{3}H]alanine in the presence of a sodium and chloride gradient. When compared to spontaneous release–which occurred when the vesicles were diluted with the uptake medium in the absence of potassium–the efflux rates were higher for both glycine and β-alanine at any $[K^+]_{out}$ assayed.

Additionally, the plasma membrane potential was monitored by the distribution in the steady-state of TPP^+ across the membrane. This ion is known to penetrate the membranes and to equilibrate according to the electrical potential across them, so that its accumulation within cells indicates an interior-negative, transmembrane potential [39]. Glycine and β-alanine effluxes induced

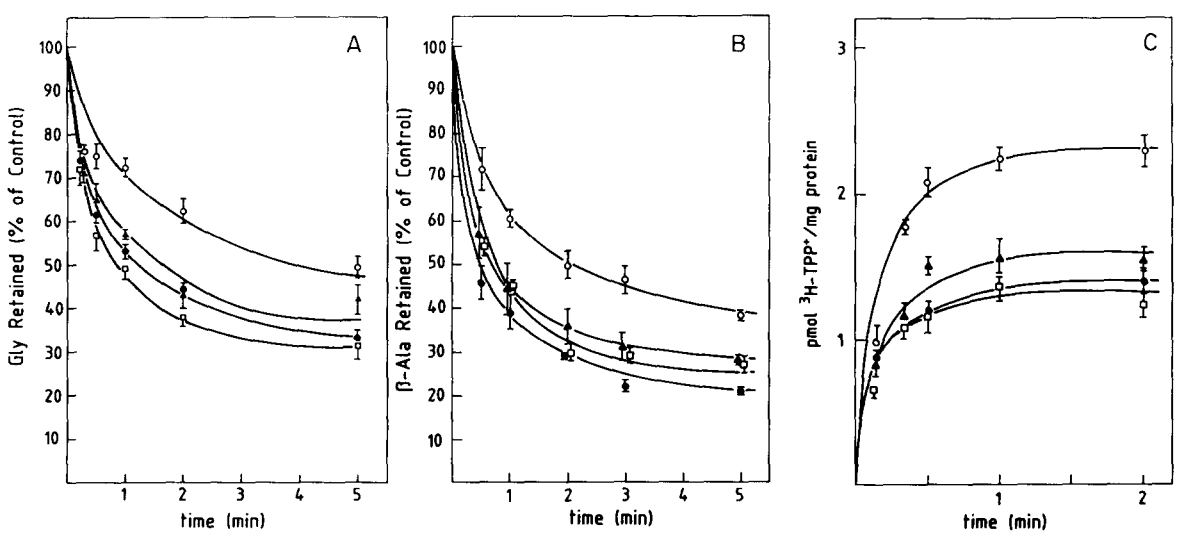

Fig. 1. Effect of $[K^+]_{out}$ on glycine and β-alanine efflux and on the membrane potential. Influx of glycine (A) and β-alanine (B) was performed as described in Materials and Methods. The efflux was initiated by adding media containing 22 mM sodium phosphate, 1 mM $MgSO_4$ and 120 mM choline chloride (○); 30 mM KCl, 90 mM choline chloride (▲); 60 mM KCl, 60 mM choline chloride (●) or 120 mM KCl (□). 100% corresponds to 126 pmol of glycine/mg of protein (A) or 81 pmol of β-alanine/mg of protein (B). Membrane vesicles preloaded with K_2SO_4 medium were diluted with the former media (○, ▲, ●, □) containing 0.2 μM [^{3}H]TPP^+ (C). Results are the mean ± S.E. of at least three experiments.

by high external potassium are correlated with the levels of TPP^+ uptake, i.e., the membrane potential (Fig. 1). It should be noted that the TPP^+ is used at low concentrations (0.2 and 1 μM) in order to monitor $\Delta\psi$ without significantly depolarizing the membrane. The $\Delta\psi$ in neuronal cells is mainly determined by the permeability of the membrane to K^+ and its concentration ratio across the membrane [40]. So, elevated $[K^+]_{out}$ acts to decrease $\Delta\psi$ by reducing the diffusion gradient for potassium ion. In our experimental conditions $[K^+]_{out}$ at 30, 60 or 120 mM decreased the concentration of TPP^+ inside the plasma membrane vesicles. This decrease, indicative of membrane depolarization, corresponds to calculated $\Delta\psi$ values of -15.0, -11.0 and -9.1 mV, respectively. These values are to be compared with a membrane potential of -24.4 mV when K^+ was absent from the external medium. The intravesicular volume considered for these $\Delta\psi$ values is 4.5 μl per mg of protein and corresponds to one minute of TPP^+ influx, steady-state distribution (Fig. 1). These observations indicated that the addition of K^+ to the external medium depolarized the synaptosomal membrane, and that the amount of glycine and β-alanine released was dependent on the $\Delta\psi$.

The dependence of glycine and β-alanine efflux on changes in $\Delta\psi$ was also investigated by using veratridine as the depolarizing agent. This alkaloid opens Na^+ channels, collapsing both the electrical and concentrative terms of the sodium electrochemical potential ($\Delta\bar{\mu}_{Na^+}$) across the synaptosomal plasma membrane [41]. Fig. 2 shows glycine and β-alanine efflux in the presence of veratridine, and the accumulation of TPP^+ as membrane potential indicator in the presence of this alkaloid. The introduction of 100 μM veratridine into the efflux medium enhanced the efflux of both amino acids. As derived from the TPP^+ accumulation data (Fig. 3), the magnitude of depolarization is represented for a decrease in the $\Delta\psi$ value, going from -18.9 mV (no veratridine) to -2.9 mV (plus veratridine). It is of interest to note that when veratridine was used as the depolarizing agent, the results showed a higher degree of variance than in other experiments. This is not unexpected since many Na^+ channels appear to be inactive in synaptosomes [42] so that varying proportions of the vesicles may be devoid of func-

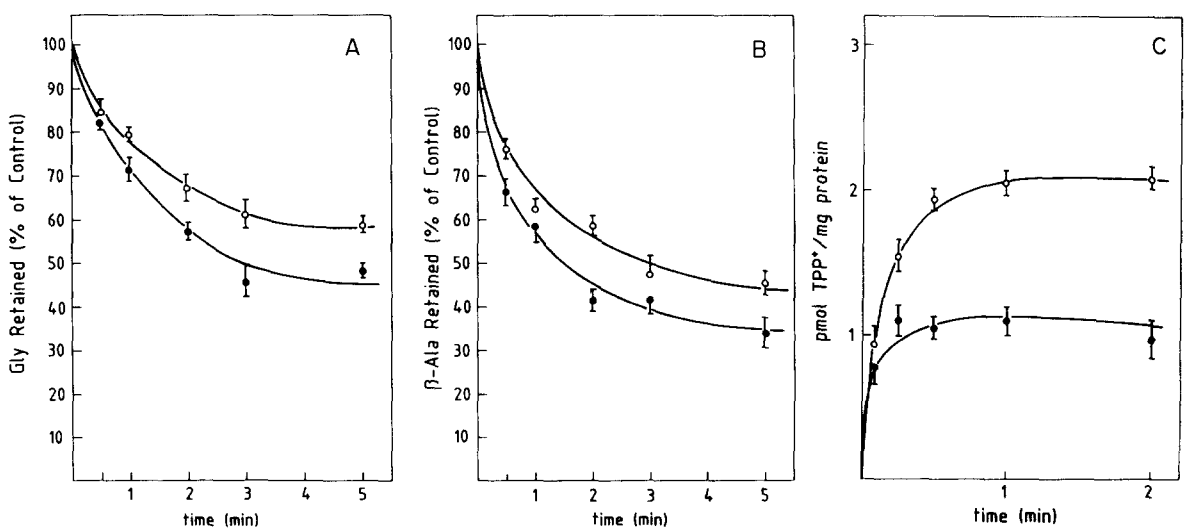

Fig. 2. Effect of veratridine on glycine and β-alanine efflux and on membrane potential. Influx of glycine (A) and β-alanine (B) was performed as described in Materials and Methods. The efflux was induced by dilution with NaCl medium and: none (○) or 100 μM veratridine (●). 100% correspond to 94 pmol of glycine/mg of protein (A) or 85 pmol of β-alanine/mg of protein (B). Membrane vesicles preloaded with K_2SO_4 medium were diluted with the former media (○, ●) containing 0.2 μM [^{3}H]TPP^+ (C). Results are representative of at least three experiments.

tional channels [43]. Depolarization induced by either high-$[K^+]_{out}$ or veratridine had no effect on the efflux of nontransmitter amino acids such as leucine and phenylalanine (data not shown).

In order to test the calcium-dependence of depolarization-induced release, glycine and β-alanine efflux were followed in the presence of Ca^{2+}, under depolarizing conditions. At the same time, the calcium ionophore A23187 was used to increase Ca^{2+} entry in the vesicles. With the aid of the ionophore (which mediates the electroneutral exchange of divalent cations for protons), influx of Ca^{2+} can be produced without depolarization [44]. However, it has been pointed out that high concentrations of A23187 induce a steady depolarization of the plasma membrane of synaptosomes [45]. In view of this, as in the above experiments, the plasma membrane potential was measured, in this case, to establish whether the amino acids efflux was related to a decreased $\Delta\psi$ or to an increased intravesicular Ca^{2+} concentration caused by the ionophore. Fig. 4 shows the results of the uptake of $^{45}Ca^{2+}$ in the presence and absence of A23187. The addition of the ionophore causes a rapid increase in the uptake of $^{45}Ca^{2+}$ into the vesicles. However, a noticeable level of $^{45}Ca^{2+}$ can already be measured in the absence of A23187; this probably represents superficial binding to the membrane. To determine whether the K^+-induced release of the amino acids was Ca^{2+}-

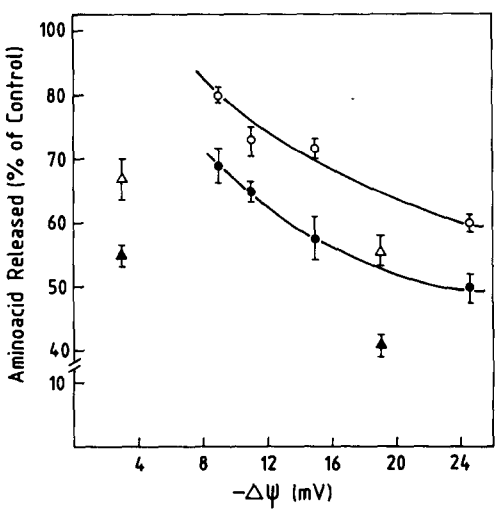

Fig. 3. Relationship between membrane potential ($\Delta\psi$) and glycine and β-alanine release. Experimental data are shown in Figs. 1 and 2. Only efflux results of glycine and β-alanine at 5 min, and influx of TPP^+ at 2 min (equilibrium conditions) are shown. $\Delta\psi$ is calculated from the TPP^+ accumulation data according to the equation in Materials and Methods. Glycine and β-alanine released in the presence of $[K^+]_{out}$ (●, ○) or in the presence of veratridine (▲, △), respectively. $-\Delta\psi$ means negative inside the vesicles.

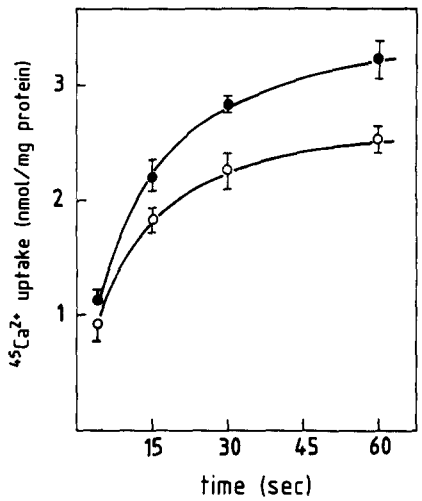

Fig. 4. Effect of ionophore A23187 on Ca^{2+} accumulation. Membrane vesicles preloaded with 150 mM potassium gluconate, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (pH 7.4) were incubated in a medium containing 150 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (pH 7.4) in the presence of 1 mM $^{45}CaCl_2$ (1.5 μCi/ml) and: none (○) or 20 μM A23187 (●). Results are the mean ± S.E. of at least three experiments.

TABLE I

EFFECT OF Ca^{2+} AND A23187 ON MEMBRANE POTENTIAL

Membrane vesicles preloaded as indicated in the legend to Fig. 5, were diluted into a medium containing 1 μM [^{3}H]TPP^+ and the following ionic composition: 120 mM mannitol, 90 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (potassium free medium), or 60 mM potassium gluconate, 90 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$, with the additions as indicated in the Table. Results are the mean ± S.E. of at least three experiments.

$[K^+]$ (mM)	$[Ca^{2+}]$ (mM)	[A23187] (μM)	Accumulation of TPP^+ (pmol/mg of protein)
0	0	0	8.0 ± 0.45
60	0	0	5.5 ± 0.25
60	0.1	20	5.0 ± 0.25
60	1.0	20	5.4 ± 0.65
60	1.5	20	5.5 ± 0.45

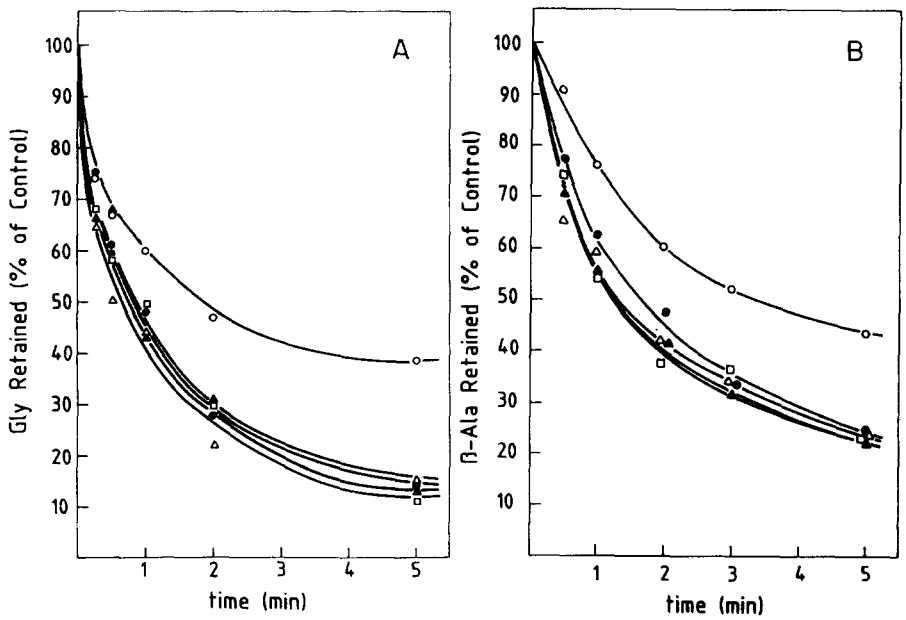

Fig. 5. Effect of Ca^{2+} and ionophore A23187 on glycine and β-alanine efflux induced by external potassium. Membrane vesicles, preloaded with150 mM potassium gluconate, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (pH 7.4) were actively loaded (during 2 min) with 150 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (pH 7.4) in the presence of 10 μM [U-^{14}C]glycine (A) or 10 μM β-[^{3}H]alanine. Composition of the efflux solution: 120 mM manitol, 90 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$ (○), or 60 mM potassium gluconate, 90 mM NaCl, 5 mM Hepes-Tris, 1 mM $MgSO_4$ with the following additions: none (●); 0.1 mM Ca^{2+} +20 μM A23187 (□); 1 mM Ca^{2+} +20 μM, A23187 (△); 1.5 mM Ca^{2+} +20 μM A23187 (▲) 100% corresponds to 64 pmol/mg protein (A) or 72 pmol/mg protein (B). Results are representative of at least three experiments.

dependent, release was measured in the presence of 0.1 to 1.5 mM Ca^{2+}. As seen in Fig. 5 release of neither glycine nor β-alanine was influenced by these concentrations of Ca^{2+}, or by 10 μM Ca^{2+} (data not shown). Table I shows the TPP^+ accumulation under these conditions. No differences in the membrane potential were observed when Ca^{2+} and A23187 were present, which correlates well with the unmodified efflux of glycine and β-alanine. Similar results were obtained in TPP^+ distribution and glycine and β-alanine efflux rates when synaptosomal membrane vesicles were depolarized by veratridine in the presence of calcium (data not shown).

Discussion

The results reported here clearly indicate that the release of glycine and β-alanine from plasma membrane vesicles derived from synaptosomes is a process depending on the depolarization of the membrane. Both high $[K^+]$ or the addition of veratridine, affect release to the extent to which they decrease the membrane potential even though they achieve this by different mechanisms. Since the high-affinity transport systems of glycine and β-alanine have been shown to be symmetrical in synaptosomal plasma membranes [33,34], and the assymetries present in active transport are only a consequence of differences between the external and internal media, it can be assumed that changes in ionic media through the membrane, and in consequence in $\Delta\psi$, will influence the carrier-mediated amino acids efflux in the same way as they influence its influx. Moreover, the calcium independence of the efflux is consistent with the release due to a thermodynamic reversal of the uptake pathway [5,9,46]. The relative Ca^{2+} independence of glycine and β-alanine release does not exclude the existence of a Ca^{2+}-dependent mechanism for these amino acids, but simply indicates that such a mechanism is not unique. The precise relationship of calcium to the transmitter function of amino acids is at present unknown

The preparations and techniques used in many studies described in the literature are so varied that a synthesis of the data into a unifying theory is still to be accomplished. Some investigators consider Ca^{2+}-dependent release essential to function as a synaptic transmitter [47,48]. However, other investigators have found amino acids release to be unchanged or increased in Ca^{2+}-free media [5,8,10,49–51]. Often the influence of calcium depends on the experimental conditions, such as preincubation times [52], or calcium concentrations [53,54]. Thus, both Ca^{2+}-dependent and -independent release have been found to occur.

One interpretation for these discrepancies is that the Ca^{2+}-independent efflux represents a process mediated by membrane transport systems from a cytoplasmic pool and that the Ca^{2+}-dependent component requires an additional intracellular apparatus (i.e. vesicles, etc.). Taking into account that the amino acids present in nerve endings are mainly found in the intracellular free amino acid pool [55,56], most of the transmitter molecules remain nearby, and thus rapidly available to the transport system.

In conclusion, the results included herein together with those previously reported from our group [33,34], suggest that the glycine and β-alanine transport systems could have a dual role in physiological conditions, helping to maintain a high amino acid concentration in the synaptic cleft during depolarization, and then mediating a rapid presynaptic reuptake when the terminals are repolarized. Such flexible transport systems are driven and controlled by the same cationic fluxes accompanying depolarization and repolarization of nerve endings, respectively.

Acknowledgement

This work was supported by a grant from the Fundación Famón Areces.

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