Biochimica et Biophysica Acta, 769 (1984) 499–504
Elsevier

BBA 71979

DEPOLARIZATION-INDUCED RELEASE OF L-GLUTAMIC ACID FROM ISOLATED-RESEALED SYNAPTIC MEMBRANE VESICLES

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(Received September 12th, 1983)

Key words: L-Glutamate efflux; Membrane potential; Depolarization; Synaptic vesicle; (Rat brain)

L-Glutamic acid actively loaded into resealed brain synaptic membrane vesicles was rapidly released into the incubation medium following the introduction of KCl and $CaCl_2$, or nigericin, or veratridine into the external medium. The KCl-induced release was enhanced by the presence of low (0.1 mM), extravesicular [Ca^{2+}]. Neither the KCl-induced nor the veratridine-stimulated L-glutamate efflux were carrier-mediated processes. Finally, the KCl-stimulated L-glutamate efflux was dependent on the ratio of intra- to extravesicular [K^+]. The observations described in this study were indicative of depolarization-induced L-glutamate release from isolated synaptic plasma membrane vesicles.

L-Glutamic acid release from mammalian central nervous system tissue has been demonstrated repeatedly and represents an important criterion for the neurotransmitter function of this amino acid. Either electrical stimulation or increases in extracellular K^+ concentration cause L-glutamate release from neuronal preparations (see, for example Refs. 1 –5). It is generally believed that the characteristics of L-glutamic acid release fit the pattern of exocytotic vesicular release and include initiation of release by depolarization, dependence on extracellular Ca^{2+}, and antagonism by elevated Mg^{2+} levels.

However, not all L-glutamate release is due to a Ca^{2+}-dependent exocytotic event [6–8]. Elevations in extracellular [K^+] that produce a decrease in the transmembrane potential ($\Delta\psi$) of brain synaptosomes (nerve ending particles) can induce also a Ca^{2+}-independent glutamate release [6–8]. Both Ca^{2+}-dependent and Ca^{2+}-independent glutamate release processes are diminished following destruction by X-irradiation of a group of cerebellar neurons [7]. Therefore, both glutamate release processes are likely to be present in the same central nervous system neurons. Furthermore, the origin of L-glutamate released from synaptosomes following elevation of extracellular [K^+] can be traced to the cytoplasmic pool of glutamate [9].

In the present study we examined the characteristics of an apparently nonvesicular L-glutamate release process from isolated-resealed synaptic plasma membrane sacs. L-Glutamate loaded into these membrane sacs either by active transport or by passive diffusion was released into the incubation medium following exposure of the membranes to elevated K^+ concentrations, veratridine or nigericin.

Methods

The method for the isolation of synaptic plasma membranes from rat brain synaptosomes and their storage at −80°C was identical to that described previously [10,11]. The protein content of all samples was measured according to Lowry et al. [12]. L-Glutamic acid decarboxylase activity was de-

termined by the method of Chude and Wu [13].

In the uptake and efflux studies, frozen synaptic membrane suspensions were thawed at 37°C (5 min) in 135 mM potassium phosphate, 0.11 M sucrose, 10 mM Tris sulfate, pH 7.4 (loading solution), according to the procedures described previously [11,14]. The final protein concentration of the suspension was 2 mg/ml. The membrane vesicles were transferred to ice and left for 1 h to reach complete ionic equilibrium prior to the addition of 100 μM unlabeled L-glutamate and 5 μCi of L-[^{3}H]glutamate (43 Ci/mmol, New England Nuclear). Direct filtration of 20-μl aliquots of the suspension was performed at the beginning and end of each assay and the L-glutamate associated with the membranes was considered as the nonspecific diffusion or binding of this amino acid to the membranes and was subtracted from all experimental data. The passive diffusion of L-glutamate across the synaptic membranes at 0°C during the period of an experiment was minimal. The L-glutamate uptake assays were initiated by transferring 20 μl of the membrane suspension to 0.5 ml of the incubation medium which contained 100 mM NaCl, 60 mM choline chloride, 10 mM Tris sulfate, pH 7.4 (uptake medium). Uptake was allowed to take place at 23°C for the indicated times. These suspensions were then diluted further with 1 ml of either the uptake medium or with 100 mM NaCl, 50 mM KCl, 10 mM choline chloride, and 10 mM Tris sulfate, pH 7.4 (efflux medium). In some experiments, various amounts of $CaCl_2$ were present in these solutions as indicated in the specific figures. All uptake and efflux incubations were stopped by filtering each sample directly through 0.45 μm Millipore filters (HAWP). The filters were washed with 3 ml of ice-cold uptake medium. The radioactivity associated with the filters was measured by scintillation spectrometry [11].

Results and Discussion

The activity of a soluble cytoplasmic enzyme marker of synaptosomes, L-glutamate decarboxylase, was not detectable in the membrane preparations used in these studies. This observation is consistent with our previous electron microscopic studies which showed that the membrane fraction used consists of resealed synaptic membrane sacs [15].

In order to study the release of intravesicular L-glutamate to the external medium, L-glutamate had to be loaded into the synaptic membrane vesicles either through a passive equilibration or an active uptake process. In experiments where passive loading was used, it was found that the equilibration of L-[^{3}H]glutamic acid was very slow and did not reach equilibrium even after 24 h incubation at 4°C. The internally loaded L-glutamate was partially released into the external medium by elevation of the extravesicular [KCl]. However, the magnitude of this release was small (approx. 25% of total L-glutamate trapped). This low level of response may have been due to the small amount of L-glutamate loaded by this technique or due to some membrane degradation that occurred during the prolonged incubation at 4°C.

Synaptic membranes have an active uptake system for L-glutamate which can be used to load them internally with this amino acid [10]. An increase in the external KCl and $CaCl_2$ concentrations caused the efflux of L-glutamate from membrane vesicles that had previously been allowed to actively take up this solute through the Na^+ gradient-dependent uptake system (Fig. 1). The amount of L-glutamate taken up internally remained constant from 2 to 5 min of incubation in the uptake

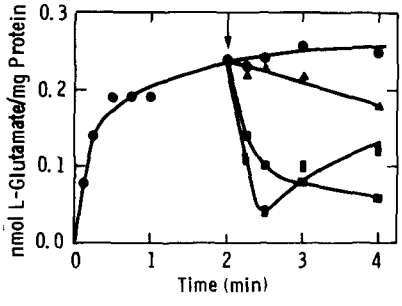

Fig. 1. L-Glutamic acid efflux induced by exposure to a $KCl/CaCl_2$ incubation medium and the effect of *p*-chloromercuribenzenesulfonic acid on this efflux. Synaptic membrane vesicles (40 μg) were preloaded with the loading solution and were diluted into 500 μl of uptake medium. After 2 min of influx, the vesicles were diluted with 1 ml of the following media: (●) uptake medium, (▲) uptake medium with 1 mM organomercurial, (▮) efflux medium, and (■) efflux medium with 1 mM organomercurial.

medium in most experiments. Occasionally, a slow efflux component was observed with continuing incubation in the uptake medium (e.g. Fig. 3 A, B). The L-glutamate efflux produced by elevation of external $KCl/CaCl_2$ concentrations was frequently followed at later time periods by a reuptake process. The presence of an inward-directed $[Na^+]$ gradient even under the conditions of L-glutamate efflux may account for the observation that dilution into the $KCl/CaCl_2$ medium did not produce a complete depletion of preloaded L-glutamate. The effect of the $KCl/CaCl_2$ medium could not be ascribed to efflux of intravesicular L-glutamate caused by a dilution effect since dilution of the membranes with the uptake medium did not produce a rapid efflux of L-glutamate.

The possibility that the glutamate efflux produced by elevation in the $KCl/CaCl_2$ concentrations was due to an inhibitory action of these ions on the L-glutamate carrier was evaluated by introducing the transport carrier inhibitor *p*-chloromercuribenzenesulfonic acid into the efflux medium (Fig. 1). The addition of this agent brought about a slow, continuous efflux in the samples incubated in the uptake medium which probably represented passive diffusion of glutamate from the intravesicular space following inhibition of the uptake carrier activity. The organomercurial also inhibited the re-uptake process seen at later time periods in the samples exposed to the $KCl/CaCl_2$. The initial rate of $KCl/CaCl_2$-induced efflux was somewhat slower in the presence of the uptake inhibitor, but the total amount of glutamate released remained fairly constant (Fig. 1). This suggests that some carrier-mediated activity contributed to the initial efflux measured in the absence of the organomercurial. Thus, elevation of the $KCl/CaCl_2$ concentration in the external medium caused the efflux of intrasynaptic glutamate through a process that was mostly unrelated to the uptake carrier activity in synaptic membranes.

The possible contribution to synaptic membrane L-glutamate efflux by the activity of the mitochondrial proton-compensated glutamate translocator was evaluated by measuring the effects of 0.5 mM bromcresol purple and *N*-ethylmaleimide on the $KCl/CaCl_2$-induced efflux. These agents are strong inhibitors of the mitochondrial translocator [16]. Neither agent had

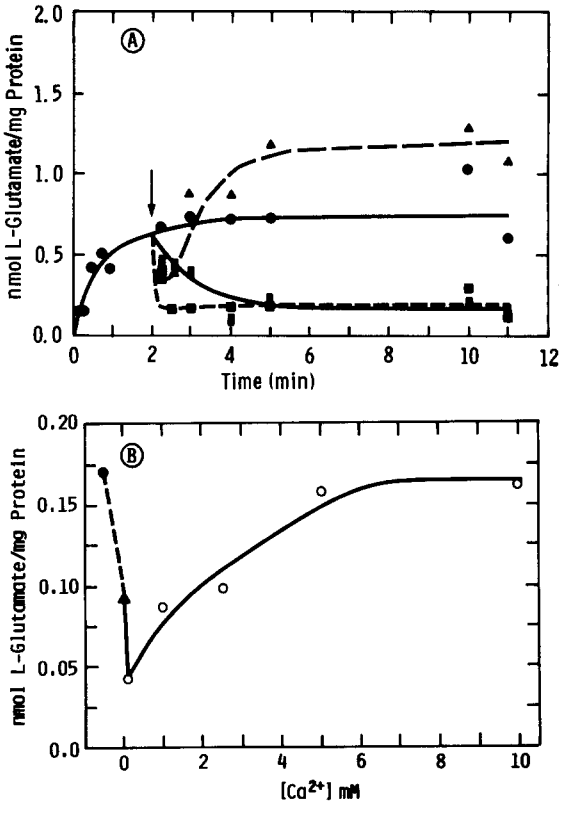

Fig. 2. Effect of $CaCl_2$ on KCl-stimulated L-glutamate efflux. Active loading of L-glutamate into the synaptic membrane vesicles was performed as in Fig. 1. (A) Kinetics of KCl-induced efflux in the presence and absence of $CaCl_2$. After 2 min of glutamate influx, the vesicles were diluted with 1 ml of the following media: (●) uptake medium, (■) efflux medium, (▲) uptake medium that contained 1 mM $CaCl_2$, and (■) efflux medium with 1 mM $CaCl_2$. (B) Effect of various Ca^{2+} concentrations on KCl-induced glutamate efflux. The membranes were actively loaded with L-glutamate for 2 min and diluted with 1 ml of uptake medium containing 0.1 mM EGTA (●), or efflux medium that contained 0.1 mM EGTA (▲), or efflux medium that contained the Ca^{2+} concentrations shown (○). All data points represent triplicate determinations of L-glutamate retained 2 min after dilution in these media. The amount of L-glutamate loaded into the vesicles at the end of the 2-min uptake period was 0.2 nmol/mg protein.

any effect on L-glutamate efflux from synaptic membranes.

Omission of Ca^{2+} from the efflux medium slowed the initial rate of KCl-evoked glutamate efflux, but had no effect on the total amount of L-glutamate released (Fig. 2A). The addition of 1 mM $CaCl_2$ in the uptake medium caused a small initial efflux of L-glutamate followed by a rela-

tively rapid re-uptake to a level above that of the control (Fig. 2A). These observations suggested that $CaCl_2$ had a small effect on both uptake and efflux processes. At a low $[Ca^{2+}]$ in the external medium (0.1 mM) there was a substantial enhancement of KCl-induced efflux even at 2 min following the addition of the efflux medium (Fig. 2B). Higher external $[Ca^{2+}]$ (> 1 mM) either did not increase the KCl-induced efflux at 2 min or appeared to reverse this effect, probably through activation of glutamate re-uptake (Fig. 2B).

The stimulation of L-glutamate uptake by Ca^{2+} was shown more clearly by direct measurements of the influence of Ca^{2+} on this uptake activity in synaptic membranes. The addition of either 1 or 3 mM $CaCl_2$ into the uptake medium caused a nearly 2-fold enhancement of L-glutamate transport from a plateau level (2–5 min) of 0.55 nmol/mg protein for the control samples to 1.0 nmol/mg for the Ca^{2+}-containing samples. No further activation was obtained when both 3 mM $CaCl_2$ and the calcium ionophore A23187 were present in the incubation medium which indicated that the presence of Ca^{2+} in the external medium is sufficient to cause activation of the carriers. The stimulation of L-glutamate transport by Ca^{2+} could not be ascribed to an increase in L-glutamate binding to the synaptic membranes since the increased amount of L-glutamate associated with the membranes could be released by elevation of the extravesicular [KCl] (e.g., Fig. 2).

In the experiments shown in Fig. 3, the intravesicular $[Na^+]$ and $[K^+]$ were altered by the introduction of nigericin, an ionophore that exchanges Na^+ in the incubation medium for K^+ or H^+ across the vesicular membrane. Efflux of L-glutamic acid from synaptic membranes following dilution either into the uptake or efflux medium was stimulated by nigericin (Fig. 3). The stimulation of efflux by nigericin is not likely to be through an activation of the reverse operation of the carrier-mediated uptake process because of the lack of a $[Na^+]$ gradient to drive the outward movement of the carriers. It is likely that under both conditions of efflux shown in Fig. 3, nigericin decreased the internal $[K^+]$ which indirectly decreased the $\Delta\psi$ (internal negative) that develops during incubation of these membranes in a NaCl medium [15].

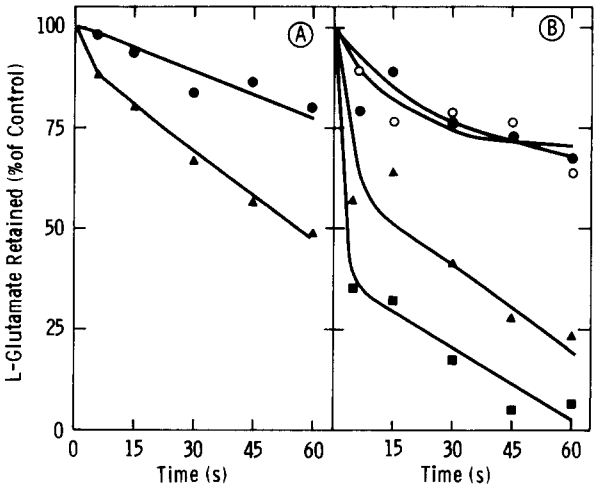

Fig. 3. Effect of nigericin on L-glutamate efflux measured in the absence (A) or presence (B) of KCl. Active loading of L-glutamate into the synaptic membrane vesicles was performed as described in Fig. 1. (A) Effect of nigericin on L-glutamate efflux into a NaCl medium. After 2 min of influx, the vesicles were diluted with 1 ml of uptake medium that contained 5 μM nigericin (▲) or did not contain nigericin (●). (B) Effect of nigericin on KCl-stimulated efflux. After 2 min of influx, the vesicles were diluted with the following media: uptake medium (●), uptake medium plus 5 μl methanol (control for the solvent used for nigericin) (○), efflux medium that did not contain $CaCl_2$ (▲), and same efflux medium without $CaCl_2$ but which included 5 μM nigericin (■).

The $\Delta\psi$ in neuronal cells is mainly determined by the permeability of the membrane to K^+ and the concentration ratio of K^+ across the membrane [17,18]. The dependence of the K^+ stimulated L-glutamate efflux on the transmembrane $[K^+]$ ratio $[K^+]_{in}/[K^+]_{out}$ was determined directly. In these studies, the initial $[K^+]_{in}$ was varied while maintaining a constant stimulus for L-glutamate efflux, i.e., an external $[K^+]$ of 33 mM. The KCl-induced efflux was increased as the ratio of the initial $[K^+]_{in}$ to the applied $[K^+]_{out}$ was increased from 1 to 6. These observations indicated that the introduction of K^+ in the incubation medium depolarized the synaptic membrane and that the amount of L-glutamate released was dependent on the magnitude of the initial $\Delta\psi$.

The dependence of L-glutamate efflux on changes in membrane $\Delta\psi$ was demonstrated also by examining the effects of veratridine on L-glutamate retention in synaptic membranes. Veratridine opens neuronal membrane channels

causing Na^+ influx and depolarization in synaptosomes [19]. The introduction of 100 μM veratridine into the incubation medium enhanced L-glutamate efflux in the absence of extravesicular KCl. The magnitude of the release caused by veratridine was approx. 25% of that induced by KCl (data not shown). Tetrodotoxin (1 μM), a Na^+-channel blocker, inhibited the action of veratridine on L-glutamate efflux. It was suspected, though, that the membrane glutamate carrier activity may have caused a rapid reduction of the $[Na^+]$ gradient at the time of the introduction of veratridine, even in those experiments where $[Na^+]_{out}$ was increased from 100 to 140 mM. Inhibition of L-glutamate transport should increase the level of depolarization induced by veratridine and should stimulate the efflux of L-glutamic acid, if the latter is sensitive to membrane depolarization. Indeed, when *p*-chloromercuribenzenesulfonic acid was included in the efflux medium veratridine produced a large release of L-glutamate which was almost equivalent to that induced by KCl (Fig. 4). Tetrodotoxin blocked the effect of veratridine almost completely. The transport inhibitor induced a slow, continuous efflux into the uptake medium similar to that observed earlier (see Fig. 1).

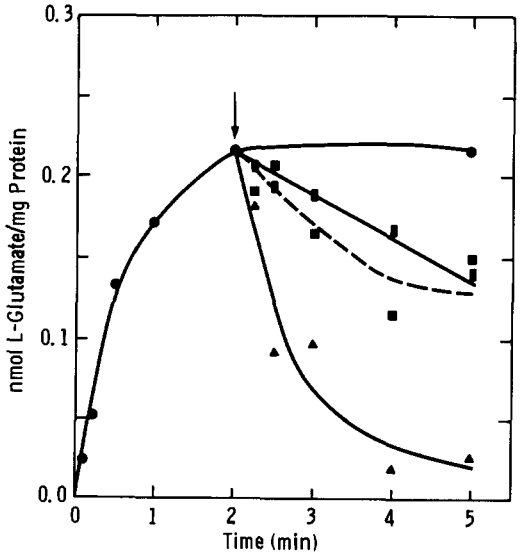

Fig. 4. Stimulation of L-glutamate efflux by veratridine and the effect of tetrodotoxin. Active loading of L-glutamate into the synaptic membrane vesicles was performed as described in Fig. 1. After 2 min of influx, the vesicles were diluted either with uptake medium or with 1 ml of the following media which contained 1 mM *p*-chloromercuribenzenesulfonic acid: (▮) 160 mM NaCl, 10 mM Tris sulfate, pH 7.4, (▲) 160 mM NaCl, 10 mM Tris sulfate, and 100 μM veratridine, (■) 160 mM NaCl, 10 mM Tris sulfate, 100 μM veratridine and 1 μM tetrodotoxin.

The enhancement of L-glutamate efflux from synaptic membrane vesicles observed following elevation of extravesicular $[K^+]$ or the introduction of nigericin or veratridine was suggestive of a common mechanism for this release process, i.e. depolarization-induced efflux of preloaded L-glutamate. This efflux process appeared to be more sensitive to low external $[Ca^{2+}]$ than the uptake system was. Since the majority of resealed membrane structures can be identified in electron microscopic examinations as presynaptic nerve endings, and since the transport activity used to load L-glutamic acid into the membranes is primarily associated with the presynaptic nerve ending structures [20], it is reasonable to conclude that the L-glutamic acid released originated from presynaptic sites. It is important to note that this release of L-glutamate from synaptic membranes may be uniquely related to this amino acid since we did not observe such $KCl/CaCl_2$-stimulated release with membranes preloaded with γ-amino [^{3}H] butyric acid [21], an inhibitory amino acid transmitter in the mammalian central nervous system.

Acknowledgements

This work was supported by grants NS 16364 from NINCDS and AA 04732 from NIAAA and DAAG 29-79-C-0156 and DAAG 29-83-K-0065 from U.S. Army Research Office. We acknowledge support provided by the Center for Biomedical Research, The University of Kansas and thank Ms. Mary Seyk and Ms. Kathy Wright for typing the manuscript.

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