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Accumulated glutamate levels in the synaptic vesicle are not maintained in the absence of active transport

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We have investigated factors which may affect accumulated glutamate levels in synaptic vesicles and glutamate efflux. Agents which dissipate the electrochemical proton gradient resulted in a rapid reduction of steady-state vesicular glutamate levels, which was prevented by *N*-ethylmaleimide. Glutamate efflux was found to occur even in the presence of an electrochemical proton gradient, but was effectively inhibited by *N*-ethylmaleimide. These results suggest that accumulated glutamate levels in synaptic vesicles are not maintained unless glutamate is taken up continuously by an active transport mechanism, and they could provide an explanation for the lack of convincing evidence for the enrichment of endogenous glutamate in isolated synaptic vesicles.

Glutamate is widely recognized as the strongest candidate for an excitatory amino acid neurotransmitter [4, 7, 25, 29, 30], and its role in neuronal plasticity and various neurological diseases has been implicated [3, 5, 9, 20, 26]. In recent years, evidence has accumulated that glutamate is taken up into isolated synaptic vesicles in an ATP-dependent manner [2, 6, 8, 13, 19, 23, 24], supporting the neurotransmitter role of glutamate. The vesicular glutamate uptake is driven by an electrochemical proton gradient, generated by a Mg-ATPase located in the vesicle membrane [19, 24, 27]. Despite the evidence for the active transport system for glutamate in the synaptic vesicle membrane, earlier investigations have failed to demonstrate that this amino acid is stored in isolated synaptic vesicles at high concentrations [15, 17]. However, immunocytochemical studies have suggested that glutamate is present in high concentrations in synaptic vesicles in certain nerve endings [28]. These apparently discrepant lines of evidence suggest that the glutamate accumulated in synaptic vesicles may not be maintained during the synaptic vesicle isolation. In an effort to understand the storage mechanism for vesicular glutamate, we have examined factors

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which affect accumulated glutamate levels in synaptic vesicles. An account of this work has been reported previously [1].

Synaptic vesicles were prepared from rat cerebrum as described by Kish and Ueda [14] and resuspended in Solution B (0.32 M sucrose, 1 mM dithiothreitol, 1 mM $NaHCO_3$). This vesicle preparation, referred to as RCSV, was used in all of the experiments to be described here. The synaptic vesicle preparation (50 μg protein) was incubated at 30°C in a solution (final volume, 100 μl) containing 10 mM HEPES (pH 7.4), 0.25 M sucrose, 4 mM KCl, 4 mM $MgSO_4$, 2 mM L-aspartate, 50 μM L-[^{3}H]glutamate (0.4 Ci/mmol) and 1 mM Tris-ATP, essentially as described previously [24]. After 10 min incubation for glutamate uptake, test agents were added in 10 μl, and incubation was allowed to continue for various periods of time. The incubation was terminated by the addition of 2 ml of ice-cold 0.15 M KCl, as described previously [24]. Each assay was carried out in duplicate, and the results were expressed as the amount (pmol/mg) of [^{3}H]glutamate retained, with the average value and the range of variation. Protein was determined according to Lowry et al. [16] with bovine serum albumin as standard.

Fig. 1A demonstrates that glutamate is taken up into the synaptic vesicle preparation in an ATP-dependent manner. The uptake reaches a steady-state level around 10 min. Previous studies have indicated that glutamate uptake is driven by an electrochemical proton gradient generated by a proton-pump Mg-ATPase in the synaptic vesicle membrane [19, 24, 27]. To determine whether the membrane potential and transmembrane pH gradient are involved in maintaining steady-state levels of glutamate in the synaptic vesicle, we examined the effect of the proton ionophore FCCP and the K^+/H^+ exchanger nigericin on the steady-state levels of glutamate in synaptic vesicles. FCCP and nigericin have been shown to dissipate the membrane potential and transmembrane pH gradient, respectively, in chromaffin granule and highly

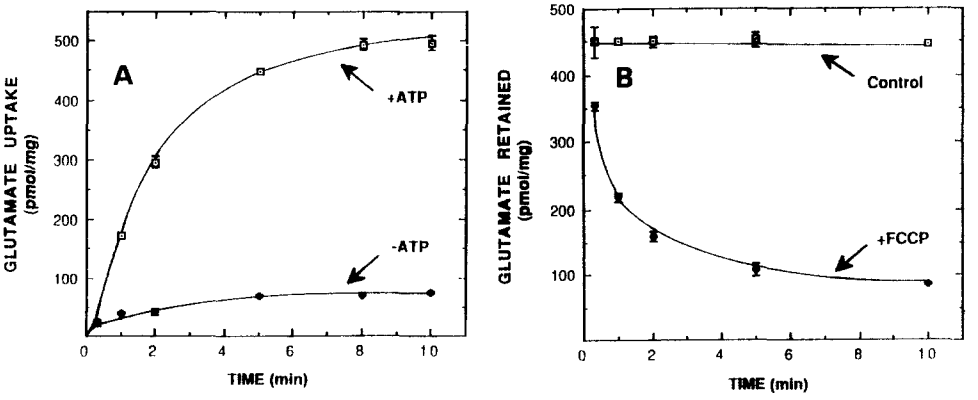

Fig. 1. Time course of vesicular glutamate uptake and the effect of FCCP on the steady-state level of glutamate. A: synaptic vesicles were incubated in the presence (■) or absence (◆) of ATP and filtered at the various times indicated. B: synaptic vesicles were preloaded for 10 min with [^{3}H]glutamate in the presence of ATP. At 10 min, either 0.5% dimethyl sulfoxide (Me_2SO) alone (control) (■) or 10 μM FCCP (in 0.5% Me_2SO) (◆) were added in small volume (10 μl/100 μl assay vol), and the samples were filtered at the various times indicated. Me_2SO (0.5%) alone had no effect on glutamate uptake activity.

purified synaptic vesicle preparations [10–12, 19, 27], as well as in the partially purified vesicle preparation used in this study (data not shown). Fig. 1B shows that the addition of FCCP (10 μM) caused a rapid reduction of the steady-state glutamate levels. Nearly identical results were also obtained with nigericin (data not shown). These results suggest that the existence of an electrochemical proton gradient across the vesicular membrane is required in order to maintain steady-state levels of vesicular glutamate.

Fig. 2 shows that the effects of FCCP and nigericin on steady-state glutamate levels were blocked by pretreatment with the sulfhydryl-modifying agent *N*-ethylmaleimide (NEM, 500 μM). In experiments not shown, we have examined the effect of FCCP on the membrane potential, which was generated upon addition of Mg-ATP, using the voltage-sensitive fluorescent dye oxonal V. We have repeatedly observed that treatment (2 min at 30°C) of the synaptic vesicles with NEM (500 μM), after the membrane potential was generated, did not prevent the ability of FCCP to dissipate the membrane potential. It may be noted that the steady-state glutamate level was hardly affected by NEM, despite its demonstrated inhibitory effects on glutamate uptake into synaptic vesicles [24] and on the proton pump ATPase [22]. These results are in accord with the notion that NEM blocks glutamate efflux regardless of electrochemical proton gradients.

In order to examine the effect of NEM and FCCP more directly on the efflux process, synaptic vesicles, which had been preloaded with [^{3}H]glutamate in the presence of ATP, were treated with NEM, then diluted 20-fold into an efflux medium devoid

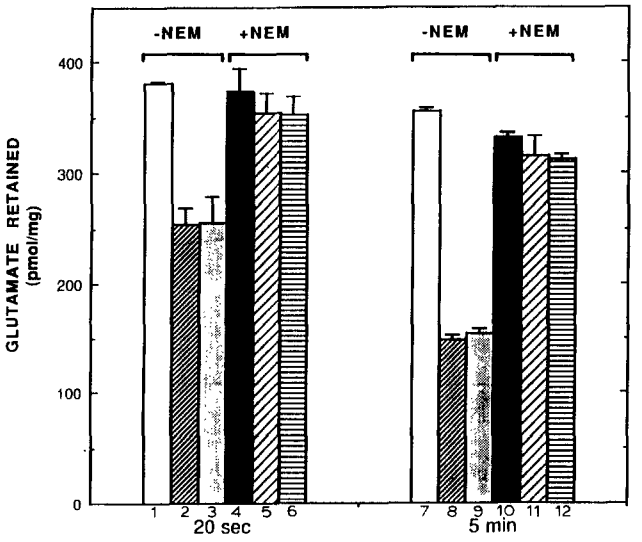

Fig. 2. Effect of NEM on steady-state vesicular glutamate levels. After synaptic vesicles were preloaded for 10 min with [^{3}H]glutamate in the presence of ATP, H_2O or 500 μM NEM were added in small volume, and the suspension was incubated for an additional 5 min at 30°C. After the 5 min treatment with H_2O (1–3, 7–9), or NEM (4–6, 10–12), either H_2O (1, 4, 7, 10), 10 μM FCCP (2, 5, 8, 11), or 20 μM nigericin (3, 6, 9, 12) were added in small volume (10 μl/100 μl assay vol), and the samples were filtered at the times indicated.

of ATP and glutamate, with or without FCCP, and further incubated. Fig. 3 demonstrates that glutamate efflux does occur at a significant rate after the 20-fold dilution. Under these conditions, the original electrochemical proton gradient was found to be maintained for at least 10 min (data not shown). The significant efflux which occurs in the presence of electrochemical proton gradients was stimulated by FCCP. Interestingly, NEM pretreatment blocked the glutamate efflux regardless of the presence of FCCP.

The results presented here indicate that the existence of an electrochemical proton gradient across the synaptic vesicle membrane is required in order to maintain steady-state glutamate levels. The experiment shown in Fig. 3 indicates that there is a rapid efflux of glutamate from these synaptic vesicles, even in the presence of electrochemical proton gradients. The rate of glutamate efflux was found to be dependent upon the glutamate concentration gradient across the vesicle membrane (Carlson, M.D. and Ueda, T., in preparation). These observations suggest that the vesicular storage mechanism of glutamate differs, at least in part, from that for catecholamines and acetylcholine [18, 21]. These amine neurotransmitters are not lost from the vesicles even if there is a large concentration gradient across the vesicle membrane, as long as the intravesicular pH is in the acidic range [18] or an electrochemical proton

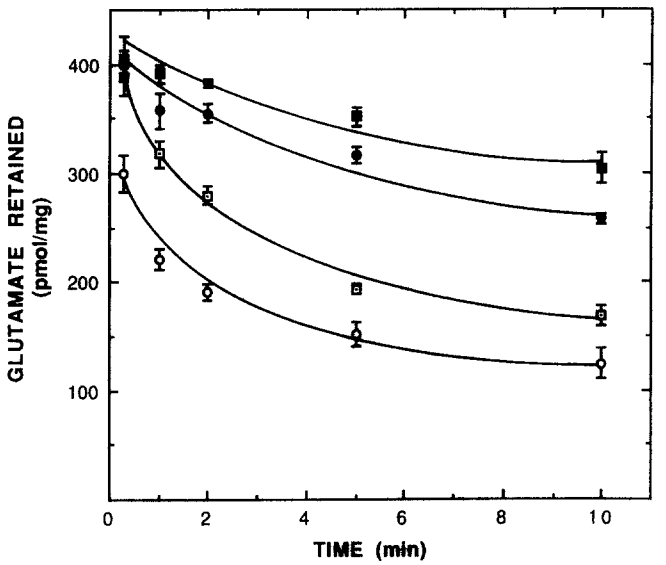

Fig. 3. Effect of NEM and FCCP on glutamate efflux from preloaded synaptic vesicles. Synaptic vesicles were preloaded with [^{3}H]glutamate in the presence of ATP. At 10 min, H_2O (■, ○) or 500 μM NEM (■, ●) were added in small volume (10 μl/100 μl assay vol), and the mixtures were incubated for an additional 5 min at 30°C. After the 5 min incubation, 1.9 ml of a solution (prewarmed at 30°C) containing the standard glutamate uptake mixture were added, except for the omission of ATP and glutamate and for the inclusion of none (control, (■), 10 μM FCCP (○), 500 μM NEM (■), or 10 μM FCCP + 500 μM NEM (●), and the incubation was allowed to continue for various periods of time. The efflux was terminated by the addition of KCl followed by filtration. The addition of the KCl solution and the immediate filtration step altogether normally took approximately 20 s (0.3 min).

gradient is maintained [21]. Although glutamate efflux does occur in the presence of an electrochemical proton gradient, the efflux is stimulated by FCCP. This suggests that an electrochemical proton gradient, particularly the membrane potential, may impose a partial kinetic barrier to glutamate efflux. Therefore, the effect of FCCP in Fig. 1B is likely due to an inhibition of uptake and a facilitation of efflux.

The inhibitory effect of NEM on the efflux of glutamate suggests that the efflux is carrier-mediated. This notion is further supported by the observations that glutamate efflux is temperature-dependent and that the glutamate efflux is reduced by a competitive inhibitor of glutamate uptake (Carlson, M.D. and Ueda, T., in preparation). The ability of NEM to achieve this inhibitory effect regardless of the presence of FCCP suggests that a cysteine sulfhydryl group is involved in a mechanism distinct from that which involves electrochemical proton gradients. Data not shown also suggest that a sulfhydryl group of the glutamate translocator is critical for the uptake (Naito and Ueda, unpublished observations). Thus, it is plausible that a cysteine sulfhydryl group plays a role in regulating steady-state glutamate levels in the synaptic vesicle in vivo.

The data presented in this study predict that rat brain synaptic vesicles would be depleted of a major portion of endogenously accumulated glutamate during the vesicle isolation, which is normally carried out under conditions in which the electrochemical proton gradient is not maintained. These considerations could provide an explanation for the apparent difficulty in demonstrating the enrichment of endogenous glutamate in isolated synaptic vesicles [15, 17].

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