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Calcium-dependent release of accumulated glutamate from synaptic vesicles within permeabilized nerve terminals

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We have studied glutamate release from synaptic vesicles in permeabilized synaptosomes, which were preloaded with [^{3}H]glutamate in an ATP-dependent manner. The release was found to be calcium-dependent and to require a heat-labile cytosolic macromolecule factor for maximum activity. Maximal release occurred at 5 μM free Ca^{2+} and within 5 min. Of the other divalent cations tested, only barium stimulated release of vesicular glutamate. The release was inhibited by *N*-ethylmaleimide. These results are characteristic of exocytotic release of monoamines and peptides observed in endocrine systems, and constitute direct evidence for the notion that calcium-dependent release of glutamate originates from the vesicular pool.

Amassed evidence indicates that glutamate serves as a major excitatory neurotransmitter in the vertebrate central nervous system (for review, see ref. 4). Despite the increased recognition of the neurotransmitter role of glutamate, the mechanism by which glutamate is released from the nerve terminal remains in large part to be elucidated. Substantial evidence now has been accumulated that glutamate is specifically taken up into highly purified isolated synaptic vesicles in an ATP-dependent manner [6, 8, 12, 15–18, 22], suggesting that glutamate could be accumulated and stored in synaptic vesicles in vivo. This is in accord with immunocytochemical evidence provided by Storm-Mathisen et al. [21] suggesting that glutamate is concentrated in certain synaptic vesicles distinct from γ-aminobutyric acid (GABA)-containing vesicles. Recent evidence has indicated that glutamate released in a calcium-dependent manner originates from a non-cytoplasmic pool [20]. These lines of evidence argue for the involvement of synaptic vesicles in synaptic release of glutamate. In this study, we have investigated the calcium-dependent release of glutamate from the synaptic vesicles in a more direct manner, using a permeabilized synaptosome preparation. Evidence is presented that glutamate is indeed released directly from the synaptic vesicle in a calcium-dependent manner, in the presence of a cytosolic factor.

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Synaptosomes were prepared from the rat (60 to 90-day-old) cerebrum according to the method of Hajos [9], and resuspended in 0.32 M sucrose at a concentration of 25 mg/ml. One ml aliquots were frozen in liquid nitrogen and thawed. The freeze/thaw step was repeated twice more to permeabilize the nerve terminal plasma membrane. Synaptic vesicles maintain ATP-dependent glutamate uptake activity even after freeze/thawing [12, 17] while synaptosomal plasma membranes are ruptured. A synaptosomal cytosol fraction was prepared from rat brain as described [23] followed by 40% ammonium sulfate precipitation. This precipitated pellet was resuspended, dialyzed with 10 mM Tris-maleate pH 7.0, and used as a partly purified cytosolic factor.

Permeabilized synaptosomes were subjected to ATP-dependent glutamate uptake under conditions modified from those described for vesicular uptake [18]. The incubation mixture contained in a final volume of 0.12 ml: permeabilized synaptosomes (250 μg protein), 10 mM Tris-maleate (pH 7.2), 4 mM KCl, 200 mM sucrose, 0.5 mM ouabain, 10 mM glucose, 20 mM aspartate (to minimize Na^+-dependent uptake and binding to glutamate receptors), 2 mM EGTA, 15 mM $MgSO_4$, 9 mM ATP, synaptosol factor (330 μg protein) and 5 μM potassium [^{3}H]L-glutamate (0.8 Ci/mmol). The ATP-dependent uptake reached a plateau by 10 min at 37°C, and the level was maintained for an additional 5 min. To study release of accumulated glutamate from permeabilized synaptosomes, 5 μl of buffer or Ca^{2+}-containing buffer solution were added to each tube at the end of 10 min of uptake, and the incubation was allowed to continue

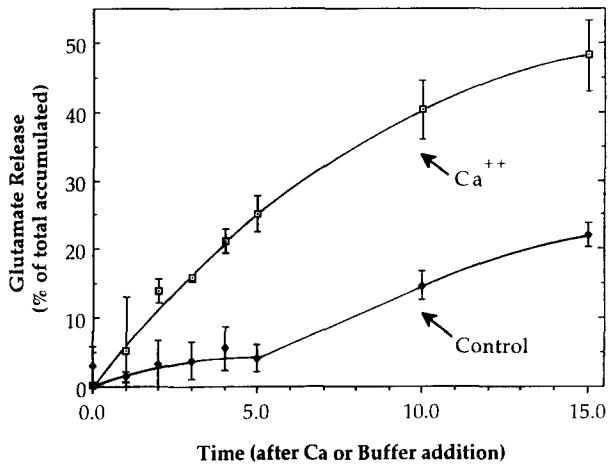

Fig. 1. Time course of glutamate from permeabilized synaptosomes. Permeabilized synaptosomes were loaded with [^{3}H]glutamate in the presence of ATP and a partially purified cytosolic factor, as described in the text, and further incubated for release for various periods of time in the presence or absence of calcium (5 μM free Ca^{2+}). The absence of calcium indicates the presence of 2 mM EGTA (free Ca^{2+}, less than 0.1 μM). The data represent mean $\pm$ S.E.M. obtained from experiments using 3 separate synaptosome and cytosolic factor preparations.

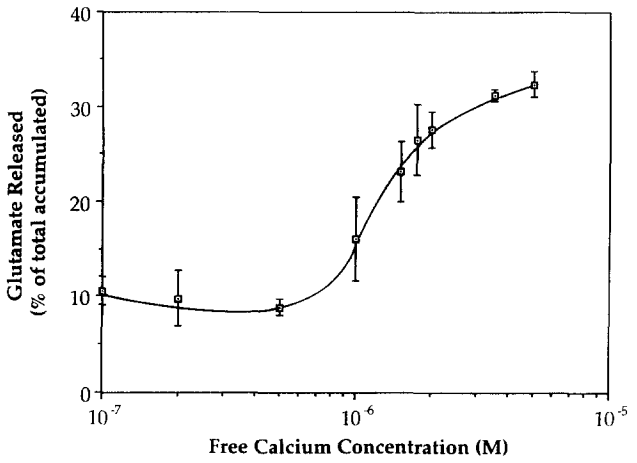

Fig. 2. Effect of various concentrations of calcium on glutamate release from permeabilized synaptosomes. The experiment was carried out as shown in Fig. 1, except that the free Ca^{2+} concentration was varied as indicated (0.1, 0.2, 0.5, 1, 1.5, 1.75, 2, 3.5, and 5 μM), and that release was allowed to occur for 5 min. Free calcium concentrations were determined using a calcium electrode calibrated against standard calcium solutions. The data represent mean $\pm$ S.E.M. obtained from experiments using 3 separate synaptosome and cytosolic factor preparations.

for 5 additional min. Each incubation was carried out in duplicate. Release was terminated by the addition of ice-cold 0.15 M KCl, followed by filtration on a glass fiber filter (GF-C). The glutamate release was expressed as % of the total [^{3}H] glutamate accumulated at the end of 10 min uptake as follows:

$$\frac{Glu_{10}-Glu_{15}}{Glu_{10}-Glu'_{10}} \times 100$$

where Glu_{10} and Glu'_{10} are the amounts of [^{3}H]glutamate accumulated in the presence of ATP at 37 and 0°C, respectively, during the 10 min incubation, and Glu_{15} is the amount of [^{3}H]glutamate retained at 37°C (in the presence of ATP) at the end of the release period. The temperature-dependent glutamate uptake in the presence of ATP was considered here to be equivalent to the ATP-dependent uptake at 37°C, since the degree of ATP-dependency was essentially the same as that of temperature-dependency in highly purified synaptic vesicles [18]. We adopted this method because the true ATP-dependent uptake activity could not be accurately determined in the permeabilized synaptosome preparation due to endogenous ATP contamination in the preparation.

The time courses of calcium-dependent and calcium-independent vesicular release are shown in Fig. 1. The vesicular pool of glutamate taken up in an energy-dependent manner was released significantly upon addition of Ca^{2+}. The Ca^{2+}-dependent release was complete by 5 min. When the cytosolic factor was omitted from the incubation, calcium-dependent release was substantially reduced (data not shown).

Fig. 2 shows that the release is dependent on the calcium concentration; maximal release occurred at approximately 5 μM free Ca^{2+}. This concentration lies in the range of free Ca^{2+} concentration (1–10 μM) which has been shown to give maximal release of other neurotransmitters and hormones, such as catecholamine from

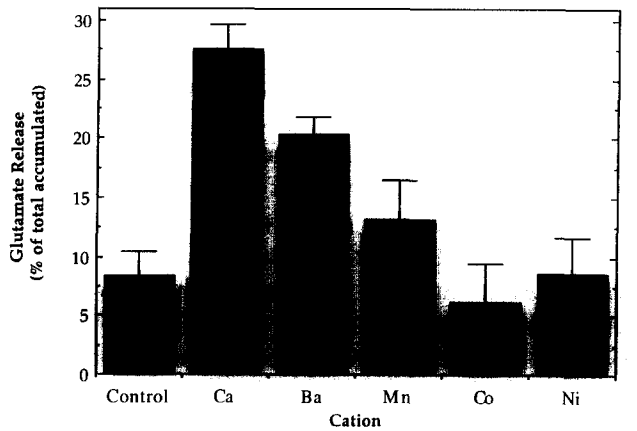

Fig. 3. Effect of various cations on glutamate release from permeabilized synaptosomes. The experiment was carried out as described in Fig. 1, except that various divalent cations were added each at 2.8 mM in the presence of 2 mM EGTA, at the beginning of the release period, and that release was terminated after 5 min incubation. For calcium, free Ca^{2+} was 3.5 μM. The data represent mean $\pm$ S.E.M. obtained from experiments using 3 separate synaptosome and cytosolic factor preparations.

permeabilized chromaffin cells [5] and histamine from mast cells [10].

The stimulatory effect of Ca^{2+} was mimicked by Ba^{2+} but not by the other divalent cations tested, which include Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Fig. 3). The divalent metal specificity is similar to that observed on catecholamine release from chromaffin granules [24]. Moreover, the Ca^{2+}-dependent release was inhibited by the sulfhydryl blocking agent *N*-ethylmaleimide (NEM) (data not shown), as in the case with exocytotic release of catecholamine from digitonin-permeabilized chromaffin cells [7, 24].

The cytosolic factor was further purified by chromatography on DEAE-Bio Gel A (150–200 mM NaCl eluate). In contrast to the permeabilized synaptosome preparation, isolated synaptic vesicles, which had been preloaded with [^{3}H]glutamate in the presence of ATP, exhibited no increase in net efflux of glutamate, in the presence of this purified factor (75 μg/0.125 μl) either in the presence or absence of 5 μM free calcium (data not shown). This purified factor was also separated from a proteinaceous substance [13] causing a decrease in net uptake into isolated synaptic vesicles (data not shown). In other experiments, the factor was shown to be a heat-labile macromolecule with a molecular weight of greater than 135,000 (data not shown).

In this study, we have provided evidence that glutamate is released from synaptic vesicles within the permeabilized synaptosome in response to an increase in calcium concentration. The glutamate release required a synaptosol factor for maximal release. We suggest that this release represents a physiologically relevant calcium-dependent release of glutamate, based upon the characteristics observed here which are similar to those observed on exocytotic release of catecholamine from chromaffin cells, histamine from mast cells, and prolactin from GH_3 cells; these include (a) sensitivity to calcium [1, 5, 10, 14], (b) cation specificity [11, 24], (c) inhibition by NEM [7, 24], and (d) marked stimulation by a synaptosomal soluble factor [14].

Calcium-dependent glutamate release using brain slices and intact synaptosomes has been well documented (for review, see ref. 19). Nicholls and colleagues have provided evidence that the calcium-dependent release is derived from a non-cytoplasmic pool [20], potentially from the synaptic vesicle pool. Our study reported here provides direct evidence that the calcium-dependent release originates from the synaptic vesicle, further supporting the vital role of synaptic vesicle in glutamate synaptic transmission. This conclusion was possible to draw since the calcium-dependent release was derived from that pool into which glutamate had previously been accumulated in an ATP-dependent, chloride-stimulated manner.

The successful demonstration of calcium-dependent release of glutamate from the permeabilized nerve terminal required two conditions. One is that release experiments were carried out under conditions which maintain active vesicular uptake. This is important since accumulated glutamate exits from the synaptic vesicle as soon as ATP and glutamate are removed from the extravesicular medium [2, 3]. The other condition was to fortify the release system with a cytosolic factor. It is interesting to note that a cytosolic protein is also required for the secretion of prolactin in a pituitary cell line [14].

The method described here or its modification provides a direct, simple biochemical assay for calcium-dependent vesicular release of glutamate, and may be useful for studying the molecular mechanism of exocytosis for glutamate and other neurotransmitters from the nerve terminal.

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