Micromolar Calcium Stimulates Proteolysis and Glutamate Binding in Rat Brain Synaptic Membranes

Abstract. *Incubation of cortical synaptic membranes with low concentrations of calcium resulted in a decrease in the amount of a high-molecular-weight doublet protein and an increase in the sodium-independent binding of glutamate. Both effects were blocked by the thiol protease inhibitor leupeptin. These results suggest that calcium-induced proteolysis of membrane components regulates the number of glutamate receptors in neuronal membranes.*

Several factors are involved in regulating the distribution and number of plasma membrane receptors and their affinity for neurotransmitters or hormones (*1*). This regulation of cell surface receptors may be one means by which the nervous system adjusts the relative efficiencies of its component circuitries. While the molecular mechanisms underlying the regulation of receptors has been well documented in isolated cells (*2, 3*), little is known of comparable mechanisms in the brain.

Recently we reported that glutamate receptor binding in hippocampal synaptic membranes was markedly enhanced by calcium ions in concentrations as low as 10 to 50 μM and that the effect was partially irreversible (*4*). On the basis of subsequent pharmacological experiments, we proposed that calcium exerted its effect on [^{3}H]glutamate binding by stimulating a membrane-associated thiol protease (*5*)—a hypothesis that has recently received experimental support from other laboratories (*6*). A neutral, calcium-activated proteolytic process that acts on neurofilament proteins has been described in the cytoplasm of neurons (*7*), but this process requires concentrations of calcium hundreds of times greater than those required to stimulate glutamate binding in hippocampal membranes. We now report that micromolar concentrations of calcium stimulate the degradation of a synaptic membrane protein from cerebral cortex and hippocampus and that this effect is blocked by the thiol protease inhibitor leupeptin. Leupeptin also inhibits the stimulatory effect of calcium ions on [^{3}H]glutamate binding. These results suggest a molecular mechanism by which calcium ions, and hence synaptic transmission, could regulate receptor number and distribution in neuronal target cells.

Since it was previously shown that the soluble, calcium-activated protease of a wide variety of systems acts on high-molecular-weight proteins (*7*), we focused our analysis on cortical synaptic membrane proteins with molecular weights above 100,000. As shown in Fig. 1, a limited number of these large proteins are reliably identified in polyacrylamide gels. Scans of the gels reveal that calcium ions at a concentration of 100 μM induce the partial disappearance of an ~ 180,000-dalton doublet protein (bands 1a and 1b in Fig. 1A). Several other proteins are not affected by this concentration of calcium ions (bands 2 and 3). Comparable results are obtained with hippocampal membranes (calcium ions at 100 μM result in a 56 ± 9 percent decrease in the doublet protein). The calcium-chelating agent EGTA prevents most of the endogenous proteolysis of the band 1 doublet protein (the absorption of band 1 in the absence of added calcium is 75 ± 5 percent of that measured in the presence of 2.5 mM EGTA). The effect of calcium ions is concentration-dependent (Fig. 1C), and the maximum effect of calcium, a 50 percent decrease in the absorbance of the doublet protein (as compared to that in the presence of 2.5 mM EGTA), is reached at a concentration of 100 μM. The apparent affinity of calcium for the protease is 28 ± 10 μM (mean ± standard error of nine experiments). The peptide leupeptin is a rather specific inhibitor of thiol proteases (*8*), and at a concentration of 80 μM it prevents the calcium-activated proteolysis of bands 1a and 1b and does

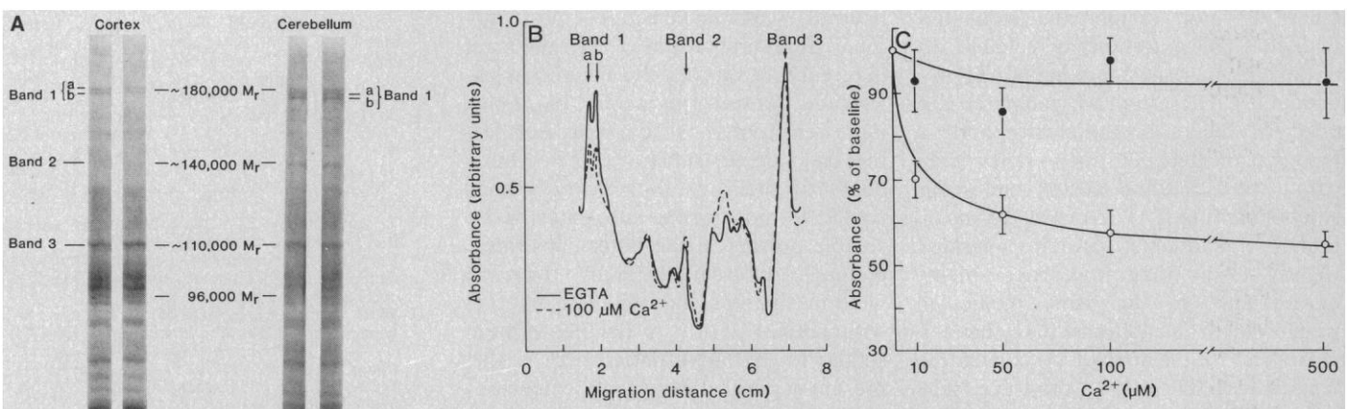

Fig. 1. Effect of calcium on the gel electrophoretic pattern of synaptic membrane proteins from rat cerebral cortex and cerebellum. Synaptic membranes were isolated (*5*) and resuspended in 50 mM tris-HCl, pH 7.4 (3 mg of protein per milliliter). Aliquots of 100 μl were then incubated with 80 μl of buffer and 20 μl of $CaCl_2$ (final concentration, 100 μM) or 20 μl of EGTA (final concentration, 2.5 mM) for 30 minutes at 30°C. Incubation was terminated by centrifugation at 10,000g for 5 minutes in a refrigerated microcentrifuge. The supernatant was removed and the remaining pellet was dissolved in 70 μl of electrophoresis buffer (60 mM tris-PO_4, pH 6.8; 1.25 mM EDTA; 2 percent sodium dodecyl sulfate; 1 percent β-mercaptoethanol; and 10 percent glycerol) and boiled for 3 minutes. Aliquots of 15 μl (70 μg of protein) were subjected to electrophoresis on 7.5 percent polyacrylamide–sodium dodecyl sulfate slab gels (*16*). The gels were stained with 0.1 percent Coomassie Brilliant Blue-R in 50 percent trichloroacetic acid, destained in 7 percent acetic acid, and scanned with an E-C Apparatus densitometer. (A) Coomassie-stained gels. Relative molecular weights (M_r) of bands 1, 2, and 3 were calculated from molecular weight standards between 96,000 and 13,000. (B) Densitometer tracing of membrane proteins isolated from cerebral cortex and incubated in the presence of 2.5 mM EGTA (solid line) or 100 μM $CaCl_2$ (dashed line). (C) Effect of leupeptin on the calcium-sensitive decrease in band 1b. Synaptic membranes from cerebral cortex were incubated in the presence of EGTA (2.5 mM) or $CaCl_2$ (10, 50, 100, and 500 μM) with 80 μM leupeptin (●; N = 6) or without it (○; N = 9). Following electrophoresis, the gels were scanned and the amount of protein was determined by measuring peak heights. Values are means for six to nine experiments. Comparable data were also obtained for band 1a.

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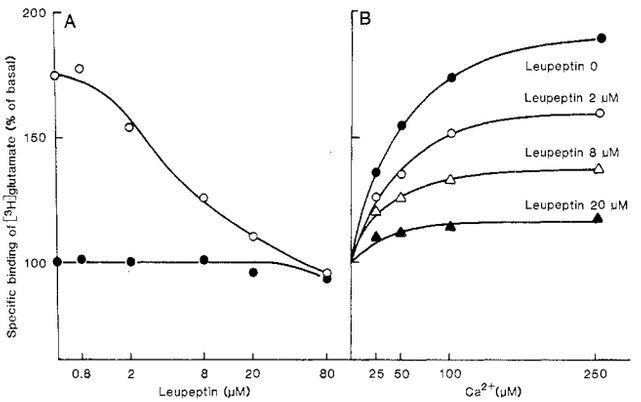

Fig. 2. Effect of leupeptin on calcium-stimulated binding of [^{3}H]glutamate in rat hippocampal membranes. The binding assay (*5*) was performed at a [^{3}H]glutamate concentration of 100 n*M*. Leupeptin and calcium were added to the membranes during the preincubation period at a concentration twice that indicated. The data (means for three different experiments which varied < 10 percent) represent specific [^{3}H]glutamate binding and are expressed as the percentage of binding in the absence of added calcium and leupeptin, which was 1.10 ± 0.05 pmole per milligram of protein. (A) Effect of increasing concentrations of leupeptin in the absence (●) or presence (○) of 250 μ*M* $CaCl_2$. (B) Effect of increasing concentrations of calcium in the presence of increasing leupeptin concentrations.

not, by itself, affect this doublet protein.

As shown in Fig. 2, leupeptin also inhibits, in a concentration-dependent manner, the calcium-induced stimulation of [^{3}H]glutamate binding in hippocampal membranes. It does not significantly affect basal [^{3}H]glutamate binding; half-maximal inhibition occurs at a leupeptin concentration of 6 μ*M*. Leupeptin also reduces the maximum effect of calcium without changing its apparent affinity for the stimulatory process (32 ± 5 μ*M*, mean of six experiments) (*9*). Similar results were recently reported by Vargas *et al.* (*6*).

Unlike hippocampal membranes, cerebellar synaptic membranes incubated with low concentrations of calcium do not exhibit increased binding of [^{3}H]glutamate (*5*). Therefore, we compared the effect of calcium on the electrophoretic patterns of synaptic membranes prepared from cerebral cortex and from cerebellum (Fig. 1A). In cerebellar membranes the doublet protein migrates slightly faster than in cortical membranes and, in the same molecular weight range, an additional fuzzy band is present above the doublet. At concentrations below 100 μ*M*, calcium does not induce detectable changes in either the fuzzy band or the doublet protein.

Thus, several lines of evidence indicate that cortical and hippocampal synaptic membranes contain a calcium-sensitive thiol protease and that proteolysis of a high-molecular-weight doublet protein (band 1) might be responsible for the calcium-induced stimulation of [^{3}H]glutamate binding to these membranes. First, chelation of calcium by EGTA reduces [^{3}H]glutamate binding (*4*) and prevents the proteolysis of this protein. Second, the apparent affinities of calcium for the protease and for the glutamate binding stimulatory process are in very close agreement. Third, leupeptin inhibits both the calcium-induced proteolysis of the doublet protein and the calcium-induced stimulation of [^{3}H]glutamate binding. Finally, the substrate protein in cerebellar membranes appears to be slightly different than in cortical membranes, and low levels of calcium ions do not stimulate its proteolysis; this is correlated with the fact that low levels of calcium ions do not stimulate [^{3}H]glutamate binding in cerebellar membranes.

Certain properties of this protease are similar to those reported for soluble, neutral, calcium-sensitive proteases found in several tissues (*7, 10*), the main difference being that the membrane-associated protease is about 100 times more sensitive to calcium ions. Further, the endogenous substrate, the band 1 doublet protein, exhibits characteristics similar to those of the substrates of the soluble proteases; the latter substrates, although different in many respects, have in common the ability to interact with actin (*7, 10*). A doublet protein similar to the one reported here is also present in purified postsynaptic densities (*11*).

We propose that the calcium-induced proteolysis of the band 1 doublet protein results in structural changes in synaptic membranes such that [^{3}H]glutamate binding sites, which were present but inaccessible, become unmasked. This hypothesis is in agreement with the postulated role of cytoskeleton-associated proteins in the regulation of the distribution of cell surface components (*2*). Finally, the presence in cortical and hippocampal membranes of both a calcium-activated protease, sensitive to micromolar concentrations of calcium, and its substrate strengthens our hypothesis concerning a possible mechanism for the long-term potentiation of synaptic transmission in rat hippocampus (*12*). Long-term potentiation in the hippocampus is accompanied by an increase in glutamate binding (*13*) and by changes in the shape of the synaptic contacts (*14*). Thus, it is possible that the proteolytic mechanism is activated by the influx of calcium which occurs at the synapse during repetitive electrical stimulation (*15*) and that the resultant proteolysis of the band 1 doublet protein triggers the changes in both the distribution of glutamate receptors and the shape of the synaptic contacts.

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