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Protease inhibitors implicate metalloendoprotease in synaptic transmission at the mammalian neuromuscular junction

(transmitter release/exocytosis/carbachol dose–response/phosphoramidon/synthetic substrate)

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ABSTRACT **Metalloendoproteases have been implicated in the calcium-dependent exocytosis of histamine from mast cells and in the calcium-dependent fusion of myoblasts. Because metalloendoproteases have also been identified in nervous tissue, we investigated the possibility that these proteases may be involved in neurotransmitter release at mammalian synapses. End-plate potentials were recorded intracellularly from mouse diaphragm/phrenic nerve preparations *in vitro*. The amplitudes of the end-plate potentials were reduced by as much as 90% during bath application of phosphoramidon, a specific inhibitor of metalloendoproteases, and by carbobenzoxy-dipeptide-amide synthetic substrates for metalloendoproteases. Only those synthetic dipeptides in which the amino group of the peptide bond was provided by a bulky hydrophobic amino acid, such as phenylalanine or leucine, which are substrates for metalloendoproteases, reduced synaptic transmission. Synthetic substrates in which proline or glycine provided the amino group of the peptide bond, which are not metalloendoprotease substrates, had little or no effect on the amplitude of end-plate potentials. The ability of synthetic substrates to reduce synaptic transmission was also dependent on the amino acid that provided the carboxyl group of the peptide bond, with glycine being more effective than tyrosine or serine. In addition, synthetic dipeptides with free carboxyl or amino termini, which have a low affinity for metalloendoproteases, also had little effect on synaptic transmission. The inhibition of synaptic transmission by phosphoramidon and the synthetic substrates occurred within 2 to 3 min and was completely reversible. Neither phosphoramidon nor the synthetic substrates altered the dose–response characteristics of the postsynaptic membrane to bath-applied carbachol. These results suggest that synaptic transmission requires the activity of a metalloendoprotease in the presynaptic nerve terminal and that proteolysis may be an important step during neurotransmitter exocytosis.**

The influx of calcium into the presynaptic nerve terminal during an action potential causes the release of transmitter into the synaptic cleft by exocytosis (1). The hypothesis that vesicles fuse with the presynaptic membrane during exocytosis is supported by morphologic and biochemical studies. Morphologic studies, using a quick-freeze technique, have identified vesicle fusion with the presynaptic membrane (2). Biochemical studies have shown that protein, unique to synaptic vesicles, appears in the presynaptic membrane after release of transmitter (3). The calcium-dependent fusion of biological membranes is not unique to neurons and occurs in many mammalian cells, including neurosecretory cells, endocrine cells, exocrine cells, and mast cells (4, 5), and in the fusion of myoblasts (6). Although the mechanisms responsible for neurotransmitter exocytosis are unresolved (7, 8), recent studies emphasize the involvement of protein in calcium-dependent membrane fusion and neurotransmitter release (9–11). The calcium-dependent exocytosis of histamine by mast cells (unpublished data) and the calcium-dependent fusion of mammalian myoblasts (12) require metalloendoprotease activity. These results, together with the fact that metalloendoproteases are found in mammalian neurosecretory (13, 14) and endocrine tissues (15), suggest the possibility that proteolysis may be involved in neurotransmitter exocytosis.

We examined the possibility that synaptic transmission requires metalloendoprotease activity by studying the amplitude of nerve-evoked end-plate potentials (EPPs) at the mammalian neuromuscular junction in the presence of inhibitors and synthetic dipeptide substrates of metalloendoproteases. The results indicate that synaptic transmission is reduced by inhibitors and synthetic substrates of metalloendoproteases. These compounds do not alter either the postsynaptic sensitivity to bath-applied carbachol or the postsynaptic excitability to direct stimulation. The observations are consistent with the hypothesis that neurotransmitter release at the presynaptic cholinergic terminal requires the activity of a metalloendoprotease.

MATERIALS AND METHODS

All experiments were carried out *in vitro*, using the mouse hemidiaphragm/phrenic nerve preparation. The hemidiaphragm was pinned out on Sylgard (Dow Corning, Midland, MI) in a 50-mm Pyrex Petri dish and maintained at room temperature. The bath volume was 5 ml, and the preparation was constantly perfused at a rate of about 5 ml/min. Two solution reservoirs, each controlled by a separate valve, fed the preparation chamber. The bath solution in the chamber was changed by switching to the desired reservoir. By introducing bath solutions containing curare and observing the subsequent changes in the EPP amplitudes, it was estimated that the bath concentration equilibrated to newly introduced compounds within 1 to 2 min.

The physiological saline used for these experiments contained 140 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM glucose, 5 mM $NaHCO_3$, 1.25 mM $NaHPO_4$, and 15 mM Hepes (Sigma) and it was adjusted with NaOH to pH 7.4 and bubbled continuously with 100% O_2. The compounds tested were dissolved in 1% dimethylformamide (Sigma) before being added to the saline. Therefore, 1% dimethylformamide (≈130 mM) was added to all control and wash salines; no osmotic compensation was made for its addition. Phosphoramidon, *N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, was obtained from Transformation Research (Framingham, MA). The L-isomer synthetic dipeptides were purchased from Vega Biochemical (Tucson, AZ).

Conventional techniques were used for nerve stimulation and

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Abbreviations: EPP, end-plate potential; Cbz, carbobenzoxy.

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for intracellular recordings of EPPs. The intracellular microelectrodes were filled with 3 M KCl and had resistances of 25–35 MΩ. The high divalent ion concentration of the saline promoted electrode sealing and allowed stable recording of membrane potentials for several hours. Muscle fibers were used only if their resting membrane potentials were −70 mV or larger. The muscle-fiber membrane potential was monitored constantly during the experiments.

For intracellular recording of the EPPs, neuromuscular transmission was decreased sufficiently to block nerve-stimulated muscle contractions by addition of (+)-tubocurare Cl (Sigma) to the saline (this usually required 10–15 μM). The phrenic nerve was drawn into a suction electrode and stimulated at 0.2 Hz. Transillumination allowed visual placement of microelectrodes near the fine nerve terminals. End plates were localized by repeated insertion of the pipettes into muscle fibers near the terminal branches until maximum amplitude and minimum time to peak of the EPPs or (in the postsynaptic carbachol-sensitivity experiments) miniature EPPs were obtained. The amplified EPPs were recorded with a FM tape recorder (dc-2,500 Hz) and later transferred to chart paper or photographed. The amplitudes of 25–50 EPPs were averaged in each experimental condition and displayed as percentage reduction from controls.

RESULTS

Effects of Metalloendoprotease Inhibitors and Synthetic Substrates on Synaptic Transmission. Proteases can be classified into four groups: serine, thiol, metal, and acid (16). Metalloendoproteases are a family of enzymes characterized by a catalytically essential metal atom in their active site (17). These proteases are further characterized by their inhibitors and synthetic substrates and by their pH optima for hydrolysis. The substrate specificities and inhibitors of proteases in the metalloendoprotease group are very similar.

Phosphoramidon is a high-affinity competitive inhibitor of metalloendoproteases and does not inhibit serine, thiol, or acid endoproteases (18, 19). Thus, since phosphoramidon is a specific inhibitor of metalloendoproteases, it can be used to determine whether this group of proteases is involved in a particular process. As shown in Fig. 1*A*, addition of 35 μM phosphoramidon to the bathing solution reduced the amplitude of the evoked EPP by 55%. The reduction in EPP amplitude was complete within 2 to 3 min after addition of phosphoramidon and remained constant as long as phosphoramidon was in the bath (up to 2 hr). The reduction in synaptic transmission by phosphoramidon was dose dependent, as shown in Fig. 1*B*. The effect of phosphoramidon was completely reversible; EPP amplitudes returned to control values within 2 to 3 min after removal of phosphoramidon from the bath (Fig. 1*A*).

The reduction in EPP amplitudes by phosphoramidon suggested that a metalloendoprotease is required for synaptic transmission. If so, synthetic substrates of known metalloendoproteases should compete with the natural protein substrate and thereby reduce synaptic transmission. Synthetic dipeptide substrates for metalloendoproteases require a dipeptide backbone in which the terminal carboxyl and amino groups are blocked (20). Metalloendoproteases hydrolyze synthetic carbobenzoxy (Cbz)-dipeptide-amides if the amino group of the dipeptide is contributed by a large aliphatic amino acid such as leucine or by a nonpolar aromatic amino acid such as phenylalanine. Hydrolysis of the synthetic substrates is also altered by the amino acid that contributes the carboxyl group, but this secondary interaction exerts a relatively small effect on hydrolysis, compared with the primary specificity.

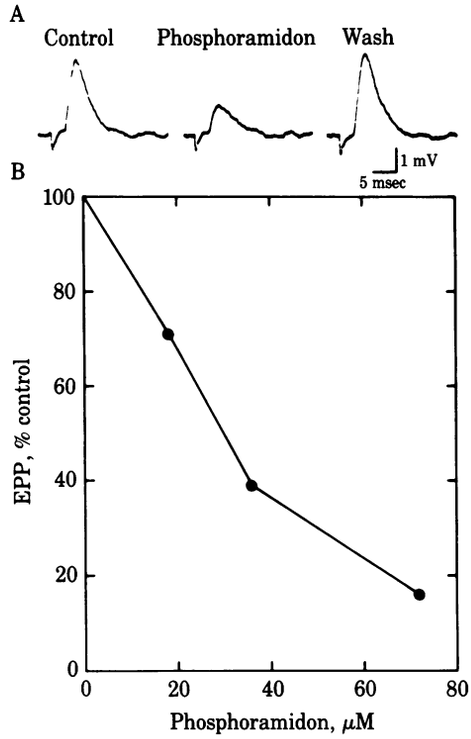

FIG. 1. Effect of phosphoramidon on evoked EPP. (*A*) Mouse hemidiaphragm preparations *in vitro* were perfused sequentially in bathing solutions containing 10 μM curare and 1% dimethylformamide (control and wash) and 10 μM curare and 35 μM phosphoramidon in 1% dimethylformamide while the muscle EPPs were recorded intracellularly during electrical stimulation of the phrenic nerve. (*B*) Effect of increasing the concentration of phosphoramidon on the amplitude of the evoked EPPs.

The effects of various Cbz-dipeptide-amides on synaptic transmission are shown in Fig. 2. The EPPs shown in Fig. 2*A* were recorded from the same muscle fiber. Bath application of 0.8 mM Cbz-Gly-Pro-amide, which *is not* a metalloendoprotease substrate, reduced EPP amplitude by ≈5%. However, bath application of 0.8 mM Cbz-Gly-Phe-amide, which *is* a metalloendoprotease substrate, reduced EPP amplitude by 60%. The amino acid specificity of the synthetic substrates that reduced synaptic transmission is shown in Fig. 2*B*. Cbz-dipeptide-amide derivatives in which leucine or phenylalanine contribute the amino group were effective in reducing EPP amplitudes. In addition to this primary specificity, greater reductions in EPP amplitudes occurred with dipeptide substrates in which glycine provided the carboxyl group than with tyrosine or serine (0.5 mM Cbz-Ser-Leu-amide reduced EPP amplitudes by 33%; data not shown). The synthetic dipeptide substrates reduced synaptic transmission in a dose-dependent manner, and the amplitude of the EPPs remained reduced as long as the compounds were present in the bath. The reduction in EPP amplitudes was complete within 2 to 3 min after introduction of the Cbz-dipeptide-amides into the bath, and inhibition by these synthetic substrates was completely reversible.

A free carboxyl- or amino-end group in a dipeptide greatly decreases hydrolysis by metalloendoproteases (20), and the ability of the synthetic substrate Cbz-Gly-Phe-amide to reduce synaptic transmission was eliminated by removing either the Cbz or the amide group (Fig. 3). Similar results were observed when the amide group was removed from Cbz-Tyr-Leu-amide. Although 0.5 mM Cbz-Tyr-Leu-amide reduced EPP amplitudes by 47–67%, 0.5 mM Cbz-Tyr-Leu had no effect on syn-

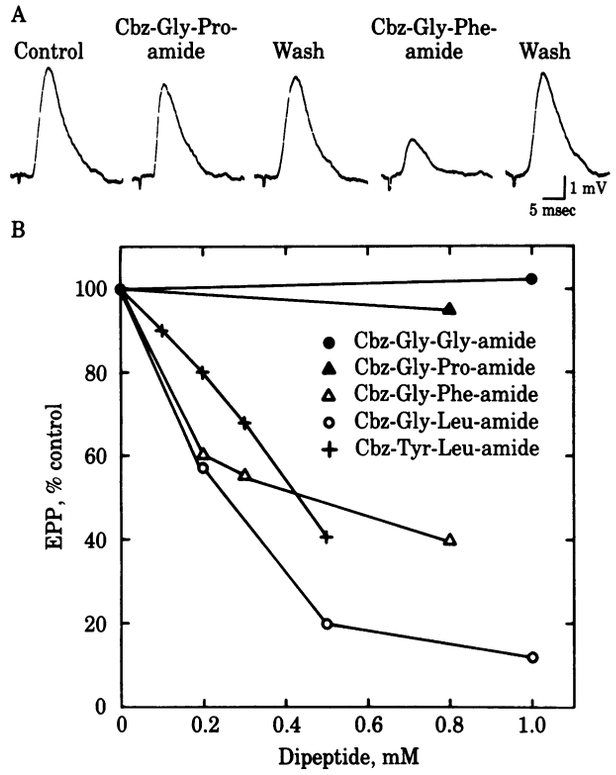

FIG. 2. Effects of synthetic dipeptides on evoked EPPs. (*A*) Intracellularly recorded evoked EPPs recorded during sequential application of bathing solution (control), 0.8 mM Cbz-Gly-Pro-amide, bathing solution (wash), 0.8 mM Cbz-Gly-Phe-amide, and bathing solution (wash). (*B*) Effects of synthetic dipeptides at various concentrations on evoked EPPs. ●, Cbz-Gly-Gly-amide; ▲, Cbz-Gly-Pro-amide; △, Cbz-Gly-Phe-amide; ○, Cbz-Gly-Leu-amide; +, Cbz-Tyr-Leu-amide; SEMs of averaged EPPs were <10% when three or more observations of averaged EPPs were made.

aptic transmission. Therefore, synaptic transmission was inhibited only by synthetic dipeptide substrates in which the amino group of the peptide bond was provided by a bulky hydrophobic amino acid and both the terminal carboxyl and the terminal amino groups were blocked. The ability of the synthetic dipeptides to inhibit synaptic transmission was similar to their reported specificity to serve as substrates for metalloendoproteases, and the concentrations of the dipeptides required for inhibition of synaptic transmission were similar to the K_m values for their interactions with known metalloendoproteases (20).

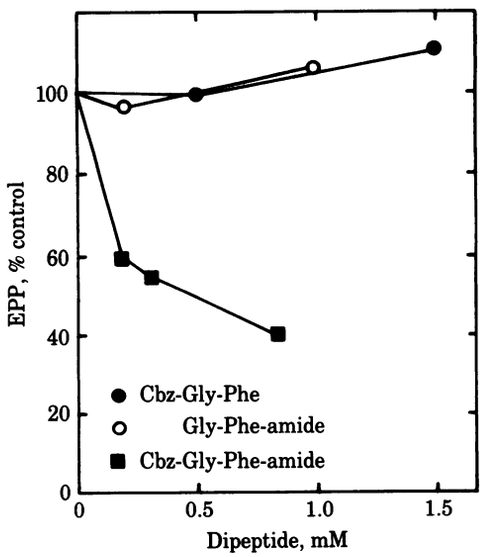

FIG. 3. Effects of amino- and carboxyl-blocking groups on inhibition of evoked EPPs by Cbz-Gly-Phe-amide. Evoked EPPs were recorded during bath application of various concentrations of Cbz-Gly-Phe-amide (■), Cbz-Gly-Phe (●), and Gly-Phe-amide (○).

The time required to inhibit synaptic transmission by phosphoramidon or by the synthetic substrates appeared to be independent of nerve stimulation. The amplitudes of the EPPs were reduced to the same extent whether the phrenic nerve was stimulated continuously at 0.2 Hz while the inhibitor was perfusing into the muscle chamber or the nerve stimulation began 2 to 3 min after introduction of the inhibitor compound. The 2- to 3-min latency to onset of inhibition most likely resulted from the time required for the compounds to reach the site of protease activity in a concentration sufficient to compete for the enzyme.

Location of Metalloendoprotease Activity. These results show that synaptic transmission is reduced by compounds that competitively inhibit metalloendoproteases. One way to determine whether this protease is required at a pre- or postsynaptic site is to test whether the inhibitors affect the postsynaptic response of the muscle to exogenous neurotransmitter. Therefore, depolarizations of muscle fibers were measured during bath application of carbachol before, during, and after the addition of protease inhibitors. Carbachol (carbamylcholine chloride; Sigma) is a nonhydrolyzable acetylcholine analog that activates postsynaptic acetylcholine receptors at the vertebrate neuromuscular junction (21–23). Thus, if the metalloendoprotease is required at a postsynaptic site, protease inhibitors should reduce the postsynaptic response to carbachol by the same amount as the EPP amplitudes. Conversely, if the metalloendoprotease is required presynaptically, no reduction in the postsynaptic carbachol response should be observed with protease inhibitors.

Hemidiaphragm preparations were maintained in control saline without curare. The location of the recording microelectrode near an end plate was verified by recording miniature EPPs. Solutions containing carbachol were perfused through the chamber for 1 min. Increasing the duration of the carbachol perfusion to 2 min did not alter the amplitude of the depolarization. Muscle fibers were perfused with a single concentration of carbachol at least three times, before, during, and after introduction of each protease inhibitor, and at least 20 min separated each carbachol application.

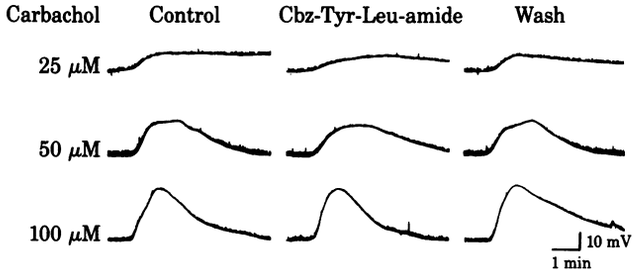

FIG. 4. Effect of Cbz-Tyr-Leu-amide on carbachol-induced EPPs. Membrane potentials of muscle fibers were recorded near the end plate during perfusion for 1 min with each of the following bathing solutions: 25, 50, and 100 μM carbachol (control). Muscle fibers were then incubated for 10 min with 0.5 mM Cbz-Tyr-Leu-amide and the membrane potential was measured after addition of 25, 50, and 100 μM carbachol. The Cbz-Tyr-Leu-amide was then removed (wash), the muscle was bathed for 10 min with control bathing solution, and the membrane potential was recorded after addition of 25, 50, and 100 μM carbachol.

Depolarizations of three different muscle fibers by three concentrations of carbachol, before, during, and after perfusion with Cbz-Tyr-Leu-amide, are shown in Fig. 4. After a 1-min carbachol perfusion, the depolarizations increased in amplitude with increasing concentrations of carbachol. Although 0.5 mM Cbz-Tyr-Leu-amide reduced EPP amplitudes by an average of 60% (Fig. 2), the depolarizations of the muscle fibers, even at low concentrations of carbachol (Fig. 4), were not altered by this dipeptide substrate. These data suggest that the inhibition of synaptic transmission by Cbz-Tyr-Leu-amide occurs at the presynaptic nerve terminal.

As additional support for this possibility, a dose–response curve was obtained for the bath application of carbachol to mouse hemidiaphragm muscle (Fig. 5). The maximum carbachol depolarization was 37 mV, and the amount of carbachol required to produce 50% of the maximum response was 55 μM. Bath application of phosphoramidon (40 μM) or of several synthetic substrates (0.5 mM) did not alter the postsynaptic carbachol dose–response curve. Because the response of the muscle fibers to carbachol at a wide range of concentrations was unaffected by protease inhibitors and the substrates, inhibition of synaptic transmission by these compounds may occur at a presynaptic site and result from a reduction in the presynaptic release of transmitter.

The metalloendoprotease inhibitor and substrates had no effect on the postsynaptic carbachol responses, and no changes were observed in the passive membrane properties of the postsynaptic cells to account for the reduction in EPP amplitudes. The resting membrane potentials of the muscle fibers ranged from −70 to −85 mV, the input resistances were 0.8–2.5 MΩ, and the time constants were 1.8–6.0 msec. Bath application of phosphoramidon or synthetic substrates, at concentrations that inhibited synaptic transmission, did not alter the resting membrane potentials of the muscle fibers, and all EPPs were recorded at membrane potentials equal to the original control values. In addition, the protease inhibitors did not change the input resistances or the time constants of the muscle fibers. Bath application of 0.5 mM Cbz-Tyr-Leu-amide produced no apparent change in the threshold or in the amplitude and duration of the muscle action potential. Thus, the reduction in EPP amplitudes by metalloendoprotease inhibitors was not accompanied by changes in the passive electrical properties of the postsynaptic cells, in the excitability of the muscle cells, or in the response to carbachol. Since no postsynaptic effects of these metalloendoprotease inhibitors could be identified, their inhibition of EPPs may occur at a presynaptic site.

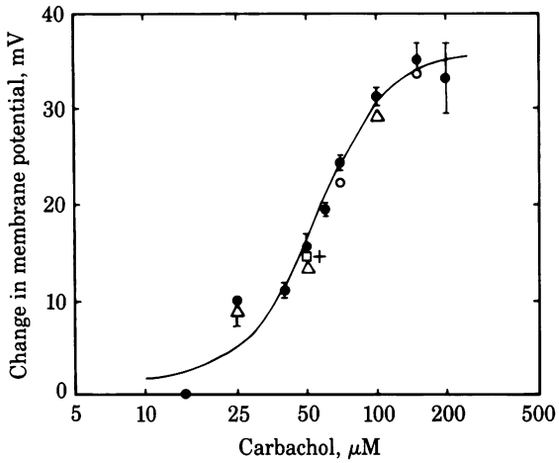

FIG. 5. Effects of metalloendoprotease inhibitor and dipeptide substrates on postsynaptic response to various concentrations of bath-applied carbachol. Muscle-fiber membrane potentials in control bath solution were recorded intracellularly near the end plate after 1 min of perfusion with carbachol (●) at various concentrations (increments of 0.5 μM). Then, the muscle fibers were incubated for 10 min with the specific compound, and the membrane potentials were recorded after 1-min perfusion with △, 0.5 mM Cbz-Tyr-Leu-amide and 25, 50, and 100 μM carbachol; ○, 0.5 mM Cbz-Gly-Leu-amide and 70 and 150 μM carbachol; □, 0.5 mM Cbz-Gly-Phe-amide and 50 μM carbachol; +, 72 μM phosphoramidon and 50 μM carbachol. The curve was generated by a four-parameter logistic function, using a computer program for a simultaneous least-squares fit (cf. ref. 24).

DISCUSSION

Nerve-evoked muscle EPPs were reduced by inhibitors of and synthetic dipeptide substrates for metalloendoproteases. The metalloendoprotease inhibitor phosphoramidon, at concentrations required to inhibit mammalian metalloendoproteases (16), decreased the amplitudes of the EPPs. The ability of the dipeptides to reduce the amplitudes of the EPPs closely parallels their specificity for metalloendoproteases. No other effects, such as changes in resting potentials, input resistances, or time constants of the muscle fibers, that could account for the observed changes in EPPs amplitudes were observed with any of the protease inhibitors. Although changes in the presynaptic action potential cannot be ruled out, the muscle-fiber action potential appeared to be unaltered by one of the more potent inhibitors of EPPs. Finally, in an attempt to localize the site at which the metalloendoprotease activity occurs in synaptic transmission, we tested the effects of protease inhibitors on the postsynaptic depolarization produced by exogenous carbachol. No changes in the postsynaptic carbachol depolarizations were observed over a broad range of carbachol concentrations, consistent with a presynaptic site for the action of the metalloendoprotease. It is important to note, however, that receptor desensitization may have affected the carbachol depolarizations and limited our measurements to the low end of the carbachol dose–response curve. A more direct demonstration of changes in transmitter release would require quantal analysis of evoked EPPs and chemical measurement of acetylcholine release.

Metalloendoproteases could be required for synaptic transmission at any step in the sequence of events during which the presynaptic terminal releases acetylcholine into the synaptic cleft. Also, the proteases could be located either intra- or extracellularly. The possible roles of the proteases include, but are not limited to, regulation of calcium influx across the presynaptic membrane, of calcium concentration in the terminal, or of binding of calcium to intracellular sites; regulation of the mechanisms by which calcium leads to vesicle fusion with the presynaptic membrane; and alteration of the number of vesicles available for release, the number of release sites, or the concentration of acetylcholine in the vesicles.

Metalloendoprotease activity is required in the calcium-dependent exocytosis of histamine by mast cells (unpublished data). The mast cell releases previously formed mediators, including histamine, by fusion of secretory vesicles with the cell plasma membrane after influx of calcium into the cell (25, 26). Metalloendoprotease inhibitors and substrates prevent mast cell exocytosis initiated by either concanavalin A or calcium ionophore A23187 and calcium. Since these protease inhibitors prevent the exocytosis caused by direct introduction of calcium, metalloendoprotease activity is required for mast cell exocytosis at a step after calcium entry. The mast cell metalloendoprotease can be identified with a fluorogenic protease substrate.

The fusion of myoblasts to multinucleate myotubes is similarly calcium dependent and requires metalloendoprotease activity at the time of fusion (12). When metalloendoprotease in-

hibitors and synthetic dipeptide substrates are added to the culture medium with calcium, myoblast fusion is prevented. We have identified and partially characterized the myoblast metalloendoprotease with a fluorogenic protease substrate, and the protease is inhibited by the same compounds that prevent fusion.

Proteolysis is also required for the fusion of the myxoviruses with their host cells (27). Influenza virus (28), for example, contains a transmembrane glycoprotein, hemagglutinin, that must be precisely hydrolyzed by a protease into two polypeptide fragments, HA1 and HA2, before the virus can fuse with its host cell. The amino terminus of the fusion-promoting HA2 peptide is a hydrophobic region that may interact with the host cell membrane.

CONCLUSIONS

Various inhibitors of metalloendoproteases reduced nerve evoked EPPs generated at mouse diaphragm/phrenic nerve preparations. The reduction in EPP amplitudes was dependent on the concentration of each inhibitor and was reversible. No postsynaptic effects of the inhibitors were observed, and thus the results are consistent with the hypothesis that nerve-evoked acetylcholine release at the neuromuscular junction requires the activity of a metalloendoprotease. Similar conclusions have been reached for the involvement of metalloendoproteases in histamine release from mast cells (unpublished data) and for the fusion of myoblasts (12). The role and location of the metalloendoproteases in these processes are unknown.

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