0270-6474/84/0412-3112$02.00/0

The Journal of Neuroscience
Vol. 4, No. 12, pp. 3112–3119
December 1984

INHIBITORS OF METALLOENDOPROTEASE ACTIVITY PREVENT K^+-STIMULATED NEUROTRANSMITTER RELEASE FROM THE RETINA OF *XENOPUS LAEVIS*[1]

JEANNE M. FREDERICK,*,[2] JOE G. HOLLYFIELD,*‡ AND WARREN J. STRITTMATTER‡§

*Cullen Eye Institute, ‡Program in Neuroscience, and §Departments of Neurology and Biochemistry, Baylor College of Medicine, Houston, Texas 77030

Received March 21, 1984; Revised June 11, 1984; Accepted June 15, 1984

Abstract

Metalloendoprotease activity was identified in retinal homogenates using a synthetic fluorogenic metalloendoprotease substrate and specific metalloendoprotease inhibitors. The requirement of metalloendoprotease activity in neurotransmitter release was examined during the depolarization-induced release of [^{3}H]glycine from the retina of *Xenopus laevis*. Neurons with high affinity uptake and calcium-dependent, K^+-stimulated release of glycine have been described previously in this retina. When isolated retinas preloaded with [^{3}H]glycine are depolarized by 22 mM K^+, the usual efflux of [^{3}H]glycine is completely abolished by the metalloendoprotease inhibitor, 1,10-phenanthroline (100 μg/ml). The inhibition of [^{3}H]glycine release by 1,10-phenanthroline is dose dependent; furthermore, 1,10-phenanthroline blocks release by chelating metal and not Ca^{2+}, since addition of equimolar calcium does not alter the inhibition. The metalloendoprotease inhibitor, carbobenzoxy (CBZ)-L-phenylalanine, also prevents release, whereas the amino acid, L-phenylalanine, has no effect. Synthetic carbobenzoxy dipeptide amides which are metalloendoprotease substrates (e.g., CBZ-Gly-Leu-amide, CBZ-Ser-Leu-amide, and CBZ-Gly-Phe-amide) also prevent [^{3}H]glycine release in a dose-dependent and reversible manner. The synthetic dipeptide CBZ-Gly-Gly-amide, however, is not a metalloendoprotease substrate and has no effect on release. The ability of synthetic dipeptides to inhibit neurotransmitter release is amino acid specific, dose dependent, reversible, and matches their ability to interact with characterized metalloendoproteases. Depolarization-stimulated transmitter release may therefore require the activity of a metalloendoprotease.

Neurotransmitter release is achieved through the fusion of synaptic vesicles with the presynaptic membrane resulting in the liberation of vesicle contents into the extracellular space. Neuronal depolarization, either by the action potential or by increasing the extracellular K^+ concentration, causes a transient influx of calcium into the presynaptic terminal and initiates the fusion of synaptic vesicles with the plasma membrane (Kelly et al., 1983). Neurotransmitter release thus involves calcium-dependent membrane fusion. The biochemical mechanisms regulating calcium-dependent membrane fusion are incompletely understood but are probably responsible for regulating the timing, extent, and site of fusion.

Metalloendoproteases are metal-dependent proteolytic enzymes which hydrolyze certain peptide bonds distant from the ends of polypeptide chains (Barrett, 1977). Activity of endogenous metalloendoproteases has been proposed as a requirement for calcium-dependent membrane fusion in the fusion of mononucleate myoblasts to form multinucleated myotubes (Couch and Strittmatter, 1983) and in neurotransmitter release at the neuromuscular junction (Baxter et al., 1983). Electrophysiologic examination of the neuromuscular junction did not directly demonstrate presynaptic inhibition of neurotransmitter release by metalloendoprotease inhibitors, and biochemical metalloendoprotease assays could not be performed on the synaptic terminal. Consequently, we are examining the possibility in the retina that metalloendoprotease activity within the presynaptic terminal may be required for neurotransmitter release.

A population of retinal neurons in *Xenopus laevis* accumulates [^{3}H]glycine by high affinity uptake and releases accumulated glycine in response to elevated K^+ in the extracellular medium (Rayborn et al., 1981). The isolated retina preparation has several advantages as a model system for studying the requirement of metalloendoprotease activity during neurotransmitter release. The glycine-accumulating neurons are intact, unlike brain slice or synaptosome preparations, and [^{3}H]glycine release can be directly and quantitatively assayed. The depolarization-induced release of retinal glycine is calcium dependent (Rayborn et al., 1981). Finally, metalloendoprotease activity can be identified and characterized in the retina.

Materials and Methods

Autoradiographic demonstration of high affinity uptake of [^{3}H]glycine by retinal neurons. Juvenile and adult toads, *Xenopus laevis*, were obtained from Nasco (Ft. Atkinson, WI) and Carolina Biological Supply

[1] This work was supported by grants from the Retina Research Foundation, Houston, Texas, by National Institutes of Health Grants EY 04757 and EY 02363, and by a Teacher-Investigator Award from the National Institute of Neurological and Communicative Disorders and Stroke (W. J. S.). J. G. H. is an Olga K. Wiess Scholar of Research to Prevent Blindness, Inc., New York, New York.

[2] To whom correspondence should be addressed.

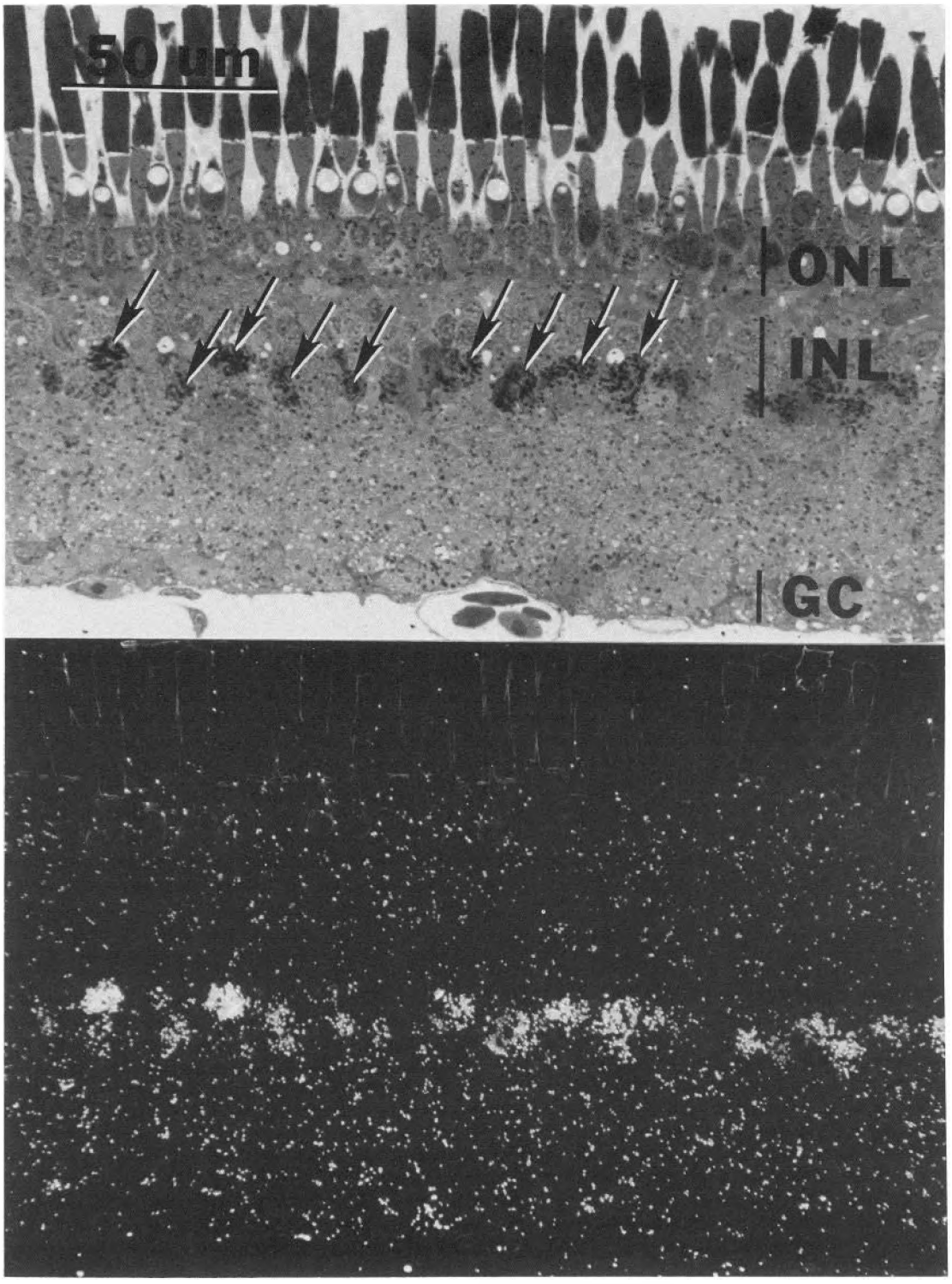

Figure 1. Localization of [^{3}H]glycine uptake by retinal neurons in *Xenopus laevis*. Photomicrographs were taken of retinas incubated for 15 min with 2 µM [^{3}H]glycine before processing for autoradiography. Heavily labeled neurons are present in the inner nuclear layer (*arrows*). The two micrographs, showing the same retinal area, are photographed with transmitted (*upper*) and darkfield (*lower*) illumination. *ONL*, outer nuclear layer, *INL*, inner nuclear layer; *GC*, ganglion cell layer.

Co. (Burlington, NC). The animals were dark-adapted overnight prior to dissection. After removing the anterior segment, each retina was isolated from its vitreous humor and pigment epithelium in minimal essential medium at 5°C (Frederick et al., 1982).

The autoradiographic methods have been described (Hollyfield et al., 1979). Briefly, isolated retinas were incubated with 2 µM [^{3}H]glycine for 15 min, rinsed in fresh medium, transferred through the serial changes of media of a release experiment (see below), fixed overnight in 2% glutaraldehyde/1% formaldehyde in 100 mM Sorenson's phosphate buffer (pH 7.35), postfixed for 30 min in 1% osmium tetroxide in the same buffer, and embedded in Epon. After polymerization, 1-µm-thick sections were cut, coated with Kodak NTB2 liquid emulsion,

and exposed for 6 hr to 3 days. The autoradiograms were developed, stained, and examined with transmitted and darkfield microscopy to identify the cells which localize [^{3}H]glycine.

Determination of [^{3}H]glycine release. Twenty retinas were transferred by pipette to a 1.5-ml conical centrifuge tube for incubation for 15 min in 200 μl of medium containing 20 μCi of [^{3}H]glycine (specific activity, 44 Ci/mmol, New England Nuclear, Boston, MA; 2 μM final concentration). The retinas were washed in three 5-ml changes of fresh medium (10 min each) with gentle agitation and were washed for an additional two 10-min intervals with either fresh medium or medium containing 1,10-phenanthroline. Fresh medium containing the carbobenzoxy (CBZ) dipeptide amides was added 6 min before depolarization. Groups of four retinas were transferred every 3 min to 2 ml of either normal medium or isotonic K^+-rich medium in which 19 mM KCl had been substituted for NaCl, for a final KCl concentration of 22 mM. Incubation in 22 mM K^+ buffer is indicated by the *bar* on the *abscissa* of each figure. One milliliter of incubation medium was mixed with 10 ml of scintillant (ACS), and the radioactivity was measured by a liquid scintillation counter (Packard Tri-Carb). The retinas were homogenized in 1 ml of medium and mixed with scintillant, and the radioactivity was determined. Release of [^{3}H]glycine into the media was calculated as a percentage of disintegrations per minute of [^{3}H]glycine released into the medium following each incubation to the disintegrations per minute of [^{3}H]glycine remaining in the tissue at the corresponding sample time. All dipeptide derivatives were obtained from Vega Biochemicals (Phoenix, AZ), and 1,10-phenanthroline was obtained from Sigma Chemical Co (St. Louis, MO).

High voltage paper electrophoresis was used to estimate the percentage of radioactivity released into the media by depolarization which was free [^{3}H]glycine. Aliquots of 22 mM K^+ buffer from release experiments were brought to dryness in a concentrator (Savant) and reconstituted with carrier solution (5 mg/ml of glycine, 5 mg/ml of choline chloride in 0.1 N HCl). Ten microliters of sample were spotted on paper for electrophoresis. After separation, glycine was identified with ninhydrin as described by Hildebrand et al. (1971) and eluted, and the radioactivity was measured.

Biochemical characterization of metalloendoprotease. The fluorescent protease substrate, 2-aminobenzoyl-alanine-glycine-leucine-alanine-4-nitrobenzylamide (AAGLAN), and the active site inhibitor, 2-(*N*-hydroxycarboxamide)-4-methylpentanoyl-L-Ala-Gly-amide, were synthesized by D. Rasnick (Enzyme Systems Products, Livermore, CA). Metalloendoprotease activity was characterized in the cytoplasmic and pellet fractions of homogenized retinas. Forty retinas from dark-adapted, adult *Xenopus* were isolated and suspended in 3.5 ml of 150 mM KCl in 10 mM HEPES, pH 7.3, at 5°C. The retinas were homogenized with 15 strokes in a glass-glass homogenizer. The homogenate was then centrifuged at 100,000 × *g* for 2 hr; the supernatant and pellet were collected and frozen at −80°C until used.

Metalloendoprotease activity was assayed in 50 μl of the supernatant containing a total of 50 μg of protein, in a final volume of 150 μl of 150 mM KCl, 10 mM HEPES, pH 7.3. The final concentration of AAGLAN was 0.3 mM in 2% dimethylformamide. After incubation at 37°C for 5 to 8 hr, the hydrolyzed substrate was measured spectrofluorometrically (Aminco Bowman Model SPF-500) with an excitation wavelength of 340 nm and an emission wavelength of 415 nm. The amount of substrate hydrolyzed was calculated by comparison with a standard curve established by complete hydrolysis of AAGLAN (0 to 1.0 mM) with 2 μg/ml of thermolysin. The results are the mean of duplicate incubations and are expressed as nanomoles of AAGLAN hydrolyzed per milligram of protein per hour of incubation.

Results

Identification of high-affinity [^{3}H]glycine uptake in retinal neurons. To localize and identify neurons which have high affinity uptake for glycine, isolated retinas were incubated with 2 μM [^{3}H]glycine and processed for autoradiography. As seen in Figure 1, [^{3}H]glycine is accumulated by perikarya of the inner nuclear layer. Silver grains also appear scattered throughout the inner and outer synaptic layers. Ultrastructural studies (Rayborn et al., 1981) have shown [^{3}H]glycine accumulation of the inner and outer plexiform layers within bulbous terminals having pre- and postsynaptic densities and synaptic vesicles. [^{3}H]Glycine is taken up with high affinity by a population of retinal neurons, and not glia.

Demonstration of Ca^{2+}-dependent, depolarization-induced release of [^{3}H]glycine. Neurons which demonstrate high affinity uptake of [^{3}H]glycine will release [^{3}H]glycine when depolarized in media containing 22 mM K^+. Figure 2 shows efflux of [^{3}H]glycine from retinas depolarized three times in succession (Fig. 2, *solid bars* on *abscissa*). However, 3 mM $CoCl_2$ in the medium blocks calcium influx and prevents depolarization-induced efflux (Fig. 2, *middle bar* labeled *K^+ and Co^{2+}*). The release appears to be calcium dependent. High voltage paper electrophoresis of media collected during K^+-stimulated efflux reveals that >50% of the released radioactivity co-migrates with free glycine. In *Xenopus*, certain retinal neurons show specific, high affinity uptake of glycine and release of accumulated glycine in response to depolarizing stimuli.

Demonstration that metalloendoprotease inhibitors prevent depolarization-induced release of the neurotransmitter, glycine. 1,10-Phenanthroline is a metal chelator which inactivates metalloproteases and has a high critical stability constant for

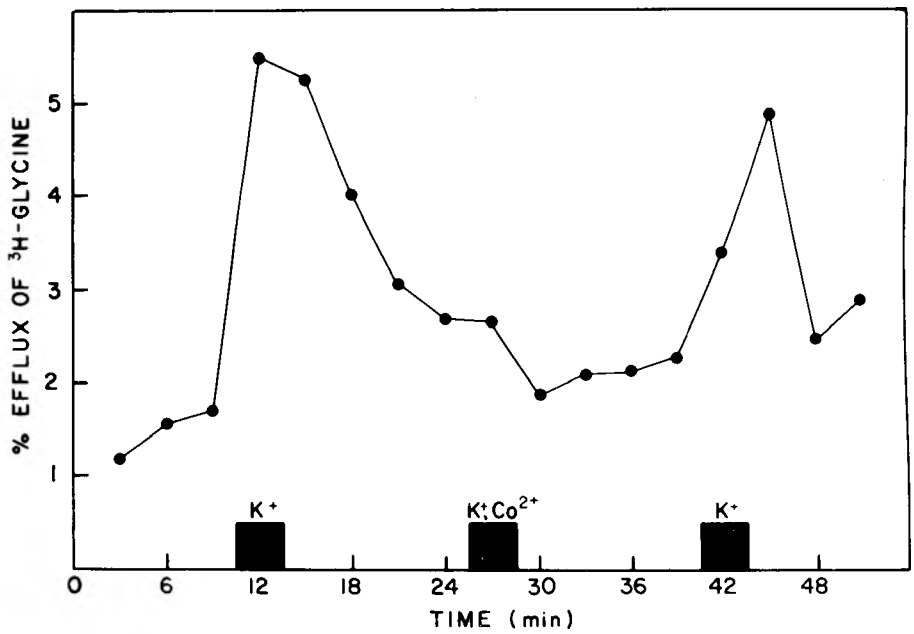

Figure 2. K^+-stimulated release of [^{3}H]glycine from retinal neurons. After preloading with [^{3}H]glycine, retinas were transferred at 3-min intervals through incubation media of either normal (3 mM) or high (22 mM, *solid bars*) K^+. The incubation solutions were collected and monitored for radioactivity. Large amounts of label were released following the first K^+-depolarization, whereas simultaneous incubation with 22 mM K^+ and 3 mM Co^{2+} caused no efflux of radioactivity above base line levels. After removing Co^{2+}, K^+-depolarization again caused the release of additional radioactivity.

metals and a low constant for calcium (for Zn^{2+} $K = 2 \times 10^6$ M; for Ca^{2+} $K = 5$ M) (Smith and Martell, 1975). When added to the medium after retinas were loaded with [^{3}H]glycine, 1,10-phenanthroline blocked the depolarization-induced release of [^{3}H]glycine (Fig. 3). To demonstrate that 1,10-phenanthroline inhibited release by chelating metal and not calcium, excess calcium equimolar to 1,10-phenanthroline was added, and release was still blocked (Fig. 3). The inhibition of release by 1,10-phenanthroline was dose dependent (Fig. 4). The concentration of 1,10-phenanthroline required to block release completely (100 μg/ml) is the same concentration required to completely inhibit the retinal metalloendoprotease (see Fig. 10). CBZ-L-Phenylalanine is a reversible inhibitor of metalloendoproteases (Burstein et al., 1974). CBZ-L-Phenylalanine prevented [^{3}H]glycine release in a dose-dependent manner, whereas L-phenylalanine, which has no effect on metalloendoprotease activity, had no effect on neurotransmitter release (Fig. 5).

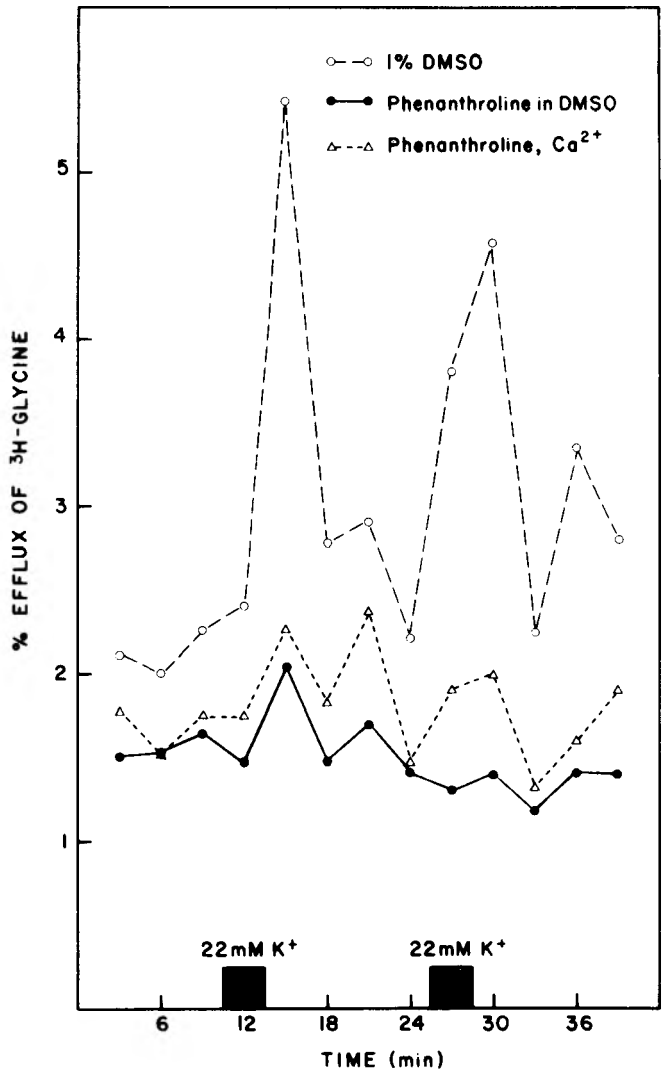

Figure 3. Inhibition of K^+-stimulated release of [^{3}H]glycine by 1,10-phenanthroline. Retinas preloaded with [^{3}H]glycine were incubated for 20 min in either 1% dimethylsulfoxide (DMSO) (○), 1,10-phenanthroline (200 μg/ml, 1.1 mM) in 1% DMSO (●), or 1,10-phenanthroline with equimolar $CaCl_2$ (△). The retinas were then transferred at 3-min intervals to incubation media with normal or 22 mM K^+ (*solid bars*); released radioactivity was later measured in the incubation solutions. Retinas incubated in 1,10-phenanthroline with DMSO fail to release [^{3}H]glycine in response to K^+-depolarization, whereas retinas incubated in DMSO alone release large amounts of radioactivity. Addition of Ca^{2+} equimolar with 1,10-phenanthroline, in addition to the normal Ca^{2+} composition of the medium, does not prevent the inhibition of [^{3}H] glycine release.

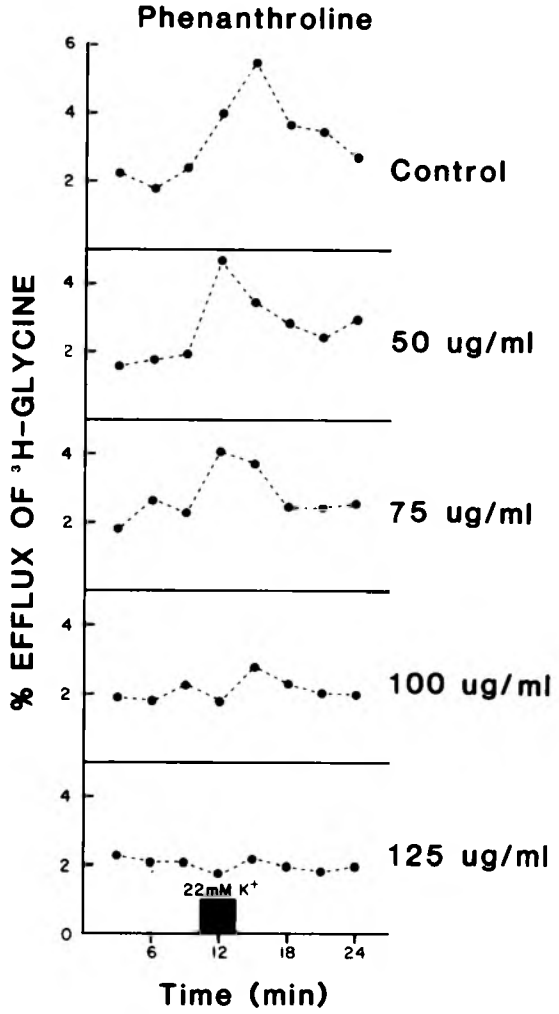

Figure 4. Concentration-dependent inhibition by 1,10-phenanthroline of [^{3}H]glycine release in response to K^+-depolarization. Retinas preloaded with [^{3}H]glycine were incubated with phenanthroline at various concentrations for 20 min before transfer to successive changes of media at 3-min intervals. Aliquots of media were counted for released radioactivity. Note the progressive reduction in the amount of radioactivity released with increasing phenanthroline concentrations.

Metalloendoproteases will hydrolyze CBZ dipeptide amides only if the derivative contains a peptide bond hydrolyzed by this protease. Metalloendoproteases hydrolyze CBZ-Gly-Leu-amide, CBZ-Ser-Leu-amide, and CBZ-Gly-Phe-amide, but not CBZ-Gly-Gly-amide (Morihara, 1974). We tested these dipeptides for their effects on the depolarization-induced release of glycine, to determine whether their ability to block neurotransmitter release paralleled the ability to interact with metalloendoproteases. The dipeptide CBZ-Gly-Phe-amide, which is a metalloendoprotease substrate, blocked depolarization-induced glycine release, whereas CBZ-Gly-Gly-amide, which is not metalloendoprotease substrate, had no effect (Figs. 6 and 7, *top*). The inhibition of [^{3}H]glycine release by CBZ-Gly-Phe-amide was reversible (Fig. 6). CBZ-Gly-Phe-amide blocked release in a dose-dependent fashion (Fig. 7). The dipeptide metalloendoprotease substrates CBZ-Gly-Leu-amide and CBZ-Ser-Leu-amide similarly blocked depolarization-induced [^{3}H]glycine release (not shown). Hence, inhibition of neurotransmitter

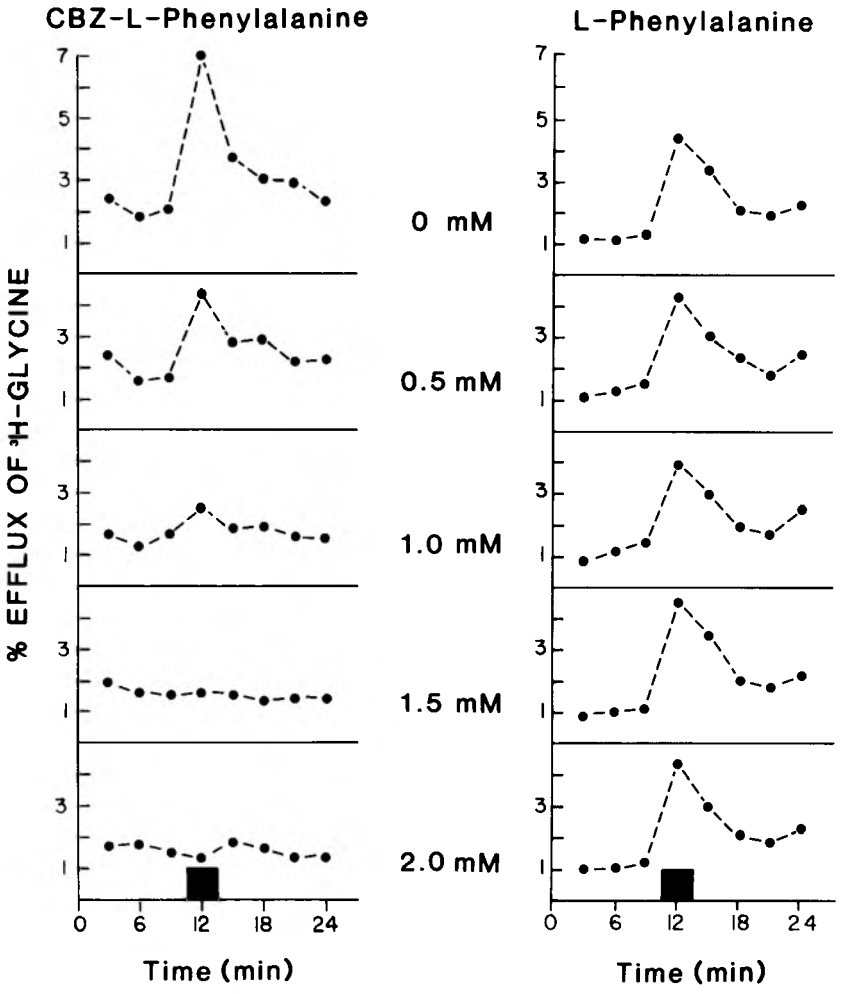

Figure 5. Specificity of a CBZ-amino acid inhibition of [^{3}H]glycine release. Retinas preloaded with [^{3}H]glycine were preincubated with CBZ-L-phenylalanine or L-phenylalanine for 12 min before K^+-depolarization. The K^+-Stimulated efflux of radioactivity was inhibited by increasing CBZ-L-phenylalanine concentrations and was unaltered by L-phenylalanine.

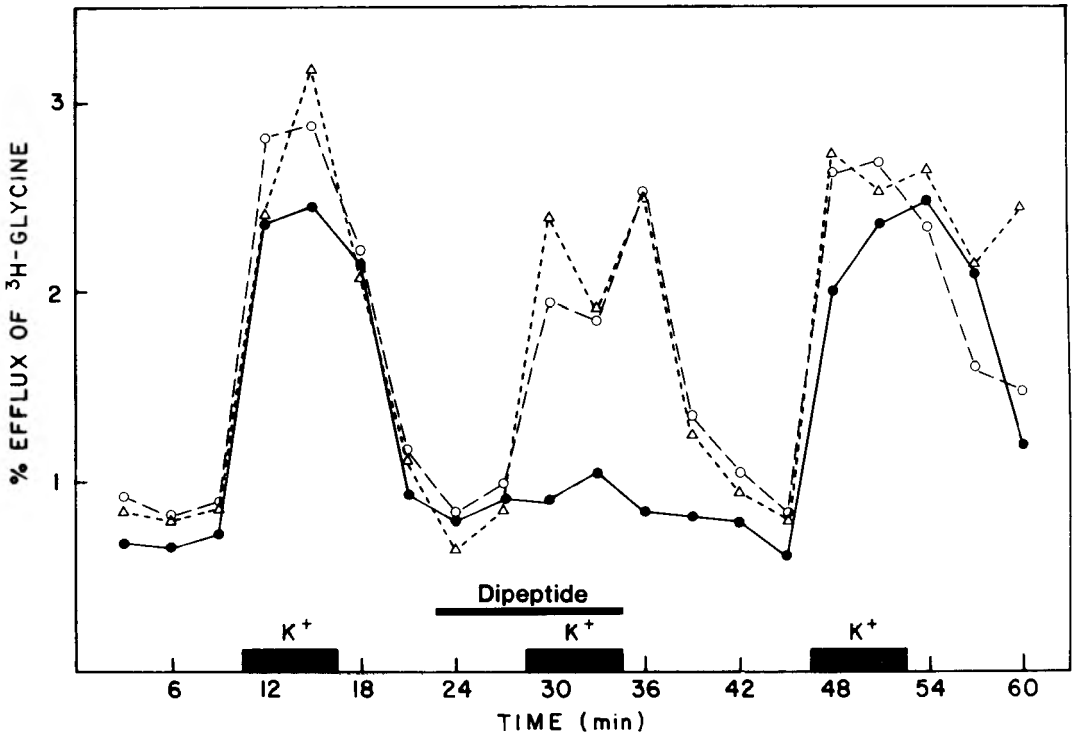

Figure 6. Specificity and reversibility of CBZ-dipeptide amides as inhibitors of K^+-stimulated [^{3}H]glycine release. Retinas preloaded with [^{3}H]glycine were transferred at 3-min intervals to media with normal or 22 mM K^+ (*solid bars*). The first and third K^+ depolarizations were performed under identical conditions and involved neither dipeptide substrates nor dimethylsulfoxide. Between times t = 21 and t = 33 min, either 1% dimethylsulfoxide (△), 1.5 mM CBZ-Gly-Gly-amide in dimethylsulfoxide (○), or 1.5 mM CBZ-Gly-Phe-amide in dimethylsulfoxide (●) were introduced into the media. CBZ-Gly-Phe-amide inhibited [^{3}H]glycine efflux during the second K^+ pulse; inhibition was completely reversed after removing CBZ-Gly-Phe-amide. In contrast, neither CBZ-Gly-Gly-amide nor dimethylsulfoxide had any effect on the efflux of radioactivity in response to K^+ stimulation.

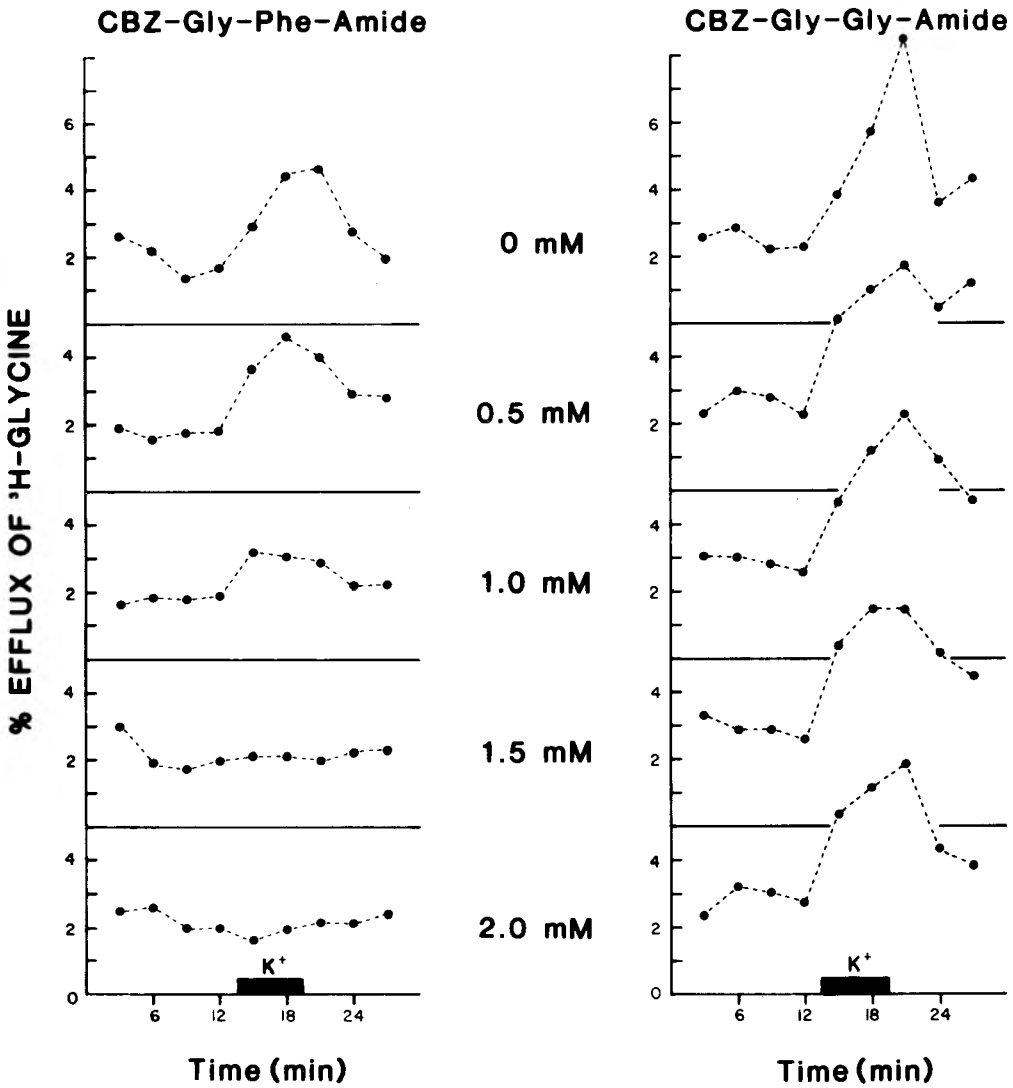

Figure 7. Specificity and dose-dependent inhibition by CBZ-dipeptide amides of K^+-stimulated [^{3}H]glycine release. Retinas preloaded with [^{3}H]glycine were incubated with either CBZ-Gly-Phe-amide (*left*) or CBZ-Gly-Gly-amide (*right*). The K^+-stimulated efflux of [^{3}H]glycine was reduced with increasing concentrations of CBZ-Gly-Phe-amide and was unaltered at any concentration of CBZ-Gly-Gly-amide.

release by synthetic metalloendoprotease substrate is amino acid specific, dose dependent, and reversible.

Identification and characterization of retinal metalloendoprotease. Assay of metalloendoprotease activity is based on hydrolysis of AAGLAN, a sensitive and specific fluorogenic synthetic protease substrate developed by Kam et al. (1979). AAGLAN contains the Gly-Leu bond hydrolyzed by metalloendoproteases. Hydrolysis of the peptide bond frees the fluorescent 2-aminobenzoyl group from intramolecular quenching by the 4-nitrobenzylamide. Fluorescence therefore quantitatively reflects the amount of substrate hydrolyzed.

The *Xenopus* retina contains soluble and membrane-associated metalloendoprotease activities which hydrolyze this fluorogenic metalloendoprotease substrate. Hydrolysis of AAGLAN by the soluble metalloendoprotease was linear for at least 8 hr (Fig. 8), was saturable with an apparent K_m of approximately 0.5 mM (Fig. 9), and was inhibited by the metal chelator 1,10-phenanthroline with an IC_{50} of approximately 20 μg/ml (Fig. 10). An active site metalloendoprotease inhibitor, 2-(*N*-hydroxycarboxamide)-4-methylpentanoyl-L-Ala-Gly-amide (Nishino and Powers, 1979), also inhibited the protease with an Ic_{50} of approximately 5 μM. Metalloendoprotease activity is found in both the cytoplasmic and pellet fractions of retinal homogenates. We do not know whether metalloendoprotease activity found in the pellet fraction is the same enzyme identified in the soluble fraction which is simply associated with the membranes, trapped in vesicles, or alternatively, a different metalloendoprotease. Studies are in progress to determine whether the soluble and membrane-associated retinal metalloendoproteases have different inhibitor specificities. Soluble (Orlowski et al., 1983) and membrane-associated (Orlowski and Wilk, 1981; Benuck et al., 1982; Matsas et al., 1983) metalloendoproteases have been documented extensively in brain.

Discussion

Metalloendoproteases are a class of soluble or membrane-bound proteases which are identified and characterized by specific inhibitors. Zinc, present in the native enzyme, is required for catalytic activity, although other metals may also confer proteolytic activity to the apoenzyme (Chlebowski and Coleman, 1976). The metal chelator, 1,10-phenanthroline, is a potent metalloendoprotease inhibitor and has received extensive use in studies of metalloendoproteases (Barrett, 1977).

In retinal homogenates we have demonstrated protease activity which hydrolyzes the fluorogenic metaloendoprotease substrate, AAGLAN, and which is inhibited by 1,10-phenanthroline. Depolarization-induced release of [^{3}H]glycine from retinal neurons *in vitro* is also inhibited by 1,10-phenanthroline. The dose-response relationships of 1,10-phenanthroline inhibition of metalloendoprotease activity in retinal homogenates and inhibition of [^{3}H]glycine release from retinal neurons were virtually identical (for comparison, see Figs. 3 and 10). 1,10-Phenanthroline does not block neurotransmitter release

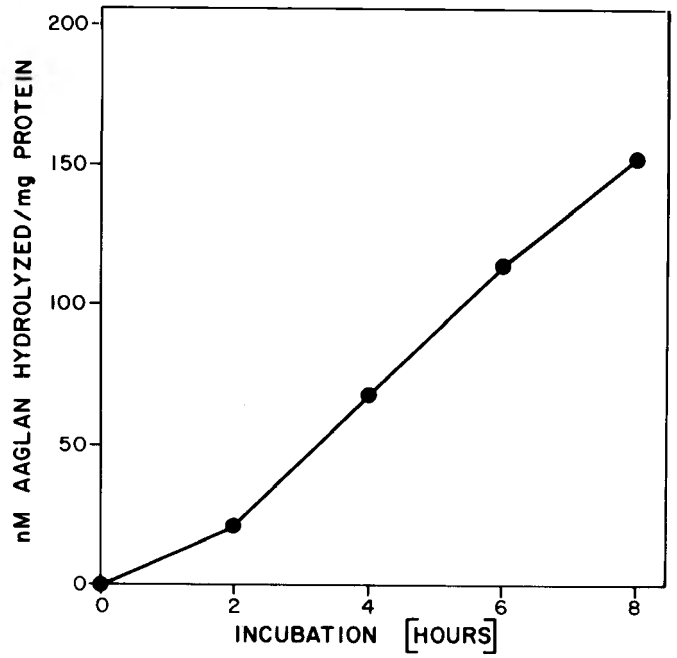

Figure 8. Hydrolysis of fluorogenic metalloendoprotease substrate, AAGLAN. Retinas were homogenized; the supernatant fraction was incubated for the times indicated with 0.3 mM AAGLAN. The amount of AAGLAN hydrolyzed was then measured spectrofluorometrically and was expressed as nanomolar concentrations of AAGLAN hydrolyzed per milligram of protein. The rate of hydrolysis was linear during the 8-hr incubation.

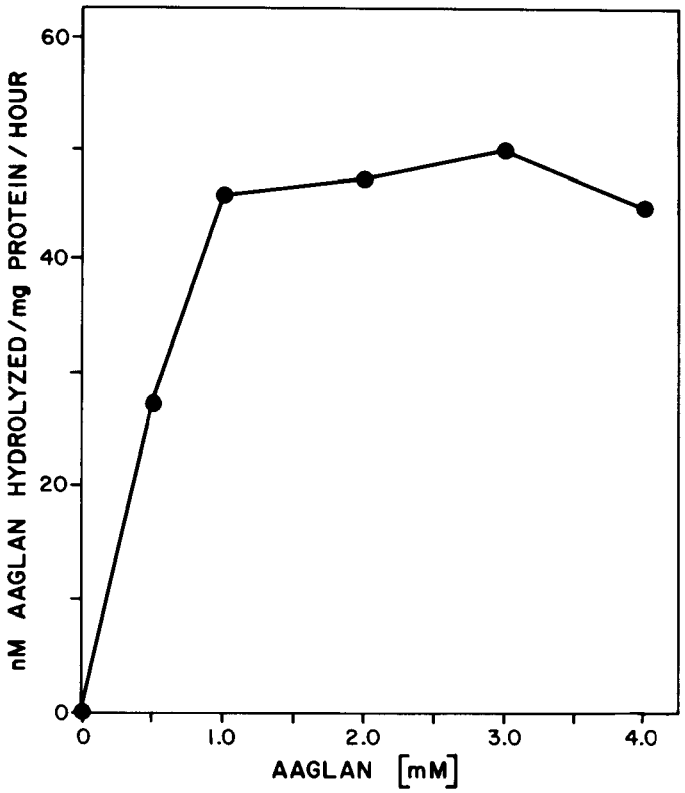

Figure 9. Saturation of soluble retinal metalloendoprotease by AAGLAN. The supernatant fraction of a retinal homogenate was incubated 6 hrs with increasing concentrations (0–4 mM) of AAGLAN. The amount of AAGLAN hydrolyzed was measured spectrofluorometrically. Hydrolysis of AAGLAN is saturable with an apparent K_m of approximately 0.5 mM.

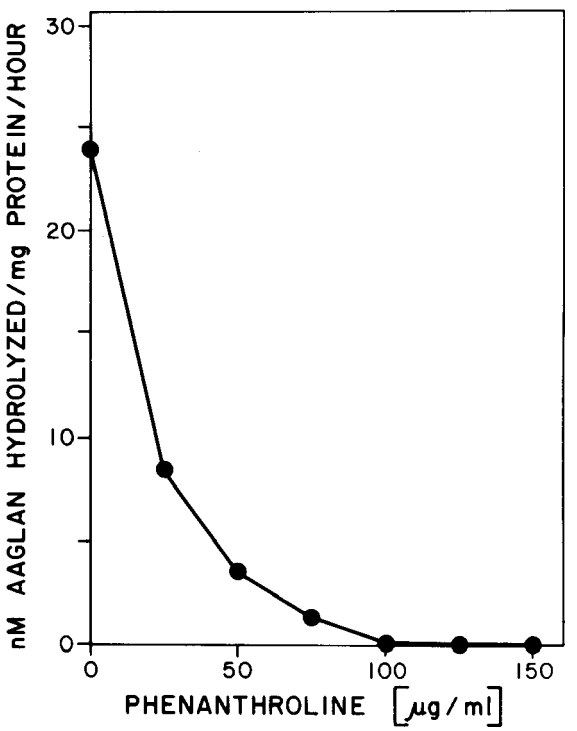

Figure 10. Inhibition of soluble retinal metalloendoprotease by 1,10-phenanthroline. Supernatant fractions of a retinal homogenate were incubated for 6 hr with 0.3 mM AAGLAN and increasing concentrations (0 to 150 μg/ml) of 1,10-phenanthroline. The hydrolysis of AAGLAN was inhibited by the metal chelator 1,10-phenanthroline with an IC_{50} of approximately 20 μg/ml. The concentration of 1,10-phenanthroline which completely inhibited hydrolysis of AAGLAN was the same concentration at which 1,10-phenanthroline inhibited K^+-stimulated [^{3}H]glycine release from retinal neurons (cf. Fig. 4).

by chelating calcium since excess calcium added to the retinal preparation does not restore neurotransmitter release. The reversible metalloendoprotease inhibitor, CBZ-L-phenylalanine, likewise inhibited [^{3}H]glycine release at concentrations inhibiting metalloendoprotease (Burstein et al., 1974), whereas L-phenylalanine, which has no effect on metalloendoproteases, had no effect on release.

The substrate specificities of metalloendoproteases are similar and have been examined extensively using synthetic dipeptide derivatives (Morihara, 1974; Barrett, 1977). Metalloendoproteases hydrolyze peptide bonds on the amino termini of nonpolar aromatic amino acids such as phenylalanine and large aliphatic amino acids such as leucine. They will not hydrolyze the peptide bonds formed by the small aliphatic amino acid, glycine. Metalloendoproteases thus hydrolyze the dipeptide substrates CBZ-Gly-Phe-amide, CBZ-Gly-Leu-amide, and CBZ-Ser-Leu-amide, but not CBZ-Gly-Gly-amide.

This paper presents evidence for the hypothesis that metalloendoproteases are involved in the release of transmitter substances at synapses. Our observations show that CBZ dipeptide amides, which are metalloendoprotease substrates, prevent [^{3}H] glycine release whereas dipeptide derivatives, which are not metalloendoprotease substrates, have no effect. CBZ dipeptide-amides, which are able to enter cells (Grinde et al., 1983), inhibit [^{3}H]glycine release, possibly by competing with the natural substrates of the metalloendoproteases. Inhibition of release by these dipeptide substrates is dose dependent, with concentrations causing half-maximal inhibition similar to the K_m concentrations for metalloendoproteases (Morihara, 1974). In addition to being amino acid specific and concentration dependent, dipeptide inhibition of transmitter release was re-

versible, since release could be restored by removing the dipeptide (Fig. 6).

Metalloendoprotease substrates and inhibitors prevent the depolarization-induced release of preload [^{3}H]glycine from a population of retinal neurons, but the mechanism of this inhibition is not understood. The inhibitors could alter the electrical properties of the neuron, prevent calcium influx, or inhibit the mechanism by which calcium initiates synaptic vesicle-plasma membrane fusion. This study implicates metalloendoprotease activity in neurotransmitter release from intact neurons of CNS derivation. Another study using a phrenic nerve-diaphragm preparation has demonstrated that metalloendoprotease inhibitors block synaptic communication from the nerve to the muscle, apparently by blocking neurotransmitter release, since they cause no changes in resting potential, transmembrane resistance, or amplitude and configuration of the action potential evoked by direct electrical stimulation of the muscle (Baxter et al., 1983). We show that the retina contains metalloendoprotease and that metalloendoprotease inhibitors prevent synaptic release of neurotransmitter.

The role of proteases in membrane fusion has been documented in viral-host cell fusion (Klenk et al., 1979). Both orthomyxovirus (influenza) and paramyxovirus (Sendai, Newcastle Disease virus) contain integral membrane protein required for fusion of the viral membrane with the host cell membrane. Viral coat protein is processed to convert the precursor protein, which cannot fuse, to a protein which can. Hemagglutinin, an integral membrane protein of influenza (Wilson et al., 1981), can mediate fusion only after cleavage of a single peptide bond to create two disulfide-linked fragments (HA_1 and HA_2) (Klenk et al., 1975). The role of hemagglutinin in membrane fusion has been shown directly by inserting the cloned hemagglutinin gene into fibroblasts (White et al., 1982). These fibroblasts will synthesize the intact viral hemagglutin and insert the protein into the plasma membrane, but the cells will not fuse. Incubation of these cells with trypsin produces the HA_1 and HA_2 fragments of hemagglutinin, mimicking *in vivo* proteolysis and causing cell-cell fusion. This and other experiments show that protein can facilitate membrane fusion and that "processing" of protein is necessary. That metalloprotease inhibitors and substrates block the depolarization-induced release of [^{3}H]glycine from retinal neurons suggests that a similar proteolytic event may be required for fusion of the synaptic vesicle with the plasma membrane.

Inhibition of transmitter release by synthetic dipeptide metalloendoprotease substrates also suggests a mechanism by which endogenous neuropeptides could modulate release. The neuropeptide β-endorphin, for example, has been shown to be hydrolyzed in brain to release the dipeptide glycyl-glutamine. Iontophoretic application of glycyl-glutamine depresses spontaneous firing of neurons in rat brain (Parish et al., 1983). Hydrolysis not only inactivates the neuropeptide β-endorphin, but produces a dipeptide which itself inhibits neuronal activity, possibly by inhibiting a proteolytic event.

In summary, retinal homogenates contain metalloendoprotease activity. Inhibitors and synthetic dipeptide substrates for metalloendoprotease prevent the calcium-dependent release of neurotransmitter from retinal neurons with the same specificity and dose dependency as their interaction with the metalloendoprotease. Proteolysis by a metalloendoprotease therefore appears to be a required step in neurotransmitter release.

References

Barrett, A. J. (1977) Introduction to proteases. *In Proteinases in Mammalian Cells and Tissues*, A. J. Barrett, ed., pp. 1–55, Elsevier-North Holland Publishing Co. Amsterdam.

Baxter, D. A., D. Johnston, and W.J. Strittmatter (1983) Protease inhibitors implicate metalloendoprotease in synaptic transmission at the mammalian neuromuscular junction. Proc. Natl. Acad. Sci. U. S. A. *80:* 4174–4178.

Benuck, M., M. J. Berg, and N. Marks (1982) Rat brain and kidney metalloendopeptidase: Enkephalin heptapeptide conversion to form a cardioactive neuropeptide, Phe-Met-Arg-Phe-amide. Biochem. Biophys. Res. Commun. *107:* 1123–1129.

Burstein, Y., K. A. Walsh, and H. Neurath (1974) Evidence of an essential histidine residue in thermolysin. Biochemistry *13:* 205–210.

Chlebowski, J., and J. E. Coleman (1976) Zinc and its role in enzymes. In *Metal Ions in Biological Systems*, H. Sigel, ed., Vol. 6, pp. 1–7, Marcel Dekker, Inc., New York.

Couch, C. B., and W. J. Strittmatter (1983) Rat myoblast fusion requires metalloendoprotease activity. Cell *32:* 257–265.

Frederick, J. M., M. E. Rayborn, A. M. Laties, D. M. K. Lam, and J. G. Hollyfield (1982) Dopaminergic neurons in the human retina. J. Comp. Neurol. *210:* 65–79.

Grinde, B., I. J. Galpin, A. H. Wilby, and R. J. Beynon (1983) Inhibition of hepatic protein degradation by synthetic analogues of chymostatin. J. Biol. Chem. *258:* 10821–10823.

Hildebrand, J. G., D. L. Barker, E. Herbert, and E. A. Kravitz (1971) Screening for neurotransmitters: A rapid radiochemical procedure. J. Neurobiol. *2:* 231–246.

Hollyfield, J. G., M. E. Rayborn, P. V. Sarthy, and D. M. K. Lam (1979) The emergence, localization and maturation of neurotransmitter systems during development of the retina in *Xenopus laevis*. I. γ-Aminobutyric acid. J. Comp. Neurol. *188:* 587–598.

Kam, C.-M., N. Nishino, and J. C. Powers (1979) Inhibition of thermolysin and carboxypeptidase A by phosphoramidates. Biochemistry *18:* 3032–3038.

Kelly, R. B., G. Miljanich, and S. Pfeffer (1983) Presynaptic mechanisms of neuromuscular transmission. In *Myasthenia Gravis*, E. X. Albuquerque and and A. T. Eldefrawi, eds., pp. 43–104, Chapman and Hall, London.

Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn (1975) Activation of influenza viruses by trypsin treatment. Virology *68:* 426–439.

Klenk, H. D., F. X. Bosch, W. Garten, T. Kohama, Y. Nagai. and R. Rott (1979) Proteolysis, a determinant for virus pathogenicity. In *Biological Functions of Proteinases*, H. Holzer and H. Tschesche, eds., pp. 139–149, Springer-Verlag, New York.

Matsas, R., I. S. Fulcher, A. J. Kenny, and A. J. Turner (1983) Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. Proc. Natl. Acad. Sci. U. S. A. *80:* 3111–3115.

Morihara, K. (1974) Comparative specificity of microbial proteinases. Adv. Enzymol. *4:* 179–243.

Nishino, N., and J. C. Powers (1979) Design of potent reversible inhibitors for thermolysin. Peptides containing zinc coordinating ligands and their use in affinity chromatography. Biochemistry *20:* 4340–4347.

Orlowski, M., and S. Wilk (1981) Purification and specificity of a membrane-bound metalloendopeptidase from bovine pituitaries. Biochemistry *20:* 4942–4950.

Orlowski, M., C., Michand, and T. G. Chu (1983) A soluble metalloendoprotease from rat brain. Eur. J. Biochem. *135:* 81–88.

Parish, D. C., D. G. Smyth, J. R. Normanton, and J. H. Wolstencroft (1983) Glycyl-glutamine, an inhibitory neuropeptide derived from β-endorphin. Nature *306:* 267–270.

Rayborn, M. E., P. V. Sarthy, D. M. K. Lam, and J. G. Hollyfield (1981) The emergence, localization and maturation of neurotransmitter systems during development of the retina in *Xenopus laevis*. II. Glycine. J. Comp. Neurol. *195:* 585–593.

Smith, R. M., and A. E. Martell (1975) *Critical Stability Constants*. Vol. 2: *Amines*, p. 251, Plenum Press, New York.

White, J., A. Helenius, and M.J. Gething (1982) Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature *300:* 558–659.

Wilson, I. A., J. J. Skehel, and D. C. Wiley (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3A resolution. Nature *289:* 366–373.