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Hydrolysis of Enkephalins in Homogenates of Anterior Segment Tissues of the Albino Rabbit Eye

Satish Dodda Kashi and Vincent H. L. Lee

The kinetics and pathways of hydrolysis of methionine enkephalin (TGGPM), leucine enkephalin (TGGPL), and [D-Ala^2] met-enkephalinamide (TAGPM) in homogenates of anterior segment tissues of the albino rabbit eye were studied using reversed phase HPLC. Both TGGPM and TGGPL were equally susceptible to hydrolysis with a half-life ranging from 11–50 min and were 11–23 times more susceptible to hydrolysis than TAGPM. All three peptides were hydrolyzed most rapidly in the corneal epithelium, followed by the iris-ciliary body, conjunctiva, corneal stroma, lens, and tears. Aminopeptidases were responsible for over 90% of the hydrolysis of TGGPM and TGGPL, while dipeptidyl peptidase and dipeptidyl carboxylpeptidase were responsible for the remainder. In contrast, dipeptidyl carboxylpeptidase was principally responsible for the hydrolysis of TAGPM, which by design is resistant to aminopeptidase action. Overall, these findings suggest that, in order to deliver short chain peptides intraocularly from topical solution instillation, it will be necessary to control the ocular tissue activity of aminopeptidases principally and, to a lesser extent, the activity of dipeptidyl peptidase and dipeptidyl carboxylpeptidase. Invest Ophthalmol Vis Sci 27:1300–1303, 1986

Enkephalins are a family of peptide neurotransmitters, first discovered in the brain, which may play a role in pain perception.[1] In recent years, a number of peptides, including enkephalins, substance P, and vasoactive intestinal peptide, have been identified in several ocular tissues,[2] but their role in the physiology of the eye and in the therapy of ocular diseases is far from understood. An earlier report[3] from this laboratory indicated that aminopeptidases, which are a family of exopeptidases that cleave peptides and proteins at their N-terminus, were unevenly distributed among the anterior segment tissues of the albino rabbit eye. It further proposed that these enzymes may play a principal role in degrading short chain biologically active peptides. This study was undertaken to test this hypothesis using three enkephalins, all pentapeptides, as model compounds. These were methionine enkephalin (Tyr-Gly-Gly-Phe-Met), leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), and [D-Ala^2]-met-enkephalinamide (Tyr-D-Ala-Gly-Phe-Met-NH_2), hereafter to be abbreviated as TGGPM, TGGPL, and TAGPM, respectively. These peptides were chosen for study primarily because their pathways of degradation in the brain and elsewhere in the body have been well characterized. In this study, the kinetics and pathway of enkephalin degradation in tears and aqueous humor, as well as homogenates of the conjunctiva, corneal epithelium, corneal stroma, iris-ciliary body, and lens of the albino rabbit were monitored over 180 min. This information would facilitate the understanding of the susceptibility of both endogenous and topically applied enkephalins to hydrolysis by aminopeptidases in anterior segment tissues.

Materials and Methods. Enkephalins were obtained commercially (Sigma Chemical Co., St. Louis, MO) and were used as received after verification of purity using reversed phase HPLC. Twelve male, albino, New Zealand rabbits (ABC Rabbitry, Pomona, CA), weighing 2–3 kg, were used for each enkephalin. Tears, aqueous humor, and anterior segment tissues were collected from these rabbits as previously described.[3] Tissue homogenates, 20% w/v, were prepared in isotonic KCl using a Potter-Elvejhem tissue homogenizer followed by centrifugation at 3,020× g in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instru-

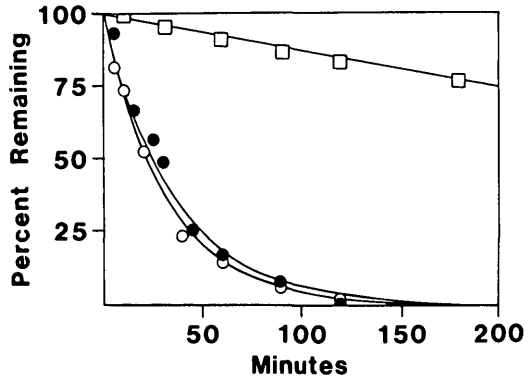

Fig. 1. Disappearance of methionine enkephalin (●), leucine enkephalin (○), and [D-Ala^2] met-enkephalinamide (□) upon incubation of 2.5 mM of each peptide in an iris-ciliary body homogenate at 37°C.

ments, Newton, CT) at 4°C for 10 min. The resulting supernatants were diluted with additional isotonic KCl to a protein concentration of 5 mg/ml.

The kinetics and pathway of degradation of enkephalins in tears, aqueous humor, and ocular tissue supernatants were studied by incubating, in triplicate, 10 μl of these fluids (containing about 50 μg of proteins) with 40 μl of a 2.5 mM enkephalin solution at 37°C for up to 180 min. At predetermined times, 75 μl of acetonitrile was added to the incubation mixture to precipitate the proteins, thereby terminating the reaction. Ten μl of a 0.25 mg/ml tryptophan solution, the internal standard, were added. The resulting mixture was centrifuged for 15 min to remove the precipitated protein and then evaporated under nitrogen to remove acetonitrile. Thereafter, 10 μl of the concentrate was injected, in duplicate, into the HPLC.

The HPLC system consisted of 2 Altex model 110A HPLC pumps, a Rheodyne model 7125 sample injector, an Axxiom model 710 HPLC controller, and an Altex Ultrasphere reverse phase ODS C-18 column (4.6 mm × 250 mm, 5 μm). The mobile phase was a binary mixture of acetonitrile and water containing 0.1% H_3PO_4 and 0.1 M $NaClO_4$. The proportion of acetonitrile in the mobile phase was increased linearly from 5–17% for the first 12 min and from 17–55% for the next 20 min at a flow rate of 1.0 ml/min. Enkephalins and their hydrolytic fragments, which eluted between 7–30 min, were monitored at 214 nm using a Kratos 773 spectrophotometric detector. The assay sensitivity was 0.1 nmole with respect to enkephalins and the majority of their hydrolytic fragments. The intra- and inter-run variations were less than 5 and 7.5%, respectively.

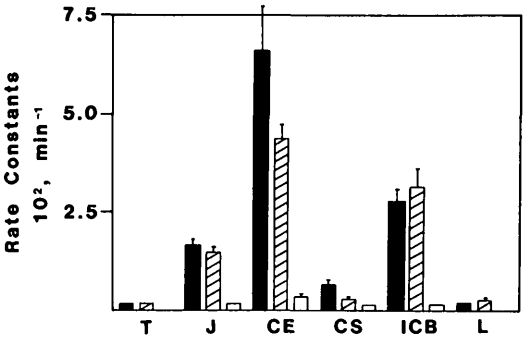

Fig. 2. First order rate constants for the hydrolysis of methionine enkephalin (■), leucine enkephalin (▨), and [D-Ala^2] met-enkephalinamide (□) in tears and homogenates of various anterior segment tissues of the albino rabbit eye. T = tears; J = conjunctiva; CE = corneal epithelium; CS = corneal stroma; ICB = iris-ciliary body; L = lens.

The investigations utilizing animals, as described in this report, conform to the ARVO Resolution on the Use of Animals in Research.

Results. Control experiments demonstrated that all three enkephalins were chemically stable over the time course of the incubation, and that the initial hydrolytic rate of these peptides was linear over the range of 10 and 100 μg of protein. Figure 1 shows a profile for the disappearance of TGGPM, TGGPL, and TAGPM in the homogenates of anterior segment tissues, specifically, iris-ciliary body. Disappearance followed first order kinetics. The rate constants for enzymatic hydrolysis of these enkephalins are displayed in Figure 2, indicating significant variations in peptidase activities among anterior segment tissues. Figure 3 shows the time course of changes in concentration of an enkephalin (TGGPM), as well as its hydrolytic fragments upon incubation with an ocular tissue homogenate (iris-ciliary body). As shown in Table 1, TGGPM and TGGPL were cleaved principally at the Tyr^1-Gly^2 bond to yield tyrosine as their principal hydrolytic product. In contrast, TAGPM was cleaved almost exclusively at the Gly^3-Phe^4 bond forming Tyr-D-Ala-Gly.

Discussion. Drug treatment of ocular diseases traditionally has involved small organic molecules. However, with the expected increasing understanding of the role of peptides in ocular physiology and pathology,[2] it is anticipated that several of these peptides would eventually be developed into therapeutic agents. Because of their molecular size and exquisite sensitivity to hydrolysis by peptidases, the successful delivery of these substances to intraocular tissues from topical administration would require an understanding of not only their ability to cross ocular membranes, but also their ability to survive the hydrolytic barrier. While there is evidence that macromolecules as large as inulin (M.W. 5,000) can penetrate the cornea,[4] the magnitude of the hydrolytic barrier is not known. Using methionine and leucine enkephalins as model peptides, this study demonstrates that such a barrier exists in certain

anterior segment tissues and that its magnitude is substantial. Both pentapeptides were hydrolyzed equally rapidly in homogenates of the corneal epithelium, iris-ciliary body, and conjunctiva with a half-life ranging from 11–50 min. In contrast, they were 10–50 times more stable to hydrolysis in tears and in homogenates of the corneal stroma and lens, and were not hydrolyzed at all in the aqueous humor. Since topically applied solutions disappear rapidly from the conjunctival sac, enkephalins so administered would probably be absorbed intact, implying that protection of these peptides from enzymatic degradation in tears is not as imperative as in the corneal epithelium, iris-ciliary body, and conjunctiva.

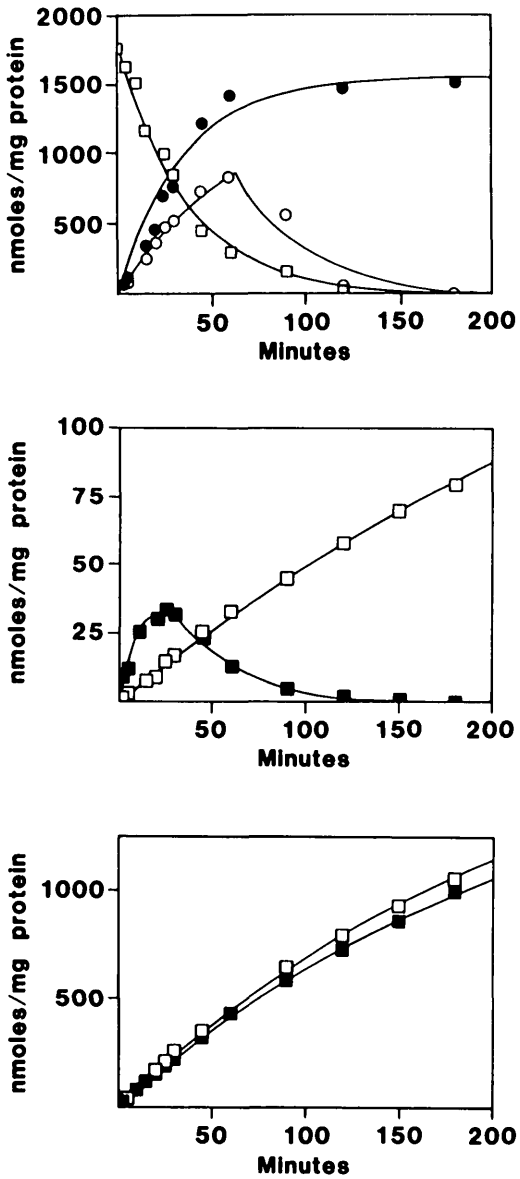

Fig. 3. Concentration-time profiles of methionine enkephalin (□), tyrosine (●), and Gly-Gly-Phe-Met (○) [top plot]; Tyr-Gly (□) and Tyr-Gly-Gly (■) [middle plot]; and Phe (□) and Met (■) [bottom plot], upon incubation of methionine enkephalin (2.5 mM) in an iris-ciliary body homogenate at 37°C.

Table 1. Percent of hydrolytic products attributed to tyrosine derived from the hydrolysis of methionine enkephalin (TGGPM), leucine enkephalin (TGGPL), and [D-Ala^2] met-enkephalinamide (TAGPM) in tears and homogenates of various anterior segment tissues of the albino rabbit eye

Tissue/Fluid	*TGGPM*	*TGGPL*	*TAGPM*
Tears	100 ± 0.02	100 ± 0.03	0
Conjunctiva	75.2 ± 1.9	98.4 ± 1.0	0
Corneal epithelium	97.4 ± 0.6	94.9 ± 1.7	1.4 ± 0.2
Corneal stroma	90.2 ± 2.8	91.7 ± 1.2	5.8 ± 0.3
Iris-ciliary body	85.5 ± 1.8	97.5 ± 2.6	1.1 ± 0.05
Lens	99.7 ± 2.3	93.3 ± 1.4	*

* No detectable hydrolysis over 180 min.

The formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly suggests that aminopeptidases, dipeptidyl peptidase, and dipeptidyl carboxylpeptidase participated in the hydrolysis of TGGPM and TGGPL in anterior segment tissue homogenates. The preponderance of Tyr indicates that, as is the case in brain synaptosome membranes[5] and homogenates of the ileum,[6] aminopeptidases were the principal peptidases involved, suggesting that this family of enzymes must be inhibited in order to minimize peptide degradation in anterior segment tissues. This is not to imply that even when the aminopeptidases were totally inhibited, a given enkephalin would remain intact. Indeed, the companion enzymes, dipeptidyl peptidase and dipeptidyl carboxylpeptidase, may now assume greater quantitative importance. This is exemplified by [D-Ala^2] met-enkephalinamide (TAGPM), which by design is resistant to aminopeptidase mediated cleavage.[7] Relative to TGGPM and TGGPL, TAGPM was 11–23 times more stable to hydrolysis in the corneal epithelium, iris-ciliary body, conjunctiva, and tears, and 2–5 times more stable to hydrolysis in the corneal stroma (Fig. 2). The principal peptidase mediating its hydrolysis is dipeptidyl carboxylpeptidase, whose presence in homogenates of anterior segment tissues is obscured by aminopeptidases against methionine and leucine enkephalins as substrates. In the brain[8] and the kidney,[9] this enzyme is identical with enkephalinase, an enzyme believed to function as a neuropeptidase in terminating the pharmacological activity of endogenous enkephalins.[10] It has yet to be established whether this is also the case in the eye.

In summary, short chain peptides as exemplified by methionine and leucine enkephalins are most susceptible to hydrolysis by aminopeptidases in homogenates

of the corneal epithelium, iris-ciliary body, and conjunctiva, although dipeptidyl peptidase and dipeptidyl carboxylpeptidase also play a role. While aminopeptidases appear to be of major quantitative importance with respect to peptide hydrolysis in tissue homogenates, in which the cellular compartmentalization of peptidases is destroyed, it is not known if this is also the case in the intact cell. Thus, further work is necessary to determine the subcellular distribution of these three peptidases in anterior segment tissues. This information would facilitate the design of a strategy to circumvent the enzymatic barrier to topical ocular peptide delivery.

Key words: enkephalins, ocular peptide hydrolysis, aminopeptidases, enkephalinase, dipeptidyl peptidase

From the University of Southern California School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California. Supported by Grant EY-06169 from the National Institutes of Health, Bethesda, Maryland. Submitted for publication: October 16, 1985. Reprint requests: Vincent H. L. Lee, University of Southern California School of Pharmacy, 1985 Zonal Avenue, Los Angeles, CA 90033.

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