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Purification, Characterization, and Localization of Neuropeptides in the Cornea

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TINSLEY, P W , G H FRIDLAND, J T KILLMAR AND D M DESIDERIO *Purification, characterization, and localization of neuropeptides in the cornea* PEPTIDES 9(6) 1373–1379, 1988 —The immunologically detected neuropeptides methionine enkephalin (ME), substance P (SP), β-endorphin (β-End), and α-melanocyte stimulating hormone (α-MSH) were purified from bovine corneal extracts by gradient, followed by isocratic, reversed phase-high perofrmance liquid chromatography (RP-HPLC) and characterized, after both chromatographic steps, by radioimmunoassay (RIA) Immunologically detected ME and SP were purified from canine corneal extracts by gradient RP-HPLC and characterized by RIA An anatomical study of the bovine cornea separated the cornea into an epithelium-enriched and a stroma-enriched portion After gradient RP-HPLC, RIA demonstrated that all the ME-like immunoreactivity was located in the corneal epithelium, whereas the SP-like immunoreactivity was distributed between the stroma and epithelium in an approximate two-to-one ratio

Cornea Methionine enkephalin Substance P β-Endorphin α-MSH HPLC Radioimmunoassay

THE cornea is unique to the peripheral nervous system, in that the neuronal construct of the cornea is dedicated primarily to sensory perception (21). Recent studies (3, 17, 21) suggest that thermal stimulation of the human cornea evokes only pain or irritation, whereas mechanical stimulation evokes predominantly corneal pain with a possibility of discriminating tactile from nociceptive stimuli The innervated cornea (22) contains a higher density of free nerve endings than several peripheral tissues such as skin and tooth pulp (27). The conduction velocities of the corneal nerves indicate the presence of both nonmyelinated C-fibers and myelinated A-delta fibers, which terminate peripherally as a free nerve ending (21,37) The response of the A-delta fibers to both mechanical and thermal stimulation is similar to that of other documented nociceptors (2,37). Another prominent function of C-fiber free nerve endings in the periphery is an axonal reflex known as neurogenic inflammation (28). Therefore, the cornea contains the appropriate components required by the gate control theory for nociceptive perception (31), as well as other forms of sensory perception, and can serve as a model tissue for the characterization of neuropeptides involved in sensory perception

Nociceptive synaptic transmission, occurring in the spinal cord and the brain, putatively utilizes the undecapeptide SP for neurotransmission (20) and several opioids for neuromodulation (14) Nociceptive neurons of the central nervous system often originate from the peripheral nervous system Therefore, a free nerve ending of a peripheral primary afferent neuron could conceivably contain the same neuropeptides utilized by that neuron at the synaptic cleft. Thus, if SP and the opioids are synaptic neuropeptides, then those same neuropeptides may well be contained within the peripheral terminals and be utilized for other purposes, such as local receptor modulation or activation. Likewise, SP is a prominent candidate for modulating the neurogenic inflammatory reflex (28), and therefore could be utilized by either the peripheral corneal terminals or the free nerve endings for that type of reflex. The opioids have also been shown to exert an antinociceptive effect on peripheral tissue (9), thus, opioid peptides could be released by the peripheral terminals for the local modulation of either nociception or inflammation.

Considering the cornea's neuronal dedication to sensory perception and the role that SP and the opioids may play in that perception, the presence, as detected by immunological methods, of the neuropeptides SP, ME, β-End, and α-MSH in the cornea was investigated Those four neuropeptides were characterized by RIA in extracts from bovine cornea at several stages of chromatographic purification SP-like and

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TABLE 1
WET WEIGHT PER CORNEA

Corneal Type	Average Wet Weight (g ± SD)	n*	Range Low–High	No of Cornea
Bovine Whole	0 53 ± 0 06	70	0 41–0 69	554
Bovine Stroma	0 51 ± 0 01	4	0 50–0 52	40
Bovine Epithelium	0 060 ± 0 015	6	0 040–0 080	64
Canine Whole	0 14 ± 0 3	12	0 088–0 19	67

*The number of experiments that contributed to the average

ME-like immunoreactivities were also examined in the whole canine cornea, and in the bovine cornea after separating the cornea into stroma-enriched and epithelium-enriched portions

METHOD

Tissue

Bovine cornea were surgically excised from eyes obtained from a local abattoir and were kept on ice (2 hr or less) Canine cornea were surgically excised from the eyes (on ice; 30 min or less) of exsanguinated laboratory dogs

For the study of examining the anatomically localized differences in the immunoreactivity of SP and ME in the bovine cornea, the cornea was separated into an anterior (epithelium-enriched) and posterior (stroma-enriched) portion The bovine cornea was obtained as above, and a scalpel blade was used to scrape the anterior portion with firm pressure until no more tissue could be removed (41). The pulp-like epithelium-enriched portion was transferred directly into 1 M acetic acid, whereas the stroma-enriched portion, which still resembled an intact cornea, was treated as if it were a whole cornea.

Because the average wet weight for both bovine and canine cornea, as well as the separated portions of the bovine cornea, have not been previously reported, we report those weights here for the first time in Table 1. Those values were used to calculate the RIA results presented in this study

Protein Precipitation

The cornea were shredded with scissors into 1 M acetic acid (10 ml/cornea) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY: setting 7, 15 sec). After an extended incubation (12 hr; 4°C) to optimize protein precipitation, the homogenate was centrifuged (2700×g, 30 min) and the peptide-rich supernatant was decanted and saved. The pellet was resuspended in 1 M acetic acid (2 ml/cornea), incubated (2 hr; 4°C), and recentrifuged. The two supernatants were combined, frozen at −70°C, and lyophilized.

Sep-Pak Chromatography

After protein precipitation, the samples were reconstituted in 2–10 ml of 0.1% trifluoroacetic acid (TFA) and eluted from a C18 Sep-Pak (Waters Assoc., Milford, MA) cartridge that had been prewashed and conditioned (4 ml methanol, 4 ml water, and 8 ml 0.1% TFA) Water-soluble salts and small polar compounds were eluted with 0 1% TFA (4 ml), and a peptide-rich fraction was eluted with the organic acetonitrile (3–4 ml of acetonitrile–0.1% TFA, 1:1, v:v) The volume of acetonitrile was reduced by a stream of nitrogen, and the sample was frozen at −70°C and lyophilized

Gradient Elution RP-HPLC

The peptide-rich fraction from the Sep-Pak elution was reconstituted in 1 ml of the volatile buffer, triethylamine formate (TEAF, pH 3.15, 40 mM), chromatographed on a Model 5020 (Varian, Walnut Creek, CA) liquid chromatographic system using a micro-Bondapak C18 (150×4.6 mm, Waters Assoc.) reversed phase analytic column with a series of linear gradients of acetonitrile in TEAF buffer The gradient was generated by increasing the acetonitrile percentage from 10 to 15% (0–18 min); 15 to 18% (18–48 min), 18 to 30% (48–72 min); held at 30% (72–80 min); and after 80 min, the gradient was terminated and the column was eluted with 100% acetonitrile for 10 min (80–90 min). The retention time for each synthetic peptide, monitored by UV at 200 nm, was calibrated in later chromatograms, and demonstrated that the gradient appropriately separates the neuropeptides ME, α-MSH, SP, and β-End (10). The flow rate was 1.5 ml/min and either 90 1-min fractions or 45 2-min fractions were collected Duplicate aliquots from the appropriate RP-HPLC regions were lyophilized in glass tubes for determination of the appropriate peptide immunoreactivity by RIA

Isocratic Elution RP-HPLC

The fractions from the gradient RP-HPLC elution that demonstrated the appropiate immunoreactivity for each individual peptide were purified further with isocratic chromatography The same liquid chromatographic system was used, but a PLRP-S (150×4.6 mm, Polymer Lab., Amherst, MA) polystyrene/divinylbenzene reversed phase analytic column was used with the appropriate percent acetonitrile-TEAF buffer system for each peptide of interest. A different column type (PLRP-S) was used for the isocratic chromatography so that the fractions from gradient chromatography would be exposed to a different matrix, and thus separate further any impurities from the peptide of interest. The PLRP-S column was also chosen to reduce column bleed, which interferes with fast atom bombardment mass spectrometry analysis. Duplicate aliquots from the appropriate regions were lyophilized in glass tubes for determination of the appropriate peptide immunoreactivity by RIA

Radioimmunoassay

Commercial RIA kits (IncStar Corp., Stillwater, MN) for the four peptides (ME, SP, β-End, and α-MSH) were utilized

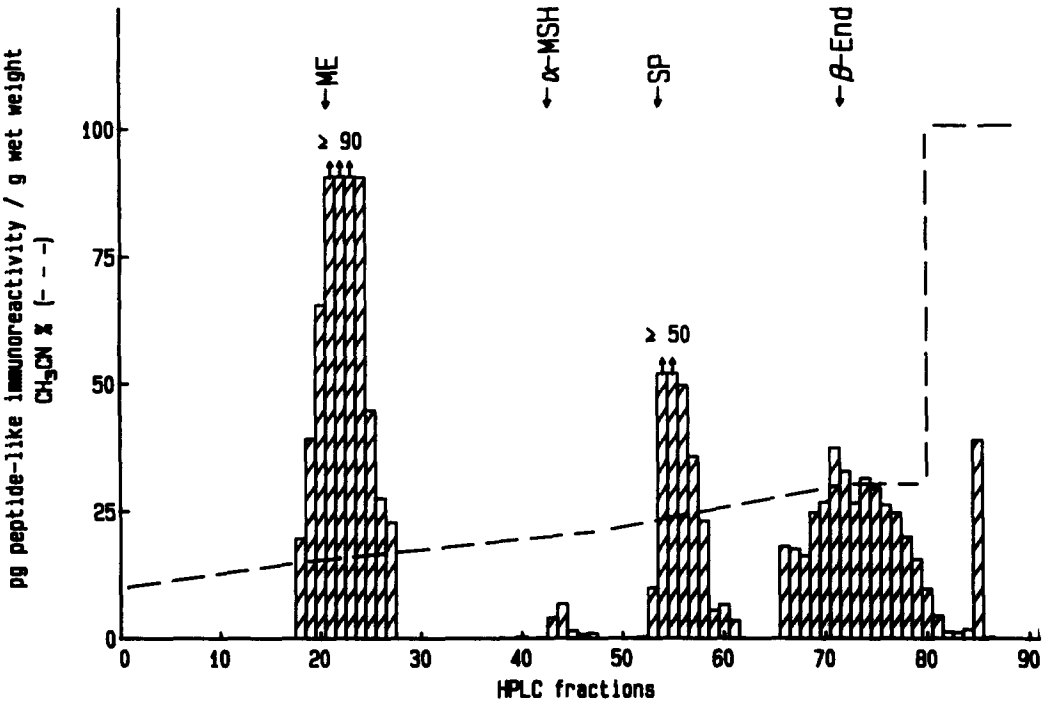

FIG 1 Profile of immunoreactivity of ME, α-MSH, SP, and β-End from whole bovine cornea after gradient elution RP-HPLC (collected in 1 min fractions) The down arrow (↓) denotes the retention time for that synthetic peptide and the up arrow (↑) signifies that the quantity indicated exceeded the upper detection limit for that RIA calibration curve ME-RIA fractions 18–27, α-MSH-RIA fractions 40–47, SP-RIA fractions 52–51, β-End-RIA fractions 66–86 The dashed line represents the HPLC gradient (% acetonitrile)

The instructions that accompanied these commercial RIA kits were followed exactly, and all assays were executed in duplicate Prior to chromatography of any biologic sample, fractions from a blank chromatogram were collected at those fractions corresponding to the retention time of each peptide of interest The volume of blank equaled that of the sample aliquot used for RIA These blank aliquots were assayed concurrently with the biological aliquots, and their immunoreactive measurement was subtracted from the final RIA results presented in this study

Synthetic Peptides

SP, ME, α-MSH, human β-End, and angiotensin I were purchased from Sigma Chemical Company.

RESULTS

RIA of the Gradient Elution RP-HPLC Fractions

To ensure that no synthetic peptide could contaminate the RP-HPLC analytic columns, neither column (C18 or PLRP-S) was exposed to synthetic peptides or standards until after all biologic samples were eluted, thus avoiding inadvertent elution or "bleed" (7) of the synthetic peptides. However, all chromatographic columns require saturation of active sites in the column with the compound of interest (7) prior to any detectable and constant elution of that compound from the column. Therefore, the results presented in this study were obtained only after extracts from over 400 bovine cornea were chromatographed to precondition the column with endogenous peptide.

A bar graph containing a composite of four different RIA results superimposed onto the RP-HPLC gradient (Fig 1) demonstrates for the whole bovine cornea an appropriate immunoreactivity corresponding to the retention time of each synthetic peptide (determined after all biologic samples were eluted) The ME, SP, and β-End results were obtained from the same chromatographic corneal sample, whereas α-MSH was from a later sample Similar determinations for the canine cornea were performed for ME and SP (Fig 2)

RIA of the Isocratic Elution of RP-HPLC Fractions

The gradient elution fractions from the bovine cornea that demonstrated the appropriate immunoreactivity were combined for each peptide, and were chromatographed isocratically with an appropriate acetonitrile percent to elute the respective peptide at between 5 to 10 min. To avoid the possible contamination of the chromatographic system and the column used for the biologic samples, the appropriate isocratic condition for each peptide was determined on a separate, yet equivalent HPLC system and analytic column

Each individual peptide from the same corneal sample after gradient elution was purified with isocratic chromatography The isocratic fractions demonstrated the appropriate immunoreactivity (Fig. 3), which correlated with the synthetic peptide's calibrated isocratic retention time and corresponding immunoreactivity.

The results from the isocratic RP-HPLC for three (ME, α-MSH, and SP) of the four peptides demonstrated the presence of a second immunoreactive peak eluting earlier than the synthetic peptide (Fig 3). We know that the more polar

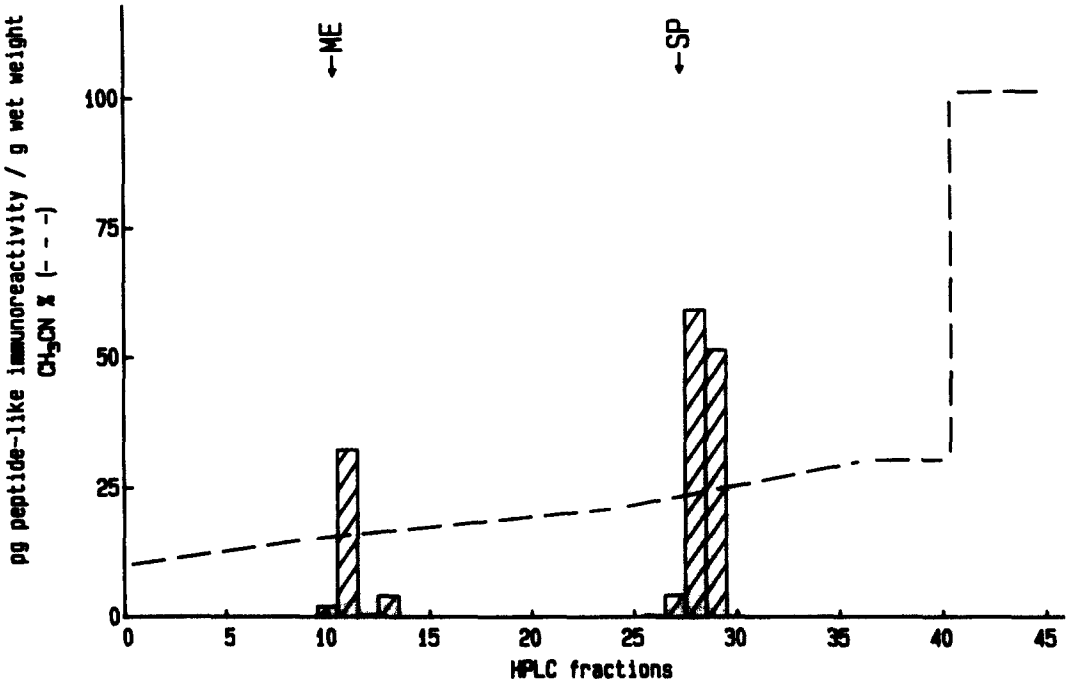

FIG 2 Profile of immunoreactivity of ME and SP from whole canine cornea after gradient elution RP-HPLC (collected in 2-min fractions) The down arrow (↓) denotes the retention time for that synthetic peptide ME-RIA fractions 10–13, SP-RIA fractions 26–29 The dashed line represents the HPLC gradient (% acetonitrile)

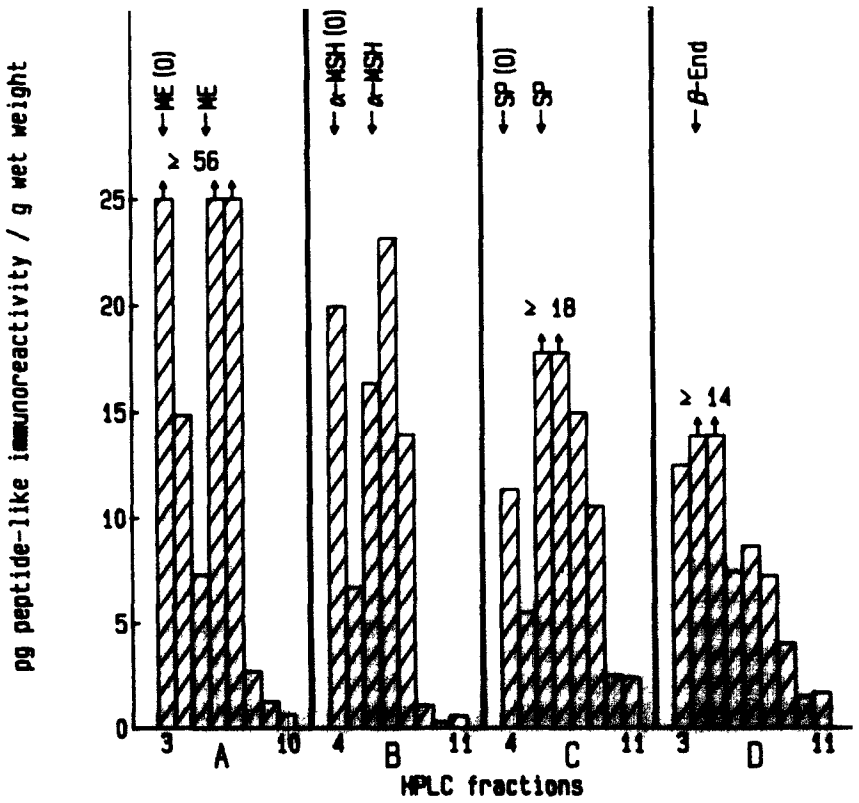

FIG 3 RIA results from whole bovine cornea after isocratic elution RP-HPLC (collected in 1-min fractions) The down arrow (↓) denotes the retention time for that synthetic peptide and the up arrow (↑) signifies that the quantity indicated exceeded the upper detection limit for that RIA calibration curve (A) ME-RIA, 15% acetonitrile; (B) α-MSH-RIA, 17% acetonitrile, (C) SP-RIA, 18% acetonitrile, (D) β-End-RIA, 22% acetonitrile

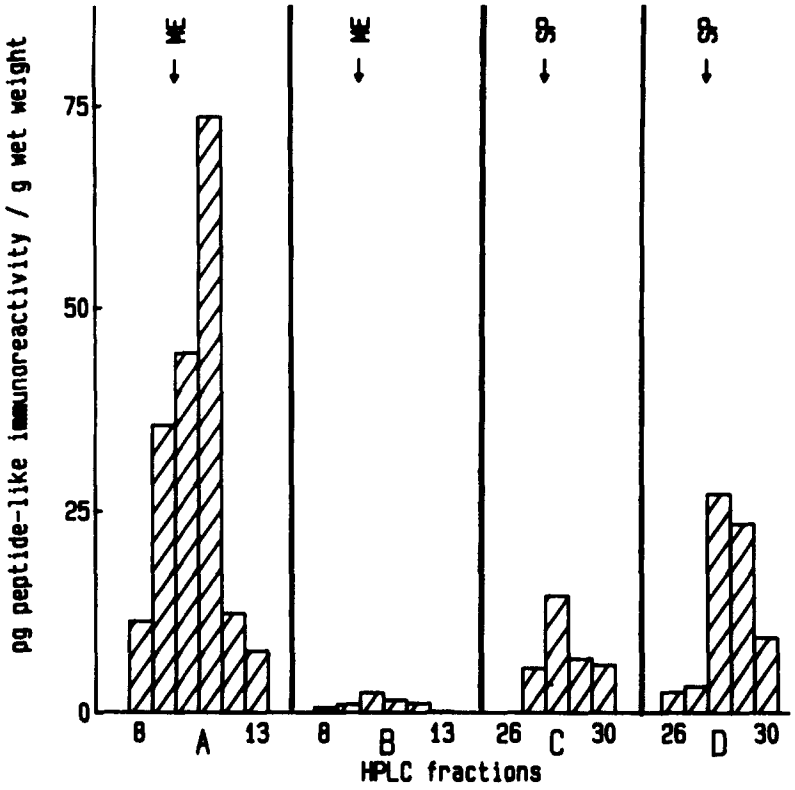

FIG 4 Comparative RIA results from the epithelium-enriched and stroma-enriched portions of the bovine cornea after gradient elution RP-HPLC (collected in 2-min fractions) The down arrow (↓) denotes the retention time for that synthetic peptide (A) ME-RIA of epithelium-enriched, (B) ME-RIA of stroma-enriched, (C) SP-RIA of epithelium-enriched, (D) SP-RIA of stroma-enriched

sulfoxide elutes earlier in the gradient than the corresponding peptide (ME, α-MSH, and SP), and are not contained in the fractions purified by isocratic-HPLC. Therefore, even though all procedures were performed with quick dispatch, air oxidation of these peptides to their corresponding sulfoxides may have occurred in the time necessary to prepare the sample for isocratic chromatography That early eluting immunoreactivity also corresponds with the retention time of the synthetic sulfoxides for each peptide, further corroborates the presence of those immunoreactive peptides and the possibility of their air oxidation

RIA of the Stroma-Enriched and Epithelium-Enriched Portions of the Bovine Cornea

The epithelium-enriched and stroma-enriched portions from the same bovine cornea, after their separate and respective gradient RP-HPLC separation, were each halved. Each half was assayed for SP-like and ME-like immunoreactivity. The RIA results (Fig 4) demonstrated that virtually all of the ME-like immunoreactivity is present in the epithelium-enriched portion, whereas at least twice the amount of SP-like immunoreactivity was measured in the stroma-enriched portion compared to the epithelium-enriched portion

DISCUSSION

SP-like immunoreactivity has been demonstrated extensively in the cornea of several species by immunohistochemical detection (5, 6, 23, 29, 30, 33, 34, 38, 39). The general anatomical findings of those studies was that large fibers containing SP-like immunoreactivity enter the stroma from the sclera and the episclera. Some of those fibers subdivide and approach the epithelium, where an abundant arborization of fine terminals containing SP-like immunoreactivity could be observed Of even more relevance to this present research is the fact that several groups have quantified SP-like immunoreactivity in the cornea by RIA (11, 16, 40) and enzyme-linked immunoabsorbent assay (32) However, all of those studies were performed on extracted samples that were not purified chromatographically; thus, no comparison can be made with the results discussed here. Without chromatographic purification, the opportunity for cross-reactivity of the assay's antibody with similar peptides is significant (4, 12, 25), as well as possible interference by extraction impurities (1,26). Thus, demonstrating the presence of SP-like immunoreactivity corresponding with the retention time of synthetic SP, at two different stages of chromatographic purification, is a significant improvement over those earlier reports

Elucidating the function of SP in the cornea was attempted by several of those groups by observing the effect of several manipulations on corneal SP-like immunoreactivity levels Thermocoagulation of the sensory trigeminal nerve (16, 23, 40), which supplies the cornea (18), diminished the corneal SP-like immunoreactivity, thus suggesting that SP is a sensory neuropeptide However, capsaicin treatment promoted pain and caused SP-like immunoreactivity to di-

minish, but did not decrease the sensitivity of the cornea to pain (11, 19, 36) Likewise, local administration of the SP-antagonist [D-Pro^2,D-Trp^{7,9}] SP did not desensitize the cornea to pain (5,13) Both results suggest that SP is not necessary for a corneal nociceptive response Thus, corneal SP probably plays a role in other types of sensory response, such as a neurogenic inflammatory-like response

ME-like, β-End-like, or α-MSH-like immunoreactivity have not been demonstrated in the cornea Drago and co-workers (8) did demonstrate by immunological methods the presence of the β-End precursor β-lipotropic hormone (β-LPH) in the bovine cornea, but was unable to demonstrate the presence of β-End-like immunoreactivity due to the possible lack of antibody specificity The desire to substantiate further the presence of β-End in the cornea prompted us to look for α-MSH, another metabolite of a β-End and β-LPH common precursor, proopiomelanocortin (24)

Thus, this study demonstrates for the first time the immunological presence of ME, β-End, and α-MSH in the bovine cornea and ME in the canine cornea These results are substantiated further by monitoring the peptide immunoreactivity in extracts from bovine cornea through two chromatographic purification steps The function of these neuropeptides in the cornea is under continuing investigation ME modulates the nociceptive transmission of SP in the central nervous system, but SP is probably not involved in nociception in the cornea, thus, this function for ME cannot be considered strongly

The results obtained with the bovine cornea anatomical study demonstrated a difference in the localization of ME-like and SP-like immunoreactivity in the epithelium-enriched versus the stroma-enriched portion of the cornea ME-like immunoreactivity was found only in the epithelium-enriched portion, whereas SP-like immunoreactivity was approximately twice as much in the stroma-enriched portion as compared to the epithelium-enriched portion No direct functional conclusions can be drawn from these results, but if the source of the immunoreactivity is assumed to be neuronal, then the following trends can be suggested Corneal SP is more prevalent in the larger nerve trunks, which then divide distally into smaller SP-containing and ME-containing nerves and nerve endings in the epithelium, in agreement with the immunohistochemical results for SP discussed earlier This reserve of SP in the larger nerve trunks also suggests that any SP-related effect of the corneal surface could be sustained longer than a corresponding ME effect

For the results examined thus far, the possible effect of endogenous corneal proteolytic enzymes has not been discussed This approach does not imply that proteolytic enzymes do not play a role in these studies, but, because these results were concerned primarily with the presence (qualitative results) and not the exact quantity of peptide present (quantitative results), the possible loss of peptide by enzymolysis was not studied But, the anatomical study was concerned with a comparison of neuropeptide amounts localized in two portions of the cornea, thus, any locally preferential proteolytic activity becomes significant. In two articles (15,35), aminopeptidase activity in the cornea was found to be more active in the epithelium than in the stroma One of those studies even used synthetic ME as the enzyme's substrate (15), thus demonstrating a more rapid proteolytic hydrolysis of ME in the epithelium as compared to the stroma. The lower proteolytic activity in the stroma helps to substantiate further the findings of this study because all the corneal ME-like immunoreactivity was localized in the epithelium where ME should have been hydrolyzed at a faster rate than in the stroma, where no ME-like immunoreactivity was demonstrated It should be noted that, whereas few reports of neuropeptidase activities in the cornea are available, many other forms of proteases could be present in the cornea and thus could have a profound effect on the concentrations on corneal ME and SP

By two different rationales, the immunological results presented here should be considered as qualitative, and the actual amounts used only to compare samples exposed to similar experimental conditions The concept of similar sample exposure applies especially to the results presented here for the bovine cornea anatomical study, because the samples were treated identically and chromatographed on a column that had already been preconditioned with 400 extracted cornea, the data are significant when compared directly

The first reason for these results to be considered qualitative is that the efficiency of the initial peptide extraction, as well as the recovery after each chromatographic step, were not corrected for with an appropriate internal standard. Because the cornea is difficult to homogenize, and the chromatographic column was preconditioned with cornea extracts but not with synthetic peptide, the loss at those steps could have been great. We deemed those losses reasonable in our present study because of the greater need to avoid misinformation from possible sample contamination by synthetic peptide For these data to be at all comparable with data obtained elsewhere, a value for overall peptide recovery on this system would be needed. A recovery study using the nonopioid peptide angiotensin I demonstrated that 87% of that synthetic peptide was recovered from a spiked corneal sample after acid extraction, Sep-Pak chromatography, and gradient RP-HPLC

A second but equally important reason for these results to be deemed qualitative is that RIA detects only immunological activity and not unambiguously the amino acid sequence of a peptide Thus, even after chromatographic purification, the compound identified by the antibody may not be the peptide of interest To alleviate this limitation, more peptide must be isolated and characterized by a procedure such as mass spectrometry, which can yield unambiguous amino acid sequence information

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