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Research Paper

Functional Identification of a Novel Transport System for Endogenous and Synthetic Opioid Peptides in the Rabbit Conjunctival Epithelial Cell Line CJVE

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Purpose. To investigate whether conjunctival epithelial cells express transport processes for opioid peptides.
Methods. We monitored the uptake of [^{3}H]deltorphin II and [^{3}H]DADLE, two hydrolysis-resistant synthetic opioid peptides, in the rabbit conjunctival epithelial cell line CJVE and elucidated the characteristics of the uptake process.
Results. CJVE cells express robust uptake activity for deltorphin II and DADLE. Both opioid peptides compete with each other for transport. Several endogenous and synthetic opioid peptides, but not non-peptide opioid antagonists, are recognized by the transport process. Though various peptides inhibit the uptake of deltorphin II and DADLE in a similar manner, the uptake of deltorphin II is partly Na^+-dependent whereas that of DADLE mostly Na^+-independent. The transport process shows high affinity for many endogenous/synthetic opioid peptides. Functional features reveal that this transport process may be distinct from the opioid peptide transport system described in the retinal pigment epithelial cell line ARPE-19 and also from the organic anion transporting polypeptides, which are known to transport opioid peptides.
Conclusions. CJVE cells express a novel, hitherto unknown transport process for endogenous/synthetic opioid peptides. This new transport process may offer an effective delivery route for opioid peptide drugs to the posterior segment of the eye.

KEY WORDS: conjunctival epithelial cell; non-peptide opioid antagonists; opioid peptides; organic anion transporting polypeptides; transport process.

INTRODUCTION

The conjunctiva constitutes an epithelial barrier in the anterior part of the eye. It lines the inside of the eyelids and covers the anterior one-third of the eyeball. It is made up of several layers of epithelial cells, the cells in the outermost layer forming a permeability barrier because of tight junctions between adjacent cells. Transfer across this epithelial barrier enables topically applied drugs to gain access to the intraocular tissues of the posterior segment of the eye. The conjunctiva thus constitutes an important non-corneal absorption route for topical ophthalmic drug delivery bypassing the anterior chamber of the eye. The pharmacologic and therapeutic potential of drug delivery across the conjunctival epithelial cell layer has prompted many investigators to identify and catalog the repertoire of transport systems in this cell type. A recent review by Hosoya *et al.* (1) provides an excellent summary of various transport systems which have been identified in conjunctival epithelial cells. The organic solutes for which transport systems have been identified in this cell layer include amino acids, nucleosides, monocarboxylates, and small peptides (i.e., dipeptides and tripeptides). In addition to these transport systems which mostly function in the influx mode, the conjunctival epithelial cells also express P-glycoprotein which functions as an efflux transporter. Many of these transport systems recognize various pharmacologically and therapeutically relevant xenobiotics as transportable substrates. This feature makes these transport systems in the conjunctival epithelial cell layer as targets for delivery of a wide variety of drugs to the posterior segment of the eye.

Peptidomimetic drugs are usually hydrophilic and therefore do not freely diffuse across cell membranes. These drugs normally penetrate cell membranes via specific transport systems. The transporters known to participate in the handling of peptidomimetic drugs include the H^+-coupled peptide transporters PEPT1 and PEPT2 (2–4), the organic anion transporting polypeptides (OATPs) (5), and the ATP-dependent efflux transporters such as P-glycoprotein, breast cancer related protein (BCRP), and multidrug resistance-associated polypeptides (MRPs) (6,7). Recently, we identified at the functional level a new transport system for a variety of

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endogenous and synthetic opioid peptides in the retinal pigment epithelial cell line ARPE-19 (8) and the neuronal cell line SK-N-SH (9). This transport system differs in several respects from the above-mentioned transporters for peptidomimetic drugs (10). PEPT1 and PEPT2 are driven by a transmembrane H^+ gradient and accept only di- and tripeptides as substrates (2–4) whereas the opioid peptide transport system handles longer peptides consisting of five or more amino acids. Di- and tripeptides are not substrates for the opioid peptide transport system, but serve as allosteric positive modulators. OATPs are anion transporters which accept, in addition to various synthetic opioid peptides, a variety of non-peptide anions and non-peptide opioid antagonists as substrates (5) whereas the opioid peptide transport system does not recognize organic anions and non-peptide opioid antagonists. The efflux transporters P-glycoprotein, BCRP, and MRPs are driven by ATP hydrolysis and handle a broad spectrum of peptide and non-peptide compounds (6, 7). In contrast, the opioid peptide transport system is an influx transporter, energized by a Na^+ gradient.

The present investigation was undertaken to examine whether conjunctival epithelial cells possess transport processes for opioid peptides. For this purpose, we used the rabbit conjunctival epithelial cell line CJVE as a model system. Here we show that the CJVE cells have robust transport activity for opioid peptides and that the transport process responsible for the activity may be distinct from the opioid peptide transport system that we described previously in ARPE-19 and SK-N-SH cells. It is also different from PEPT1, PEPT2, OATPs, and the efflux transporters. The opioid peptide transport process identified in CJVE cells is novel and has never been described in any other cell type. This novel transport process has potential for topical ophthalmic delivery of peptide-based opioidergic drugs.

MATERIALS AND METHODS

Materials

The synthetic opioid peptides DPDPE, DADLE, and DAMGO were obtained either from the National Institute on Drug Abuse Research Resources (National Institutes of Health, Bethesda, MD) or from Bachem Americas, Inc. (Torrance, CA). All other opioid peptides were obtained from the American Peptide Company, Inc. (Sunnyvale, CA). The non-peptide opioid antagonists naloxone and naltrexone, non-opioid peptides, dimethylamiloride, and the pan-cytokeratin mouse monoclonal antibody (clone C-11) were obtained from Sigma-Aldrich (St. Louis, MO). The amino acid sequences of various opioid peptides used in the present study are given in Table I.

[tyrosyl-3,5–^{3}H(N)]Deltorphin II (specific radioactivity, 38.5 Ci/mmol) and [tyrosyl-3,5–^{3}H(N)]DADLE (specific radioactivity, 45.7 Ci/mmol) were purchased from PerkinElmer (Boston, MA).

Cell Line

The rabbit conjunctival epithelial cell line CJVE used in the present study was kindly provided by Dr. K. Araki-Sasaki (Osaka University Graduate School of Medicine, Osaka, Japan). This cell line was developed by immortalization of rabbit conjunctival epithelial cells by a procedure similar to the one used for the development of rabbit corneal epithelial cell lines (11,12). Briefly, primary cultures of rabbit conjunctival epithelial cells were incubated with a recombinant SV40-adenovirus vector for 1 h at a multiplicity of 1:100. After infection, cells were cultured in hormone-supplemented epithelial medium. Immortalized cells were then cloned by the filter paper technique (12). The cell line was maintained in Minimum Essential Medium, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin. The components of the cell culture medium were obtained from the following sources: Minimum Essential Medium from Invitrogen (Carlsbad, CA), fetal bovine serum from HyClone (Logan, UT), and non-essential amino acids and penicillin/streptomycin from Mediatech, Inc. (Manassas, VA).

Table I. Amino Acid Sequences of the Endogenous and Synthetic Opioid Peptides Used in the Present Study

Opioid peptide	Amino acid sequence
Met-Enkephalin	Tyr-Gly-Gly-Phe-Met
Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-Enkephalin (des-Tyr1)	Gly-Gly-Phe-Leu
Met-Enkephalinamide	Tyr-Gly-Gly-Phe-Met-NH_2
Enkephalin (Met5, Arg6, Gly7, Leu8)	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
Dynorphin (1–7)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg
Dynorphin (1–9)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
β-Lipoprotein (61–64)	Tyr-Gly-Gly-Phe
Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
DPDPE	Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH
DADLE	Tyr-D-Ala-Gly-Phe-D-Leu
DAMGO	Tyr − D − Ala − Gly− N^{α} − Me − Phe − Gly − ol

D-Pen D-Penicillamine

Morphological and Immunofluorescence Studies

To characterize the morphology of the CJVE cells in culture, phase-contrast images were obtained using a Nikon Eclipse TE300 microscope equipped with a Photometrics CoolSNAP digital camera and RSImage v1.9.2 imaging software (Roper Scientific, Inc.). For immunofluorescence analysis, cells grown on coverslips in a 24-well plate were fixed with ice-cold methanol. Prior to treatment with the primary antibody, coverslips were treated with Image-iT FX Signal Enhancer (Invitrogen) for 1 h. The primary antibody (mouse pan-monoclonal antibody against cytokeratins), diluted 1 in 250 in 1× Universal Blocking Reagent (BioGenex, San Ramon, CA), was added to the coverslips which were then incubated at 4°C overnight. After the treatment, the coverslips were washed with phosphate-buffered saline and treated again with Image-iT FX Signal Enhancer. The secondary antibody (goat anti-mouse IgG), conjugated with Oregon Green 488, was then added (1 in 1,500 dilution). The treatment with the secondary antibody was for 30 min at room temperature. A mounting solution (Vectashield Hard Set mounting solution, Vector Laboratories, Burlingame, CA)

containing the nuclear stain DAPI (4′,6-diamidino-2-phenylindole) was then added. Coverslips were then examined using a Zeiss Axioplan 2 fluorescent microscope equipped with AxioCamHR digital camera and AxioVision 4.3 software.

Uptake Measurements

CJVE cells were seeded in 24-well culture plates at an initial density of 1×10^6 cells/well and cultured for 4 days to obtain confluent cultures. The culture medium was replaced with freshly prepared medium every other day. Uptake of [^{3}H]deltorphin II and [^{3}H]DADLE in these cells was measured as described previously (8,9). The medium was removed by aspiration and the cells washed with uptake buffer once. Uptake was initiated by adding 0.25 ml of uptake buffer containing 0.2 μCi of [^{3}H]deltorphin II or [^{3}H]DADLE. Concentration of these peptides during uptake varied between 10 and 20 nM depending on the experiment. Initial experiments were carried out to determine the time course of uptake. Subsequent uptake measurements were made with 15 min incubation. At the end of the incubation period, uptake was terminated by aspirating out the uptake buffer from the wells. The cell monolayers were quickly washed twice with ice-cold uptake buffer without the radiolabeled substrates. Cells were then lysed in 0.5 ml of 2% SDS/2N NaOH and the radioactivity associated with the cells was counted by scintillation spectrophotometry. The composition of the uptake buffer in most experiments was: 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. When Na^+-free buffer was used, NaCl in the uptake buffer was replaced iso-osmotically with *N*-methyl-D-glucamine chloride (NMDGCl). Non-mediated diffusional component of uptake was determined by measuring the uptake of radiolabeled deltorphin II or DADLE in the presence of excess (2.5 mM) of unlabeled deltorphin II or DADLE respectively. For both peptides, the diffusional component represented less than 5% of measured total uptake. Saturation kinetics was analyzed by measuring the uptake with increasing concentrations of the substrate. The Michaelis constant and the maximal velocity were determined by fitting the Michaelis-Menten equation describing a single saturable transport system to the data: $v = V_{max}\cdot S/(K_t+S)$ where v is the uptake rate, S is the substrate concentration, K_t is the Michaelis constant, and V_{max} is the maximal velocity. The kinetic nature of interaction between deltorphin II and DADLE was evaluated by investigating the effect of DADLE on the saturation kinetics of deltorphin II uptake and the effect of deltorphin II on the saturation kinetics of DADLE uptake.

Data Analysis

The kinetic parameters (K_t and V_{max}) were determined using the computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL). These determinations were made by nonlinear regression analysis and the values confirmed by linear regression analysis according to the Eadie-Hofstee transformation of the Michaelis-Menten equation. The kinetic nature of interaction between deltorphin II and DADLE was analyzed by determining the effects of the inhibitor (DADLE or deltorphin II) on the K_t and V_{max} values for the uptake of deltorphin II or DADLE. The IC_{50} values (i.e., concentrations of inhibitors necessary for 50% inhibition) were calculated from dose-dependence studies. Statistical analysis was made using the Student's t test, and a $p<0.05$ was taken as statistically significant. Experiments were repeated two or three times and measurements were made in duplicate for each experimental condition. Data are presented as means ± S. E.

RESULTS

Characterization of CJVE Cells

Figure 1A describes the morphological features of confluent cultures of CJVE cells by phase-contrast microscopy. The cells assume the cobblestone appearance when confluent. We then examined the expression of cytokeratins, an epithelial cell marker (13), in these cells. For this, we used a pan-

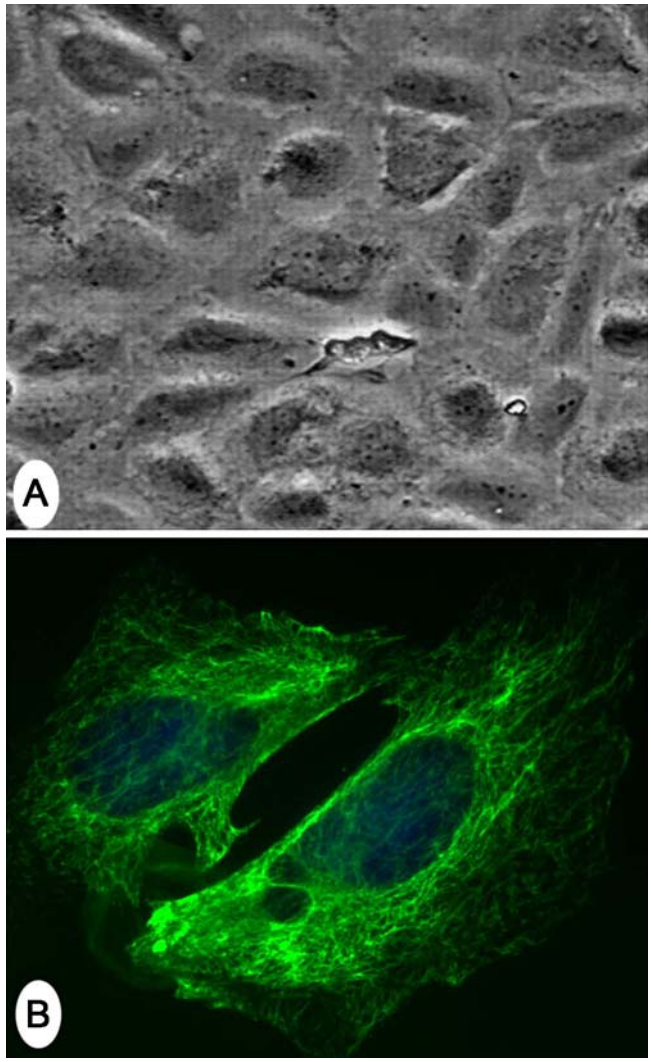

Fig. 1. Morphology and cytokeratin expression in rabbit CJVE cells. **A** Phase-contrast micrograph of confluent cultures of CJVE cells, demonstrating the cobblestone appearance. **B** Immunofluorescence analysis of cytokeratin expression in CJVE cells using a pan-cytokeratin monoclonal antibody. Green fluorescence, cytokeratin; Blue fluorescence, nuclear stain DAPI.

cytokeratin antibody which recognizes a wide variety of cytokeratins. The CJVE cells were positive for cytokeratins (Fig. 1B). These data show that CJVE cell line is an appropriate model system for conjunctival epithelial cells.

Time Course and Kinetic Features of Deltorphin II Uptake in CJVE Cells

Deltorphin II is a linear heptapeptide (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) which is resistant to mammalian peptidases. The presence of a D-amino acid at position 2 makes it resistant to aminopeptidases and the presence of an amide at the C-terminus makes it resistant to carboxypeptidases. It is one of the opioid peptides secreted from the skin glands of *Phyllomedusa* amphibians and has very high affinity and selectivity for δ opioid receptor (14,15). We examined the uptake of this opioid peptide in CJVE cells. Figure 2 describes the time course of deltorphin II uptake in the presence and absence of Na^+. CJVE cells were found to have robust uptake activity for this peptide. The uptake process was partly Na^+-dependent as evidenced from the significant difference in uptake when monitored in the presence and absence of Na^+. The uptake measured in the presence of NaCl was saturable (Fig. 3A). The kinetic parameters (Michaelis constant, K_t and Maximal velocity, V_{max}) for this process were 45±4 μM and 20. 6±0.5 nmol/mg of protein/15 min, respectively. Interestingly, the uptake in the absence of Na^+ was also saturable (Fig. 3B). Even though the uptake activity was lower with a V_{max} of 12.9±0.7 nmol/mg of protein/15 min, the K_t value (57±6 μM) was comparable to the value determined in the presence of Na^+. A caveat in these experiments is that the uptake measurements were made using a 15 min incubation, a time point which does not provide linear uptake rates. The high cost of the radiolabeled peptide precluded the use of shorter time periods for most uptake experiments. Because the experimental conditions employed in kinetic studies are not ideal, the values calculated for the kinetic parameters from these studies may only approximate the true values.

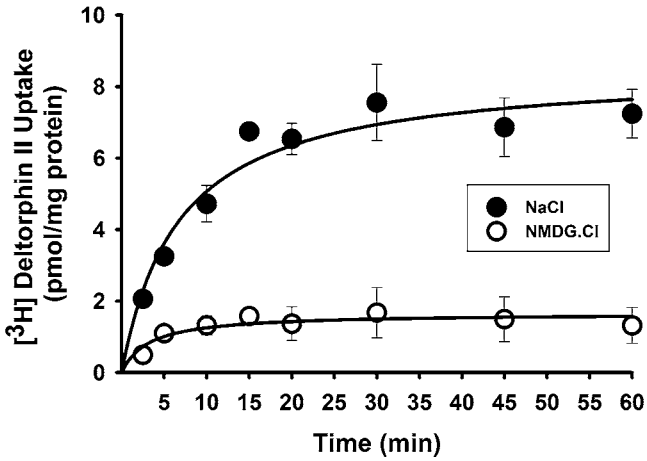

Fig. 2. Time course of deltorphin II uptake in rabbit CJVE cells. Uptake of [^{3}H]deltorphin II (10 nM) was measured in confluent cultures of CJVE cells for varying periods of time in the presence (NaCl) or absence (NMDGCl) of Na^+. The experiment was repeated three times, each done in duplicate. Data represent means ± S. E.

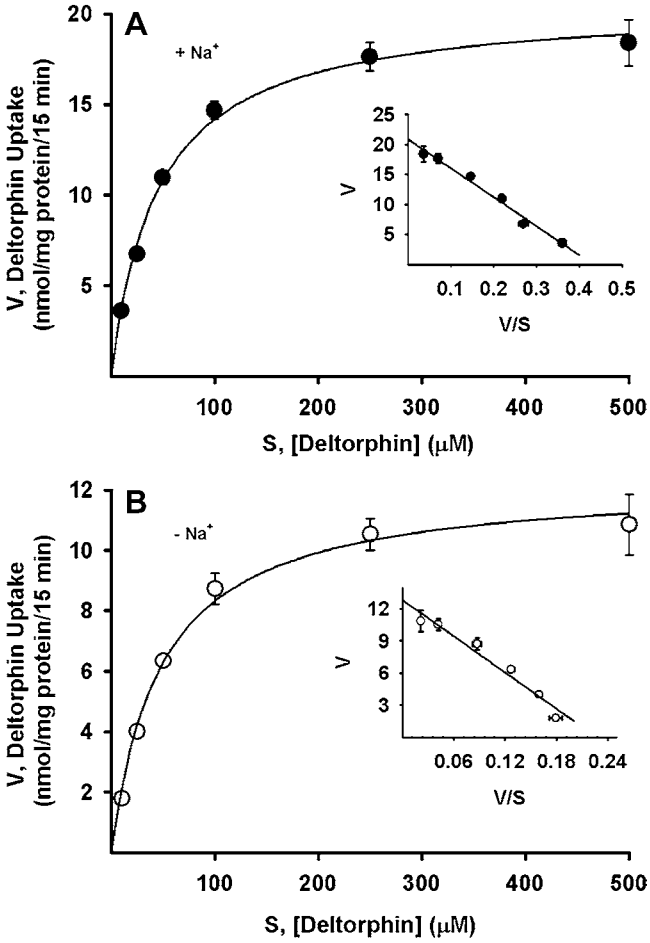

Fig. 3. Saturation kinetics of deltorphin II uptake in rabbit CJVE cells in the presence or absence of Na^+. **A** Uptake was measured in the presence of [^{3}H]deltorphin II (20 nM) and increasing concentrations of unlabeled deltorphin II in a NaCl-containing medium for 15 min. *Inset*: Eadie-Hofstee transformation of the data given in the main figure. **B** Uptake was measured for 15 min in the presence of [^{3}H]deltorphin II (20 nM) and increasing concentrations of unlabeled deltorphin II in a medium in which NaCl was replaced with NMDGCl iso-osmotically. *Inset*: Eadie-Hofstee transformation of the data given in the main figure. The experiment was repeated twice, each in duplicate. Data (means ± S. E.) are for these replicate values.

Inhibition of Deltorphin II Uptake by Endogenous and Synthetic Opioid Peptides

We then examined the ability of a variety of endogenous and synthetic opioid peptides to compete with [^{3}H]deltorphin II for the uptake process in CJVE cells (Table II). At a concentration of 0.25 mM, all opioid peptides examined inhibited the uptake of deltorphin II (10 nM) markedly. Since the uptake of deltorphin II was saturable in the presence and absence of Na^+, we compared the ability of all these opioid peptides to inhibit deltorphin II uptake in the presence of NaCl as well as in the presence of NMDGCl (Table II). The peptides were equally effective in inhibiting the uptake in the presence and absence of Na^+. In contrast, the non-peptide opioid antagonists naloxone and naltrexone did not compete with deltorphin II for the uptake process. In fact, these antagonists had significant stimulatory effect on deltorphin II uptake ($p<0.05$). We then determined the IC_{50}

Table II. Inhibition of [^{3}H]-deltorphin II Uptake in a Rabbit Conjunctival Epithelial Cell Line (CJVE) by Endogenous and Synthetic Opioid Peptides and Non-peptide Opioid Antagonists

	[^{3}H]-Deltorphin II uptake			
	NaCl		NMDGCl	
Compound	pmol/mg protein/15 min	%	pmol/mg protein/15 min	%
Control	2.76±0.19	100	1.04±0.09	100
Met-Enkephalin	0.07±0.01	3	0.06±0.01	5
Leu-Enkephalin	0.07±0.01	3	0.05±0.01	4
Met-Enkephalin (des-Tyr1)	0.20±0.01	7	0.12±0.01	11
Met-Enkephalinamide	0.09±0.01	3	0.06±0.01	6
Enkephalin (Met5, Arg6, Gly7, Leu8)	0.05±0.01	2	0.04±0.01	4
Dynorphin (1–7)	0.04±0.01	2	0.04±0.01	4
Dynorphin (1–9)	0.04±0.01	2	0.03±0.01	2
Endomorphin-1	0.71±0.12	26	0.42±0.06	41
β-Lipotrophin (61–64)	0.38±0.08	14	0.30±0.02	29
Deltorphin I	0.17±0.01	6	0.08±0.01	8
Deltorphin II	0.30±0.02	11	0.13±0.01	12
DPDPE	0.43±0.04	16	0.35±0.01	33
DADLE	0.08±0.01	3	0.06±0.01	5
DAMGO	0.18±0.02	7	0.11±0.01	10
Naloxone	3.36±0.13	122	1.53±0.09	146
Naltrexone	3.27±0.23	119	1.34±0.16	128

Uptake of [^{3H}]-deltorphin II (10 nM) was measured in confluent cultures of CJVE cells for 15 min in the presence (NaCl) or absence (NMDGCl) of Na^+. The concentration of the competitors was 250 μM. Data are means ± S.E. for four measurements from two independent experiments.

values for selected opioid peptides for the inhibition of deltorphin II uptake from dose-response studies (Fig. 4). We used four endogenous opioid peptides (Met-enkephalin, Leu-enkephalin, dynorphin 1–7, and dynorphin 1–9) and three synthetic opioid peptides (DADLE, DAMGO, and DPDPE). The IC_{50} values for the endogenous peptides were 12.9±3.5, 8.8±2.2, 12.3±2.9, and 7.8±1.0 μM, respectively. The IC_{50} values for the synthetic peptides were 6.0±1.9, 9.0±3.0, and 99.2±32.5 μM, respectively. Thus, all four endogenous opioid peptides showed comparable affinity for the deltorphin II transport system in these cells. Among the synthetic opioid peptides, DADLE and DAMGO had comparable affinity while DPDPE showed a 10-fold lower affinity.

Influence of Non-opioid Peptides on Deltorphin II Uptake in CJVE Cells

We have previously shown that the opioid peptide transport system identified in SK-N-SH cells was markedly stimulated by dipeptides and tripeptides (9). Therefore, in the present study we evaluated the influence of several non-opioid peptides on deltorphin II uptake in CJVE cells (Table III). At a concentration of 1 mM, none of the peptides examined had a stimulatory effect on deltorphin II uptake. In fact, most of these peptides had significant inhibitory effect on deltorphin II uptake ($p<0.05$). This was an unexpected result. Since the opioid peptide transport system expressed in SK-N-SH cells is also present in ARPE-19 cells, we compared the influence of these peptides on deltorphin II uptake in CJVE cells and ARPE-19 cells under identical conditions (Table III). As expected, most of the peptides stimulated the uptake of deltorphin II in ARPE-19 cells. This stimulatory effect was observed under identical conditions in which the same peptides inhibited deltorphin II uptake in CJVE cells. The tetrapeptide Gly-Pro-Gly-Gly did not have much effect on deltorphin II uptake in ARPE-19 cells as well as in CJVE cells. The pentapeptide Gly-Gly-Gly-Gly-Gly did not affect deltorphin II uptake in ARPE-19 cells but caused significant inhibition in CJVE cells. These data indicated for the first time that the opioid peptide transport process in CJVE cells may be distinct from the opioid peptide transport process in ARPE-19 and SK-N-SH cells.

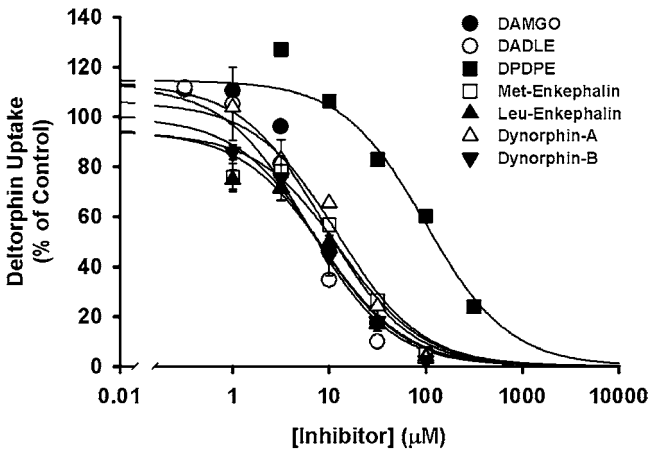

Fig. 4. Dose-response relationship for the inhibition of deltorphin II uptake by various endogenous and synthetic opioid peptides in rabbit CJVE cells. Uptake of [^{3}H]deltorphin II (10 nM) was measured for 15 min in a NaCl-containing medium in the presence of increasing concentrations of the opioid peptides. Uptake measured in the absence of competing opioid peptides was taken as 100%. Data (means ± S. E.) are for 4 independent measurements from two separate experiments.

Table III. Influence of Non-opioid Peptides on Deltorphin II Uptake in the Rabbit Conjunctival Epithelial Cell Line CJVE and the Human Retinal Pigment Epithelial Cell Line ARPE-19

	Deltorphin II uptake			
	CJVE		ARPE-19	
Non-opioid peptide	pmol/mg protein/15 min	%	pmol/mg protein/15 min	%
Control	3.13±0.22	100	3.00±0.34	100
Gly-Gly-Ala	2.29±0.18	73	6.06±0.18	202
Gly-Gly-Leu	2.00±0.16	64	9.69±0.32	323
Gly-Gly-His	1.55±0.02	49	8.64±0.76	288
Gly-Gly-Ile	1.80±0.08	57	13.70±0.30	457
Gly-Gly-Phe	0.98±0.08	31	7.92±0.81	264
Leu-Gly-Gly	1.08±0.10	35	7.44±0.22	248
Val-Tyr-Val	0.92±0.10	29	8.19±1.44	273
Gly-Gly-Gly-Gly	1.98±0.07	63	4.86±0.57	162
Gly-Pro-Gly-Gly	2.67±0.19	85	2.49±0.47	83
Gly-Gly-Gly-Gly-Gly	1.29±0.08	41	2.83±0.24	94

Uptake of [^{3}H]-deltorphin II (10 nM) was measured in confluent cultures of CJVE and ARPE-19 cells for 15 min in the presence of NaCl. The concentration of non-opioid peptides was 1 mM. Data are means ± S.E. for four measurements from two independent experiments.

Influence of Prototypic Substrates of OATPs on Deltorphin II Uptake in CJVE Cells

OATPs are organic anion transporters which mediate the cellular uptake of a variety of organic anions in a Na^+-independent manner (5). Some of the members of the OATP family (e.g., OATP1A2, OATP1B1, and OATP1B3) transport deltorphin II (16–19). But this transport process is inhibitable by non-peptide opioid antagonists naloxone (16–19). The Na^+-dependent nature of the deltorphin II uptake system in CJVE cells and the lack of inhibition of this process by naloxone and naltrexone indicate that the transporter(s) responsible for this uptake process is/are distinct from OATPs. To further confirm this, we evaluated the influence of two prototypical anionic substrates of OATPs, namely taurocholate and estrone-3-sulfate, on deltorphin II uptake in CJVE cells. Taurocholate (100 μM) and estrone-3-sulfate (250 μM) failed to inhibit deltorphin II uptake in these cells (data not shown). At the concentrations employed in the present study, taurocholate and estrone-3-sulfate are known to have marked inhibitory effect on the transport process mediated by OATPs (16–19). These data clearly show that the opioid peptide transport system expressed in CJVE cells is distinct from OATPs.

Uptake of DADLE in CJVE Cells

Competition studies described in Fig. 4 showed that DADLE inhibits deltorphin II uptake with an IC_{50} of 6.0± 1.9 μM. Among the opioid peptides examined in the present study, DADLE showed the highest affinity for the opioid peptide transport process in CJVE cells. DADLE is an analog of Leu-enkephalin with D-Ala at amino acid position 2 and D-Leu at amino acid position 5. It is resistant to hydrolysis by peptidases and it is available in radiolabeled form. Therefore, we examined directly the transport of DADLE in CJVE cells. We detected robust uptake activity in these cells for DADLE. Figure 5A describes the time course of DADLE uptake in the presence and absence of Na^+. In contrast to deltorphin II whose uptake was markedly stimulated by Na^+, the uptake of DADLE was influenced by Na^+ only minimally. The uptake process was saturable, with a K_t value of 8.7±0.6 μM and a V_{max} value of 11.1±0.2 nmol/mg of protein/15 min (Fig. 5B). The Michaelis constant for DADLE uptake is similar to the IC_{50} value calculated for the inhibition of deltorphin II uptake by DADLE (6.0± 1.9 μM), indicating that both peptides may be transported by the same process. We then investigated the characteristics of DADLE uptake. First, the uptake was not sensitive to inhibition by organic anions such as taurocholate and estrone-3-sulfate (data not shown), showing that OATPs are not responsible for the observed uptake. Second, the various tripeptides which inhibited deltorphin II uptake also inhibited DADLE uptake (Table IV). Third, many endogenous and synthetic opioid peptides which competed with deltorphin II for the uptake process also competed with DADLE during uptake (Table IV). Fourth, the non-peptide opioid antagonists naloxine and naltrexone, which failed to inhibit deltorphin II, also did not inhibit DADLE uptake (Table IV). Thus, the characteristics of DADLE uptake are very similar to those of deltorphin II uptake, except for the difference in Na^+-dependence.

We then investigated the dose-response relationship for the inhibition of DADLE uptake by deltorphin II. The IC_{50} value for the inhibition was 70±8 μM (data not shown). This value is different from the Michaelis constant determined for the transport of deltorphin II (45±4 μM). We then analyzed the kinetics of inhibition of deltorphin II transport by DADLE and of the inhibition of DADLE transport by deltorphin II (Fig. 6). We found the inhibition in both cases to be competitive, the presence of the inhibitor resulting in an increase in Michaelis constant without having any significant effect on the maximal velocity.

Influence of Dimethylamiloride, an Inhibitor of Na^+/H^+ Exchanger, on the Uptake of Deltorphin II and DADLE

In the case of deltorphin II as well as DADLE, we found a significant fraction of uptake to be Na^+-independent. This fraction was saturable, suggesting that it represents a carrier-

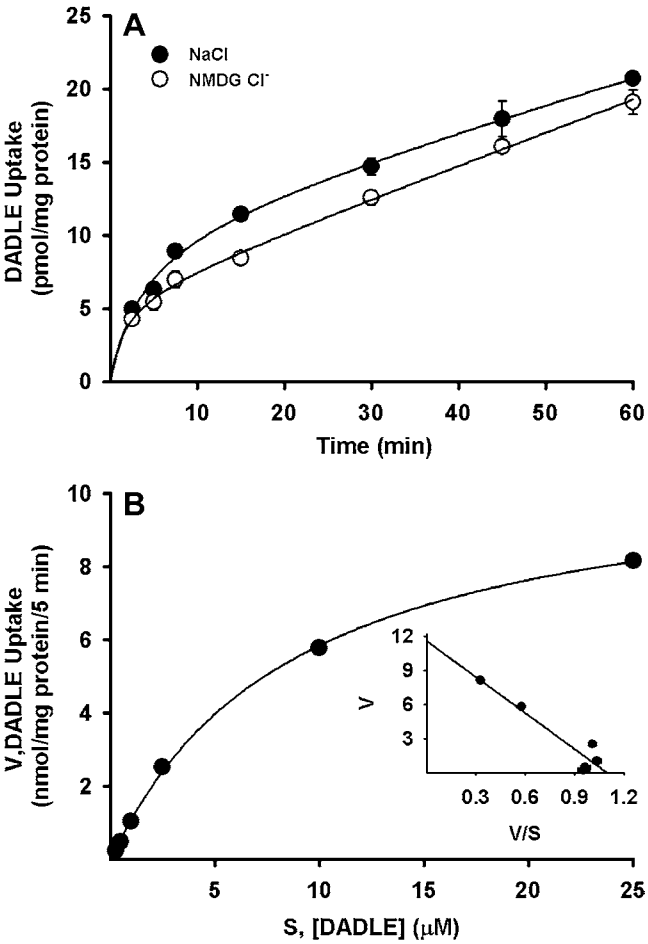

Fig. 5. Characteristics of the uptake of DADLE in rabbit CJVE cells. **A** Uptake of [^{3}H]DADLE (10 nM) was measured in confluent cultures of CJVE cells for varying periods of time in the presence (NaCl) or absence (NMDGCl) of Na^+. **B** Saturation kinetics of DADLE uptake was evaluated by monitoring the uptake in a NaCl-containing medium with varying concentrations of DADLE. The concentration of [^{3}H]DADLE was kept constant at 10 nM and the concentration was varied by the addition of unlabeled DADLE. *Inset*: Eadie-Hofstee transformation of the data given in the main figure. The experiments were repeated twice, each done in duplicate. Data are means ± S. E. for these replicate values.

mediated process. The partial dependence of these uptake activities on Na^+ might be explained if these transport processes are H^+-dependent and Na^+/H^+ exchanger is functionally coupled to these transport processes. We investigated this possibility by examining the effect of dimethylamiloride on the uptake of deltorphin II and DADLE. Dimethylamiloride is a potent inhibitor of Na^+/H^+ exchanger. If the exchanger is coupled functionally to the uptake of deltorphin II and DADLE, the presence of dimethylamiloride would be expected to inhibit the uptake when measured in a Na^+-containing medium. We found no evidence of such inhibition. The values for deltorphin II (10 nM) uptake in the absence and presence of dimethylamiloride (100 μM) were 2.90±0.10 and 3.10±0.16 pmol/mg of protein/15 min, respectively. The difference between these two values was not statistically significant. Similarly, the values for DADLE (10 nM) uptake in the absence and presence of dimethylamiloride (100 μM) were 8.20±0.35 and 7.74±0.40 pmol/mg of protein/15 min, respectively. The difference between these two values was also not statistically significant. These data show that the partial dependence of these transport processes on Na^+ is not due to functional coupling with Na^+/H^+ exchanger.

DISCUSSION

Here we describe the uptake characteristics of two hydrolysis-resistant opioid peptides in the rabbit conjunctival epithelial cell line CJVE. The peptide substrates are deltorphin II and DADLE. The uptake of deltorphin II in these cells is predominantly Na^+-dependent. However, even though the uptake is reduced considerably when measured in the absence of Na^+, the uptake process is saturable not only in the presence of Na^+ but also in the absence of Na^+. These data show that a carrier-mediated process is responsible for the Na^+-dependent as well as for the Na^+-independent uptake. This is supported further by the ability of a variety of endogenous and synthetic opioid peptides to inhibit deltorphin II uptake irrespective of whether the uptake is monitored in the presence or absence of Na^+. The Michaelis constant for the uptake process is ~50 μM. Interestingly, the substrate affinity is not altered by the presence or absence of Na^+. This is surprising because, with most Na^+-coupled transport systems, the presence of Na^+ increases the substrate affinity as has been observed, for example, with the Na^+-coupled glucose transport system (20) and the Na^+-coupled proline transport system (21).

We have recently reported on the identification of a Na^+-coupled transport system for deltorphin II in the human retinal pigment epithelial cell line ARPE-19 and also in the human neuronal cell line SK-N-SH (8,9). However, the transport process discovered in the rabbit conjunctival cells seems to be distinct from the one reported in ARPE-19 and SK-N-SH cells. This conclusion is based on the observed differences in the effects of various tripeptides. In SK-N-SH cells, the tripeptides have a marked stimulatory effect on deltorphin II uptake. This is also true in ARPE-19 cells as evidenced in the present study. However, under identical conditions, the uptake of deltorphin II in CJVE cells is inhibited by the tripeptides.

In the previous study with ARPE-19 cells (8), we examined substrate selectivity of the opioid peptide uptake system using mostly endogenous opioid peptides. These studies showed that the transport system in these cells is able to handle opioid peptides consisting of five or more amino acids. The interaction of synthetic opioid peptides with this transport system was not examined. In the present study, we evaluated the ability of not only endogenous opioid peptides but also of synthetic opioid peptides to compete with deltorphin II for the uptake process in CJVE cells. These studies have shown that the new opioid peptide transport process expressed in the rabbit CJVE cell line is also able to handle a wide variety of endogenous opioid peptides consisting of five or more amino acids. In addition, the synthetic opioid peptides DADLE, DPDPE, and DAMGO are also able to compete with deltorphin II for the uptake process very effectively. In fact, DADLE is among the opioid peptides which exhibit the highest affinity for the transport process. Its affinity is comparable to that of the endogenous

Table IV. Influence of Tripeptides, Opioid Peptides, and Non-peptide Opioid Antagonists on [^{3}H]-DADLE Uptake in the Rabbit Conjunctival Epithelial Cell Line CJVE

Compound	[^{3}H]DADLE uptake	
	pmol/mg protein/15 min	%
Control	8.90±0.30	100
Tripeptides		
Gly-Gly-Ile	0.89±0.04	10
Gly-Gly-Phe	0.38±0.02	4
Gly-Gly-Leu	2.16±0.17	24
Gly-Gly-His	2.44±0.05	27
Opioid peptides		
Deltorphin II	4.21±0.19	47
DADLE	0.65±0.05	7
DAMGO	0.93±0.04	10
Leu-Enkephalin	0.08±0.01	1
Met-Enkephalin	0.08±0.01	1
Non-peptide opioid antagonists		
Naloxone	10.89±0.17	122
Naltrexone	9.89±0.28	111

Uptake of [^{3}H]-DADLE (10 nM) in confluent cultures of CJVE cells was measured for 15 min in a NaCl-containing medium. The concentration of tripeptides was 1 mM and that of opioid peptides and non-peptide opioid antagonists was 250 μM. Data are means ± S. E. for four measurements from two independent experiments

opioid peptides Leu-enkephalin, Met-enkephalin, and dynorphins. Since DADLE is also a hydrolysis-resistant peptide, we investigated the uptake characteristics of this synthetic peptide in rabbit CJVE cells. We expected the uptake process to be Na^+-dependent as we found for deltorphin II uptake, but the results were different. The uptake of DADLE in CJVE cells is mostly Na^+-independent, but saturable. This finding raised the question whether or not DADLE is transported via the same system that is responsible for deltorphin II uptake. We addressed this question by using a kinetic approach, known as A-B-C test (22,23). We have used this approach successfully in our previous studies to show that the dipeptide glycylsarcosine and the tripeptide Phe-Pro-Ala are transported via a common system in the kidney (24). According to the A-B-C test, if two compounds, 'A' and 'B', are transported via the same system, then the uptake characteristics for these two compounds must satisfy the following criteria: (a) increasing concentrations of 'A' should be able to inhibit the uptake of 'B' completely and increasing concentrations of 'B' should be able to inhibit the uptake of 'A' completely, (b) 'A' should function as a competitive inhibitor of 'B', and 'B' should function as a competitive inhibitor of 'A', (c) the IC_{50} value for 'A' to inhibit the uptake of 'B' should be similar to the Michaelis constant for the uptake of 'A', and the IC_{50} value for 'B' to inhibit the uptake of 'A' should be similar to the Michaelis constant for the uptake of 'B', and (d) the effects of a third set of compounds, denoted as 'C', should be similar for the uptake of 'A' and 'B'. The uptake characteristics of deltorphin II and DADLE in CJVE cells meet most of these criteria. Increasing concentrations of DADLE are able to block the uptake of deltorphin II completely (Fig. 4) and increasing concentrations of deltorphin II are able to block the uptake of DADLE completely (data not shown). DADLE and deltorphin II inhibit the uptake of each other in a competitive manner, by increasing the value for K_t without having any significant effect on V_{max} (Fig. 6). The IC_{50} value for DADLE to inhibit deltorphin II uptake (6.0±1.9 μM) is similar to the K_t value determined for DADLE uptake (8.7±0.6 μM). But, the IC_{50} value for deltorphin II to inhibit DADLE uptake (70±8 μM) is significantly different from the K_t value determined for deltorphin II uptake (45±4 μM). The IC_{50} values determined under the experimental conditions employed in the present study are almost equal to the inhibition constant (K_i) as the concentration of deltorphin II or DADLE used for measurement of their uptake was 10 nM, a value 2 to 3 orders of magnitude less than their corresponding K_t values. Finally, the inhibitory effects of various endogenous and synthetic opioid peptides on the uptake of deltorphin II and DADLE are comparable (Tables II and IV). The same is true for the non-peptide opioid antagonists naloxone and naltrexone

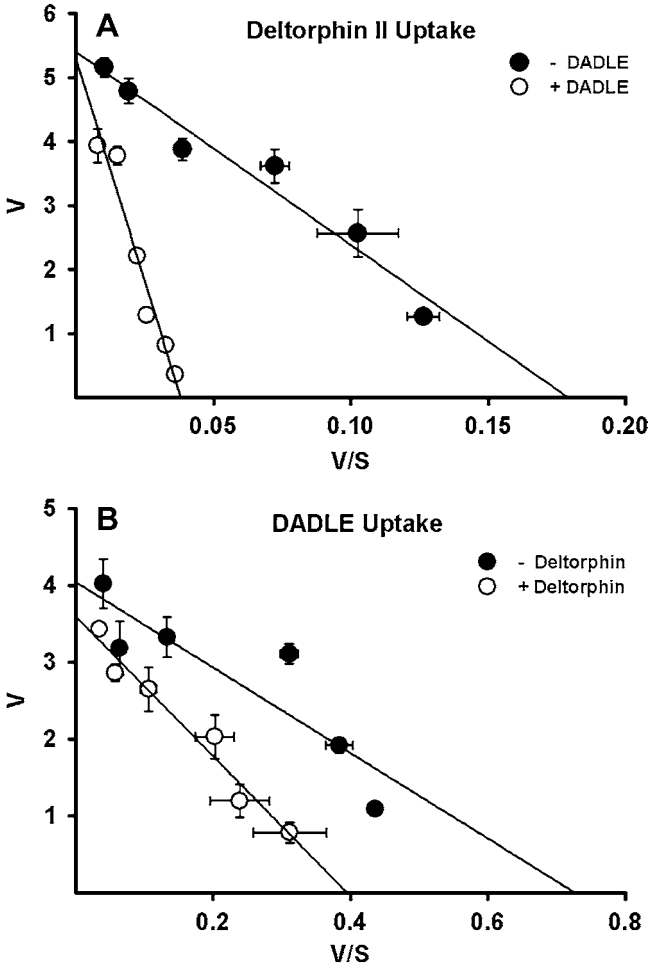

Fig. 6. Kinetic nature of the interaction between deltorphin II and DADLE for the uptake process in rabbit CJVE cells. **A** Uptake of deltorphin II was measured for 15 min in a NaCl-containing medium with increasing concentrations of deltorphin II in the presence or absence of 7.5 μM DADLE. The concentration of [^{3}H]deltorphin was kept constant at 20 nM. **B** Uptake of DADLE was measured for 15 min in a NaCl-containing medium with increasing concentrations of DADLE in the presence or absence of 60 μM deltorphin II. The concentration of [^{3}H]DADLE was kept constant at 15 nM. In both cases, experiments were repeated twice, each in duplicate. Data (means ± S. E.) are presented in the form of Eadie-Hofstee plots.

(Tables II and IV). However, there are significant differences in the functional features of deltorphin II uptake and DADLE uptake, which suggest that more than one transport system may be involved in the uptake of these peptides in CJVE cells. Thus, the A-B-C test turned out to be insufficient to conclude whether or not a single transport system is responsible for the observed uptake of deltorphin II and DADLE in these cells. Additional studies are needed to address this issue.

In summary, the studies reported here identify a novel transport process for opioid peptides. To date, this transport process has not been described in any other cell type. It handles a variety of endogenous and synthetic opioid peptides. It shows high affinity for these peptides, with Michaelis constant in low micromolar range. Thus, mammalian cells express more than one transport system for opioid peptides, all of them recognizing as substrates a variety of opioid peptides consisting of five or more amino acids. The principal distinguishing feature between the transport system described previously in the human RPE cell line ARPE-19 and the transport process identified in the rabbit conjunctival cell line CJVE in the present study seems to be with regard to the effects of non-opioid peptides. The transport system in ARPE-19 cells is markedly stimulated by tripeptides whereas the transport process in CJVE cells in the present study is inhibited by tripeptides. Whether or not the inhibitory tripeptides serve as transportable substrates for the process in CJVE cells remains to be determined. The non-availability of a suitable hydrolysis-resistant tripeptide in radiolabeled form precludes such studies at this time. Though these differences strongly suggest that the opioid transport processes expressed in human ARPE-19 cells and in rabbit CJVE cells are mediated by different transporters, it is possible that these differences may simply reflect species-specific changes in functional features of the same transport system.

The pharmacologic and therapeutic potential of this novel transport process in conjunctival epithelial cells for the ocular delivery of opioid peptides is obvious. There is evidence for the clinical utility of opiates in the treatment of a variety of ocular diseases such as glaucoma (25,26), eye pain (27), and ocular infection of herpes simplex virus (HSV-1) (28). The functional identification of a robust transport process for opioid peptides in conjunctival epithelial cells may provide a rationale for targeting the conjunctiva as an effective delivery route for these peptides. Permeability studies have in fact shown that opioid peptides do get across the conjunctival barrier much more readily than predicted by simple diffusion (29). The pharmacologic and therapeutic potential of this transport process for the delivery of peptides other than opioids remains to be investigated. Even though we primarily focused on opioid peptides as potential substrates for this transport process in the present study, it is possible that non-opioid peptides may also be transported via the same process. If this is true, it broadens the scope of this transport process for the ocular delivery of a wide range of peptidomimetic drugs. The physiologic function of the opioid peptide transport process in the conjunctiva remains unknown. Exogenous opioid peptides regulate conjunctival epithelial cell proliferation (30). Conjunctiva expresses preproenkephalin mRNA, suggesting that enkephalins are constitutively synthesized in this tissue (31). These opioid peptides may function in an aurocrine manner in the control of cell proliferation under physiologic and pathologic conditions. Another potential area in which the transport process may be involved is the communication between bacteria and the host. Gram-positive bacteria generate specific peptides which play a critical role in quorum sensing (32). Recent studies have shown that these peptides also have marked biologic effects on host cells. In intestinal and colonic epithelial cells, the quorum sensing peptides induce the expression of the heat shock protein HSP27 and modify stress response of these cells (33). These peptides contain five or more amino acids. Studies from our laboratory have shown that Glu-Arg-Gly-Met-Thr, a pentapeptide produced by *B. subtilis* as a quorum sensing peptide, serves as a substrate for the opioid peptide transport system expressed in intestinal and colonic epithelial cells (unpublished data). We speculate that the opioid peptide transport process expressed in the conjunctival epithelial cells may serve a similar role in the communication between bacteria and the host. The transport process may facilitate the entry of bacterial peptides into the host cells to elicit biologic effects, and hence may function as an essential and integral component for communication between the host and the commensal and pathogenic bacteria in the anterior outer surface of the eye.

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