Identification of a Novel Sodium-Coupled Oligopeptide Transporter (SOPT2) in Mouse and Human Retinal Pigment Epithelial Cells

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PURPOSE. A sodium-coupled oligopeptide transporter (SOPT1) was described originally in ARPE-19 cells. The transporter is inducible by HIV-1 Tat. Recent studies of conjunctival epithelial cells have identified a second oligopeptide transporter (SOPT2). This study was conducted to determine whether the newly discovered SOPT2 is expressed in ARPE-19 cells, to examine whether the new transporter is also inducible by HIV-1 Tat, and to find out whether this transporter is expressed in primary RPE cells.

METHODS. The transport activity of SOPT2 was monitored in control and Tat-expressing ARPE-19 cells and in primary mouse and human fetal RPE cells by the uptake of the synthetic opioid peptide DADLE ((H-Tyr-D-Ala-Gly-Phe-D-Leu-OH) and by its susceptibility to inhibition by small peptides. Substrate selectivity was examined by competition studies and kinetic parameters were determined by saturation analysis.

RESULTS. ARPE-19 cells express DADLE uptake activity that is inhibited by small peptides, indicating expression of SOPT2 in these cells. The activity of SOPT2 is induced by HIV-1 Tat. SOPT2 accepts endogenous and synthetic opioid peptides as substrates, but nonpeptide opiate antagonists are excluded. An 11-amino-acid HIV-1 Tat peptide also serves as a high-affinity substrate for the transporter. Primary cultures of mouse and human fetal RPE cells express SOPT2. The transporter is partially Na⁺-dependent with comparable substrate selectivity and inhibitor specificity in the presence and absence of Na⁺.

CONCLUSIONS. ARPE-19 cells as well as primary mouse and human fetal RPE cells express the newly discovered oligopeptide transporter SOPT2, and the transporter is induced by HIV-1 Tat in ARPE-19 cells. (*Invest Ophthalmol Vis Sci.* 2010;51:413–420) DOI:10.1167/iovs.09-4048

Recently we made a serendipitous discovery that a novel Na⁺-coupled transport system for opioid peptides is expressed in the human retinal pigment epithelial cell line ARPE-19.¹ This transport system accepts as substrates a wide variety

of opioid peptides consisting of 5 to 13 amino acids. Nonpeptide opiate antagonists naloxone and naltrexone do not interact with the transport system. Subsequently, we showed that the transport system is also expressed in the human neuronal cell line SK-N-SH and that the activity of the transport system is stimulated markedly by dipeptides and tripeptides but inhibited by the free amino acid lysine.² The dipeptides, tripeptides, and lysine are not transportable substrates for the transport system. These compounds merely function as modulators of the transport system. Kyotorphin (Tyr-Arg) is a dipeptide with opioid activity. Although an opioid peptide, this dipeptide is not a transportable substrate for the Na⁺-coupled opioid peptide transport system but is a potent stimulator of the transport system similar to other dipeptides and tripeptides.³ The unique features of this new transport system for opioid peptides differentiate it from other systems known to transport opioid peptides (e.g., P-glycoprotein and the OATPs [organic anion-transporting polypeptides]).⁴

We routinely monitor the activity of the Na⁺-coupled opioid peptide transport system using deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂) as a model substrate.^{1–3} Recently, we investigated the interaction of several synthetic opioid peptides with the transport system responsible for deltorphin II uptake in the rabbit conjunctival epithelial cell line CJVE.⁵ Deltorphin II was taken up into these cells in a Na⁺-coupled manner, but it was surprising that the transport activity was not stimulated by dipeptides and tripeptides; rather, it was inhibited. These unexpected findings suggested the existence of a second Na⁺-coupled opioid peptide transport system in mammalian cells. Several synthetic opioid peptides such as DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH), DPDPE (H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH; D-Pen, D-penicillamine), DALCE (H-Tyr-D-Ala-Gly-Phe-Leu-Cys-OH), DAMGO (H-Tyr-D-Ala-Gly-N^α-Me-Phe-Gly-ol), and DSLET (H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH) interact with this new transport system with high affinity. DADLE showed the highest affinity among these peptides (K_m , ~5 μ M). We characterized the kinetic features of this transport system using DADLE as the substrate. The newly discovered second opioid peptide transport system also does not interact with the nonpeptide opiate antagonists naloxone and naltrexone, but accepts a wide variety of opioid peptides as substrates. Thus, the two opioid peptide transport systems exhibit similar substrate selectivity; however, these two systems can be differentiated based on the opposing modulatory effects of dipeptides and tripeptides.

The present investigation was undertaken with two specific goals. First, we wanted to know whether the second opioid peptide transport system is expressed in ARPE-19 cells, and if it is, whether the activity of the transport system is induced by HIV-1 Tat. Tat is the major transactivator of gene expression in HIV-1 human immunodeficiency virus and is coded by the

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HIV-1 genome.^{6,7} This viral protein, released into the circulation of patients with HIV-1 infection, exerts a variety of biological effects on mammalian cells.^{6,7} The rationale for the present studies was our original finding that the transport of deltorphin II in ARPE-19 cells is enhanced markedly by HIV-1 Tat.¹ For this, we compared DADLE uptake characteristics between ARPE-19 cells and Tat-ARPE-19 cells (cells stably expressing HIV-1 Tat through transfection with pcDNA-HIV-1 Tat construct). Second, we wanted to know whether the transport system is expressed normal retinal pigment epithelial cells. For this, we studied the uptake of DADLE in primary cultures of mouse and human fetal RPE cells.

METHODS

Materials

The synthetic opioid peptides DPDPE, DADLE, DAMGO, DSLET, and DALCE 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 and nonopioid peptides were obtained either from the American Peptide Company, Inc. (Sunnyvale, CA) or from Sigma-Aldrich (St. Louis, MO). The development of the stable ARPE-19 cell line expressing the HIV-1 Tat gene is described elsewhere.^{1,8} HIV-1 Tat peptide Tat₄₇₋₅₇ (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) was from American Peptide Company, Inc., and FITC-conjugated Tat peptide Tat₄₇₋₅₇ was from AnaSpec, Inc. (San Jose, CA).

[Tyrosyl-3,5-³H(N)]deltorphin II (specific radioactivity, 38.5 Ci/mmol) and [tyrosyl-3,5-³H(N)]DADLE (specific radioactivity, 45.7 Ci/mmol) were from PerkinElmer (Boston, MA).

Uptake Measurements

ARPE-19 (vector control) and Tat-ARPE-19 cells were seeded in 24-well culture plates at an initial density of 0.5 to 1 × 10⁶ cells/well and cultured in the presence of the antibiotic G418 (0.1 mg/mL) for 4 days to obtain confluent cultures. The culture medium was replaced with freshly prepared medium every other day. Uptake of [³H]deltorphin II and [³H]DADLE in these cells was measured as described previously.^{1-3,5} 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 0.1 to 0.25 μCi of [³H]deltorphin II or [³H]DADLE. Concentration of these peptides during uptake was 10 to 25 nM, depending on the experiment. Initial experiments were performed to determine the time course of uptake. Subsequent uptake measurements were made with 30 minutes' incubation representing initial uptake rates. Uptake was terminated by aspiration of 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 quantified. 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 CaCl₂, 0.8 mM MgSO₄, 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). Nonmediated diffusional component of uptake was determined by measuring the uptake of radiolabeled deltorphin II or DADLE in the presence of excess (1 mM) of unlabeled deltorphin II or DADLE respectively. For both peptides, the diffusional component represented less than 5% of measured total uptake. Saturation kinetics were analyzed by measuring the uptake with increasing concentrations of the substrate. The *K_m* and the maximum 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 *K_m*, and *V_{max}* is the maximal velocity. The *IC*₅₀ (i.e., concentration of various peptides necessary to cause 50% inhibition of deltorphin II uptake or DADLE uptake) was calculated from dose-response experiments.

Data Analysis

The kinetic parameters (*K_t* and *V_{max}*) were determined by nonlinear regression analysis and the values confirmed by linear regression analysis according to the Eadie-Hofstee transformation of the Michaelis-Menten equation (Sigma Plot, ver. 6.0; SPSS, Inc., Chicago, IL). Statistical analysis was performed with either the paired Student's *t*-test or the one-way ANOVA followed by Tukey's post hoc test. A *P* < 0.05 was taken as statistically significant. Experiments were repeated three times, and measurements were made in duplicate for each experimental condition. Data are presented as the mean ± SE.

Establishment of Primary Cultures of Mouse RPE Cells

The protocol for the use of mice in these studies has been approved by the institutional Committee for Animal Use in Research and Education and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

We have an established procedure for the isolation of RPE cells from mouse retinas.⁹⁻¹¹ This method was adapted from that described for isolation of rat RPE.¹² Three-week-old mice were used for preparation of primary RPE cell cultures. Briefly, enucleated mouse eyes were rinsed in 5% povidone-iodine solution, followed by rinsing with sterile Hanks' balanced salt solution (HBSS). The eyes were placed in cold RPE cell culture medium that consisted of DMEM/F12 medium, supplemented with 25% fetal bovine serum, gentamicin (0.1 mg/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). They were incubated in HBSS, containing collagenase (19.5 U/mL) and testicular hyaluronidase (38 U/mL) for 40 minutes at 37°C, followed by incubation in HBSS containing 0.1% trypsin (pH 8) for 50 minutes at 37°C. The eyes were dissected to separate RPE from neural retina. Isolated RPE cells were collected in a 15 mL centrifuge tube and centrifuged at 1200 rpm for 10 minutes, followed by resuspension in RPE cell culture medium. RPE cells were cultured at 37°C. Purity of the culture was verified by immunodetection of RPE65 (retinal pigment epithelial protein 65) and CRALBP (cellular retinaldehyde binding protein), proteins widely used as markers of RPE. The anti-RPE65 antibody was provided by T. Michael Redmond (Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, Bethesda, MD) and the anti-CRALBP antibody was provided by John C. Saari (Department of Ophthalmology, University of Washington, Seattle, WA).

For uptake measurements, RPE cells were seeded (0.1 × 10⁶ cells/well) in 24-well culture plates and cultured for 4 days. Culture medium was changed every other day and uptake measurements were performed on the fifth day. Cells in passages 2 to 4 were used in these studies. The protocols for uptake measurements were exactly as described for ARPE-19 cells.

Establishment of Primary Cultures of Human Fetal RPE Cells

The protocol for preparation and use of cultured human RPE cells was approved by the University of Southern California Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. RPE cells were isolated from human fetal eyes (gestational age, 18–20 weeks) obtained from Advanced Bioscience Resources, Inc (Alameda, CA). Informed consent was obtained by Advanced Bioscience Resources, Inc. from the mothers of the eye tissue donors. Eyes were collected by the personnel at Advanced Biosciences Resources, Inc. The time span between death of the donor and tissue preservation was 2 to 4 hours. The eyes were shipped in RPMI medium at 4°C to the University of Southern California on the same day and processed immediately. Primary cultures of RPE cells were established as described previously.^{13,14} The purity of the cultures was established by immunohistochemical staining of cytokeratin, a marker for RPE cells. Greater than 95% of cells were cytokeratin positive, indicating epithelial origin, whereas no cells were found positive for macrophage marker CD11 or for the endothelial cell marker von Willebrand factor.

Frozen vials of RPE cells were then shipped to the Medical College of Georgia where the cells were used for uptake measurements. Experiments were performed using RPE cells that had been passaged 2 to 4 times. Cells were seeded in 24-well culture plates (0.1×10^6 cells/well) and cultured for 4 days. Culture medium was changed every other day, and uptake measurements were performed on the 5th day.

Uptake of FITC-Tat₄₇₋₅₇ in Primary Cultures of Mouse and Human Fetal RPE Cells

Cells were seeded in chamber slides (Nalge Nunc International, Chicago, IL) at a density of 5000 cells/chamber and cultured for 24 hours. They were then washed with phosphate-buffered saline twice and subsequently incubated with fluorescein isothiocyanate-conjugated Tat₄₇₋₅₇ (FITC-Tat; 5 nM) for 15 minutes in the absence or presence of 10 μ M DADLE. Cells were washed with phosphate-buffered saline and then fixed with 4% paraformaldehyde for 5 minutes at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes. The cells were then washed with water, and the slides were mounted (Gel Mount; Sigma-Aldrich). The entry of FITC-Tat into cells was detected by epifluorescence with a fluorescence microscope.

RESULTS

Characteristics of DADLE Uptake in ARPE-19 Cells

The original opioid peptide transport system, identified in ARPE-19 and Tat-ARPE-19 cell lines and subsequently in the SK-N-SH cell line, was characterized using deltorphin II as a model substrate.¹⁻³ The second opioid peptide transport system identified in the CJVE cell line was characterized with DADLE used as a model substrate.⁵ Both systems have overlapping substrate specificity, accepting deltorphin II, DADLE, and a variety of endogenous opioid peptides as transportable substrates, but are modulated differentially by small nonopioid peptides.^{1-3,5} To determine whether ARPE-19 cells express the newly discovered second opioid peptide transport system, we first studied the effects of DADLE and other synthetic opioid peptides on deltorphin II uptake in Tat-ARPE-19 cells, which have robust deltorphin II uptake activity. DADLE and other opioid peptides (DALCE, DPDPE, DAMGO, and DSLET) effectively competed with deltorphin II for uptake in ARPE-19 cells (Fig. 1). At a concentration of 25 μ M, these peptides caused 40% to 90% inhibition of deltorphin II (25 nM) uptake. DADLE

FIGURE 1. Inhibition of deltorphin II uptake by synthetic opioid peptides in Tat-ARPE-19 cells. Uptake of [³H]deltorphin II (25 nM) was measured for 30 minutes in Tat-ARPE-19 cells in a Na⁺-containing medium in the absence (control) or presence of synthetic opioid peptides (25 μ M). Data are presented as percentage of control uptake (100%). The difference in uptake in the absence and presence of synthetic opioid peptides was statistically significant in all cases (a, $P < 0.001$).

FIGURE 2. Saturation kinetics of DADLE uptake in Tat-ARPE-19 cells. Uptake of DADLE was measured in Tat-ARPE-19 cells for 30 minutes in the presence (NaCl) and absence of Na⁺ (NMDG-Cl). The concentration of radiolabeled tracer was 25 nM and the concentration of DADLE was varied in the range of 0.5 to 25 μ M. Inset: Eadie-Hofstee plot.

was among the peptides that caused the most inhibition. We then studied the uptake of DADLE directly using [³H]DADLE as the substrate. There was robust uptake of DADLE in Tat-ARPE-19 cells. The uptake process was partially Na⁺-dependent, with the presence of Na⁺ enhancing the uptake modestly (1.4–2.2-fold in different experiments). The uptake was linear at least up to 45 minutes in the presence or absence of Na⁺. The uptake process was saturable, in both the presence and the absence of Na⁺ (Fig. 2). The kinetic parameters K_t (K_m) and V_{max} (maximal velocity) for the uptake process in the presence of Na⁺ were 5.0 ± 1.0 μ M and 1.1 ± 0.1 nanomoles/mg of protein/30 minutes. The corresponding values in the absence of Na⁺ were 6.8 ± 0.8 μ M and 0.8 ± 0.2 nanomoles/mg of protein/30 minutes. The value for K_t was different between the two experimental conditions (i.e., with or without Na⁺; $P < 0.05$).

To determine whether the uptake of DADLE in ARPE-19 cells was influenced by HIV-1 Tat, we compared DADLE uptake between control ARPE-19 cells and Tat-expressing ARPE-19 cells (Table 1). As a positive control for Tat-induced uptake, we studied deltorphin II uptake under similar conditions. As shown previously,¹ the uptake of deltorphin II was stimulated by HIV-1 Tat in these cells in both the presence and the absence of Na⁺. The uptake of DADLE was also stimulated several-fold by HIV-1 Tat in the presence as well as absence of Na⁺. There was, however, one noticeable difference between deltorphin II uptake and DADLE uptake. The uptake of deltor-

TABLE 1. Comparison of Deltorphin II and DADLE Uptake between ARPE-19 and Tat-ARPE-19 Cells

	ARPE-19	Tat-ARPE-19
Deltorphin II		
NaCl	0.21 ± 0.03	2.30 ± 0.71
NMDG-Cl	0.06 ± 0.01	0.17 ± 0.01
DADLE		
NaCl	0.57 ± 0.07	16.32 ± 1.07
NMDG-Cl	0.31 ± 0.02	6.86 ± 1.79

Uptake of [³H]deltorphin II (25 nM) and [³H]DADLE (25 nM) was measured for 30 minutes in vector-transfected ARPE-19 cells and HIV-1 Tat cDNA-transfected ARPE-19 cells in the presence (NaCl) or absence (NMDG-Cl) of Na⁺. Data are expressed as the mean picomoles per milligram protein per 30 minutes $\pm$ SE.

phin II was mostly Na^+ -dependent in control ARPE-19 cells and in Tat-ARPE-19 cells; in contrast, the uptake of DADLE was only partially Na^+ -dependent.

Deltorphin II uptake in SK-N-SH cells is stimulated by small nonopioid peptides such as Gly-Gly-Ile and kyotorphin,^{2,3} whereas DADLE uptake in CJVE cells is inhibited by these peptides.⁵ These opposing effects of Gly-Gly-Ile, kyotorphin, and other small peptides were the basis for the conclusion that DADLE uptake and deltorphin II uptake are mediated by two separate transport systems. Since the present studies have shown that ARPE-19 cells possess transport activity for both opioid peptides, we wanted to know whether these cells express both transport systems. For this, we studied the effects of Gly-Gly-Ile and kyotorphin on the uptake of deltorphin II and DADLE in Tat-ARPE-19 cells (Table 2). As shown previously,^{2,3} deltorphin II (20 nM) uptake was stimulated by both small peptides (1 mM), and the stimulation was observed in the presence as well as the absence of Na^+ . Under identical conditions, the uptake of DADLE was markedly inhibited by the same small peptides. Lysine, which is an inhibitor of deltorphin II uptake in SK-N-SH cells, inhibited deltorphin II uptake and DADLE uptake in ARPE-19 cells.

Interaction of Endogenous Opioid Peptides with the DADLE Uptake System in Tat-ARPE-19 Cells

We then studied the ability of enkephalins (25 μM) and dynorphins (25 μM) to compete with DADLE (10 nM) for the uptake process (Fig. 3A). Leu-enkephalin, Met-enkephalin, dynorphin 1-7, dynorphin 1-9, and dynorphin 1-13 were all able to inhibit DADLE uptake almost completely. In contrast, the nonpeptide opiate antagonists naloxone and naltrexone did not inhibit DADLE uptake.

Fragments of HIV-1 Tat peptide are being used for delivery of drugs (e.g., peptides, nucleotides) into mammalian cells.¹⁵ The ability of these peptide fragments to cross the plasma membrane of mammalian cells is related to their highly cationic nature; however, the exact mechanism by which the peptide fragments gain access to cells is not known. Since the transport system responsible for DADLE uptake in Tat-ARPE-19 cells interacts with peptides consisting of 5 to 13 amino acids, we wanted to find out whether Tat peptide fragments interact with the DADLE uptake system. We selected the 11-amino-acid HIV-1 Tat peptide₄₇₋₅₇ for our studies. This peptide was able to compete with DADLE for the transport process with high potency. We monitored the potency of the HIV-1 Tat peptide for inhibition of DADLE uptake in the presence and absence of Na^+ (Fig. 3B). In the presence of Na^+ , the Tat peptide inhibited DADLE uptake with an IC_{50} of $0.6 \pm 0.1 \mu\text{M}$. The inhibitory potency was similar in the absence of Na^+ (IC_{50} , $0.45 \pm$

FIGURE 3. Interaction of endogenous opioid peptides, nonpeptide opiate antagonists, and a synthetic HIV-1 Tat peptide (Tat₄₇₋₅₇) with the DADLE uptake system in Tat-ARPE-19 cells. (A) Uptake of [^3H]DADLE (10 nM) was measured for 30 minutes in Tat-ARPE-19 cells in the absence (control) and presence of endogenous opioid peptides (25 μM) and nonpeptide opiate antagonists (500 μM). Data are presented as the percentage of control uptake (100%). The difference in uptake in the absence and presence of synthetic opioid peptides was statistically significant in all cases (a, $P < 0.001$). In contrast, there was a small, but statistically significant, stimulation of DADLE uptake in the presence of naloxone and naltrexone ($P < 0.05$). (B) Uptake of [^3H]DADLE (10 nM) was measured for 30 minutes in Tat-ARPE-19 cells in the absence (control) and presence of increasing concentrations of HIV-1 Tat₄₇₋₅₇ peptide. Two uptake buffers were used: with Na^+ (NaCl) and without Na^+ (NMDG chloride). Data are presented as a percentage of control uptake (100%).

TABLE 2. Effects of Small Peptides and Lysine on Deltorphin II and DADLE Uptake in Tat-ARPE-19 Cells

	DADLE Uptake		Deltorphin II Uptake	
	NaCl	NMDG-Cl	NaCl	NMDG-Cl
Control	100 $\pm$ 3	100 $\pm$ 6	100 $\pm$ 12	100 $\pm$ 6
Gly-Gly-Ile	18 $\pm$ 1	14 $\pm$ 1	257 $\pm$ 14	471 $\pm$ 17
Kyotorphin	2 $\pm$ 1	1 $\pm$ 1	217 $\pm$ 8	427 $\pm$ 23
Lysine	21 $\pm$ 1	12 $\pm$ 1	11 $\pm$ 1	61 $\pm$ 5

Uptake of [^3H]deltorphin II (25 nM) and [^3H]DADLE (25 nM) was measured for 30 minutes in HIV-1 Tat-expressing ARPE-19 cells in the absence (control) and presence of Gly-Gly-Ile (1 mM), kyotorphin (1 mM), or lysine (1 mM). Uptake was measured in the presence (NaCl) or absence (NMDG-Cl) of Na^+ . Data are expressed as the mean percentage $\pm$ SE of the respective control uptake values (100%).

$0.08 \mu\text{M}$). There was no statistically significant difference between the two values ($P > 0.05$).

Characteristics of DADLE Uptake in Primary Cultures of Mouse and Human RPE Cells

We have described the expression of two distinct opioid peptide transport systems in mammalian cells,^{1-3,5} but all these studies were performed with transformed cell lines (ARPE-19, SK-N-SH, CJVE). The existence of these transport systems has not yet been described in any normal tissue or in the primary cultures of any normal cell. Therefore, we studied DADLE uptake in primary cultures of mouse and human fetal RPE cells to see whether these normal cells express the transport system. DADLE uptake was robust in both cell types (Fig. 4A). In

FIGURE 4. Na^+ -dependence, saturation kinetics, and substrate selectivity of DADLE uptake system in primary cultures of mouse and human fetal RPE cells. (A) Uptake of DADLE (25 nM) was measured for 30 minutes in mouse and human fetal RPE cells in the presence (NaCl) and absence (NMDG-Cl) of Na^+ . The difference in uptake in the presence and absence of Na^+ was statistically significant in both mouse and human RPE cells (b, $P < 0.01$; c, $P < 0.001$). (B) Uptake of DADLE was measured in human fetal RPE cells for 30 minutes in the presence (NaCl) and absence of Na^+ (NMDG-Cl). The concentration of radiolabeled tracer was 25 nM, and the concentration of DADLE was varied in the range of 0.5 to 25 μM . (C) Eadie-Hofstee transformation of the kinetic data. (D) Uptake of [^3H]DADLE (25 nM) was measured for 30 minutes in mouse and human fetal RPE cells in the absence (control) and presence of endogenous opioid peptides (25 μM in all cases except for deltorphin II which was used at a concentration of 250 μM) and nonpeptide opiate antagonists (500 μM). Unlabeled DADLE (25 μM) was also used as a competitive inhibitor of [^3H]DADLE uptake. Data are presented as percentage of control uptake (100%). The difference in uptake in the absence and presence of synthetic opioid peptides was statistically significant in all cases (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$).

mouse as well as human fetal RPE cells, DADLE uptake was partially Na^+ dependent; the uptake was stimulated approximately twofold in the presence of Na^+ . The uptake was saturable in human RPE cells in both the presence and the absence of Na^+ (Figs. 4B, 4C). K_t and V_{max} were $2.8 \pm 0.5 \mu\text{M}$ and $0.95 \pm 0.06 \text{ nmol/mg of protein/30 minutes}$ in the presence of Na^+ and $6.4 \pm 1.1 \mu\text{M}$ and $0.78 \pm 0.05 \text{ nmol/mg of protein/30 minutes}$ in the absence of Na^+ . K_t and V_{max} were different between the two experimental conditions (i.e., with or without Na^+ ; $P < 0.05$). In both cell types, the transport system accepted the endogenous opioid peptides enkephalins and dynorphin 1-13 as well as the synthetic opioid peptides deltorphin II, DALCE, and DSLET as substrates as evidenced from the competition of these compounds with [^3H]DADLE for the uptake process (Fig. 4D). Unlabeled DADLE also competed effectively with [^3H]DADLE for the uptake process. Nonpeptide opiate antagonists naloxone and naltrexone did not inter-

act with the transport system. In fact, there was a significant stimulation of DADLE uptake in the presence of naloxone in mouse and human RPE cells and also in the presence of naltrexone in human RPE cells. We then investigated the effects of kyotorphin and small nonopioid peptides (1 mM) on DADLE uptake (10 nM) in human fetal RPE cells (Fig. 5). The dipeptide kyotorphin and the tripeptides Gly-Gly-Leu, Gly-Gly-Ile, Gly-Gly-Phe, and Gly-Gly-His inhibited DADLE uptake. The inhibition was observed in both the presence and the absence of Na^+ . Similarly, lysine also inhibited DADLE uptake.

Transport of HIV-1 Tat₄₇₋₅₇ Peptide Via the DADLE Transport System in Primary Cultures of Mouse and Human Fetal RPE Cells

Since the 11-amino-acid HIV-1 Tat₄₇₋₅₇ peptide interacts with the DADLE uptake system in ARPE-19 cells, we wanted to

FIGURE 5. Influence of small peptides and lysine on DADLE uptake in primary cultures of human fetal RPE cells. Uptake of [3 H]DADLE (10 nM) was measured for 30 minutes in human fetal RPE cells in the absence (control) and presence of lysine and small peptides (1 mM). Data are presented as a percentage of control uptake (100%). The difference in uptake in the absence and presence of lysine and small peptides was statistically significant (a, $P < 0.001$).

know whether it is also true in primary RPE cells. For this, we first examined the effect of the Tat peptide on DADLE uptake in these cells (Figs. 6A, 6B). In mouse RPE cells, the Tat peptide competed with DADLE for the uptake process with high potency. The IC_{50} for inhibition of DADLE uptake in the presence and absence of Na^+ were 0.65 ± 0.15 and 0.41 ± 0.12 μ M, respectively. The corresponding values for human fetal RPE cells were 0.40 ± 0.14 and 0.67 ± 0.06 μ M. There was no statistically significant difference between the IC_{50} measured in the presence and absence of Na^+ . The inhibition of DADLE uptake by the Tat peptide was competitive in human RPE cells (Fig. 6C). K_i in the absence and presence of Tat peptide (0.5 μ M) were 2.8 ± 0.5 and 7.1 ± 1.5 μ M, respectively. The difference between these two values was statistically significant ($P < 0.05$). The corresponding V_{max} was 0.95 ± 0.05 and 1.02 ± 0.09 nmol/mg of protein/30 minutes. The difference between these two values was not statistically significant ($P > 0.05$). To determine whether the HIV-1 Tat₄₇₋₅₇ peptide fragment is actually transported into RPE cells via the DADLE transport system, we used FITC-labeled Tat₄₇₋₅₇ and observed the cellular entry of the peptide by monitoring fluorescence in cells (Fig. 6D). When the mouse and human RPE cells were incubated with FITC-Tat₄₇₋₅₇ (5 nM), the fluorescent peptide entered the cells. However, this entry was completely blocked when the peptide was incubated with the cells in the presence of DADLE (25 μ M), indicating competition between the two peptides for the uptake process.

DISCUSSION

The existence of two distinct peptide transporters, identified as PEPT1 and PEPT2, in mammalian tissues has been described in the literature.¹⁶⁻¹⁸ These two transporters accept dipeptides and tripeptides as substrates; longer peptides are excluded. Furthermore, the transport process mediated by PEPT1 and PEPT2 is energized by a transmembrane electrochemical H^+ gradient. There is no direct role for Na^+ in the process. PEPT1 and -2 do not recognize peptides larger than dipeptides and tripeptides. Despite this restriction in substrate specificity, PEPT1 and -2 have often been called oligopeptide transporters with no convincing rational basis. Some organic anion transporting polypeptides (OATPs) transport large peptides, but

they do not function solely as peptide transporters.¹⁹⁻²¹ The majority of substrates for OATPs are nonpeptide organic anions.

A Na^+ -coupled transport system for large peptides was originally described in the human RPE cell line ARPE-19.¹ It is also expressed in other mammalian cell lines.^{2,3} In view of our recent discovery of a second Na^+ -coupled peptide transport system for large peptides in the conjunctival epithelial cell line,⁵ we have named the original transporter SOPT1 (sodium-coupled oligopeptide transporter 1) and the second transporter SOPT2. SOPT1 and -2 exhibit overlapping substrate selectivity, but differ in modulation by small peptides. SOPT1 is stimulated markedly by dipeptides and tripeptides, whereas SOPT2 is inhibited by the same small peptides. Lysine inhibits both transporters. These characteristics of SOPT1 and -2 are markedly different from those of PEPT1, PEPT2, and OATPs.

The present study was undertaken to determine whether the newly discovered SOPT2 is expressed in RPE. These studies provide clear evidence for expression of this transporter in ARPE-19 cells. SOPT2 in ARPE-19 cells accepts a wide variety of endogenous and synthetic opioid peptides consisting of 5 to 13 amino acids as substrates. Naloxone and naltrexone, which are opiate antagonists but not peptides, are not recognized by SOPT2 as substrates. Small nonopioid peptides containing two or three amino acids inhibit the transport function of SOPT2, but are not transportable substrates. Even though most of the substrates of SOPT2 examined in the present study are opioids, it is important to note that the 11-amino-acid HIV-1 Tat peptide (Tat₄₇₋₅₇) is a transportable substrate for SOPT2. We speculate that SOPT1 and -2 may not be specific for opioid peptides, but actually may transport a variety of oligopeptides.

An interesting distinguishing feature between SOPT1 and -2 is their Na^+ -dependence. SOPT2 is only partially Na^+ -dependent whereas SOPT1 is predominantly Na^+ -dependent. Even though there is substantial DADLE uptake in ARPE-19 cells in the absence of Na^+ , it is unlikely that another transport system different from SOPT2 is responsible for this process. This conclusion is based on the findings that the substrate selectivity and the inhibitor specificity are exactly the same for the uptake process, irrespective of whether the transport activity is monitored in the presence or absence of Na^+ . In most cases of Na^+ -coupled transport systems, the presence of Na^+ increases the substrate affinity. This effect of Na^+ also seems to be true in the case of SOPT2. The affinity of SOPT2 for DADLE is significantly higher in the presence of Na^+ than in the absence of Na^+ . Of note, the Tat peptide fragment Tat₄₇₋₅₇ exhibits similar affinity for the transport system in the presence and absence of Na^+ , perhaps because the affinity of Tat₄₇₋₅₇ is much higher than that of DADLE. In other words, the influence of Na^+ on substrate affinity seems to decrease with increasing substrate affinity. The partial dependence of SOPT2 on Na^+ is not unique. There are other transporters that exhibit this interesting phenomenon (e.g., the carnitine transporter CT2).²² Despite the distinct nature of SOPT1 and -2, it is interesting to note that the activity of both transporters is enhanced by pretreatment of ARPE-19 cells with HIV-1 Tat.

The present studies demonstrate for the first time the expression of SOPT2 in primary RPE cells. This is an important finding. Until now, the Na^+ -coupled oligopeptide transport activity has been demonstrated only in cell lines but never in any normal tissue or in any primary cultures of normal cells. In this study, we have shown that mouse and human primary RPE cells express transport activity ascribable to SOPT2. All endogenous opioid peptides consisting of five or more amino acids are recognized as substrates by SOPT2. The transporter is likely to play an important role in the handling of these peptides by the outer blood-retinal barrier, which is formed solely by RPE cells. The finding that the nonopioid peptide Tat₄₇₋₅₇ is a

FIGURE 6. Uptake of the HIV-1 Tat peptide Tat₄₇₋₅₇ via the DADLE uptake system in primary cultures of mouse and human fetal RPE cells. **(A)** Uptake of [³H]DADLE was measured for 30 minutes in mouse RPE cells in the absence (control) and presence of increasing concentrations of Tat₄₇₋₅₇ peptide under two different conditions (NaCl and NMDG-Cl). Data are presented as the percentage of control uptake (100%). **(B)** Uptake of [³H]DADLE was measured for 30 minutes in human fetal RPE cells in the absence (control) and presence of increasing concentrations of Tat₄₇₋₅₇ peptide under two different conditions (NaCl and NMDG-Cl). Data are presented as percentage of control uptake (100%). **(C)** Competitive inhibition of DADLE uptake by Tat₄₇₋₅₇ peptide in human fetal RPE cells. Uptake of DADLE was measured for 30 minutes over a concentration range of 0.5 to 25 μM in the absence and presence of Tat₄₇₋₅₇ peptide (0.5 μM). *Inset:* Eadie-Hofstee plot. **(D)** Uptake of FITC-Tat₄₇₋₅₇ peptide in mouse and human fetal RPE cells. Cells were incubated with FITC-Tat₄₇₋₅₇ peptide (5 nM) for 15 minutes in a Na⁺-containing medium in the absence or presence of DADLE (25 μM). Cells incubated under similar conditions but in the absence of FITC-Tat₄₇₋₅₇ peptide served as the negative control. After the incubation, the cells were washed with ice-cold uptake buffer, stained with DAPI (nuclear stain), and observed with a fluorescence microscope (blue, nuclear stain; green, FITC-Tat₄₇₋₅₇).

transportable substrate for SOPT2 suggest that other nonopioid peptides (e.g., angiotensin, bradykinin, and cholecystokinin) may serve as substrates for SOPT2. If this is indeed the case, the transporter may have a biological role in RPE that goes beyond the handling of the opioid peptides.

SOPT1 and -2 represent a novel class of transporters that have not been described in the literature previously. However, these transporters have been characterized only at the functional level. There is no information available at present on the molecular nature of either of these two transporters. Further characterization of these two transporters in RPE and other cell types, both at the functional level and molecular level, is warranted because of their potential role in vivo in the handling of opioid peptides and other peptide hormones. These transporters also hold great potential for the delivery of peptide drugs and peptidomimetic drugs. Since peptides and peptidomimetic drugs are generally hydrophilic, they cannot per-

meate the plasma membrane of mammalian cells simply by diffusion. The functional features of SOPT1 and -2 suggest that these transporters can be exploited for the delivery of such drugs into mammalian cells. The present findings that FITC-coupled Tat₄₇₋₅₇ is in fact a transportable substrate for SOPT2 suggest that the peptide substrates of this transporter can be used as a carrier of a variety of drugs into cells. Additional studies are needed to fully evaluate the pharmacologic potential of these transporters as delivery systems for large peptides and peptidomimetic drugs.

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