ACCELERATED PUBLICATION

Identification of a novel Na⁺- and Cl⁻-coupled transport system for endogenous opioid peptides in retinal pigment epithelium and induction of the transport system by HIV-1 Tat

Huankai HU*, Seiji MIYAUCHI*, Christy C. BRIDGES*, Sylvia B. SMITH† and Vadivel GANAPATHY*¹

*Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta GA 30912, U.S.A., and †Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, GA 30912, U.S.A.

The endogenous opioid peptides enkephalins, dynorphins and endorphins consist of five or more amino acids. These peptides participate in a multitude of biological functions in mammalian cells by interacting with different subtypes of opiate receptors located on the plasma membrane and in the nucleus. Here we report on the identification of a new peptide transport system in the human retinal pigment epithelial (RPE) cells that transports a variety of endogenous opioid peptides with high affinity. We identified this novel, hitherto unrecognized, transport system when we were analysing the differential effects of Tat, the trans-acting factor encoded by HIV-1, on various transport processes in RPE cells. This transport system is markedly induced by Tat. This opioid transport system is energized by transmembrane Na⁺ and Cl⁻ gradients and is distinct from any of the previously

identified transport systems for opioid peptides in mammalian cells. Free amino acids, dipeptides, tripeptides and non-peptide opiate receptor antagonists are excluded by this newly identified transport system. The affinities of endogenous opioid peptides for this system are in the range of 0.4–40 µM. The identification of the high-affinity Na⁺- and Cl⁻-coupled transport system in mammalian cells that is specific for endogenous opioid peptides and is induced by HIV-1 Tat is of significance not only to the biology of opioid peptides but also to the pathology of HIV-1 infection in humans.

Key words: active transport, dynorphins, enkephalins, opioid peptides.

INTRODUCTION

HIV-1 genome-encoded Tat is the major transactivator of the virus expression and is released into the circulation of persons with HIV-1 infection via secretion by, or lysis of, infected cells. HIV-1 Tat exerts a variety of biological effects on mammalian cells [1,2]. Recently, we initiated studies to investigate the influence of HIV-1 Tat on gene expression in retinal pigment epithelium (RPE), with special emphasis on the expression of the genes coding for membrane transporters. One of the important functions of RPE is to mediate the transcellular transfer of nutrients from choroidal blood into the subretinal space to nourish the photoreceptor cells [3]. In these studies, we stably expressed HIV-1 Tat protein in the human RPE cell line ARPE-19 and monitored the differential expression of genes in control ARPE-19 cells and in Tat-expressing ARPE cells by microarray analysis. We found that expression of several transporter genes was affected markedly by HIV-1 Tat. This included the up-regulation of the genes coding for the amino acid transporters ATB⁰ and xCT and for the creatine transporter and the down-regulation of the gene coding for the organic anion transporting polypeptide OATP-A. In the case of the up-regulated genes, we have corroborated the microarray data with functional analysis by demonstrating an increase in the transport activity for ATB⁰, xCT (the transport system consisting of xCT as the light chain), and creatine transporter in Tat-expressing ARPE cells compared with control cells stably transfected with vector alone [4–6]. We then initiated studies to analyse the transport function for the down-regulated

OATP-A. Since OATP-A transports opioid peptides such as DPDPE, deltorphin II, and Leu-enkephalin [7], we used deltorphin II as the substrate in transport assays in an attempt to confirm the microarray data, expecting a decrease in the transport activity in Tat-expressing cells compared with control cells. To our surprise, the transport of this opioid peptide was many-fold higher in Tat-expressing ARPE-19 cells than in control cells. Furthermore, while the transport of deltorphin II via OATP-A occurs via a Na⁺-independent process [7], we found the transport of this peptide in control and Tat-expressing ARPE-19 cells to be obligatorily dependent on the presence of Na⁺ and Cl⁻. Substrate specificity studies have shown that the transport of deltorphin II in these cells is effectively inhibited by a variety of endogenous opioid peptides consisting of 4–13 amino acids. To our knowledge, this is the first evidence for the existence of a Na⁺- and Cl⁻-coupled active transport system for endogenous opioid peptides in mammalian cells. The identification of this novel transport system and the findings that this transport system is up-regulated by HIV-1 Tat may have important physiological and clinical implications.

MATERIALS AND METHODS

Materials

The HIV-1 Tat cDNA construct in pGEM2 vector (catalogue no. 909) and the monoclonal antibody to HIV-1 Tat (catalogue no. 4138) were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD, U.S.A.). The human

Abbreviations used: ATB⁰, amino acid transporter B⁰; xCT, light chain of the amino acid transport system xCT; OATP, organic anion transporting polypeptide; RPE, retinal pigment epithelium; RT-PCR, reverse transcriptase-PCR; Tyr-MIF-1, tyrosine melanocyte-stimulating hormone inhibitory factor 1; DPDPE, [D-penicillamine^{2,5}]enkephalin; PTS, peptide transport system; PEPT, peptide transporter; OPT, oligopeptide transport.

¹ To whom correspondence should be addressed (e-mail vganapat@mail.mcg.edu).

RPE cell line ARPE-19 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). FITC-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Unlabelled opioid peptides and the opioid receptor antagonists (naloxone and naltrexone) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Tyr-D-Ala²-[3,5-³H]deltorphin II (specific radioactivity 38.5 Ci/mmol; referred to subsequently as [³H]deltorphin II) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.).

Stable transfection of ARPE-19 cells with pcDNA-Tat cDNA

The pGEM2-Tat cDNA construct contained as the insert a 295 bp fragment encoding the first exon of HIV-1 Tat gene joined to a 145 bp pET3A transcription terminator. The entire insert (440 bp) was removed from the construct by digestion with *Pst*I and *Eco*RI and ligated into the multiple cloning region of pcDNA3.1(−) vector at *Pst*I/*Eco*RI site so that the insert is downstream of the cytomegalovirus (CMV) promoter. The resultant construct was sequenced to confirm the orientation of the insert. This construct was electroporated into ARPE-19 cells and the stably transfected cell clones were isolated in the presence of Geneticin (G418). To serve as a control, empty pcDNA3.1 vector was electroporated into ARPE-19 cells in an identical manner and the clones harboring the vector were selected by G418 resistance.

The expression of HIV-1 Tat in the stably transfected cell line (Tat-ARPE-19) was confirmed by the analysis of Tat mRNA and Tat protein. Total RNA was isolated from control ARPE-19 cells and Tat-ARPE-19 cells and used as the template for reverse transcriptase-PCR (RT-PCR) to monitor the expression of Tat mRNA. The primers used for RT-PCR were: 5'-GTCA-ACATAGCAGAATAGGCAT-3' (sense) and 5'-GTACCCATC-CGGATATAGTTC-3' (antisense). These primers encompassed the entire insert in the pcDNA-Tat construct and the expected size of the RT-PCR product was 441 bp. The expression of the Tat protein in the stably transfected cell line was monitored by immunofluorescence using a monoclonal antibody specific for HIV-1 Tat and a FITC-conjugated secondary antibody.

Uptake measurements in control ARPE-19 cells and Tat-ARPE-19 cells

Control ARPE-19 cells and Tat-ARPE-19 cells were maintained in 75-cm² culture flasks in Dulbecco's modified Eagle's medium/F₁₂ medium (1:1, v/v) in the presence of fetal bovine serum (10%) and G418 (100 µg/ml). For uptake measurements, cells were released by trypsin treatment and seeded in 24-well culture plates at an initial density of 0.1×10^6 cells/well. The culture medium was replaced with fresh medium on the second day following the initial seeding and uptake measurements were made on the third day. The medium was removed by aspiration and uptake buffer containing [³H]deltorphin II was added to the cells to initiate uptake. After incubation at 37 °C for a desired time, uptake was terminated by the removal of the medium and washing of the cells with ice-cold uptake buffer. The cells were then dissolved in 1% SDS in 0.2 M NaOH and used for measurement of radioactivity. The uptake buffer in most experiments was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. When the influence of Na⁺ on the uptake process was investigated, the concentration of NaCl in the uptake buffer was adjusted, as desired, by iso-osmotically replacing NaCl with *N*-methyl-D-glucamine chloride. To assess the influence of Cl[−] on the uptake process, the composition of the uptake medium was modified by replacing KCl and CaCl₂ with equimolar concentrations of

Figure 1 Analysis of the expression of Tat mRNA and Tat protein in pcDNA-ARPE-19 and Tat-ARPE-19 cells

(A) The expression of Tat mRNA was evaluated by RT-PCR using total RNA prepared from pcDNA-ARPE-19 cells and Tat-ARPE-19 cells (lane 1, RNA from Tat-ARPE-19 cells; lane 2, RNA from pcDNA-ARPE-19 cells). (B) The expression of Tat protein was evaluated by immunofluorescence using a Tat-specific monoclonal antibody (a, Tat-ARPE-19 cells; b, pcDNA-ARPE-19 cells).

potassium gluconate and calcium gluconate and the concentration of NaCl was adjusted, as desired, by iso-osmotically replacing NaCl with sodium gluconate. Saturation kinetics were analysed by fitting the data to the Michaelis–Menten equation. The Michaelis–Menten constant, K_m , was calculated by non-linear regression analysis and then confirmed by linear regression. Na⁺- and Cl[−]-activation kinetics were analyzed by fitting the data to the Hill equation and the Hill coefficients for Na⁺ and Cl[−] (h ; the number of Na⁺ and Cl[−] ions involved in the activation process) and the $K_{0.5}$ values (the concentration of Na⁺ and Cl[−] needed for half-maximal activation) were determined from the analysis. Again, these constants were first calculated by non-linear regression methods and subsequently confirmed by linear regression methods. Experiments were repeated three times in duplicate and the results are given as means $\pm$ S.E.M.

RESULTS

Evidence for the expression of HIV-1 Tat in ARPE-19 cells stably transfected with pcDNA-Tat construct

Control ARPE-19 cells (i.e., cells stably transfected with pcDNA vector alone) and Tat-ARPE-19 cells were compared for expression of HIV-1 Tat by RT-PCR and immunofluorescence. RT-PCR was performed using total RNA isolated from control and Tat-ARPE-19 cells as the template and primers specific for the cDNA insert in pcDNA-Tat construct. An RT-PCR product of expected size (441 bp) was obtained only with RNA prepared from Tat-ARPE-19 cells (Figure 1A). RNA from control cells did not yield any product. The expression of the Tat protein was analysed by immunofluorescence using a monoclonal antibody specific for Tat. The protein was detectable in Tat-ARPE-19 cells but not in control cells (Figure 1B). These data demonstrate that ARPE-19 cells stably transfected with pcDNA-Tat construct express the HIV-1 Tat protein.

Figure 2 Time course and ion-dependence of deltorphin uptake in pcDNA-ARPE-19 and Tat-ARPE-19 cells

(A) Uptake of [3 H]deltorphin II (50 nM) was measured in pcDNA-ARPE-19 and Tat-ARPE-19 cells for different periods in the presence of NaCl. (B) Uptake of [3 H]deltorphin II (50 nM) was measured in pcDNA-ARPE-19 and Tat-ARPE-19 cells for 30 min in the presence of Na $^+$ and Cl $^-$ (NaCl), in the absence of Na $^+$ but in the presence of Cl $^-$ (NMDG chloride), or in the absence of Cl $^-$ but in the presence of Na $^+$ (Na gluconate).

Deltorphin II uptake in control ARPE-19 cells and in Tat-ARPE-19 cells

Figure 2(A) describes the time course of deltorphin II uptake in control ARPE-19 cells and in Tat-ARPE-19 cells. When measured in the presence of NaCl, deltorphin uptake was linear up to 30 min in both cell lines. But, the uptake in Tat-expressing cells was approx. 6–10-fold higher than in control cells. The uptake in both cell lines was obligatorily dependent on Na $^+$ as well as Cl $^-$ (Figure 2B). Removal of Na $^+$ or Cl $^-$ from the uptake medium resulted in an 85–95% decrease in deltorphin uptake in control cells and in Tat-expressing cells. These data show that the deltorphin uptake detected in ARPE-19 cells occurs via a Na $^+$ - and Cl $^-$ -dependent process irrespective of whether or not the cells express HIV-1 Tat. We conclude that ARPE-19 cells express a Na $^+$ - and Cl $^-$ -dependent transport system for deltorphin II and that the expression of HIV-1 Tat leads to the up-regulation of this transport system.

Kinetic characteristics of the deltorphin transport system

Since the Tat-expressing cells exhibit much higher activity of the deltorphin transport system than the control cells, we used the former to characterize the saturation kinetics and Na $^+$ - and Cl $^-$ -activation kinetics of the transport system. In the presence of NaCl, deltorphin II uptake was saturable with a Michaelis–Menten constant (K_t) of $46 \pm 5 \mu\text{M}$ (Figure 3A). With the concentration of Cl $^-$ kept constant at 140 mM, increasing concentrations of Na $^+$ increased the uptake in a sigmoidal manner (Figure 3B). The Hill coefficient (h) for the Na $^+$ -activation process was 3.0 ± 0.7 . With the concentration of Na $^+$ kept constant at

Figure 3 Saturation kinetics and Na $^+$ - and Cl $^-$ -activation kinetics of deltorphin uptake in Tat-ARPE-19 cells

(A) Uptake of deltorphin II was measured in the presence of NaCl for 30 min over a deltorphin II concentration range of 10–1000 μM . Inset: Eadie–Hofstee plot. (B) Uptake of [3 H]deltorphin II (50 nM) was measured for 30 min in the presence of varying concentrations of Na $^+$ (2.5–140 mM), with Cl $^-$ concentration kept constant at 140 mM. Inset: Hill plot. (C) Uptake of [3 H]deltorphin II (50 nM) was measured for 30 min in the presence of various concentrations of Cl $^-$ (10–140 mM), with Na $^+$ concentration kept constant at 140 mM. Inset: Hill plot.

140 mM, increasing concentrations of Cl $^-$ also increased the uptake, but the relationship between the uptake rate and Cl $^-$ concentration was hyperbolic (Figure 3C). The value for the Hill coefficient (h) for the Cl $^-$ -activation process was 1.2 ± 0.5 . These data show that the Na $^+$:Cl $^-$:deltorphin stoichiometry for the transport process is 2 or 3:1:1.

Substrate specificity of the deltorphin transport system

The substrate selectivity of the deltorphin II uptake process was first investigated in Tat-expressing ARPE-19 cells by competition studies (Table 1). The uptake of [3 H]deltorphin II (50 nM) was measured in the absence and presence of a wide variety of opioid peptides and related compounds (1 mM). Free amino acids (L-tyrosine, L-proline and glycine), dipeptides (carnosine and Tyr-Pro), and tripeptides (Gly-Gly-Gly) did not compete with deltorphin for the uptake process to any significant extent. In fact,

Table 1 Substrate selectivity of the deltorphin II transport system in Tat-ARPE-19 cells

Uptake of [^3H]deltorphin II (50 nM) was measured in Tat-ARPE-19 cells for 30 min in the presence of NaCl. The final concentration of inhibitor was 1 mM. * $P < 0.05$ compared with control.

Inhibitor	Structure	[^3H]Deltorphin II uptake	
		(pmol/mg protein/30 min)	(%)
Control		7.76 ± 0.27	100
Gly-Gly-Gly		$9.68 \pm 0.39^*$	125
L-Tyrosine		7.52 ± 0.15	97
L-Proline		7.31 ± 0.20	94
Glycine		7.23 ± 0.14	93
Carnosine	β -Ala-His	$6.21 \pm 0.08^*$	80
Tyr-Pro		$5.72 \pm 0.09^*$	74
D-Tyrosine		$4.05 \pm 0.09^*$	52
Tyr-MIF-1	Tyr-Pro-Leu-Gly-NH ₂	$9.20 \pm 0.15^*$	119
β -Lipotropin	Tyr-Gly-Gly-Phe	$6.26 \pm 0.31^*$	81
β -Casomorphin	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	$2.03 \pm 0.05^*$	26
Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH ₂	$0.34 \pm 0.00^*$	4
[des-Tyr ¹]Met-enkephalin	Gly-Gly-Phe-Met	$0.74 \pm 0.03^*$	10
Met-enkephalin	Tyr-Gly-Gly-Phe-Met	$0.50 \pm 0.01^*$	6
Met-enkephalinamide	Tyr-Gly-Gly-Phe-Met-NH ₂	$0.69 \pm 0.01^*$	9
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	$0.15 \pm 0.01^*$	2
α -Neo-endorphin(1–6)	Tyr-Gly-Gly-Phe-Met-Lys	$0.19 \pm 0.01^*$	3
Dynorphin A(1–6)	Tyr-Gly-Gly-Phe-Leu-Arg	$0.12 \pm 0.00^*$	2
[Arg ⁶ ,Phe ⁷]Met-enkephalin	Tyr-Gly-Gly-Phe-Met-Arg-Phe	$0.11 \pm 0.01^*$	1
[Arg ⁶ ,Gly ⁷ ,Leu ⁸]Met-enkephalin	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu	$0.09 \pm 0.01^*$	1
Dynorphin A(1–7)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg	$0.10 \pm 0.02^*$	1
Dynorphin B(1–9)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe	$0.08 \pm 0.01^*$	1
Dynorphin A(1–13)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys	$0.07 \pm 0.01^*$	1
Naloxone	Opiate receptor antagonist	8.38 ± 0.10	108
Naltrexone	Opiate receptor antagonist	8.22 ± 0.15	106

the tripeptide Gly-Gly-Gly caused a significant stimulation of deltorphin uptake ($P < 0.05$). Interestingly, D-tyrosine inhibited the uptake by about 50%. The most interesting aspect of substrate selectivity of the transport system is that almost all of the endogenous opioid peptides tested showed marked competition with deltorphin for the uptake process. These peptides consisted of 4–13 amino acids. The only exceptions were β -lipotropin (Tyr-Gly-Gly-Phe) and Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH₂), which showed no or little inhibition of deltorphin uptake. As expected, unlabelled deltorphin II competed with radiolabelled deltorphin II for the uptake process. The other opioid peptides that showed marked inhibition of deltorphin uptake included Leu-enkephalin, Met-enkephalin, Met-enkephalinamide, [des-Tyr¹]Met-enkephalin, Met-enkephalin extended at the C-terminus by two or three amino acids, α -neo-endorphin, and various forms of dynorphin. Of note are the findings that naloxone and naltrexone, two of the well known non-peptide opiate antagonists (Figure 4), did not compete with deltorphin for the uptake process. These data show that the deltorphin transport system identified in Tat-ARPE-19 cells preferentially recognize endogenous opioid peptides as substrates. This unique substrate selectivity of the transport system is also seen in control ARPE-19 cells (Table 2).

Relative affinities of endogenous opioid peptides for the transport system

To determine the relative affinities of various endogenous opioid peptides for the transport system, we investigated the dose-response relationship for the inhibition of the uptake of [^3H]deltorphin II by these peptides in Tat-ARPE-19 cells (results not shown). The IC₅₀ values, calculated from these dose-response curves, are given in Table 3. Dynorphin A containing

Figure 4 Chemical structures of naloxone and naltrexone

13 amino acids showed highest affinity for the transport system with an IC₅₀ value of $0.38 \pm 0.03 \mu\text{M}$. This was followed by [Arg⁶,Gly⁷,Leu⁸]Met-enkephalin, dynorphin B(1–9), dynorphin A(1–6), dynorphin A(1–7), [Arg⁶,Phe⁷]Met-enkephalin, Leu-enkephalin, Met-enkephalinamide, [des-Tyr¹]Met-enkephalin, Met-enkephalin, and deltorphin II. The IC₅₀ values for these peptides were in the range of 2.5–40 μM .

DISCUSSION

Here we show for the first time that human RPE cells express a Na⁺- and Cl[−]-coupled active transport system for a variety of opioid peptides consisting of 4–13 amino acids. To our

Table 2 Substrate selectivity of the deltorphin II transport system in control ARPE-19 cells

ARPE-19 cells stably transfected with pcDNA vector alone were used as control cells. Uptake of [3 H]deltorphin II (50 nM) was measured in these cells for 30 min in the presence of NaCl. Final concentration of opioid peptides/antagonists was 1 mM. * $P < 0.05$ compared with control.

Opioid peptide/antagonist	[3 H]Deltorphin II uptake	
	(pmol/mg protein/30 min)	(%)
Control	1.08 $\pm$ 0.015	100
Deltorphin II	0.17 $\pm$ 0.006*	16
Met-enkephalin	0.06 $\pm$ 0.004*	5
[Arg ⁶ ,Phe ⁷]Met-enkephalin	0.05 $\pm$ 0.001*	4
[Arg ⁶ ,Gly ⁷ ,Leu ⁸]Met-enkephalin	0.04 $\pm$ 0.001*	4
Dynorphin A(1–6)	0.06 $\pm$ 0.001*	5
Dynorphin A(1–7)	0.05 $\pm$ 0.002*	5
Dynorphin A(1–13)	0.04 $\pm$ 0.001*	4
Dynorphin B(1–9)	0.06 $\pm$ 0.000*	5
Naltrexone	1.12 $\pm$ 0.02	104
Naloxone	1.01 $\pm$ 0.00	94

Table 3 Relative affinities of endogenous opioid peptides for the deltorphin II transport system in Tat-ARPE-19 cells

IC₅₀ values (i.e., concentration of the peptide needed for 50 % inhibition) were calculated from the dose-response curves in Figure 4 for the inhibition of [3 H]deltorphin II (25 nM) uptake by the peptides.

Opioid peptide	IC ₅₀ (μ M)
Dynorphin A13	0.4 $\pm$ 0.1
[Arg ⁶ ,Gly ⁷ ,Leu ⁸]Met-enkephalin	2.5 $\pm$ 0.4
Dynorphin B(1–9)	3.1 $\pm$ 0.6
Dynorphin A(1–6)	4.2 $\pm$ 0.6
Dynorphin A(1–7)	7.3 $\pm$ 1.9
[Arg ⁶ ,Phe ⁷]Met-enkephalin	9.1 $\pm$ 1.7
Leu-enkephalin	9.5 $\pm$ 1.8
Met-enkephalinamide	13.0 $\pm$ 2.7
[des-Tyr ¹]enkephalin	31.9 $\pm$ 5.5
Met-enkephalin	33.2 $\pm$ 4.4
Deltorphin II	38.8 $\pm$ 4.6

knowledge, such a transport system has not been identified previously in any mammalian cell. A family of transport systems for bioactive peptides has been described in the blood/brain barrier and this family consists of at least four distinct subtypes, known as peptide transport systems 1–4 (PTS-1, PTS-2, PTS-3, and PTS-4) [8,9], but none of these transport systems exhibits the ion-dependence and substrate selectivity characteristics of the opioid peptide transport system described in this paper. Among the four subtypes of PTS family, PTS-1 is almost exclusively responsible for the transport of enkephalins [10]. However, the transport system reported in the present study is not related to PTS-1, because there are marked differences between the two systems in terms of energy dependence and substrate specificity. PTS-1 is an energy-independent facilitative transport system [8–10], whereas the transport system described in the present study is a Na⁺- and Cl[−]-coupled transport system. The opioid-related peptide Tyr-MIF-1 is widely used as a selective model substrate for PTS-1 [9], but this peptide is not recognized by the opioid peptide transport system in RPE cells. In addition, dynorphin A13 exhibits almost 10-fold higher affinity than the shorter dynorphins, which are made of only six to nine amino acids, for the opioid peptide transport system in RPE cells whereas,

among the various dynorphins tested, dynorphin 1–8 shows the highest affinity for PTS-1 [10]. These data show that none of the peptide transport systems, known to exist in the blood/brain barrier, represents the transport system described in the current study. Another transport system for the synthetic opioid peptide [D-penicillamine^{2,5}]enkephalin (DPDPE) has been described in the blood/brain barrier [11,12]. But, unlike the opioid peptide transport system in RPE cells, this system does not recognize Leu-enkephalin as a substrate. The organic anion transporting polypeptides such as rat Oatp1 and Oatp2 and human OATP-A and OATP8 can transport Leu-enkephalin and deltorphin II as does the opioid peptide transport system in RPE cells, but these transport systems are Na⁺-independent [7,13,14]. Furthermore, the non-peptide opioid receptor antagonist naloxone is a substrate for these organic anion transporting polypeptides [7], whereas the opioid peptide transport system identified in RPE cells in the present study does not interact with this compound. PEPT1 and PEPT2, expressed in the mammalian intestine, kidney, brain, and lung, are peptide transport systems, but these transporters recognize only dipeptides and tripeptides as their substrates and exclude peptides larger than tripeptides [15–18]. Moreover, PEPT1 and PEPT2 are Na⁺-independent and are driven by a transmembrane H⁺ gradient. Finally, an oligopeptide transport system (OPT1) has been described in *Saccharomyces cerevisiae* that can transport enkephalins, but this yeast transport system is H⁺-coupled and recognizes not only enkephalins but also the non-peptide opiate receptor antagonist naloxone [19]. Thus, based on the ion-dependence and substrate selectivity characteristics, the opioid peptide transport system described in RPE cells in the present study is not identical with any of the previously identified transport systems in mammalian cells that can interact with opioid peptides. Since opioid peptides are involved in important biological functions in mammalian tissues, the newly identified ion-coupled active transport system, selective for a variety of endogenous opioid peptides, is likely to play a crucial role in the biology of these peptides.

As important as the discovery of this new opioid peptide-selective active transport system in mammalian cells is the finding that HIV-1 Tat up-regulates this transport system. We have identified this transport system in RPE cells. We do not know yet the distribution pattern of this transport system in tissues other than RPE in mammals. Since endogenous opioid peptides function as neurotransmitters, we speculate that the newly identified transport system may also be expressed in the brain to modulate the extracellular levels of these peptides. Endogenous opioid peptides include Leu-enkephalin, Met-enkephalin, and dynorphins, all of which are high-affinity substrates for the newly identified transport system. The plasma levels of enkephalin-like material in man are in the range of 0.1–1 nM [20] and the plasma half-lives for these peptides are in the range of 2–10 min [21, 22]. These peptides are present in the central nervous system and gastrointestinal tract. The concentrations of enkephalins within the synapses of enkephalinergic neurons are likely to be several-fold higher than the plasma levels. It is currently believed that the half-lives of enkephalins in the plasma are determined primarily by peptidases in the circulation [23]. The results of the present study suggest that the newly identified opioid transport system is also likely to play a significant role in the disposal of enkephalins from the circulation by mediating their entry into cells. These peptides serve as ligands for different classes of opiate receptors that are expressed on the plasma membrane of target cells [24,25]. In addition to these plasma membrane receptors, mammalian cells express a nuclear receptor for enkephalins [26]. This receptor, known as opioid growth factor receptor (OGFr), plays a crucial role in cell proliferation and wound healing. The opioid peptide

transport system might serve as an important determinant of not only the ligand concentration in the extracellular medium for interaction with the plasma membrane receptors, but also the ligand concentration in the intracellular medium for interaction with the nuclear receptor. Changes in the expression levels of this transport system are likely to lead to significant alterations in cellular signalling mediated by these receptors and consequently in the biological functions of opioid peptides. Therefore, the up-regulation of the transport system by HIV-1 Tat may have important clinical and pathological consequences in patients infected with HIV-1.

REFERENCES

- 1 Gaynor, R. B. (1995) Regulation of HIV-1 gene expression by the transactivator protein Tat. *Curr. Top. Microbiol. Immunol.* **193**, 51–77
- 2 Gallo, R. C. (1999) Tat as one key to HIV-induced immune pathogenesis and Tat toxoid as an important component of a vaccine. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 8324–8326
- 3 Hughes, B. A., Gallemore, R. P. and Miller, S. S. (1998) Transport mechanisms in retinal pigment epithelium. In *The Retinal Pigment Epithelium: Current Aspects of Function and Disease* (Marmor, M. F. and Wolfensberger, T. J., eds.), pp. 103–134, Oxford University Press, Oxford
- 4 Ganapathy, V., Bridges, C. C., Subramanian, R. K., Ganapathy, M. E. and Smith, S. B. (2002) Induction of the cystine/glutamate transporter x_c^- by HIV-1 transactivator protein TAT in the human retinal pigment epithelial cell line ARPE-19. *Invest. Ophthalmol. Vis. Sci.* **43**, E-abstract 4565
- 5 Hu, H., Miyauchi, S., Smith, S. B. and Ganapathy, V. (2003) Induction of ATB⁰, an amino acid transporter/retroviral receptor by HIV-1 Tat in RPE. *Invest. Ophthalmol. Vis. Sci.* **44**, E-abstract 4616
- 6 Ganapathy, V., Miyauchi, S., Hu, H. and Smith, S. B. (2003) Regulation of expression of creatine transporter in ARPE-19 cells by HIV-1 Tat protein. *Invest. Ophthalmol. Vis. Sci.* **44**, E-abstract 2275
- 7 Gao, B., Hagenbuch, B., Kullak-Ublick, G. A., Benke, D., Aguzzi, A. and Meier, P. J. (2000) Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J. Pharmacol. Exp. Ther.* **294**, 73–79
- 8 Banks, W. A. and Kastin, A. J. (1990) Peptide transport systems for opiates across the blood-brain barrier. *Am. J. Physiol.* **259**, E1–E10
- 9 Banks, W. A., Kastin, A. J. and Michals, E. A. (1987) Tyr-MIF-1 and Met-enkephalin share a saturable blood-brain barrier transport system. *Peptides* **8**, 899–903
- 10 Banks, W. A., Kastin, A. J., Fischman, A. J., Coy, D. H. and Strauss, S. L. (1986) Carrier-mediated transport of enkephalins and N-Tyr-MIF-1 across blood-brain barrier. *Am. J. Physiol.* **251**, E477–E482
- 11 Thomas, S. A., Abbruscato, T. J., Hruby, V. J. and Davis, T. P. (1997) The entry of [D-penicillamine^{2,5}]enkephalin into the central nervous system: saturation kinetics and specificity. *J. Pharmacol. Exp. Ther.* **280**, 1235–1240
- 12 Williams, S. A., Abbruscato, T. J., Hruby, V. J. and Davis, T. P. (1996) Passage of a delta-opioid receptor selective enkephalin, [D-penicillamine^{2,5}]enkephalin, across the blood-brain and the blood-cerebrospinal fluid barriers. *J. Neurochem.* **66**, 1289–1299
- 13 Cattori, V., van Montfort, J. E., Stieger, B., Landmann, L., Meijer, D. K., Winterhalter, K. H., Meier, P. J. and Hagenbuch, B. (2001) Localization of organic anion transporting polypeptide 4 (Oatp4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2 and Oatp3. *Pflügers Arch.* **443**, 188–195
- 14 Kullak-Ublick, G. A., Ismail, M. G., Stieger, B., Landmann, L., Huber, R., Pizzagalli, F., Fattinger, K., Meier, P. J. and Hagenbuch, B. (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**, 525–533
- 15 Leibach, F. H. and Ganapathy, V. (1996) Peptide transporters in the intestine and the kidney. *Annu. Rev. Nutr.* **16**, 99–119
- 16 Daniel, H. (1996) Function and molecular structure of brush border membrane peptide/H⁺ symporters. *J. Membr. Biol.* **154**, 197–203
- 17 Inui, K. and Terada, T. (1999) Dipeptide transporters. *Pharmaceut. Biotechnol.* **12**, 269–288
- 18 Ganapathy, V. and Miyauchi, S. (2003) *Am. Pharmaceut. Rev.* **6**, 14–18
- 19 Hauser, M., Donhardt, A. M., Barnes, D., Naider, F. and Becker, J. M. (2000) Enkephalins are transported by a novel eukaryotic peptide uptake system. *J. Biol. Chem.* **275**, 3037–3041
- 20 Ryder, S. W. and Eng, J. (1982) Radioimmunoassay of leucine-enkephalin like substance in human and canine plasma. *J. Clin. Endocrinol. Metab.* **52**, 367–369
- 21 Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. (1976) Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. *Nature* **262**, 782–783
- 22 Roda, L. G., De Marco, V. and Possenti, R. (1983) Stability of peripheral enkephalins. In *Degradation of Endogenous Opioid* (Ehrenpreis, S. and Sicuteri, F., eds.), pp. 25–42, Raven Press, New York
- 23 Roda, L. G., Roscetti, G., Possenti, R., Venturelli, F. and Vita, F. (1986) Control mechanisms in the enzyme hydrolysis of adrenal-released enkephalins. In *Enkephalins and Endorphins. Stress and the Immune System* (Plotnikoff, N. P., Faith, R. E., Murgio, A. J. and Good, R. A., eds.), pp. 17–33, Plenum Press, New York
- 24 Chaturvedi, K., Christoffers, K. H., Singh, K. and Howells, R. D. (2000) Structure and regulation of opioid receptors. *Biopolymers* **55**, 334–346
- 25 Kieffer, B. L. and Gaveriaux-Ruff, C. (2002) Exploring the opioid system by gene knockout. *Prog. Neurobiol.* **66**, 285–306
- 26 Zagon, I. S., Verderame, M. F. and McLaughlin, P. J. (2002) The biology of the opioid growth factor receptor (OGFr). *Brain Res. Rev.* **38**, 351–376

Received 15 July 2003/7 August 2003; accepted 19 August 2003

Published as BJ Immediate Publication 19 August 2003, DOI 10.1042/BJ20031059