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Differential Modulation of Sodium- and Chloride-Dependent Opioid Peptide Transport System by Small Nonopioid Peptides and Free Amino Acids

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ABSTRACT

We recently identified a novel opioid peptide transport system in the retinal pigment epithelium that transports opioid peptides by a Na^+/Cl^--dependent process. Here we describe a similar transport system expressed in SK-N-SH cells (a human neuronal cell line) and show for the first time that the activity of the transport system is modulated differentially by lysine and small nonopioid peptides. The transport process in SK-N-SH cells, monitored with deltorphin II as the substrate, is Na^+/Cl^--dependent and interacts with several opioid peptides, consisting of 5 to 13 amino acids. The activity of this transport system is markedly stimulated by specific dipeptides and tripeptides, with significant stimulation observable at low micromolar concentrations. The ion dependence, Na^+/Cl^--activation kinetics, and opioid peptide selectivity of the transport system, however, remain unchanged. The stimulation by the modulatory peptides is associated with an increase in maximal velocity with no change in substrate affinity of the system. Amino acids have no or little effect on the transport system, with the exception of lysine. This cationic amino acid inhibits the transport system, with significant inhibition occurring at physiologic concentrations of the amino acid. The inhibitory effect is primarily associated with a decrease in the maximal velocity of the transport system with little change in substrate affinity. Methyl and ethyl esters of lysine retain the inhibitory potency, but most other structural analogs have no effect. The differential modulation of the transport system by lysine and specific small peptides has important implications in the biology and pharmacology of opioid peptides.

Opioidergic neurotransmission plays a critical role in a variety of biologic processes, including analgesia, constipation, respiration, euphoria, sedation, and meiosis (Akil et al., 1984; De Luca and Coupar, 1996; Okada et al., 2002; Bodnar and Hadjimarkou, 2003). Three distinct types of opiate receptors have been identified at the molecular level: μ, δ, and κ (Waldhoer et al., 2004). These receptors exhibit differential involvement in eliciting the above-mentioned biologic processes. The action of opiates in inducing analgesia has tremendous therapeutic applications as evidenced from the current use of various opiate agonists as potent analgesics. There are several synthetic opiate agonists that include morphine, codeine, heroin, meperidine, and methadone. The remarkable ability of these agents to induce analgesia is also at least partly responsible for the abuse potential of such drugs. Available evidence indicates that the analgesic effects of opiates are mediated by μ and δ receptors (Waldhoer et al., 2004). The discovery of various receptors for exogenous opiates such as morphine led to the identification of endogenous opiates that function as physiologic ligands for these receptors. All endogenous opiates known to date are peptides. There are four classes of endogenous opioid peptides: enkephalins, endomorphins, dynorphins, and endorphins. These peptides are produced in vivo from different precursor proteins, which are found primarily in the brain and gastrointestinal tract.

Any classic neurotransmission process involving a specific neurotransmitter consists of four distinct components: synthesis of the neurotransmitter by the presynaptic neurons, release of the neurotransmitter at the synaptic cleft upon stimulation of the presynaptic neuron, interaction of the neurotransmitter with specific receptors on postsynaptic neurons or on the motor end-plate to initiate neurotransmis-

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ABBREVIATIONS: NMDG, *N*-methyl-D-glucamine; Sar, sarcosine (*N*-methylglycine); PEPT, peptide transporter; PTS, peptide transport system.

sion, and removal of the neurotransmitter from the synaptic cleft leading to the termination of neurotransmission. Different mechanisms participate in the removal of different neurotransmitters. For monoamines such as serotonin, dopamine, and norepinephrine, Na^+/Cl^--coupled active transport systems are responsible for the removal process (Nelson, 1998). For acetylcholine, it is not a transporter but an enzyme (acetylcholinesterase) that mediates the clearance of the neurotransmitter by hydrolysis (Lane et al., 2004). What is the mechanism for the clearance of opioid peptides from the synapse? It is currently believed that peptidases are responsible for this process. This belief is based on identification of various peptidases that possess the ability to hydrolyze enkephalins. These peptidases include neutral endopeptidase, aminopeptidase N, angiotensin-converting enzyme, and dipeptidylaminopeptidase (Roques and Fournie-Zaluski, 1986; Roques, 2000). But, do these enzymes possess the ability to degrade opioid peptides other than enkephalins? Is the enzyme-dependent degradation of opioid peptides the only mechanism for the termination of opioidergic neurotransmission? Are there additional mechanisms for the clearance of opioid peptides from the synapse? Answers to these questions are sorely needed to fully exploit the opioidergic neurotransmission for therapeutic purposes.

Recently we made a serendipitous discovery that a Na^+- and Cl^--coupled transport system mediates the entry of the synthetic opioid peptide deltorphin II into a human retinal pigment epithelial cell line (ARPE-19) (Hu et al., 2003). Such a transport system had not been described previously in the literature. The transport system interacts with a variety of opioid peptides containing 5 to 13 amino acids. We believe that the newly discovered transport system may have relevance to the biology of opioidergic neurotransmission. Because the transport system is energetically coupled to transmembrane gradients of Na^+ and Cl^-, such a system is ideal for effective clearance of opioid peptides from the synapse. To provide evidence in support of a role for the transport system in opioidergic neurotransmission, in the present study we tested whether SK-N-SH cells, a human neuronal cell line, express this transport system. These studies show that the neuronal cell line does indeed possess the Na^+/Cl^--coupled transport activity for opioid peptides as monitored by the uptake of deltorphin II. More importantly, the activity of the transport system is modulated differentially by lysine and small nonopioid peptides. These findings have important implications in the biology and pharmacology of opioid peptides.

Materials and Methods

Materials. The SK-N-SH cell line was obtained from the American Type Culture Collection (Manassas, VA). Tyr-D-Ala^2-[3,5-^{3}H]deltorphin II (specific radioactivity, 45 Ci/mmol), referred to subsequently as [^{3}H]deltorphin II, was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). The primary structure of deltorphin II is Tyr-D-Ala-Phe-Glu-Val-Val-Gly. Unlabeled opioid peptides, amino acids, lysine analogs, and lysine esters were obtained either from Sigma-Aldrich (St. Louis, MO) or from American Peptide Co., Inc. (Sunnyvale, CA). Unlabeled dipeptides and tripeptides were obtained either from Sigma Chemical or from Bachem Biosciences (King of Prussia, PA).

Culture of SK-N-SH Cells. SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium, containing 1% nonessential amino acids, 1 mM pyruvate, 0.15% sodium bicarbonate, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Uptake Measurements. Cells were seeded in 24-well culture plates at an initial density of 0.1×10^6 cells/well and cultured for 4 days. The culture medium was replaced with fresh medium on the 2nd day and uptake measurements were carried out on fourth day. To initiate uptake, the culture medium was aspirated out, and uptake buffer containing [^{3}H]deltorphin II (25 nM; ~0.25μCi/assay) was added to the cells. Uptake was allowed to proceed for different time periods at 37°C, after which the 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/0.2 M NaOH and radioactivity associated with the cells was measured. Most studies were done with a 30-min incubation period as uptake was found to be linear at least up to 30 min. 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 $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. When the influence of Na^+ on the uptake process was studied, the concentration of NaCl was adjusted by replacing NaCl with *N*-methyl-D-glucamine (NMDG) chloride iso-osmotically. When the influence of Cl^- on the uptake process was studied, the uptake buffer was modified by replacing KCl and $CaCl_2$ with equimolar concentrations of respective gluconate salts, and the concentration of NaCl was adjusted by replacing NaCl with sodium gluconate iso-osmotically.

Kinetic Analyses. Saturation kinetics was analyzed by fitting the data from uptake versus concentration experiments to the Michaelis-Menten equation, describing a single saturable transport system. The diffusional component was negligible (<5% total uptake). The Michaelis constant (K_t), the concentration of deltorphin II necessary for half-maximal uptake, was calculated by nonlinear regression analysis and then confirmed by linear regression using the Eadie-Hofstee equation. 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^+ or Cl^- ions involved in the activation process) and the $K_{0.5}$ values (the concentration of Na^+ and Cl^- necessary for half-maximal activation) were first calculated by nonlinear regression analysis and confirmed by linear regression.

Conformational Analysis. The most probable conformations of Tyr-Pro and Tyr-Leu (in vacuo, 25°C) were deduced by 20,000-step Monte Carlo conformational search using a Merck molecular force field. MacroModel software (version 8.6; Schrödinger LLC, San Diego, CA) was used for this purpose.

Data Analyses. Uptake measurements were made at least three times with independent cell cultures, and each experiment was done in duplicate or triplicate. Results from these replicates are given as means ± S.E. Statistical analysis was done by Student's t test, and a p value

Results

General Features of the Deltorphin II Transport System in SK-N-SH Cells. Figure 1 describes the ion dependence and opioid peptide selectivity of the transport system responsible for the uptake of deltorphin II in SK-N-SH cells. The uptake of [^{3}H]deltorphin II (25 nM), measured in the presence of NaCl, was linear over a time period of 1 to 30 min, and the uptake was negligible when NaCl in the uptake buffer was replaced with NMDG chloride (Fig. 1A). The transport system was also dependent on the presence of Cl^- as the uptake was almost completely abolished when NaCl in the uptake buffer was replaced with sodium gluconate (Fig. 1B). Unlabeled opioid peptides such as enkephalins, dynorphins, and casomorphin were able to compete with [^{3}H]deltorphin II effectively at a concentration of 1 mM (Fig. 1C). Unlabeled deltorphin II was also able to compete with [^{3}H]deltorphin II for the uptake process, and the magnitude

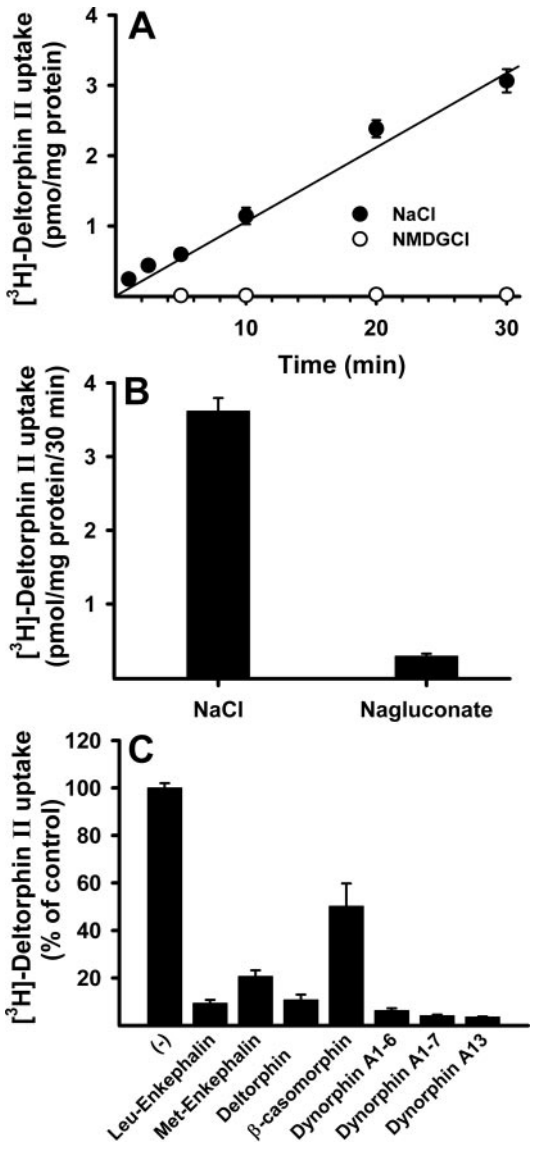

Fig. 1. Time course, ion dependence, and opioid peptide selectivity of the deltorphin II transport system in SK-N-SH cells. A, uptake of [^{3}H]deltorphin II (25 nM) was measured for different periods of time in the presence of NaCl (presence of Na^+ and Cl^-) or NMDG chloride (absence of Na^+ but presence of Cl^-). B, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl (presence of Na^+ and Cl^-) or sodium gluconate (presence of Na^+ but absence of Cl^-). C, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl with or without various opioid peptides (1 mM). Uptake measured in the absence of competing peptides, denoted as (−), was taken as the control (100%), and uptake measured in the presence of competing peptides is given as a percentage of this control value. All seven peptides examined inhibited the uptake of deltorphin II significantly ($p < 0.001$).

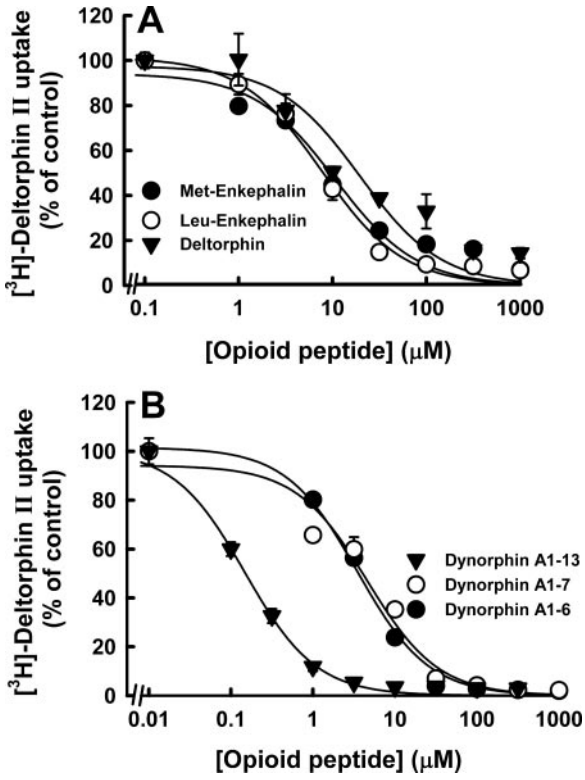

Fig. 2. Dose-response relationship for the inhibition of deltorphin II uptake by opioid peptides. Uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl with or without increasing concentrations of competing opioid peptides. A, dose-response relationship for inhibition of [^{3}H]deltorphin II (25 nM) uptake by Met-enkephalin, Leu-enkephalin, and unlabeled deltorphin II. B, dose-response relationship for inhibition of [^{3}H]deltorphin II (25 nM) uptake by three different dynorphins. Uptake measured in the absence of competing peptides was taken as the control (100%) and uptake measured in the presence of competing peptides is given as a percentage of this control value.

of inhibition was comparable with that of enkephalins. Figure 2 describes the dose-response relationship for the inhibition of [^{3}H]deltorphin II uptake by enkephalins, dynorphins, and unlabeled deltorphin II. The IC_{50} values (i.e., concentration of peptides necessary for 50% inhibition) calculated from these studies were as follows: Leu-enkephalin, 7.8 ± 1.2 μM; Met-enkephalin, 10.7 ± 3.1 μM; deltorphin II, 19.5 ± 7.8 μM; dynorphin A1-6, 3.5 ± 0.4 μM; dynorphin A1-7, 4.6 ± 1.1 μM; and dynorphin A1-13, 0.15 ± 0.01 μM.

Modulation of the Opioid Peptide Transport System by Small Nonopioid Peptides. Because the competition studies revealed that the uptake of deltorphin II was effectively inhibited by several opioid peptides consisting of five or more amino acids, we asked whether the transport system interacts with di- and tripeptides. To address this question, we tested the effects of 24 different dipeptides and 5 different tripeptides on the uptake of [^{3}H]deltorphin II (25 nM) (Fig. 3). Peptides were present at 1 mM during uptake. About half of the dipeptides tested did not have any significant effect on [^{3}H]deltorphin II uptake. These peptides were Gly-(*N*-methyl)glycine (Gly-Sar), Gly-Pro, Pro-Gly, Pro-Ala, Pro-Leu, Pro-Phe, Leu-Pro, Phe-Pro, and *N*-β-Ala-His (carnosine), suggesting that the transport system does not interact with these peptides. Two of the peptides, Lys-Asp and Tyr-Pro, caused significant inhibition of uptake (~40%). On the other hand, some dipeptides stimulated the uptake of [^{3}H]deltorphin II to a marked extent. These peptides were Ala-Gly, Ser-Tyr, Met-Ala, Leu-Gly, Ala-Met, Ser-Val, Met-Phe, Ser-Leu, His-Ala, Met-Glu, His-His, Ala-Ala, and Tyr-Leu. The stimulation varied over the range of 2.4- to 5.0-fold. Among the five tripeptides examined, all of them were found to have a marked stimulatory effect on [^{3}H]deltorphin II uptake. These peptides were Gly-Gly-Ile, Gly-Gly-Phe, Gly-Gly-Gly, Tyr-Gly-Gly, and Glu-Gly-Phe. The stimulation varied over the range of 2.4- to 7.8-fold. The stimulation of [^{3}H]deltorphin II uptake by these dipeptides and tripeptides suggests that the

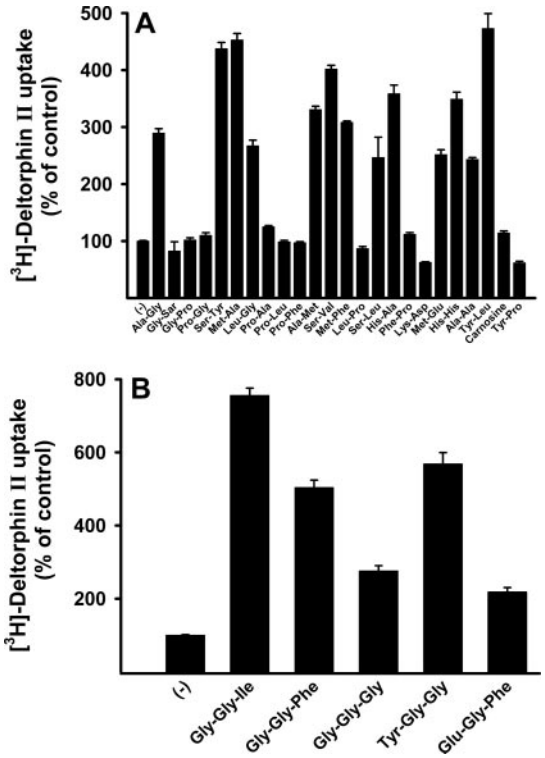

Fig. 3. Modulation of opioid peptide transport system by dipeptides and tripeptides. Uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl with or without various dipeptides (A) and tripeptides (B). Final concentration of the peptides during uptake was 1 mM. Uptake measured in the absence of peptides, denoted as (−), was taken as the control (100%), and uptake measured in the presence of peptides is given as the percentage of this control value. Significant stimulation ($p < 0.001$) was observed with the dipeptides Ala-Gly, Ser-Tyr, Met-Ala, Leu-Gly, Ala-Met, Ser-Val, Met-Phe, Ser-Leu, His-Ala, Met-Glu, His-His, Ala-Ala, and Tyr-Leu and with all tripeptides. Significant inhibition ($p < 0.001$) was observed with Lys-Asp and Tyr-Pro. All other dipeptides examined had no significant effect ($p > 0.05$).

transport system possesses an allosteric modulatory site that binds to these stimulatory peptides to produce their effects.

It was noteworthy that none of the dipeptides containing proline stimulated the transport activity. In fact, some of them (e.g., Tyr-Pro) caused significant inhibition. Proline is unique in that it possesses an imino group rather than an amino group. When present in peptides, proline forms an *N*-substituted peptide bond and exerts substantial influence on the conformation of the peptides. Gly-Sar, which also has an N-substituted peptide bond, has no stimulatory effect. These data suggest that the presence of a regular peptide bond (–CO–NH–) may be obligatory for the stimulatory effect. Interestingly, Lys-Asp, which has a regular peptide bond, caused inhibition rather than stimulation, indicating that other conformational features play a critical role in determining the influence of these peptides. With those peptides that stimulated the transport activity, analysis of their size, charge, and lipophilicity and the corresponding magnitude of stimulation failed to show any relationship between these parameters and the influence on the transport activity. Among the dipeptides examined, Tyr-Pro showed maximal inhibition and Tyr-Leu showed maximal stimulation. Therefore, we deduced the conformation of these two dipeptides for comparison (Fig. 4).

Figure 5 describes the dose-response relationship for the

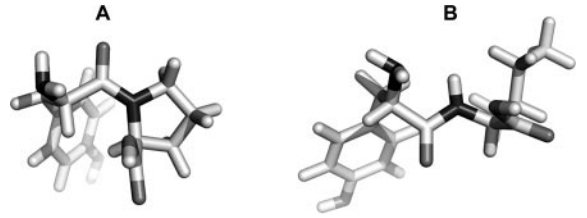

Fig. 4. Conformational models for Tyr-Pro, an inhibitory dipeptide (A), and Tyr-Leu, the most potent stimulatory dipeptide (B).

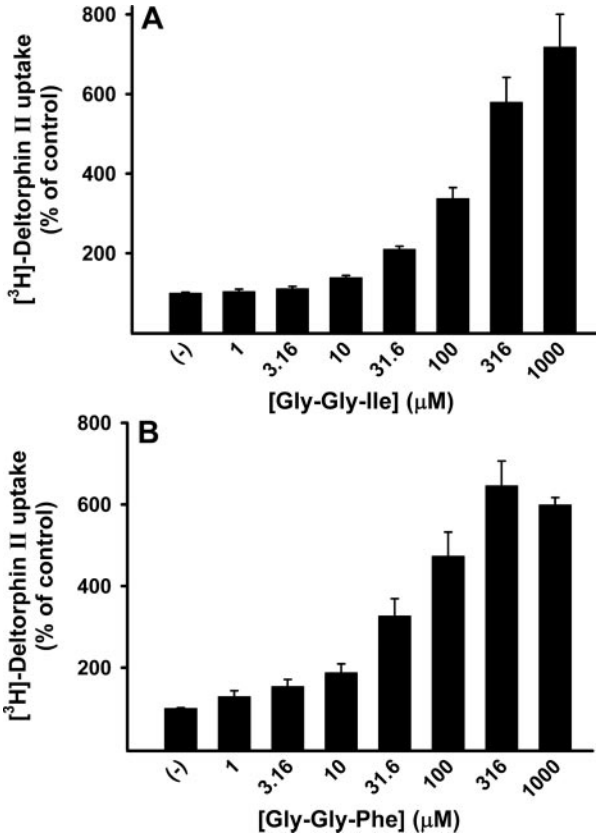

Fig. 5. Dose-response relationship for the stimulation of deltorphin II uptake by Gly-Gly-Ile (A) and Gly-Gly-Phe (B). Uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl with or without increasing concentrations of the tripeptides. Uptake measured in the absence of tripeptides, denoted as (−), was taken as the control (100%) and uptake measured in the presence of tripeptides is given as a percentage of this control value. Significant stimulation ($p < 0.05$) was observed with Gly-Gly-Ile at concentrations ≥10 μM and with Gly-Gly-Phe at concentrations ≥3.16 μM.

stimulation of the transport system by two tripeptides (Gly-Gly-Ile and Gly-Gly-Phe). Both peptides stimulated [^{3}H]deltorphin II uptake 6- to 8-fold at a concentration of 1 mM. More importantly, the stimulation by these peptides was observed even at micromolar concentrations. At a concentration of 31.6 μM, Gly-Gly-Phe and Gly-Gly-Ile stimulated the uptake by 3- and 2-fold, respectively. Again, as with dipeptides, there was no discernible relationship between the size or lipophilicity of these tripeptides and the magnitude of stimulation. We also examined whether the time of incubation had any influence on the extent of stimulation by these tripeptides. For this, we monitored the uptake of deltorphin II in the absence or presence of Gly-Gly-Ile (1 mM) with a 2.5-min incubation rather than a 30-min incubation. These studies showed that, even with the shorter incubation period,

the tripeptide stimulated the uptake of deltorphin II to the same extent (6.7-fold) as seen with a 30-min incubation.

Characteristics of the Opioid Peptide Transport System in the Presence or Absence of the Stimulatory Peptide Gly-Gly-Ile. We then compared the ion specificity, opioid peptide selectivity, saturation kinetics, Na^+-activation kinetics, and Cl^--activation kinetics of the [^{3}H]deltorphin II uptake system in the absence and presence of 1 mM Gly-Gly-Ile. The transport system was Na^+-dependent as well as Cl^--dependent both in the absence and presence of the stimulatory peptide (Fig. 6A). The opioid peptide selectivity remained the same whether or not the activity of the transport system was monitored in the absence or presence of the stimulatory peptide. Even in the presence of Gly-Gly-Ile, the uptake of [^{3}H]deltorphin II was effectively inhibited by enkephalins, dynorphins, and unlabeled deltorphin II (Fig. 6B). Free amino acids (Gly, Pro, Ala, and D-Ala) did not interact with the transport system in the absence or presence of the stimulatory peptide. Saturation kinetics of the transport system revealed that the process was saturable with or without Gly-Gly-Ile. Under both conditions, the uptake process followed Michaelis-Menten kinetics, indicating the involvement of a single saturable transport system (Fig. 7). The stimulation by Gly-Gly-Ile was associated with an increase in the maximal velocity (V_{max}) and with no change in the Michaelis constant (K_t). The V_{max} values in the absence and presence of the stimulatory peptide (1 mM) were 2.7 ± 0.2 and 8.6 ± 0.8 nmol/mg of protein/30 min, respectively. The corresponding values for K_t were 17.5 ± 2.6 and 14.2 ± 2.2 μM. The Na^+-activation kinetics and Cl^--activation kinetics also remained the same in the absence and presence of Gly-Gly-Ile (Fig. 8). Both in the absence and presence of the tripeptide, the activation of [^{3}H]deltorphin II uptake by Na^+ exhibited sigmoidal kinetics, indicating participation of >1 Na^+ ion in the activation process. The maximal stimulation of the uptake process by Na^+ increased in the presence of Gly-Gly-Ile, but the values for the Hill coefficient (h) and $K_{0.5}$ for Na^+ remained essentially the same (h, 2.2 ± 0.5 and 2.9 ± 0.2 in the absence and presence of the tripeptide; $K_{0.5}$, 38 ± 7 and 34 ± 3 mM in the absence and presence of the tripeptide). Likewise, both in the absence and presence of the tripeptide, the activation of [^{3}H]deltorphin II uptake by Cl^- exhibited hyperbolic kinetics, indicating participation of 1 Cl^- ion in the activation process. The maximal stimulation of the uptake process by Cl^- increased in the presence of Gly-Gly-Ile, but the h values and $K_{0.5}$ for Cl^- remained essentially the

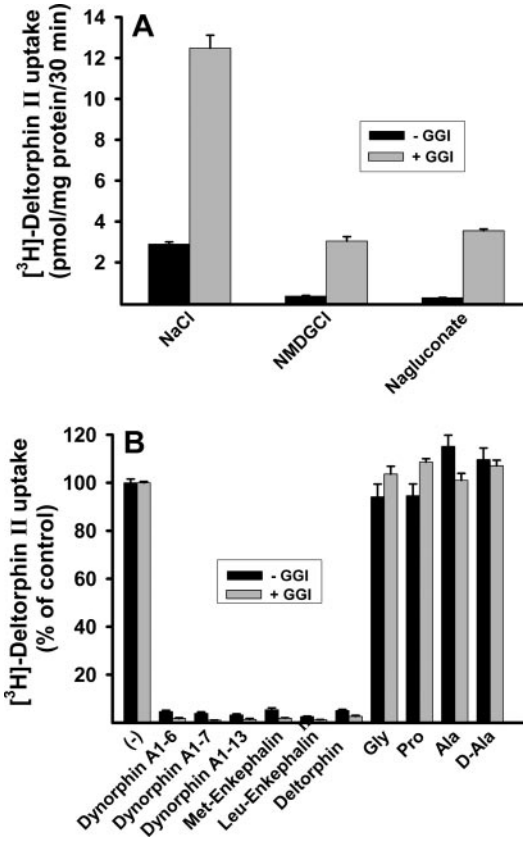

Fig. 6. Ion-dependence and opioid peptide selectivity of the deltorphin II transport system in the absence and presence of Gly-Gly-Ile. A, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl (presence of Na^+ and Cl^-), NMDG chloride (absence of Na^+ but presence of Cl^-), or sodium gluconate (presence of Na^+ but absence of Cl^-). Uptake measurements were done in the absence (−GGI) or presence (+GGI) of Gly-Gly-Ile (1 mM). B, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of NaCl with or without various opioid peptides or amino acids (1 mM). Uptake measured in the absence of peptides/amino acids, denoted as (−), was taken as the control (100%) and uptake measured in the presence of peptides/amino acids is given as a percentage of this control value. Uptake measurements were made in the absence (−GGI) or presence (+GGI) of Gly-Gly-Ile (1 mM).

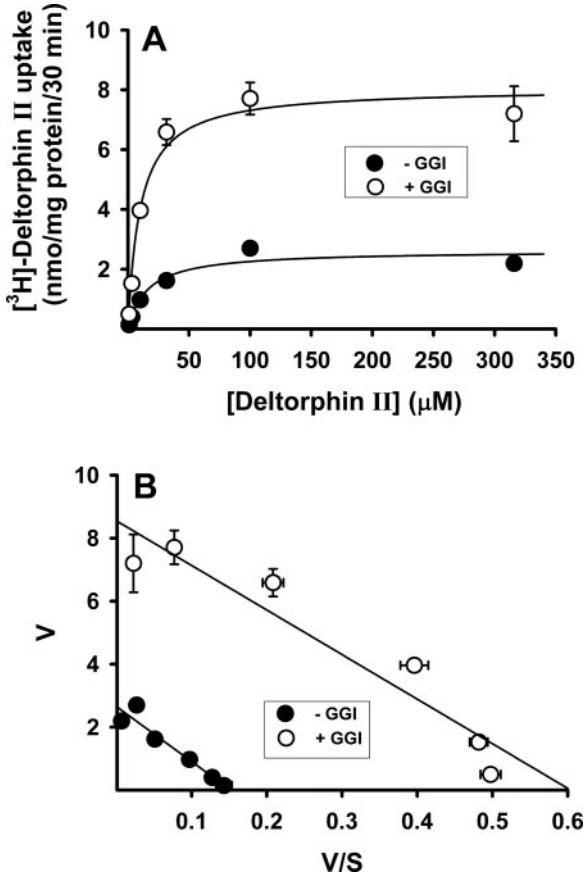

Fig. 7. Saturation kinetics of opioid peptide transport system, monitored with deltorphin II as a model substrate, in the absence or presence of Gly-Gly-Ile. Uptake of deltorphin II was measured for 30 min in the presence of NaCl at increasing concentrations of deltorphin II (5–316 μM). Radiolabeled deltorphin II was present at tracer concentrations (25 nM). Uptake measurements were made in the absence (−GGI) or presence (+GGI) of Gly-Gly-Ile (1 mM). A, deltorphin II uptake versus deltorphin II concentration. B, Eadie-Hofstee plot [deltorphin II uptake (V) versus deltorphin II uptake/deltorphin II concentration (V/S)].

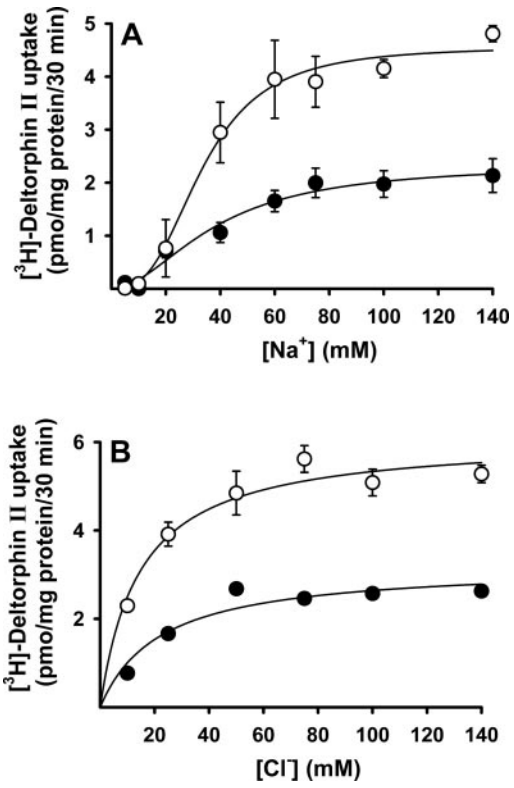

Fig. 8. Na^+-activation kinetics and Cl^--activation kinetics of opioid peptide transport system in the absence or presence of Gly-Gly-Ile. A, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of increasing concentrations of Na^+ (5–140 mM) but in the presence of a fixed concentration of Cl^- (140 mM). The concentration of Na^+ was varied by replacing NaCl with NMDG chloride iso-osmotically. Uptake measurements were made in the absence (●) or presence (○) of Gly-Gly-Ile (1 mM). Data represent only Na^+-dependent uptake, which was calculated by subtracting uptake measured in the absence of Na^+. B, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the presence of increasing concentrations of Cl^- (10–140 mM) but in the presence of a fixed concentration of Na^+ (140 mM). The concentration of Cl^- was varied by replacing NaCl with sodium gluconate iso-osmotically. Uptake measurements were made in the absence (●) or presence (○) of Gly-Gly-Ile (1 mM). Data represent only Cl^--dependent uptake, which was calculated by subtracting uptake measured in the absence of Cl^-.

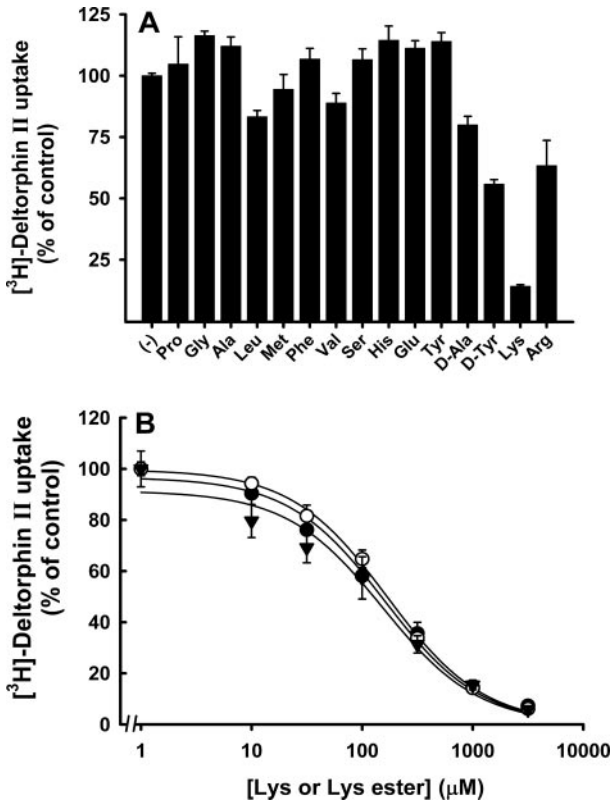

Fig. 9. Effects of free amino acids on the opioid peptide transport system. A, uptake of [^{3}H]deltorphin II (25 nM) was measured for 30 min in the absence or presence of free amino acids (1 mM). Uptake measured in the absence of amino acids, denoted as (−), was taken as the control (100%), and uptake measured in the presence of amino acids is given as a percentage of this control value. Significant inhibition ($p < 0.05$) was observed with Leu, Val, D-Ala, D-Tyr, Lys, and Arg. A small but statistically significant ($p < 0.05$) stimulation was seen with some amino acids (Gly, Ala, His, Glu, and Tyr). B, dose-response relationship for the inhibition of [^{3}H]deltorphin II (25 nM) uptake by lysine (●), lysine methyl ester (○), and lysine ethyl ester (▼). Uptake measured in the absence of lysine or its esters was taken as the control (100%), and uptake measured in the presence of lysine or its esters is given as a percentage of this control value.

same (h, 1.1 ± 0.2 and 1.0 ± 0.2 in the absence and presence of the tripeptide; $K_{0.5}$, 22 ± 8 and 15 ± 4 mM in the absence and presence of the tripeptide).

Modulation of the Opioid Peptide Transport System by Free Amino Acids. The data in Fig. 6 showed that the uptake of [^{3}H]deltorphin II was not influenced by small neutral amino acids. We then extended this study to all other amino acids to assess the influence of large aliphatic amino acids, aromatic amino acids, anionic amino acids, and cationic amino acids on the transport system (Fig. 9A). At a concentration of 1 mM, most of the amino acids tested had either no effect or minimal inhibitory or stimulatory effect on [^{3}H]deltorphin II uptake. However, the cationic amino acid lysine had profound inhibitory effect, causing ~90% inhibition. Because lysine was unique among the amino acids to show this inhibitory effect, we screened various structural analogs and derivatives of lysine for their influence on [^{3}H]deltorphin II uptake (Table 1). These studies produced interesting results in terms of structural features necessary for the inhibitory effect. The ability of arginine and ornithine to inhibit the uptake process was much less than that of lysine, indicating that it was not just the cationic nature of the amino acids that was the basis for the effect. Lysine was inhibitory whereas lysinamide was not. This result would suggest the essential nature of the free carboxyl group of lysine for the inhibitory effect, but it does not seem to be the case because the methyl and ethyl esters of lysine were as potent as lysine as inhibitors of [^{3}H]deltorphin II uptake. However, acetylation of the α-amino group or the ε-amino group of lysine abolished the inhibitory effect almost completely. Polyamines such as diaminopentane and hexamethylenediamine and zwitterionic compounds such as γ-aminobutyric acid, δ-aminolevulinic acid, and 6-aminohexanoic acid showed little or no effect. Because lysine and its methyl and ethyl esters showed marked effects at a concentration of 1 mM, we performed dose-response studies for these three compounds (Fig. 9B). All three compounds had comparable potencies as inhibitors of [^{3}H]deltorphin II uptake, with an IC_{50} value in the range of 150 to 180 μM. We also evaluated the influence of lysine on the kinetic parameters of the transport system (data not shown). The inhibition caused by lysine was primarily associated with a decrease in the maximal velocity (V_{max}) with no significant change in substrate affinity (K_t).

TABLE 1

Influence of lysine and its structural analogs and derivatives on the opioid peptide transport system in SK-N-SH cells

Uptake of [^{3}H]deltorphin II (25 nM) was measured in SK-N-SH cells for 30 min in the presence of NaCl with or without lysine or its structural analogs and derivatives. Final concentration of these compounds during uptake was 1 mM. Data are presented as means ± S.E.

Compound	Deltorphin II Uptake	% Control
	pmol/mg protein/30 min	
None	3.00 ± 0.19	100
L-Lys	0.26 ± 0.01	9
L-Arg	1.46 ± 0.12	49
γ-Aminobutyrate	2.62 ± 0.13	87
L-Ornithine	1.87 ± 0.08	62
L-Carnitine	2.34 ± 0.12	78
L-Citrulline	2.18 ± 0.06	73
D-Lys	2.60 ± 0.31	87
Trimethyl L-Lys	2.39 ± 0.11	80
L-Lysinamide	2.92 ± 0.09	97
L-Lys-methyl ester	0.28 ± 0.01	9
L-Lys-ethyl ester	0.55 ± 0.05	18
α-*N*-Acetyl-L-Lys	2.82 ± 0.08	94
ϵ-*N*-Acetyl-L-Lys	2.38 ± 0.13	79
α-*N*-Acetyl-L-Lys-methylester	2.37 ± 0.20	79
δ-Aminolevulenic acid	2.68 ± 0.18	89
N-Iminoethyl-L-Lys	2.40 ± 0.15	80
1,5-Diaminopentane	3.24 ± 0.16	108
Hexamethylenediamine	2.83 ± 0.13	94
6-Aminohexanoic acid	2.92 ± 0.18	97

We then evaluated the effect of lysine on deltorphin II uptake in the absence and presence of Gly-Gly-Ile (1 mM) to see whether lysine would have a similar inhibitory effect even in the presence of the stimulatory peptide. These studies showed that lysine (1 mM) caused 85% inhibition in the absence of Gly-Gly-Ile and 73% inhibition in the presence of Gly-Gly-Ile, indicating that the inhibitory effect of lysine is not altered by the presence of the stimulatory peptide.

Discussion

Here we report on the constitutive expression of a Na^+- and Cl^--dependent opioid peptide transport system in SK-N-SH cells, a human neuronal cell line. The existence of this novel transport system in mammalian cells was originally discovered in the human retinal pigment epithelial cell line ARPE-19 (Hu et al., 2003). Because opioid peptides are found at high levels in the brain and are known to play an important role as modulators of pain perception, we thought that the transport system might be expressed in neurons. This led to the present report on the constitutive expression of the transport system in SK-N-SH cells.

The characteristics of the transport system in SK-N-SH cells are comparable with those in ARPE-19 cells. The transport process is coupled to Na^+ and Cl^- and selectively interacts with opioid peptides. Dynorphin A1-13 is the most potent inhibitor of deltorphin II uptake via the transport system. The Na^+/Cl^-/opioid peptide stoichiometry is 2 or 3:1:1. The opioid peptide selectivity and order of their IC_{50} values to inhibit deltorphin II uptake in SK-N-SH cells are similar to those in ARPE-19 cells.

The Na^+/Cl^--dependent opioid transport system identified in ARPE-19 cells (Hu et al., 2003) and in SK-N-SH cells (the present study) is distinct from any of the transport systems described previously for opioid peptides (Ganapathy and Miyauchi 2005). Four different transport systems that recognize opioid peptides have been identified in the blood-brain barrier (Banks and Kastin, 1990). These transport systems, known as peptide transport system (PTS)-1, PTS-2, PTS-3, and PTS-4, are Na^+-independent and show no involvement of metabolic energy. Another transport system that is specific for endomorphins has also been described in the blood-brain barrier (Kastin et al., 2001; Somogyvari-Vigh et al., 2004). A distinct transport system specific for deltorphins, which shows partial Na^+ dependence but with no evidence of concentrative uptake, has been described in brain microvessels (Fiori et al., 1997). None of these transport systems has been identified at the molecular level. Transport of opioid peptides has been demonstrated via some of the cloned transporters. This includes P-glycoprotein/ABCB1 (Sarkadi et al., 1994; Chen and Pollack 1998, 1999; Thompson et al., 2000; King et al., 2001; Oude Elferink and Zadina, 2001), organic anion transporting polypeptides (Gao et al., 2000; Cattori et al., 2001; Kullak-Ublick et al., 2001; Nozawa et al., 2003; Lee et al., 2005), and multidrug resistance-related protein MRP2/ABCC2 (Hoffmaster et al., 2004, 2005). None of these transport systems is Na^+-dependent. There are two peptide transporters, PEPT1/SLC15A1 and PEPT2/SLC15A2, which handle dipeptides and tripeptides in mammalian tissues (Ganapathy and Miyauchi, 2003; Daniel and Kottra, 2004), but these transporters are H^+-coupled and do not interact with opioid peptides. Thus, the Na^+/Cl^--dependent opioid transport system identified in our laboratory is novel and is distinct from any of the previously described transport systems for opioid peptides.

The present studies have also unraveled an interesting, and pharmacologically and therapeutically relevant feature of this transport process. The transport system is modulated differentially by small nonopioid peptides and free amino acids. Specific dipeptides and tripeptides stimulate the transport system whereas lysine and its methyl and ethyl esters inhibit the transport system. The stimulation by small peptides is marked (2–8-fold), and significant stimulation is observed at micromolar concentrations of these peptides. Among the 24 dipeptides examined, proline-containing peptides were noticeably with no effect, whereas peptides containing hydrophobic amino acids (aliphatic and/or aromatic) are the ones with marked stimulatory effect. The same seems to be the case with tripeptides even though proline-containing tripeptides have not been tested. Because these dipeptides and tripeptides do not compete with deltorphin II for the uptake process, these peptides do not interact with the substrate-binding site. It is likely that the transport system possesses a modulatory site where these peptides bind and stimulate the transport activity. This is the first indication that it is possible to stimulate the uptake of opioid peptides into neurons using small molecules. These findings have pharmacologic implications because modulation of the transport system may mean modulation of opioidergic bioactivity. Stimulation of the opioid peptide transport system by nonopioid small peptides is expected to decrease the extracellular levels of opioid peptides, attenuating or terminating the activation of opiate receptors. Dipeptides and tripeptides are found in the brain (Teuscher et al., 2001). PEPT2, which transports dipeptides and tripeptides into cells, is expressed abundantly in the brain (Wang et al., 1998; Shen et al., 2004). Variations in the expression and activity of this transporter may lead to changes in the extracellular concentrations of specific small peptides which may function as the stimulators

of the opioid peptide transport system. These findings have pharmacologic and therapeutic significance. It may be possible to design and develop small molecules with specific and potent stimulatory activity on the opioid peptide transport system. Such small molecules may be used as therapeutic agents to modulate opioidergic activity in vivo. Opioid peptides are found at high levels in the brain and in the gastrointestinal tract (Sundler et al., 1987), suggesting an important role for these peptides in these tissues. Because pharmacologic activation of the transport system is expected to decrease the bioactivity of endogenous opioid peptides, such therapeutic agents may be beneficial in the management of opiate addiction. In the gut, opioids promote constipation as evident from the therapeutic efficacy of opiate agonists (e.g., loperamide) as antidiarrheal agents. Therapeutic activation of the opioid peptide transport system may be useful to reduce the activity of endogenous opioid peptides and hence in the management of constipation.

The inhibition of the opioid peptide transport system by lysine may also have therapeutic relevance. Enhancement of the bioactivity of endogenous opioid peptides by interfering with the clearance of extracellular opioid peptides via inhibition of the transport system has potential in the management of pain. In the gut, lysine and similar small molecules that inhibit the transport system may have therapeutic use in the control of diarrhea. Therapeutic use of this naturally occurring amino acid to enhance opioidergic bioactivity will produce "physiologic" effects in terms of blunting the pain perception and preventing diarrhea. The main advantage of this approach is that the effects of lysine would be entirely due to potentiation of the actions of endogenous opioid peptides. These effects would involve only those opiate receptors that are tonically stimulated by endogenous opiates. On the contrary, exogenous opiate agonists are expected to stimulate all opiate receptors irrespective of the magnitude and duration of the interaction of these receptors with endogenous opioid peptides. Indiscriminate activation of opiate receptors may underlie the unwanted side effects such as drug dependence and withdrawal. A recent study by Smriga and Torii (2003) found lysine to be therapeutically useful as a partial serotonin receptor 4 antagonist. This study suggested that nutritional supplementation with lysine may be beneficial in the management of stress-induced anxiety and serotonin-mediated diarrhea-prone intestinal dysfunctions. Our data suggest that lysine-induced enhancement of opioidergic neurotransmission in the enteric nervous system may also play a role in this phenomenon.

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