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Stimulation of Na^+/Cl^--coupled Opioid Peptide Transport System in SK-N-SH Cells by L-kyotorphin, an Endogenous Substrate for H^+-coupled Peptide Transporter PEPT2

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Summary: We have recently identified a Na^+/Cl^--coupled transport system in mammalian cells for endogenous and synthetic opioid peptides. This transport system does not transport dipeptides/tripeptides, but is stimulated by these small peptides. Here we investigated the influence of L-kyotorphin (L-Tyr-L-Arg), an endogenous dipeptide with opioid activity, on this transport system. The activity of the transport system, measured in SK-N-SH cells (a human neuronal cell line) with deltorphin II as a model substrate, was stimulated ~2.5-fold by L-kyotorphin, with half-maximal stimulation occurring at ~100 μM. The stimulation was associated primarily with an increase in the affinity for deltorphin II. The stimulation caused by L-kyotorphin was stereospecific; L-Tyr-D-Arg (D-kyotorphin) had minimal effect. The influence of L-kyotorphin was observed also in a different cell line which expressed the opioid peptide transport system. While L-kyotorphin is a stimulator of opioid peptide transport, it is a transportable substrate for the H^+-coupled peptide transporter PEPT2, which is expressed widely in the brain. Since the activity of the opioid peptide transport system is modulated by extracellular L-kyotorphin and since PEPT2 is an important determinant of extracellular L-kyotorphin in the brain, the expression/activity of PEPT2 may be a critical factor in the modulation of opioidergic neurotransmission in vivo.

Keywords: opioid peptide transport; kyotorphin; SK-N-SH cell line; PEPT2; opioidergic neurotransmission; stereospecificity

Introduction

The endogenous opioid peptides enkephalins, dynorphins and endomorphins, are derived from proenkephalin, prodynorphin, and proopiomelanocortin.[1–3] Proenkephalin is the precursor for Met-enkephalin (a pentapeptide), Leu-enkephalin (a pentapeptide), and longer Met-enkephalins containing 7 or 8 amino acids with Arg-Phe or Arg-Gly-Leu extensions at the C-terminus. Prodynorphin is the precursor for dynorphin 1–8, dynorphin 1–17, α-neoendorphin, and β-neoendorphin. Proopiomelanocortin is the precursor for β-endorphin. All these endogenous opioid peptides consist of five or more amino acids. These peptides produce their biologic effects by serving as ligands for opioid receptors.[4] In addition to their role in neuromodulation/neurotransmission,[5] opioid peptides also play important regulatory roles in development,[6,7] cell survival[8,9] or proliferation.[10–13]

The biologic activity of opioid peptides mediated through activation of plasma membrane opioid receptors depends on extracellular concentrations of these peptides. It is currently believed that peptidase-mediated degradation is the principal mechanism for the regulation of extracellular concentrations of opioid peptides.[14] There was no evidence for the existence of active reuptake mechanisms for these peptides as has been shown for other neurotransmitters such as serotonin, dopamine, and norepinephrine. Recently, we discovered a novel Na^+- and Cl^--coupled transport system specific for opioid peptides in the human retinal pigment epithelial cell line ARPE-19[15] and the human neuronal cell line SK-N-SH.[16] This transport system is highly energy-dependent and concentrative, and exhibits high affinity for endogenous opioid peptides. It recognizes enkephalins (5 amino acids) and larger opioid peptides including dynorphin 1–13. Small peptides consisting of 2 or 3 amino

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acids are not substrates for the transport system, but these peptides have marked stimulatory effect on the activity of the opioid peptide transport system.[16] On the other hand, lysine inhibits the transport system. The expression of the transport system is upregulated by the transactivating protein Tat of the human immunodeficiency virus HIV-1.[15] While the role of this novel transport system in the cellular uptake of opioid peptides is clearly evident, the relevance of this transport system to opioidergic neurotransmission remains to be established. Such a role will depend on whether or not this transport system is expressed at the synapse in opioidergic neurons. Furthermore, the existence of the transport system has been demonstrated only at the functional level. Nothing is known at present on the molecular nature of this transport system.

There are several small neuropeptides identified in the central nervous system. These include L-kyotorphin (L-Tyr-L-Arg), carnosine (β-Ala-His), Cys-Gly, Gly-Gln, and *N*-acetyl-Asp-Glu (NAAG). Since the opioid peptide transport system is markedly stimulated by small peptides, it would be of biologic and pharmacologic importance to know if any of these endogenous neuropeptides influences the activity of the opioid peptide transport system. Some of these small neuropeptides are excellent substrates for another peptide transport system, known as PEPT2, which is distinct from the opioid peptide transport system. PEPT2 is a H^+-coupled transporter which recognizes small peptides consisting of 2 or 3 amino acids as high-affinity substrates[17–19] and it is expressed in the central nervous system, detectable in astrocytes, neurons, and choroidal epithelium.[20] PEPT2 transports many of the small neuropeptides found in the brain.[21–25] L-Kyotorphin has been shown to interact with PEPT2 with high affinity,[26,27] even though the transport of this dipeptide via PEPT2 has not been demonstrated directly. The activity of PEPT2 in the central nervous system is therefore likely to be an important determinant of extracellular concentrations of these small peptides in the brain. If these peptides modulate the activity of the opioid peptide transport system, it would suggest that PEPT2 in the brain may have an important role in the regulation of the activity of the opioid peptide transport system. With this rationale, the present investigation was undertaken to investigate the influence of various endogenous small neuropeptides on the activity of the opioid peptide transport system.

Methods

Cell culture: The human neuronal cell line SK-N-SH and the human RPE cell line ARPE-19 were obtained from American Type Culture Collection (Manassas, VA, USA). Generation of stable ARPE-19 cell line expressing HIV-1 Tat has been described previously.[15] SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. ARPE-19 cells were cultured in RPMI medium. Tyr-D-Ala^2-[3,5-^{3}H] deltorphin II (specific radioactivity, 45 Ci/mmol), heretofore referred to as [^{3}H]deltorphin II, was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Unlabeled deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly) was obtained from American Peptide Company Inc. (Sunnyvale, CA, USA). L-Tyr-L-Arg (L-kyotorphin) was procured from MP Biomedicals (Solon, OH, USA). L-Tyr-D-Arg (D-kyotorphin) and L-Arg-L-Tyr were purchased from Bachem Biosciences (King of Prussia, PA, USA). All other non-opioid dipeptides and tripeptides were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or MP Biomedicals (Solon, OH, USA).

Uptake measurements: Uptake experiments were carried out as described previously.[15,16] Cells were seeded in 24-well culture plates at an initial density of 0.2×10^6 cells/well and cultured for 3 days. The culture medium was replaced with fresh medium every two days, and uptake measurements were made on the fourth day. To initiate uptake, the culture medium was aspirated out, and uptake buffer containing [^{3}H]deltorphin II (25 nM; $\sim$0.25 μCi/assay) was added to the cells. Uptake was allowed to proceed at 37 °C for 30 min as uptake was found to be linear during this time period. Uptake was terminated by removal of the uptake buffer, and the cells were washed with ice-cold uptake buffer without the radiolabeled substrate. Cells were then lysed and dissolved in 1% SDS/0.2 M NaOH, and the radioactivity of the lysate was measured. The uptake buffer used in the experiments was 25 mM Hepes/Tris (pH 7.4), containing 140 mM NaCl (or iso-osmotic substitution with *N*-methyl-D-glucamine chloride to determine the dependence of the transport process on Na^+), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose.

Kinetic studies: Saturation kinetics of the opioid peptide transport system was performed in the presence and absence of modulating peptides, and data were analyzed according to the Michaelis-Menten equation describing a single saturable process. The Michaelis constant (K_t) (the concentration of deltorphin II necessary for half maximal uptake) and the maximal velocity (V_{max}) were calculated by nonlinear regression and confirmed by linear regression. The effects of L-kyotorphin on Na^+- and Cl^--activation kinetics of deltorphin II uptake were determined by fitting the experimental data to the Hill equation. The Hill coefficients for the activation of the uptake process by Na^+ and Cl^- (h, number of Na^+ or Cl^- ions involved in the activation process) were calculated using nonlinear and linear regression analyses.

Treatment with peptides: When the effects of various peptides on the uptake of deltorphin II were evaluated in SK-N-SH cells, the peptides were added at indi-

cated concentrations along with [^{3}H]deltorphin II simultaneously. There was no pre-treatment of the cells with these peptides. The same was true in the kinetic studies where the effects of di- and tripeptides on kinetic parameters of deltorphin II uptake were analyzed.

Analysis of L-kyotorphin transport via human PEPT2 in *X. laevis* oocyte expression system: The experimental procedures with *X. laevis* were performed in accordance with the United States National Institutes of Health guidelines and with institutional policies governing appropriate care and use of animals in research. Capped cRNA from hPEPT2 cDNA,[28,29] cloned in pGH19, an *X. laevis* oocyte expression vector,[30,31] was synthesized using the mMESSAGE-mMACHINE kit (Ambion, Austin, TX, USA). Mature oocytes from *X. laevis* were isolated by treatment with collagenase A (1.6 mg/mL), manually defolliculated and maintained at -18 °C in modified Barth's medium, supplemented with 25 mg/mL gentamicin as described previously.[32] On the following day, oocytes were injected with 50 ng of cRNA. Water-injected oocytes served as control. The oocytes were used for electrophysiological studies 5–6 days after cRNA injection. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method. Oocytes were superfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Mes/Hepes/Tris, pH 7.5), followed by the same buffer containing Gly-Sar (1 mM) or L-kyotorphin (1 mM) at pH 7.5 and pH 5.5. The pH of the buffer was altered by adjusting the relative concentrations of Mes, Hepes, and Tris. The membrane potential was clamped at –50 mV and the currents induced by Gly-Sar and L-kyotorphin were monitored. The H^+-activation kinetics of L-kyotorphin-induced currents were studied by monitoring L-kyotorphin (1 mM)-induced currents at different pH and then analyzing the effects of varying concentrations of H^+ on L-kyotorphin-induced currents by the Hill equation. The Hill coefficient for the activation of the transport process by H^+ was determined by nonlinear and linear regression analyses. The experiments were repeated five times with different oocytes.

Statistical analysis: Statistical analysis was made using the Student's t test, and a $p < 0.05$ was taken as statistically significant. Experiments were repeated two or three times and measurements were made in duplicate or triplicate for each experimental condition. Data are presented as means ± S.E.

Results

Influence of endogenous small neuropeptides on deltorphin II uptake: We examined the influence of various small neuropeptides (1 mM) on the activity of the Na^+/Cl^--coupled opioid peptide transport system by monitoring the uptake of [^{3}H]deltorphin II (25 nM) in SK-N-SH cells in the presence of NaCl. We used the dipeptide Met-Ala and the tripeptide Gly-Gly-Ile as positive controls as these small peptides are known to have marked stimulatory effect on the transport system.[16] As expected, Met-Ala and Gly-Gly-Ile stimulated [^{3}H]deltorphin II uptake by ~3-fold (**Fig. 1**; $p < 0.05$). Under the same conditions, carnosine and NAAG inhibited the uptake (40% and 60%, respectively; $p < 0.05$), and Gly-Glu had no significant effect. In contrast, Gly-Gln, Cys-Gly, and L-kyotorphin (L-Tyr-L-Arg) showed significant stimulatory effect ($p < 0.05$), L-kyotorphin being the most effective among these three dipeptides. The stimulation was ~2.5-fold at 1 mM L-kyotorphin.

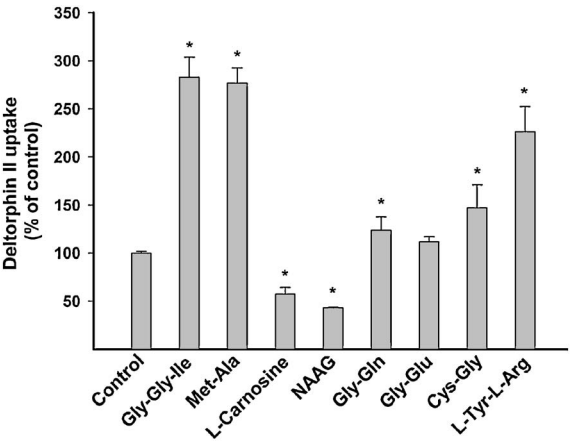

Fig. 1. Influence of small neuropeptides on the transport of deltorphin II in SK-N-SH cells. Uptake of [^{3}H]deltorphin II (25 nM) was measured in confluent cultures of SK-N-SH cells in the absence or presence of various neuropeptides (1 mM). The uptake buffer contained NaCl and the incubation time was 30 min. Gly-Gly-Ile and Met-Ala were used as positive controls for the stimulation of deltorphin II uptake by small peptides. Data (means ± S.E.) are presented as percent of control uptake, measured in the absence of small peptides. *, $p < 0.05$ compared to control uptake.

Kinetics of L-kyotorphin-induced stimulation of [^{3}H]deltorphin II uptake: **Figure 2** describes the dose-response relationship for the stimulation of the opioid peptide transport system by L-kyotorphin. When studied over a concentration range of 1–1000 μM, the stimulatory effect was significant at L-kyotorphin concentrations ≥ 30 μM ($p < 0.05$), and half-maximal stimulation was observed at ~100 μM. The uptake of [^{3}H]deltorphin II followed Michaelis-Menten kinetics in the absence and presence of L-kyotorphin (1 mM) (**Fig. 3**). The stimulatory effect of kyotorphin was associated primarily with an increase in substrate affinity. The values for Michaelis constant (K_t) for deltorphin II were 44 ± 7 μM in the absence of L-kyotorphin and 5.2 ± 1.5 μM in the presence of L-kyotorphin. There was no significant change in V_{max} (5.5 ± 0.3 and 5.0 ± 0.2 nmol/mg of protein/30 min in the absence and presence of L-kyotorphin, respectively; $p > 0.05$). We then examined the influence of L-kyotorphin on the Na^+- and Cl^--activation kinetics

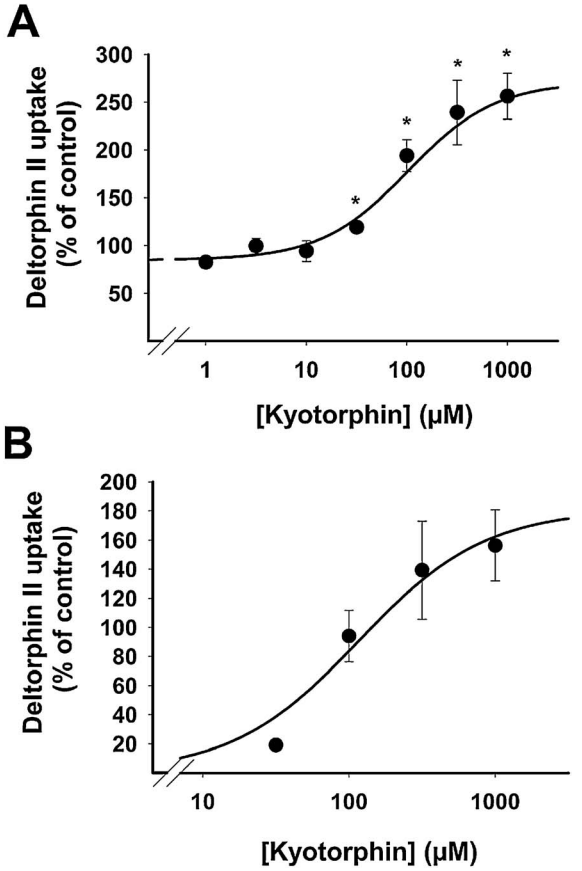

Fig. 2. Dose-response relationship for the stimulation of deltorphin II transport by L-kyotorphin in SK-N-SH cells. Uptake of [^{3}H]deltorphin II (25 nM) was measured in confluent cultures of SK-N-SH cells in the presence of increasing concentrations of L-kyotorphin (L-Tyr-L-Arg). The uptake buffer contained NaCl and the incubation time was 30 min. Data (means ± S.E.) are presented as percent of control uptake, measured in the absence of L-kyotorphin (A) or as percent increase in uptake above the control uptake (B). *, $p<0.05$ compared to control uptake in the absence of kyotorphin.

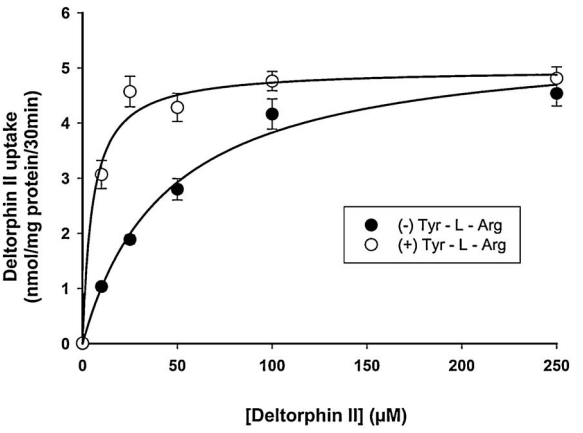

Fig. 3. Influence of L-kyotorphin on the kinetic parameters of deltorphin II uptake in SK-N-SH cells. Uptake of deltorphin II was measured in confluent cultures of SK-N-SH cells in the absence or presence of L-kyotorphin (1 mM) with varying concentrations of deltorphin II. The concentration of [^{3}H]deltorphin II was kept constant at 25 nM. Uptake measurements were made in the presence of NaCl with a 30 min incubation time.

of deltorphin II uptake (**Fig. 4**). The Na^+-activation kinetics was sigmoidal both in the absence and presence of L-kyotorphin, and the stimulatory effect of L-kyotorphin was observed at all concentrations of Na^+ examined. The values for Hill coefficient were similar in the absence and presence of L-kyotorphin (2.8 ± 0.4 in the absence of L-kyotorphin and 3.6 ± 1.4 in the presence of L-kyotorphin). The Cl^--activation kinetics was hyperbolic both in the absence and presence of L-kyotorphin with no significant change in Hill coefficient. The values were 0.9 ± 0.2 and 0.8 ± 0.1 in the absence and presence of kyotorphin, respectively. These data show that a minimum of 2 Na^+ and 1 Cl^- are involved in the activation of the transport system and that the activation kinetics is not altered in the presence of L-kyotorphin.

Influence of Gly-Gly-Phe and Ser-Tyr on kinetic parameters of deltorphin II uptake: Our previous studies have shown that the tripeptide Gly-Gly-Ile stimulated deltorphin II uptake primarily by increasing the value for V_{max} without having any significant effect on K_t.[16] The results of the present study with L-kyotorphin are different from these earlier findings. Therefore, we examined the influence of two additional small peptides (Gly-Gly-Phe and Ser-Tyr) on the kinetic parameters of deltorphin II uptake (**Fig. 5**). These two peptides were selected based on our previous studies[16] in which both peptides enhanced deltorphin II uptake markedly in SK-N-SH cells. Here we confirmed these previous findings. Both peptides showed marked stimulatory effect on deltorphin II uptake. However, in contrast to our previous observations with Gly-Gly-Ile, the dipeptide and tripeptide used in the present study showed significant effects on Michaelis constant as well as maximal velocity. In experiments analyzing the effects of the tripeptide, the control values for Kt and Vmax were 33 ± 1 μM and 4.2 ± 0.1 nmol/mg protein/30 min; the corresponding values in the presence of Gly-Gly-Phe (1 mM) were 13 ± 3 μM and 6.0 ± 0.3 nmol/mg of protein/30 min. The difference in these two kinetic parameters between the absence and presence of the tripeptide was statistically significant ($p<0.05$). In experiments analyzing the effects of the dipeptide, the control values for K_t and V_{max} were 25 ± 1 μM and 2.8 ± 0.1 nmol/mg protein/30 min; the corresponding values in the presence of Ser-Tyr (1 mM) were 6.8 ± 2.1 μM and 4.6 ± 0.3 nmol/mg of protein/30 min. The difference in K_t and V_{max} between the absence and presence of the dipeptide was statistically significant ($p<0.05$). These studies show that even though dipeptides and tripeptides stimulate the opioid peptide transport system, the influence on kinetic parameters is differ-

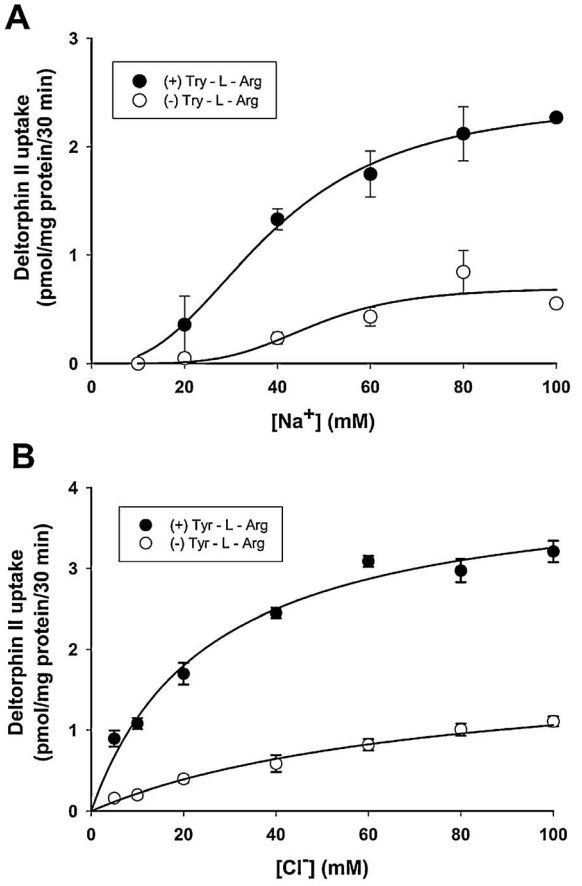

Fig. 4. Influence of L-kyotorphin on the Na^+- and Cl^--activation kinetics of deltorphin II uptake in SK-N-SH cells. Uptake of [^{3}H]deltorphin II (25 nM) was measured in confluent cultures of SK-N-SH cells in the absence or presence of L-kyotorphin (1 mM) using a 30 min incubation time. For Na^+-activation kinetics, the concentration of Na^+ was varied over a range of 0–100 mM, using N-methyl-D-glucamine chloride to substitute for NaCl isoosmotically. For Cl^--activation kinetics, the concentration of Cl^- was varied over a concentration range of 0–100 mM, using sodium gluconate to substitute for NaCl isoosmotically.

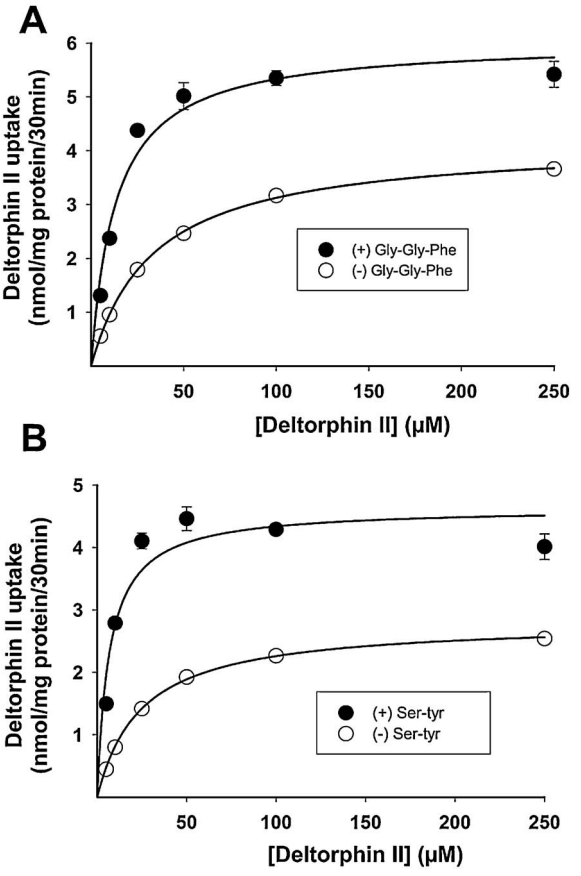

Fig. 5. Influence of Gly-Gly-Phe and Ser-Tyr on the kinetic parameters of deltorphin II uptake in SK-N-SH cells. Uptake of deltorphin II was measured in confluent cultures of SK-N-SH cells in the absence or presence of Gly-Gly-Phe (1 mM) (A) or Ser-Tyr (1 mM) (B) with varying concentrations of deltorphin II. The concentration of [^{3}H]deltorphin II was kept constant at 25 nM. Uptake measurements were made in the presence of NaCl with a 30 min incubation time.

ent for each of the stimulatory peptide. In addition, the values for K_t and V_{max} in control experiments also varied significantly depending on the culture conditions which include passage number and confluency state. Nonetheless, the conclusions on the effects of the stimulatory peptides on K_t and V_{max} are dependable because the kinetic parameters in these experiments were analyzed in the absence and presence of the stimulatory peptides under identical culture conditions, thus eliminating the variability due to differences in passage number and confluency state. We therefore conclude that the effects of the stimulatory peptides on the kinetic parameters of the opioid peptide transport system depend on the individual peptide, most likely determined by the structure and stereochemistry of the constituent amino acids in these peptides.

Differential effects of L-Tyr-L-Arg (L-kyotorphin) and L-Tyr-D-Arg (D-kyotorphin) on the opioid peptide transport system: The potency of kyotorphin as an analgesic agent differs markedly between the L- and D-isomers. D-Kyotorphin (L-Tyr-D-Arg) exhibits several fold higher analgesic activity than L-kyotorphin (L-Tyr-L-Arg).[33] Therefore, we compared the effects of L- and D-kyotrophins on deltorphin II uptake (**Fig. 6**). In SK-N-SH cells, L- kyotorphin at a concentration of 1 mM stimulated the opioid peptide transport activity by 2.5-fold, when monitored in the presence of NaCl. Under identical conditions, the ability of the D-isomer to stimulate deltorphin II uptake was significantly less ($p < 0.05$). Interestingly, switching the amino acids at the C- and N-termini of the L-isomer did not affect the stimulatory potency as evident from the greater than 2-fold stimulation observed with L-Arg-L-Tyr. We also dissected the effects of these three peptides on Na^+-dependent and Na^+-independent deltorphin II uptake, even though the uptake mediated

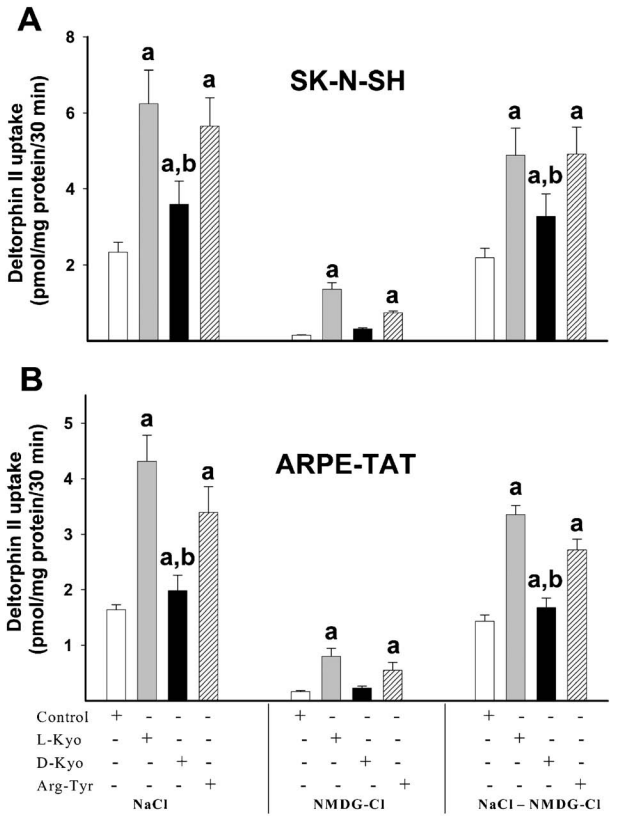

Fig. 6. Influence of L-kyotorphin (L-Tyr-L-Arg), D-kyotorphin (L-Tyr-D-Arg), and L-Arg-L-Tyr on deltorphin II transport in SK-N-SH cells and in ARPE-TAT cells. Uptake of [^{3}H]deltorphin II (25 nM) was measured in confluent cultures of SK-N-SH cells (A) and ARPE-TAT cells (B) in the absence or presence of the three peptides (1 mM). Uptake measurements were made in the presence of either NaCl or *N*-methyl-D-glucamine chloride (NMDG-Cl). a, $p<0.05$ compared to control uptake. b, $p<0.05$ compared to uptake in the presence of L-kyotorphin.

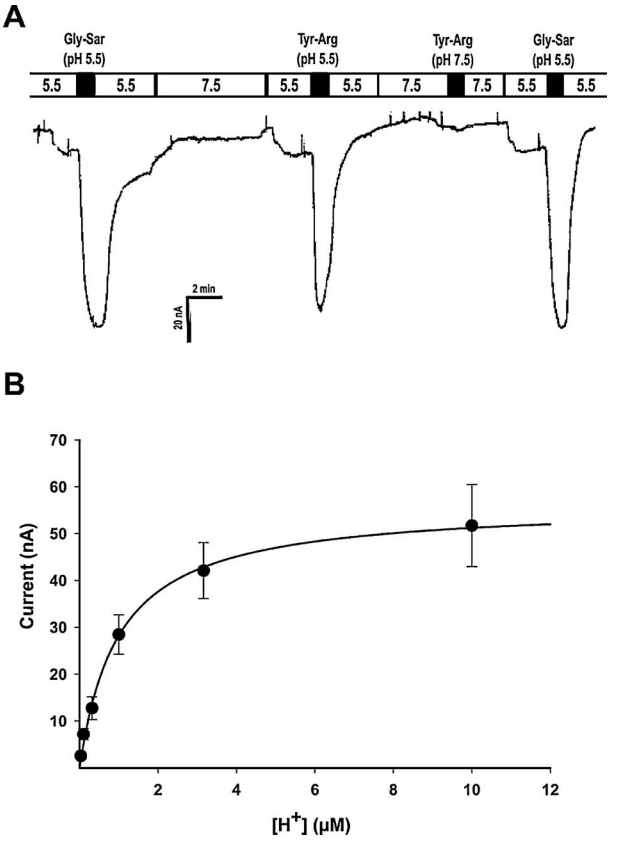

Fig. 7. Direct demonstration of L-kyotrophin transport via human PEPT2 in the *X. laevis* oocyte expression system. Human PEPT2 was expressed in *X. laevis* oocytes by injection of human PEPT2 cRNA. Transport of L-kyotorphin via PEPT2 was measured by monitoring the peptide-induced inward currents under voltage-clamp conditions. (A) Currents were monitored when the oocytes were superfused with either glycylsarcosine (Gly-Sar) (1 mM) or L-kyotorphin (L-Tyr-L-Arg) (1 mM) at two different pH (7.5 and 5.5). Gly-Sar was used as a positive control for a transportable substrate via PEPT2. (B) L-Kyotorphin (1 mM)-induced currents were measured at different pH and the magnitude of currents was plotted as a function of H^+ concentration. Data were analyzed using the Hill equation to determine the Hill coefficient for H^+ activation and the H^+ concentration necessary for half-maximal stimulation.

by the transport system is largely Na^+-dependent. The Na^+-dependent uptake was stimulated by L-Tyr-L-Arg and L-Arg-L-Tyr but significantly less by L-Tyr-D-Arg. Uptake of deltorphin II measured in the absence of Na^+ also exhibited similar profile with regard to these three peptides, suggesting that the uptake of deltorphin II in the absence of Na^+ was also mediated predominantly by the same transport system. Similar results were obtained when deltorphin II uptake was monitored in a different cell line which expresses the opioid peptide transport system robustly. We have shown earlier that ARPE-TAT, the human retinal pigment epithelial cell line stably expressing the transactivator protein Tat of the human immunodeficiency virus type 1, possesses robust uptake activity for the opioid peptide deltorphin II.[15] L-Tyr-L-Arg and L-Arg-L-Tyr stimulated deltrorphin II uptake in this cell line markedly, both in the presence and absence of Na^+. The stimulatory potency of D-kyotorphin was significantly less, as observed in SK-N-SH cells.

Transport of L-Tyr-L-Arg via PEPT2, a H^+-coupled peptide transporter: L-Kyotorphin is a dipeptide which has been shown to interact with PEPT2, a high-affinity H^+-coupled peptide transporter expressed widely in the brain.[26,27] These studies have demonstrated that L-kyotorphin competes with the dipeptide glycylsarcosine (Gly-Sar) for transport via PEPT2 with an inhibition constant of $\sim 30\ \mu$M.[26,27] However, the actual transport of L-kyotorphin via PEPT2 has not yet been demonstrated directly. The activity of PEPT2 is likely to be an important determinant of extracellular levels of L-kyotorphin in the brain. Since the opioid peptide transport activity is stimulated by extracellular L-kyotorphin, we believe that PEPT2 activity is an important factor in the stimulation of opioid peptide uptake by L-kyotorphin in

vivo. Therefore, we investigated the transport of L-kyotorphin by human PEPT2 directly by monitoring the peptide-induced inward currents in *X. laevis* oocytes expressing human PEPT2 (**Fig. 7**). Since the transport process mediated by PEPT2 is electrogenic, we expected inward currents in these oocytes in response to L-kyotorphin if this peptide is a transportable substrate of PEPT2. Exposure of oocytes expressing human PEPT2 to L-kyotorphin induced marked inward currents at an extracellular pH of 5.5, demonstrating that the dipeptide is indeed a transportable substrate for PEPT2. The dipeptide Gly-Sar was used as a positive control. Such currents were not detectable in water-injected oocytes (data not shown). We then analyzed the H^+-activation kinetics for this process. We found that the transport of L-kyotorphin via PEPT2 increased as the concentration of extracellular H^+ increased. The relationship was hyperbolic, suggesting involvement of 1 H^+ in the activation process with a H^+:L-kyotorphin stoichiometry of 1:1. The concentration of H^+ necessary for half-maximal stimulation of the transport process was 1.2 ± 0.2 μM.

Discussion

The expression of the novel Na^+/Cl^--dependent opioid peptide transport system in SK-N-SH cells and its modulation by small peptides have already been reported.[16] However, none of the modulatory peptides tested in this previous report is biologically relevant to the brain because none of them is likely to be present in this tissue at significant concentrations. To understand the biologic importance of the observed modulation of the opioid peptide transport system by small peptides in the brain, we initiated the present study. The purpose of the study was to examine the effects of specific small peptides, which are known to occur in the brain tissue at significant concentrations, on the opioid peptide transport system in SK-N-SH cells. These peptides include carnosine, glycylglutamine, glycylglutamate, cysteinylglycine, *N*-acetyl aspartylglutamate, and L-kyotorphin. Among these, L-kyotorphin (L-Tyr-L-Arg) was found to have the maximal stimulatory effect on the activity of the opioid peptide transport system, monitored with the hydrolysis-resistant synthetic opioid peptide deltorphin II as the model substrate. The activity of the transport system was stimulated $\sim$2.5-fold in the presence of 1 mM L-kyotorphin. This effect was primarily associated with an increase in substrate affinity, with little or no effect on the maximal velocity. The Na^+-activation kinetics and the Cl^--activation kinetics remained unaffected in the presence of this activating peptide. However, the stimulatory effect was not specific for L-kyotorphin. Even when the sequence of the amino acids was switched, there was significant stimulation of the opioid peptide transport system as evidenced with L-Arg-L-Tyr. But, the effect was stereospecific. While the naturally occurring L-kyotorphin (L-Tyr-L-Arg) was effective, L-Tyr-D-Arg (D-kyotorphin) was not.

The effects of L-kyotorphin on the kinetic parameters of the transport system were surprising. We have reported previously that the tripeptide Gly-Gly-Ile stimulated the opioid peptide transport system in the same cells, primarily enhancing the maximal velocity with little effect on the substrate affinity. Here we found L-kyotorphin to influence the substrate affinity rather than the maximal velocity. Therefore, we tested two new small peptides (Gly-Gly-Phe and Ser-Tyr) and found their effects to be mixed. These two peptides stimulated the opioid peptide transport system by enhancing the substrate affinity as well as the maximal velocity. It thus seems that the effects on the transport system are not the same for all stimulatory peptides. We believe that the observed effects are allosteric and that whether or not a stimulatory peptide will affect the substrate affinity or the maximal velocity will depend on the interaction of the stimulatory peptide with the allosteric binding site. This interaction will definitely be influenced by the amino acid sequence and structural aspects of the stimulatory peptide. This is clearly evident from the lack of stimulation with carnosine (β-Ala-His) and *N*-acetyl aspartylglutamate. In fact, these two peptides caused inhibition of the transport system. The inhibition was significant at 1 mM of these peptides. Even though this may not be physiologically relevant to carnosine which is unlikely to be present at these concentrations in brain, the findings may have biologic significance in the case of *N*-acetyl alspartylglutamate since this peptide occurs in brain at low millimolar concentrations.[34] In addition, even though the other four dipeptides examined (Gly-Gln, Gly-Glu, Cys-Gly, and L-Tyr-L-Arg) stimulated the transport system, the magnitude of stimulation was very different. These data suggest that the sequence, stereochemistry, and structural aspects of small peptides play as important determinants of not only the magnitude of the stimulatory effect but also the effects on the kinetic parameters of the transport system associated with the stimulation.

The findings that L-kyotorphin is a stimulator of the opioid peptide transport system in the neuronal cell line are interesting and may be of physiologic importance. L-Kyotorphin is a naturally occurring dipeptide in the brain. The present studies show that L-kyotorphin is able to stimulate deltorphin II uptake significantly at concentrations as low as 30 μM. There is very little information available in the literature on the extracellular concentration of this neuropeptide. Ueda et al.[35] have determined the concentration of this peptide in rat brain and spinal cord. The concentration was highest in the midbrain ($\sim$2.2 μM), followed by pons/medulla, spinal cord, hypothalamus, and cortex. But, we do not expect the dipeptide to be uniformly distributed in the brain. There is evidence that L-kyotorphin is concentrated in the syn-

aptosomal fraction.[36] Therefore, it is very likely that the concentration of the dipeptide is several-fold higher in specific regions of the brain and that the significant stimulation of deltorphin II uptake with $\sim 30\,\mu$M L-kyotorphin observed in the present study is physiologically relevant. In addition, the extracellular concentration of L-kyotorphin in the brain is influenced by the activity of the high-affinity peptide transporter PEPT2. L-Kyotorphin is a high-affinity substrate for this transporter. Polymorphisms in the gene coding for PEPT2 exist in humans which affect the activity of the transporter significantly,[37,38] indicating that significant individual variations may occur in extracellular levels of L-kyotorphin in the brain. This would mean that the magnitude of the stimulation of the opioid peptide transport system is also likely to differ from individual to individual. What this means in terms of individual variations in opioid peptide biology remains to be seen.

L-Kyotorphin is a nociceptive peptide, which produces its analgesic effects by inducing the release of enkephalins from enkephalinergic neurons.[39,40] This effect involves depolarization of nerve terminals and calcium influx. The efficacy of L-kyotorphin as a nociceptive agent is dependent on its stability to the actions of peptidases. D-Kyotorphin (L-Tyr-D-Arg), which is resistant to hydrolysis by peptidases, is more potent than L-kyotorphin (L-Tyr-L-Arg) in inducing enkephalin release.[33] Therefore, the kyotorphin receptor, which mediates the effects of this kyotorphin, does not differentiate between L- and D-kyotorphins. Our studies show that L-kyotorphin enhances the uptake of opioid peptides and that D-kyotorphin does not. This means that the observed effects of L-kyotorphin on the opioid peptide transport system are not mediated through the kyotorphin receptor. This is supported further by the stimulatory effects of various small peptides, which are biologically and pharmacologically unrelated to L-kyotorphin, on the opioid peptide transport system. Therefore, we suggest that L-kyotorphin and other small peptides stimulate the opioid peptide transport system by directly acting on the transporter as allosteric modulators. It is interesting to note that L-kyotorphin induces the release of enkephalins and at the same time stimulates the opioid peptide transport system. The opioid peptide transport system is likely to be involved in the clearance of enkephalins. The physiologic significance of these apparently paradoxical effects of kyotorphin remains to be determined.

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