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A Novel Nonopioid Action of Enkephalins: Competitive Inhibition of the Mammalian Brain High Affinity L-Proline Transporter

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SUMMARY

The high affinity L-proline transporter (PROT) is a member of the family of Na^+ (and Cl^-)-dependent plasma membrane transport proteins that comprises transporters for several neurotransmitters, osmolytes, and metabolites. The brain-specific expression of PROT in a subset of putative glutamatergic pathways implies a specialized function for this novel transporter and its presumed natural substrate L-proline in excitatory synaptic transmission. However, definitive studies of the physiological role(s) of high affinity L-proline uptake have been precluded by the lack of specific uptake inhibitors. Here, we report that Leu- and Met-enkephalin and their des-tyrosyl derivatives potently and selectively inhibited high affinity L-proline uptake in rat hippocampal synaptosomes and in PROT-transfected HeLa cells. High concentrations of the opiate receptor antagonist naltrexone did not block the inhibitory actions of these peptides, arguing against an involvement of opioid receptors. Des-tyrosyl-Leu-enkephalin elevated the apparent K_m of L-proline transport in transfected HeLa cells without altering the V_{max}. PROT-transfected HeLa cells did not accumulate [^{3}H]Leu-enkephalin above background levels, demonstrating that enkephalins are not substrates for PROT. These findings indicate that enkephalins competitively inhibit mammalian brain PROT through a direct interaction with the transporter protein at or near the L-proline binding site. The high potency and specificity of des-tyrosyl-Leu-enkephalin make this compound a useful tool for elucidating the structure-function properties and physiological role(s) of PROT.

The discovery more than 20 years ago that mammalian brain synaptosomes express a high affinity, Na^+-dependent L-proline uptake activity, similar to the synaptosomal uptake activities identified for recognized neurotransmitters, suggested that L-proline may be a neurotransmitter or neuromodulator in the mammalian central nervous system (1–5). However, definitive studies of the synaptic role(s) of L-proline have been hampered by the inability to associate this imino acid with specific nerve pathways, by its complex excitatory and/or inhibitory actions on neurons in different brain regions (for a review, see Ref. 6), and by the lack of specific inhibitors that block its biosynthesis or high affinity transport in nervous tissue.

Recently, we isolated rat and human brain cDNAs that code for a high affinity Na^+ (and Cl^-)-dependent PROT (7, 8). This novel transporter exhibits significant amino acid sequence conservation (42–50%) with the other members of a gene family of Na^+ (and Cl^-)-dependent plasma membrane transport proteins that includes transporters for several neurotransmitters (norepinephrine, dopamine, serotonin, γ-aminobutyric acid, glycine), osmolytes (betaine, taurine), and metabolites (creatine) (for a review, see Ref. 9). The substrate specificity, ion dependence, and kinetics of L-proline uptake in HeLa cells transiently expressing the PROT cDNA (7, 8) clearly distinguish this carrier from the other widely expressed mammalian Na^+-dependent plasma membrane carriers that transport L-proline, including the intestinal brush border "imino" carrier (for a review, see Ref. 10) and the system "A" and system "ASC" neutral amino acid carriers (for a review, see Ref. 11).

The brain-specific expression of PROT in subpopulations of putative glutamatergic neurons in rat brain (7, 12, 13) warrants the consideration of a specialized role for this trans-

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ABBREVIATIONS: PROT, L-proline transporter; YGGFL, Leu-enkephalin; YGGFM, Met-enkephalin; GGFL, des-Tyr-Leu-enkephalin; YGGFLR, dynorphin $A_{(1-6)}$; GGFL-NH_2, des-Tyr-Leu-enkephalinamide; NMDA, *N*-methyl-D-aspartate; GFL, Gly-Phe-Leu; FL, Phe-Leu; YGGF, des-Met-Met-enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GABA, γ-aminobutyric acid; g_{av}, average gravity.

porter and its presumed natural substrate, L-proline, in the modulation of excitatory synaptic transmission. Within the rat hippocampal formation, high affinity L-proline uptake is confined to a subset of glutamatergic nerve terminals; the Schaffer collateral-commissural, lateral perforant path, and dentate associational-commissural projections take up 3,4-L-[^{3}H]proline, whereas the mossy fibers and medial perforant path do not (12). Thus, high affinity L-proline uptake may modulate in some way excitatory transmission at specific glutamatergic synapses.

To facilitate studies of the physiological role(s) of PROT in the mammalian central nervous system, a potent and specific inhibitor is necessary. Previously, Rhoads *et al.* (14) reported that the opioid pentapeptides YGGFL and YGGFM, and their des-tyrosyl derivatives, inhibited the uptake of L-proline by rat cerebral cortical synaptosomes but not the uptake of other neurotransmitters or amino acids. Interestingly, the opioid receptor antagonist naloxone did not block the inhibition of synaptosomal L-proline uptake by the enkephalins, indicating that opioid receptors did not mediate this effect (14). These results were interpreted to indicate that a specific population of nerve terminals exist in rat brain that contain both a high affinity L-proline transport system and a novel "nonopioid" binding site for the enkephalins (14). However, the molecular mechanism by which the enkephalins inhibited synaptosomal L-proline uptake was not established. In principal, enkephalins could inhibit high affinity synaptosomal L-proline uptake through a receptor-mediated second messenger pathway as originally proposed (14), through alteration of transmembrane ion gradients, or through a direct interaction with the transporter protein.

Here, we elucidate the mechanism underlying this novel nonopioid action of the enkephalins. We demonstrate that the endogenous opioid peptides YGGFL and YGGFM potently and selectively inhibit high affinity L-proline uptake in mammalian brain PROT-transfected HeLa cells and in rat hippocampal synaptosomes. Furthermore, we demonstrate that GGFL elevates the apparent K_m of L-proline uptake in PROT-transfected HeLa cells, without altering the V_{max}. We conclude that enkephalins competitively inhibit mammalian brain PROT through a direct interaction with the transporter protein at or near the substrate binding site.

Materials and Methods

Preparation of synaptosomes and assay of amino acid uptake. Synaptosomes were prepared and amino acid uptake was assayed as described previously (5). Briefly, adult male Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, PA) were killed by decapitation after being anesthetized with ether, and the hippocampi were dissected and pooled in ice-cold 0.32 M sucrose. All subsequent steps were performed at 4°. The hippocampi were homogenized in ≥20 volumes of sucrose through five passes of a loose-fitting Teflon pestle. The homogenate was centrifuged at 1,000 × g_{av} for 10 min. The supernatant (S_1) was divided into several fractions and centrifuged at 20,000 × g_{av} for 20 min. The resulting pellet from each fraction was resuspended in incubation buffer consisting of either 5 ml of Na^+ buffer (122 mM NaCl, 3.1 mM KCl, 25 mM HEPES, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM dextrose, adjusted to pH 7.4 with NaOH) or 5 ml of Li^+ buffer (identical to Na^+ buffer except that NaCl and NaOH were replaced with equimolar concentrations of LiCl and LiOH) and centrifuged again at 20,000 × g_{av} for 20 min. The crude synaptosomal pellets (P_2s) were resuspended in the same buffers (70–200 μg/ml of protein) and analyzed immediately for amino acid uptake.

Synaptosomes (1.0-ml aliquots of each synaptosomal fraction) were brought to 25° and then incubated for 2 min with radiolabeled amino acids in the presence or absence of the indicated concentration of GGFL. Uptake was terminated through vacuum filtration over Whatman GF/C filters that were pretreated with 0.1% polyethyleneimine. The synaptosomes were rapidly washed with 20 ml of the appropriate ice cold incubation buffer (<30 sec) and solubilized in Protosol (New England Nuclear, Boston, MA), and the radioactivity was determined with a liquid scintillation counter (Beckman). Na^+-dependent uptake was calculated as the difference between radiolabeled amino acid accumulated in Na^+ buffer and in Li^+ buffer. Kinetic parameters (K_m, V_{max}) were calculated with the EBDA-LIGAND multiple linear regression program (15) modified for the analysis of uptake data. Under these conditions, uptake is linear with time and protein concentration, and negligible metabolism of the radiolabeled amino acids occurs (5). Protein concentration was determined according to a modification of the Lowry procedure (16) with bovine serum albumin as a standard. Radiolabeled amino acids were purchased from New England Nuclear.

Transient expression of plasma membrane transporter cDNAs in HeLa cells. A T_7 vaccinia virus transient expression system (17) was used to express the indicated plasma membrane transporter cDNAs in HeLa cells. The full-length coding sequences of cDNAs for the rat and human high affinity PROTs (7, 8), human dopamine transporter (18), rat GABA transporter subtype (rGAT-1) (19), human serotonin transporter (20), human norepinephrine transporter (21), rat creatine transporter (22), human glycine transporter subtype (hGlyt1B) (23), and the rat glutamate transporter subtype (EAAC1) (24) were subcloned in the sense orientation downstream of the T_7 promoter in pBluescript plasmids (Stratagene, La Jolla, CA). HeLa cells (~150,000/well in 24-well plates) were infected with recombinant vaccinia virus strain VTF_{7-3} (10 plaque-forming units/cell) in serum-free Optimem media (Life Technologies), followed 30 min later with liposome-mediated (3 μg/well; Lipofectin; Life Technologies) transfection of cDNA from the indicated plasma membrane transporter (1 μg/well). Uptake assays were conducted 8–10 hr after transfection in Krebs-Ringer Tris-HEPES uptake medium as described previously (7). Assays were terminated and washed three times with 1.0 ml of ice-cold Krebs-Ringer Tris-HEPES uptake medium; cells were solubilized with 500 ml of 1% sodium dodecyl sulfate; and accumulated radioactivity was determined through scintillation counting. A minimum of three control wells on each 24-well plate were transfected with pBluescript II SK(−) to determine nonspecific transport values, which were subtracted from signals obtained with the indicated transporter cDNA. To examine the ability of selected pharmacological agents to inhibit uptake, transport assays were conducted for 20 min in the presence or absence of the indicated concentrations of the agent added just before the addition of the radiolabeled substrate. Naltrexone, YGGFL, YGGFM, GGFL, GFL, FL, YGGFLR, and dynorphin $A_{(1-17)}$ were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

GGFL selectively inhibits the high affinity component of synaptosomal L-proline uptake. Previously, GGFL was found to be the most potent inhibitor of L-proline uptake by rat cerebral cortical synaptosomes (14). Initially, we confirmed that GGFL inhibited the uptake of 3,4-L-[^{3}H]proline by rat hippocampal synaptosomes in a concentration-dependent manner (Fig. 1). Inhibition of L-proline uptake was observed at GGFL concentrations as low as 10 nM (data not shown). High concentrations of GGFL, however, did not completely inhibit Na^+-dependent L-proline uptake. GGFL concentrations exceeding 100 μM inhibited L-proline

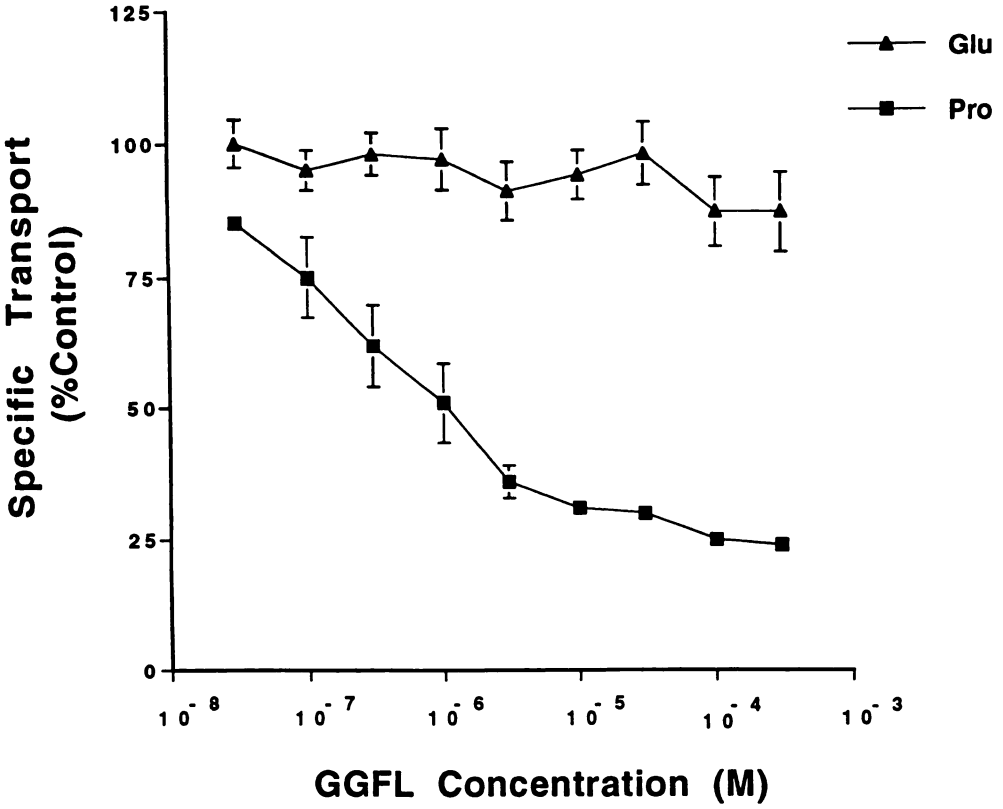

Fig. 1. Inhibition of synaptosomal L-proline transport by GGFL. Synaptosomes were prepared from rat hippocampus, and uptake assays were conducted as described in Experimental Procedures. Synaptosomes were incubated simultaneously with 3,4-L-[^{3}H]proline (90 nM) and L-[^{14}C]glutamate (90 nM) in the presence or absence of the indicated concentrations of GGFL. Data represent the mean ± standard error of three separate determinations.

uptake by ~75% (Fig. 1). Half-maximal inhibition of L-proline uptake occurred at a GGFL concentration of 340 nM. In contrast, GGFL did not significantly alter the simultaneously measured uptake of L-[^{14}C]glutamate at any concentration examined (Fig. 1).

Rat hippocampal synaptosomes accumulate 3,4-L-[^{3}H]proline by two distinct Na^+-dependent processes: one with high affinity and low capacity and one with low affinity and high capacity (5). Fig. 2 shows that Eadie-Hofstee plots of data from experiments that measured the velocity of L-proline uptake as a function of substrate concentration were curvilinear. The data were consistent with the existence of two distinct Na^+-dependent uptake processes. Kinetic parameters (Table 1) were estimated through multiple nonlinear regression with the EBDA-LIGAND program (15). Based on these kinetic parameters, we estimated that at the 3,4-L-[^{3}H]proline concentration used in the experiment shown in Fig. 1 (90 nM), ~75–80% of the total accumulated L-proline is taken up by the high affinity process. We reasoned that GGFL may selectively inhibit the high affinity synaptosomal L-proline uptake process. Accordingly, the remaining 25–30% of L-proline uptake that is not inhibited by maximal concentrations of GGFL could represent the persistence of the low affinity uptake process.

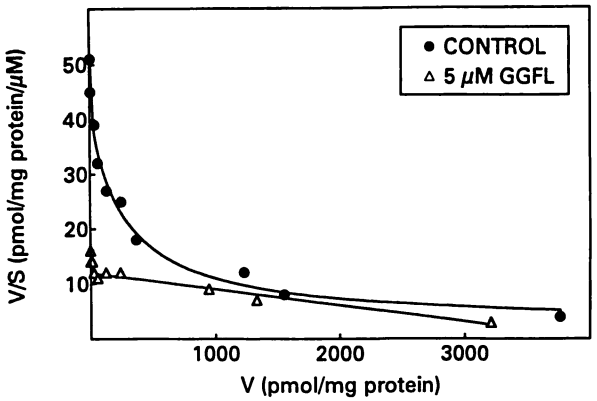

Fig. 2. GGFL selectively inhibits the high affinity component of hippocampal synaptosomal L-proline uptake. Eadie-Hofstee transformation of initial velocity data for the Na^+-dependent uptake of 3,4-L-[^{3}H]proline in the absence or in the presence of 5 μM GGFL. Data represent the mean of three separate experiments. Kinetic parameters calculated from these data are presented in Table 1.

TABLE 1

Kinetic parameters of Na^+-dependent L-proline uptake in rat hippocampal synaptosomes

	Control (−GGFL) (two components)		+5 μM GGFL (one component)
	High	Low	
K_m	4.2 ± 0.6 (5)	310 ± 70 (5)	300 ± 60 (3)
V_{max}	340 ± 50 (5)	9000 ± 1900 (5)	9000 ± 1800 (3)

Rat hippocampal synaptosome fractions were assayed for Na^+-dependent L-proline uptake as described in Materials and Methods. Kinetic parameters were estimated by multiple nonlinear regression with the EBDA-LIGAND program (15). In the absence of GGFL (Control), the data were consistent with the existence of two distinct Na^+-dependent uptake processes. In the presence of GGFL (5 μM), the data were consistent with the existence of a single low affinity uptake process. Kinetic parameters determined for L-proline uptake in the presence of 5 μM GGFL did not differ significantly from those determined for the low affinity component observed in the absence of GGFL. Data represent mean ± standard error of the numbers of experiments given in parentheses. Units are μM for K_m and pmol/mg protein/min for V_{max}.

To test this hypothesis, we conducted synaptosomal uptake assays with various concentrations of L-proline (0.1–1000 μM) in the presence or absence of 5 μM GGFL. Eadie-Hofstee transformation of data obtained from three uptake assays conducted in the presence of 5 μM GGFL were consistent with

the existence of a single, low affinity interaction (Fig. 2). The kinetic parameters determined for L-proline uptake in the presence of GGFL were not significantly different than those of the low affinity component of L-proline uptake observed in the absence of GGFL (Table 1). These results indicate that GGFL selectively inhibited the high affinity component of Na^+-dependent L-proline uptake by rat hippocampal synaptosomes.

Enkephalins inhibit L-proline uptake by PROT-transfected HeLa cells in a naltrexone-insensitive manner. Fig. 3 shows that YGGFL, YGGFM, and the opiate receptor-inactive fragments GGFL and GFL (25) inhibited 3,4-L-[^{3}H]proline uptake by HeLa cells transfected with mammalian brain PROT cDNAs. The inhibitory potency of these peptides (IC_{50} values from 0.26–4.6 μM) (Table 2) exceeded the affinity of PROT for its presumed natural substrate, L-proline (K_m of about 10 μM) (7, 8). The potent and nonselective opiate receptor antagonist naltrexone only weakly inhibited PROT-induced 3,4-L-[^{3}H]proline uptake by HeLa cells (IC_{50} >100 μM) (Table 2). Furthermore, coincubation with naltrexone (100 μM) did not alter the inhibitory potency of GGFL, arguing against the involvement of opioid receptors (data not shown).

We investigated the structural requirements for high affinity inhibition of L-proline uptake by the enkephalins and related peptides (Fig. 3 and Table 2). GGFL exhibited the highest inhibitory potency (IC_{50} = 0.26 μM), followed by YGGFL (IC_{50} = 2.1 μM), YYGFM (IC_{50} = 4.1 μM), and GFL (IC_{50} = 4.6 μM). GGFL-NH_2, YGGFLR, dynorphin $A_{(1-8)}$, and des-Tyr-dynorphin $A_{(1-8)}$ were less potent inhibitors, exhibiting IC_{50} values of 16–70 μM (Table 2). Removal of the carboxyl-terminal residue of enkephalin, yielding YGGF, dramatically reduced the inhibitory potency (IC_{50} ≫ 100 μM) (Fig. 3 and Table 2).

GGFL specifically inhibits mammalian brain PROT. Mammalian brain PROT exhibits significant amino acid sequence conservation (42–50%) with the other members of the Na^+ (and Cl^-)-dependent plasma membrane transporter gene family. Therefore, we investigated the ability of YGGFL, YGGFM, and GGFL to inhibit the transport activities of several other plasma membrane transporters. Fig. 4 shows that 100 μM concentrations of these peptides only weakly inhibited the transport activities of the other transporters examined.

GGFL competitively inhibits 3,4-L-[^{3}H]proline uptake by PROT-transfected HeLa cells. Of the peptides examined, GGFL was the most potent and selective inhibitor of mammalian brain PROT (Fig. 3 and Table 2). Therefore, to explore further the mechanism of inhibition of PROT by the enkephalins, we examined the effect(s) of GGFL on the kinetics of L-proline uptake in PROT-transfected HeLa cells. In five experiments, each conducted in triplicate, GGFL elevated the apparent K_m of L-proline uptake (Fig. 5A) without altering the V_{max} (Fig. 5B), which is consistent with competitive inhibition.

Finally, to examine the possibility that enkephalins may be substrates for PROT, PROT-transfected HeLa cells were incubated with [^{3}H]YGGFL in the presence or absence of 0.1% bovine serum albumin. Fig. 6 shows that PROT-transfected HeLa cells did not accumulate [^{3}H]YGGFL above plasmid vector-transfected control levels.

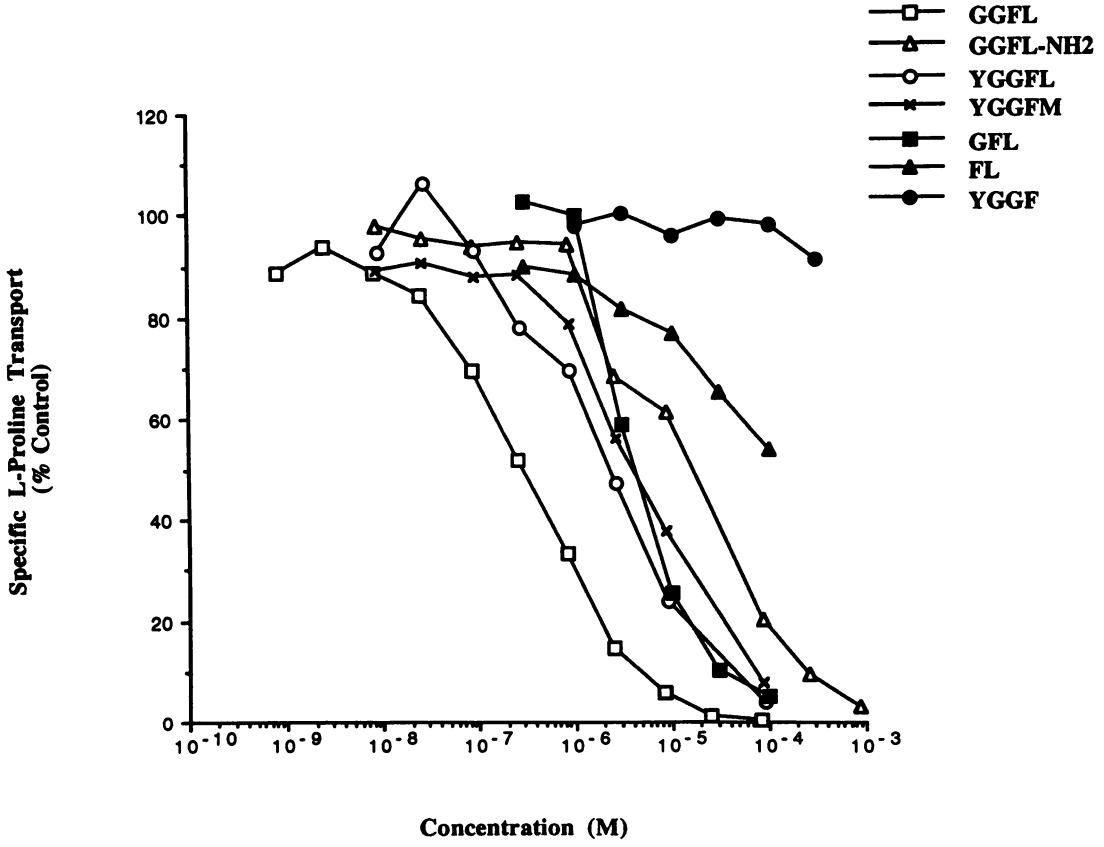

Fig. 3. Inhibition of L-proline uptake by enkephalins in PROT-transfected HeLa cells. Vaccinia virus (VTF_{7-3})-infected HeLa cells were transiently transfected with rat PROT cDNA, and proline transport assays (50 nM 3,4-L-[^{3}H]proline) were conducted for 20 min with or without increasing concentrations of the indicated peptides. Nonspecific transport was determined for each assay by a parallel transfection of the pBluescript II SK(−) vector, and values were subtracted from signals obtained with rat PROT. Data represent the mean of three to six determinations of the percentage of 3,4-L-[^{3}H]proline uptake obtained with labeled substrate alone.

TABLE 2
Inhibition of Na^+-dependent L-proline uptake in PROT-transfected HeLa cells

	IC_{50}
	μM
Peptide	
GGFL	0.26
YGGFL	2.1
YGGFM	4.1
GFL	4.6
GGFL-NH_2	12
YGGFLR	16
YGGFLRRI (dynorphin A_{1-8})	53
GGFLRRI (des-Tyr-dynorphin A_{1-8})	70
YGGF	>100
GGF	>100
GG	>100
GF	>100
GGYR	>100
GGR	>100
GGH	>100
FL	>100
[D-Ala^2,D-Leu^5]Leu-enkephalin	>100
YGGFLRRIRPKLK (dynorphin A_{1-13})	>100
YGGFLRRIRPKLKWDNQ (dynorphin A_{1-17})	>100
GGFLRRIRPKLKWDNQ (dynorphin A_{2-17})	>100
YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ (β-endorphin)	>100
pGHP-NH_2 (thyrotropin-releasing hormone)	>100
cyclo-HisPro	>100
Angiotensin II	>100
Neurotensin	>100
β-AlaHis	>100
Nonpeptide	
Naloxone	>100
Naltrexone	>100

Discussion

Enkephalins inhibit mammalian brain PROT through a direct interaction with the transporter protein. Our results demonstrate that the endogenous opioid pentapeptides YGGFL and YGGFM potently inhibit high affinity L-proline uptake in rat hippocampal synaptosomes and in mammalian brain PROT-transfected HeLa cells. This inhibitory action of the enkephalins was not mediated by opioid receptors, was specific for the high affinity L-proline transporter, and seems to result from a direct interaction with the substrate recognition domain on the mammalian brain PROT protein.

Several lines of evidence indicate that this inhibitory action of the enkephalins was not mediated by opioid receptors. First, the potent and nonselective opiate receptor antagonist naltrexone could not block the inhibitory action of GGFL. Second, GGFL does not bind to opioid receptors (25). Third, transient expression of the PROT cDNA in HeLa cells, which lack endogenous opioid receptors (26), conferred high affinity Na^+ (and Cl^-)-dependent L-proline uptake that could be completely blocked by micromolar concentrations of enkephalins (see Fig. 3).

Potent inhibition of substrate translocation by the enkephalins was specific for mammalian brain PROT. Enkephalins only weakly inhibited uptake of appropriate radiolabeled substrates by HeLa cells transiently expressing several other plasma membrane transporters that share significant amino acid sequence homology with PROT (IC_{50} >100 μM). Therefore, the enkephalin-mediated inhibition of PROT is not likely to result from an indirect effect such as an alteration of transmembrane ion gradients, an effect that would disrupt transport by all of the plasma membrane transporters that we examined.

The proposed direct interaction of enkephalins with the substrate recognition domain of PROT is based on our data showing that GGFL is a competitive inhibitor of L-proline uptake in PROT-transfected HeLa cells. We observed that GGFL significantly increased the K_m for L-proline transport but had little effect on the V_{max} (Fig. 5). Due to their hydrophilic nature, enkephalins are not likely to diffuse freely across the plasma membrane. Therefore, the enkephalin binding site may reside on an extracellular domain of the PROT protein or in an aqueous pore formed by abutting transmembrane elements within the transporter.

GGFL selectively inhibited the high affinity Na^+ (and Cl^-)-dependent component of synaptosomal L-proline uptake (Fig. 2 and Table 1). Because the pharmacological properties, ionic requirements, and kinetics of L-proline uptake in PROT-transfected HeLa cells are similar to those of the high affinity component of synaptosomal L-proline uptake (for a review, see Ref. 8), we conclude that mammalian brain PROT represents the high affinity Na^+ (and Cl^-)-dependent L-proline uptake system in mammalian nervous tissue. Our observation that micromolar concentrations of GGFL completely inhibited PROT-mediated high affinity L-proline uptake in transfected HeLa cells supports this conclusion (Fig. 3). Recently, we observed that the low affinity component of synaptosomal L-proline uptake (K_m = 310 μM) (Table 2) is Cl^- independent.[1] Thus, based on differences in ionic requirements and apparent substrate affinity, we conclude that the low affinity synaptosomal L-proline uptake system represents a distinct transporter protein that is not inhibited by the enkephalins.

Structure-activity relationships. At least 14 peptides have been identified that exhibit opioid activity (for a review, see Ref. 27). These peptides are derived through proteolytic processing from three distinct polyhormone precursors that are the products of separate genes. The precursor proteins are proenkephalin, which gives rise to YGGFL, YGGFM, and several peptides with carboxyl-terminal extensions of YGGFM; prodynorphin, which gives rise to dynorphin $A_{(1-8)}$, dynorphin $A_{(1-17)}$, the neoendorphins, and leumorphin; and proopiomelanocortin, which gives rise to the endorphins. These peptides all contain YGGFL or YGGFM at their amino terminus. However, we demonstrate that potent inhibition of high affinity L-proline uptake is a specific property of the enkephalins. The dynorphins and β-endorphin were orders of magnitude less potent than the enkephalins (Table 2). Similarly, the opiate alkaloid antagonists, naloxone and naltrexone, and several other peptide compounds, including thyrotropin-releasing hormone, angiotensin II, neurotensin, carnosine, and cyclohistidylproline, failed to inhibit high affinity L-proline uptake (IC_{50} >100 μM) (Table 2).

Assessment of the relative inhibitory potencies of several enkephalin peptide fragments revealed specific structural requirements for potent inhibition of mammalian brain PROT. Our results demonstrate that the nonopioid inhibitory potency resides in the carboxyl-terminal domain of the enkephalin sequence. Removal of the amino-terminal tyrosine from YGGFL, yielding GGFL, increased the inhibitory potency by almost 1 order of magnitude. GGFL was equipo-

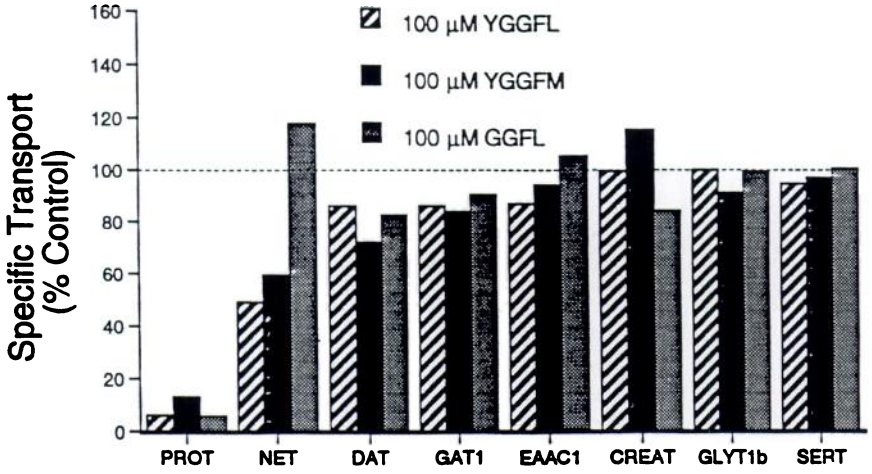

Fig. 4. Specificity of inhibition of mammalian brain PROT by the enkephalins. HeLa cells were transiently transfected with cDNAs encoding the following plasma membrane transporters: the human high affinity proline transporter (*PROT*) (8), human norepinephrine transporter (*NET*) (21), human dopamine transporter (*DAT*) (18), rat brain GABA transporter subtype 1 (*GAT1*) (19), rat brain glutamate transporter subtype EAAC1 (*EAAC1*) (24), rat brain homolog of the rabbit creatine transporter (*CREAT*) (22), human glycine transporter subtype 1b (*GLYT1b*) (23), and human serotonin transporter (*SERT*) (20). Transport of the appropriate radiolabeled substrates were assayed as described in the presence or absence of 100 μM of YGGFL, YGGFM, or GGFL. Data represent triplicate determinations of the percentage of specific uptake of the appropriate radiolabeled substrate obtained in the absence of peptide.

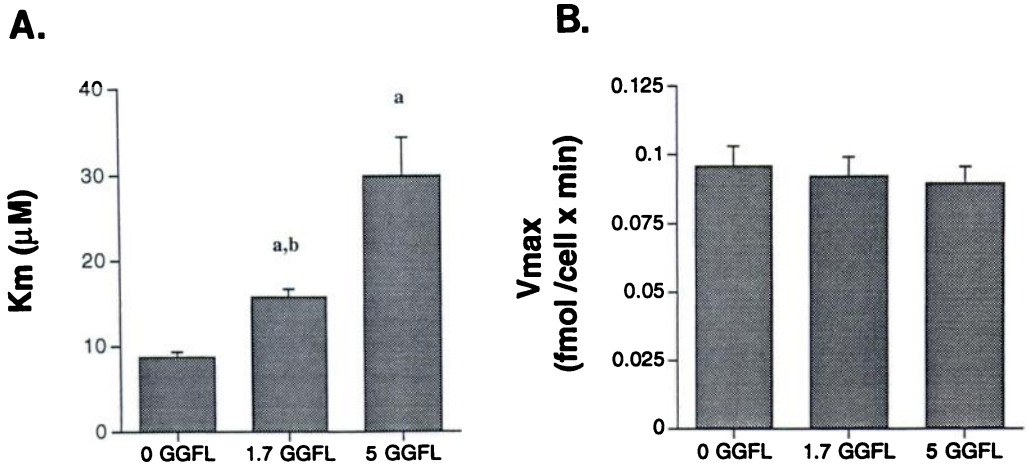

Fig. 5. Competitive inhibition of 3,4-L-[^{3}H]proline uptake by GGFL in PROT-transfected HeLa cells. The effects of GGFL on kinetic parameters of L-proline uptake in PROT-transfected HeLa cells were determined. Data represent the mean ± standard error of four to six independent estimations of the K_m and V_{max} of L-proline uptake in rat PROT-transfected HeLa cells (see Experimental Procedures). Each independent determination of kinetic parameters was based on triplicate measurements of uptake velocity at four different 3,4-L-[^{3}H]proline concentrations (1, 3, 10, and 50 μM) at the indicated GGFL concentration. Analysis of variance indicated that GGFL significantly increased the K_m for L-proline transport at both the 1.7 μM and 5 μM concentrations (A, $p < 0.001$ compared with 0 GGFL; B, $p < 0.025$ compared with 5 μM GGFL). In contrast, neither concentration of GGFL significantly altered the V_{max} of L-proline transport ($p > 0.25$ for all comparisons).

tent at blocking human and rat brain PROTs. Amidation of the carboxyl-terminal leucine residue of GGFL, yielding GGFL-NH_2, reduced the inhibitory potency by 46-fold. Removal of the carboxyl-terminal residue of enkephalin, yielding YGGF, dramatically reduced the inhibitory potency (IC_{50} ≫100 μM). Addition of amino acids to the carboxyl terminus of YGGFL or YGGFM (as in the various dynorphin fragments tested) decreased the inhibitory potency (see Table 2). Finally, the smallest enkephalin fragment that retained significant inhibitory potency was GFL (IC_{50} = 4.6 μM). In contrast, the tripeptide GGF and the dipeptides GG, GF, and FL failed to inhibit high affinity L-proline uptake with IC_{50} values of >100 μM (Table 2). These results suggest that the enkephalin molecule contains two distinct biologically active sites: an opiate site that mediates binding to opiate receptors, and a nonopiate site that seems to interact directly with the the substrate recognition and/or translocation domain of PROT.

Mammalian brain PROT is a representative member of a family of proteins that transduce free energy stored in an electrochemical gradient into work in the form of a concentration gradient. Our understanding of the molecular mechanisms that couple substrate translocation to the energy stored in transmembrane ion gradients is at a rudimentary stage. In contrast, we gained an increasingly sophisticated understanding of the molecular mechanisms underlying ion conduction through transmembrane ion channels, due in large part, to the discovery of peptide toxins that block voltage-gated and/or ligand-gated ion channels (for a review, see Ref. 28). These toxins have proved to be valuable molecular probes with which to explore the biochemical and structure-function properties of these important synaptic proteins. For

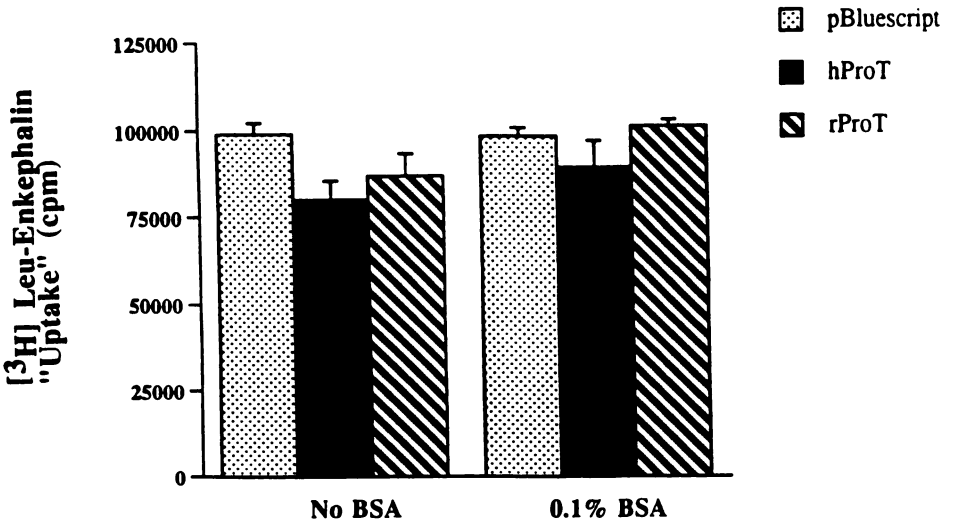

Fig. 6. [^{3}H]YGGFL is not transported by mammalian brain PROT. HeLa cells were transfected with human PROT or with the pBluescriptII SK(−) vector, and uptake assays were conducted using [^{3}H]YGGFL as the potential substrate, in the presence or absence of 0.1% bovine serum albumin. Data represent the mean ± standard error of triplicate determinations. Note that transfected HeLa cells did not accumulate [^{3}H]YGGFL above plasmid vector control levels.

example, the identification of specific amino acids residues in the *Shaker*-type K^+ channel that constitute the binding domain for charybdotoxin led to the discovery of the ion conduction pore of voltage-gated K^+ channels (29). Similarly, we anticipate that the enkephalins may represent valuable molecular probes with which to explore the structural and functional properties of mammalian brain PROT.

Physiological implications. The physiological role or roles of high affinity L-proline uptake in mammalian nervous tissue are enigmatic. The brain-specific expression of PROT in subpopulations of putative glutamatergic pathways (7, 12, 13) is consistent with a specialized role for this novel transporter and its presumed natural substrate, L-proline, in excitatory neurotransmission. We recently obtained direct ultrastructural evidence that PROT immunoreactivity is selectively localized in a subset of presynaptic axon terminals forming asymmetric "excitatory-type" synapses with postsynaptic dendritic spines in the rat caudate-putamen and somatosensory cortex (30). Therefore, high affinity L-proline uptake may regulate the concentration of this imino acid in specific glutamatergic synapses. In this regard, recent studies have demonstrated that physiologically relevant concentrations of L-proline (3–30 μM) enhance synaptic transmission at one glutamatergic synapse where high affinity L-proline uptake exists: the Schaffer collateral-commissural synapse in hippocampal area CA1 (31). L-Proline-induced potentiation of excitatory transmission at this synapse is long lasting, requires the activation of NMDA receptors, and is separable from electrically evoked long term potentiation (31). The high potency and specificity of GGFL make this a useful pharmacological agent with which to elucidate the physiological role(s) of PROT at this synapse.

We do not know whether the direct block of high affinity L-proline uptake by the enkephalins has a physiological role(s) in synaptic signaling. Nevertheless, our data suggest that consideration of such a role is warranted. This inhibition was specific with respect to both the transporter (PROT) and the inhibitory peptide (enkephalins). Furthermore, both rat and human brain PROTs were potently inhibited by the enkephalins, suggesting that this inhibitory effect may be evolutionarily conserved. Enkephalins have the potential to influence high affinity L-proline uptake in a wide variety of neuronal pathways. Immunocytochemical studies have revealed a widespread distribution of neuronal circuits at virtually all levels of the neuroaxis that exhibit enkephalin immunoreactivity (for a review, see Ref. 32). In particular, there are several brain regions in which a good correspondence exists between the distribution of enkephalin-immunoreactive nerve fibers and high levels of expression of mammalian brain PROT; these include the olfactory bulb; frontal, piriform, and entorhinal cortices; amygdala; caudate-putamen; and hippocampus (30). However, several other brain regions do not exhibit such a close correspondence. For example, the globus pallidus contains high levels of enkephalin immunoreactive nerve fibers (32) but only low levels of PROT immunoreactivity (30).

One neuroanatomic circuit in which enkephalins could modulate high affinity L-proline uptake in glutamatergic nerve terminals is the lateral perforant path projection from the entorhinal cortex to the outer molecular layer of the dentate gyrus and the stratum lacunosum-moleculare of the hippocampal CA3 field. Gall *et al.* (33) identified enkephalin immunoreactivity in the lateral perforant path, and Nadler *et al.* (12) demonstrated that high affinity L-proline uptake is enriched in the terminal fields of this pathway. Xie and Lewis (34) observed that enkephalins, released during high frequency stimulation, facilitate induction of long-term potentiation of excitatory transmission at the lateral perforant path-dentate granule cell synapses. These effects of the enkephalins were partially blocked by bath application of the opiate receptor antagonist naloxone (see Fig. 2B of Ref. 34). Because activation of μ and δ receptors reduces GABA-mediated inhibitory postsynaptic currents but has no direct enhancing effects on excitatory postsynaptic currents, Xie and Lewis (34) proposed that endogenous enkephalins facilitate long-term potentiation at lateral perforant path synapses by suppressing GABAergic inputs to the granule cell (disinhibition). Our data raise the possibility that enkephalin-mediated inhibition of high affinity L-proline uptake may also contribute to the long-lasting facilitation of excitatory synaptic transmission at lateral perforant path synapses.

We observed significant inhibition of L-proline uptake in PROT-transfected HeLa cells at submicromolar levels of YGGFL and YGGFM (Fig. 3), whereas the IC_{50} values were in the low micromolar range (Table 2). These values are within the upper limit of what is considered to be physiological.

Enkephalins are stored and released from large, dense-cored vesicles in the mammalian central nervous system (35). The concentration of enkephalins contained in these vesicles has been estimated to be between 0.4 and 1.8 mM (36). Therefore, based on the geometry of a typical excitatory synapse (average width, ~20 nm; average diameter, ~2 μm) (37), the synaptic concentration of enkephalins bathing the high affinity L-proline transporter could reach tens of μM (see Ref. 38), well within the range that inhibits mammalian brain PROT. Furthermore, it is generally accepted that the synaptic actions of the enkephalins are terminated by enzymatic hydrolysis. Thus, an aminopeptidase has been purified from bovine brain that catalyzes the hydrolysis of the amino-terminal tyrosine from YGGFL or YGGFM, generating the destyrosyl derivatives (39). Our data indicate that the resulting opiate receptor-inactive (25) peptide fragments GGFL and GGFM retain the ability to inhibit high affinity L-proline uptake (Fig. 3 and Table 2). Therefore, inhibition of high affinity L-proline uptake may persist after the opioid actions of the enkephalins have been terminated.

Finally, mouse brain synaptosomes metabolize L-proline to L-glutamate through the sequential actions of proline oxidase and Δ-1-pyrroline-5-carboxylate dehydrogenase (40). Therefore, high affinity L-proline uptake may modulate the synthesis and/or release of L-glutamate in specific excitatory nerve terminals in the mammalian central nervous system. However, these enzymes have been found in glial cells, not neurons (for a review, see Ref. 7). Future studies are necessary to determine the metabolic fate of L-proline taken up through the high affinity mammalian brain PROT. It may also be illuminating to examine the effect(s) of inhibiting high affinity synaptosomal L-proline uptake on releasable pools of L-glutamate.

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