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Iminoglycine Transport System in Synaptosomes and Its Interaction with Enkephalins†

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ABSTRACT: Evidence is presented which suggests that proline, pipecolic acid, and glycine are accumulated by a common transport system in rat brain cortical synaptosomes and synaptosomal plasma membrane vesicles. This system is Na^+ dependent and appears to be similar to the iminoglycine transport system present in renal tubules and in renal brush border membranes. The opioid pentapeptides Leu- and Met-enkephalin specifically inhibit the uptake of these three imino/amino acids, presumably by interaction with a nonopioid receptor, since the inhibition is not affected by the opiate antagonist naloxone and occurs with des-tyrosyl enkephalins as well as with the intact pentapeptides.

Proline, hydroxyproline, and glycine have been shown to be transported via a common transport system in the renal tubule. Evidence for the presence of this system, commonly referred to as the iminoglycine transport system, stems from in vitro studies as well as from clinical studies on familial iminoglycinuria, an autosomal recessive inborn error of membrane transport characterized by an exaggerated renal clearance of proline, hydroxyproline, and glycine (Scriver et al., 1961; Scriver & Wilson, 1964; Rosenberg et al., 1968; Scriver, 1983). Iminoglycinuria appears to be the result of inactivation or defect in the membrane transport protein. From their studies on proline transport by isolated brain capillaries, Hwang et al. (1983) suggested that inherited abnormalities of renal transport such as iminoglycinuria may have a cerebral counterpart and that genetic regulation in the two tissues may be related. Other evidence suggests that pipecolic acid, a six-membered analogue of proline, shares a common transport system with proline in mouse brain synaptosomes (Nomura et al., 1980) and in rabbit brush border membrane vesicles (Ganapathy et al., 1983) where it was suggested that pipecolic acid is accumulated via the iminoglycine transport system.

We have recently shown (Rhoads et al., 1983c) that synaptosomal uptake of proline is strongly inhibited by micromolar concentrations of Leu- and Met-enkephalins and that this inhibition cannot be reversed by naloxone. We have also shown that enkephalins have no effect on several other putative or established neurotransmitters such as glutamic acid, aspartic acid, taurine, and γ-aminobutyric acid. In the present study, evidence is presented that in both synaptosomes and synaptosomal plasma membrane vesicles, proline, pipecolic acid, and glycine are accumulated via a common iminoglycine transport system and that Leu- and Met-enkephalins inhibit specifically this system presumably by interaction with a nonopioid receptor.

Experimental Procedures

Materials. L-[U-^{14}C]Proline and L-[U-^{14}C]glycine were purchased from New England Nuclear Corp. (Boston, MA) and had the following specific activities (millicuries per millimole): proline, 293; glycine, 104. DL-[^{3}H]Pipecolic acid (2.98

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Table I: Characteristics of Pipecolic Acid Uptake by Rat Brain Cortical Synaptosomes[a]

inhibitor (concn)	% inhibn of uptake of pipecolic acid
veratridine (100 μM)	77 ± 6
Tityus toxin (5 μg/mL)	64 ± 8
tetrodotoxin (5 μg/mL)	0
veratridine (100 μM) + tetrodotoxin (5 μg)	31 ± 5
Tityus toxin (5 μg) + tetrodotoxin (5 μg)	13 ± 7
$18:1^{\Delta 9}$ (10 μM)	48 ± 5
18:0 (10 μM)	8 ± 3

[a] Pipecolic acid accumulation was determined after a 10-min incubation as described under Experimental Procedures. Each value represents the means ± SEM of three determinations. [b] Control value for uptake was 29.3 ± pmol/mg of protein.

Ci/mmol) was obtained by custom tritium labeling and was generously provided by Dr. E. Giacobini (Southern Illinois University School of Medicine). The enkephalins, β-endorphins, and scorpion (*Tityus serrulatus*) venom were purchased from Sigma Chemical Co. (St. Louis, MO) and Bachem (Torrance, CA). Veratridine (as veratrine) was obtained from Dr. M. Anthony Verity, University of California, Los Angeles, and naloxone and dynorphin 1-13 were from Dr. Horace Loh, Department of Pharmacology, University of California at San Francisco. Membrane filters (0.45 μm) were obtained from Micro Filtration Systems (Dublin, CA).

Methods. Synaptosomal fractions were prepared from cerebral cortices of adult Sprague-Dawley rats by methods described previously (Rhoads et al., 1983a,b). Synaptosomal fractions (0.1 mg of protein) were incubated at 25 °C with 0.1 μCi of ^{14}C-labeled glycine or proline or with 1.25 μCi of ^{3}H-labeled pipecolic acid (0.3–0.5 nmol) in 1 mL of a medium containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM KCl, and 1 mM $CaCl_2$. To study the Na^+ dependence of uptake, NaCl in the incubation medium was replaced by 300 mM sucrose or 150 mM choline chloride, and uptake in this medium was defined as Na^+-independent uptake. The difference in uptake between Na^+-containing medium and Na^+-free medium was taken as the strictly Na^+-dependent uptake. Following incubation, the synaptosomal particles were collected on membrane filters, the filters were washed with 20 mL of the ice-cold incubation medium, and the washed filters were dissolved in scintillation fluid for assay of radioactivity (Peterson & Raghupathy, 1972). Washing of the filters with distilled water instead of the isotonic buffer led to the loss of more than 90% of the radioactivity collected on the filters, indicating that the amino/imino acids are accumulated within membrane components which are osmotically sensitive. In other studies, we have established that under the incubation conditions employed (low protein concentration, short incubation time, and no added cofactors), little or no metabolism of the added radiolabeled amino acids takes place (Peterson et al., 1972). Under these experimental conditions, we have earlier observed that the concentration gradient (tissue:medium) was about 30:1 for most amino acids (Peterson & Raghupathy, 1972).

Plasma membrane vesicles were prepared from synaptosomal fractions by methods previously described (Rhoads et al., 1982b). In transport studies with this system, the vesicles were loaded with 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM $MgSO_4$. The incubation buffer contained 150 mM NaCl, 1 mM $MgSO_4$, 5 mM Tris-HCl (pH 7.4), 0.1 μCi of [^{14}C]glycine or [^{14}C]proline, and 0.1–0.2 mg of vesicle protein [determined by the method of Lowry et al. (1951)], all in a final volume of 1 mL. Incubation conditions and postincubation procedures were as described previously (Rhoads et al., 1982b). In experiments not described in this paper, it has been shown that in the absence of Na^+ in the incubation medium little or no uptake of glycine occurs in this system.

Results

In initial experiments, we compared the synaptosomal uptake of DL-pipecolic acid in the presence and absence of Na^+ in the incubation medium. It was observed that in the absence of external Na^+, little or no uptake of pipecolic acid occurred. Similar results for mouse synaptosomes have been reported by Nomura et al. (1980). As is the case with other amino acids (Rhoads et al., 1983b), the synaptosomal uptake of pipecolic acid reached a maximum by approximately 10 min of incubation.

The general characteristics of Na^+-dependent pipecolic acid uptake by rat brain cortical synaptosomes are shown in Table I. The uptake is strongly inhibited by veratridine and by *Tityus serrulatus* venom, and this inhibition is reversed by tetrodotoxin. These observations suggest that the pipecolic acid transport system is located primarily in compartments also containing veratridine-sensitive Na^+ channels, which, like the veratridine-sensitive transport systems of other neuroactive amino acids (Kanner, 1980; Rhoads et al., 1982b,c), is evidence of their association with the presynaptic membrane. As has been shown with other Na^+-dependent amino acid transport systems (Rhoads et al., 1982a), oleic acid at 10 μM strongly inhibits the uptake of pipecolic acid while the saturated fatty acid palmitic acid has little or no effect on the uptake.

The data in Table II clearly show that proline, pipecolic acid, and glycine mutually inhibit the uptake of each other in synaptosomal preparations. The uptake of pipecolic acid is strongly inhibited by proline. At 10 μM concentration, proline inhibits the uptake of pipecolic acid by almost 50%, and at 100 μM, the inhibition is about 75%. The uptake of proline is also inhibited by pipecolic acid, and as much as 80% in-

Table II: Mutual Inhibition of Synaptosomal Proline, Pipecolic Acid, and Na^+-Dependent Glycine Uptake

inhibitory amino acid	concn (μM)	% inhibn of uptake of[a]			
		[^{14}C]proline	[^{3}H]pipecolic acid	[^{14}C]glycine	
				Na^+ independent	Na^+ dependent
proline	10		45 ± 7	0	13 ± 5
	100		75 ± 6	4 ± 1	51 ± 8
pipecolic acid	10	52 ± 4		2 ± 3	17 ± 6
	100	81 ± 2		4 ± 5	49 ± 9
glycine	10	25 ± 2			
	100	44 ± 3	40 ± 2		

[a] Means ± SEM of three independent determinations.

Table III: Inhibition of Imino Acid and Na^+-Dependent Glycine Uptake in Synaptosomal Plasma Membrane Vesicles[a]

inhibitory amino acid[b]	% inhibn of [^{14}C]Pro uptake[c]	% inhibn of [^{14}C]Gly uptake[d]
L-proline		17 ± 1
DL-pipecolic acid	77 ± 8	16 ± 3
glycine	18 ± 3	

[a] Each value represents the mean ± SEM of three determinations. [b] Inhibitor amino acid concentration was 100 μM. [c] Control value for uptake of proline was 11.9 ± 1.0 pmol/mg of protein. [d] Control value for the Na^+-dependent uptake was 33.5 ± 2.2 pmol/mg of protein.

hibition is seen at 0.1 mM pipecolic acid concentration. Glycine has been shown to be taken up by two distinct mechanisms—one strictly Na^+ dependent and the other Na^+ independent (Peterson & Raghupathy, 1973). Only the Na^+-dependent component of glycine uptake has been shown to be inhibited by veratridine (Rhoads et al., 1982c). The data in Table II clearly show that the Na^+-independent glycine uptake in cortical synaptosomes is unaffected by proline and pipecolic acid. In contrast, the strictly Na^+-dependent glycine uptake is substantially inhibited by both imino acids (Table II). Glycine also inhibits the synaptosomal uptake of proline and pipecolic acid although the sensitivity of this uptake to glycine is much less. This lesser sensitivity to glycine is consistent with the results obtained in other systems (Hwang et al., 1983; Ganapathy et al., 1983).

Mutual inhibition between the imino acids and glycine can be seen in synaptosomal plasma membrane vesicles as well. In this system, the uptake of proline and glycine is driven by an artificially imposed Na^+ gradient. As can be seen from the results presented in Table III, the uptake by glycine by vesicles is inhibited by both proline and pipecolic acid. At a concentration of 100 mM, proline inhibits the Na^+-driven glycine uptake by 17% while at the same concentration pipecolic acid inhibits it by 16%. Furthermore, glycine at a concentration of 100 mM inhibits the vesicular uptake of proline by 17%. The generally lesser sensitivity of glycine to inhibition by the imino acids as well as the lower inhibitory effect on glycine on the uptake of imino acids is in line with other studies (Hwang et al., 1983; Ganapathy et al., 1983).

We have earlier demonstrated (Peterson & Raghupathy, 1977; Raghupathy & Peterson, 1977) that the synaptosomal uptake of proline is inhibited competitively by neutral amino acids. Double-reciprocal plots of the inhibition of glycine uptake by proline, however, yielded a complex picture not amenable to simple interpretation. This is due in part to the error structure inherent in the calculations made to obtain the strictly Na^+-dependent glycine uptake, since there are two separate systems, a Na^+-dependent and a Na^+-independent system, involved (Peterson & Raghupathy, 1973). A further complicating factor is that synaptosomal preparations contain more than one strictly Na^+-dependent glycine transport system as well (Mayor et al., 1981), only one of which may compete for the iminoglycine transport site.

We have recently shown (Rhoads et al., 1983c) that Leu- and Met-enkephalins strongly inhibit the synaptosomal uptake of proline but have no effect on the uptake of several other putative neurotransmitters or on the Na^+-independent uptake of the neuroinactive amino acid leucine. The Na^+-dependent uptake of pipecolic acid by brain cortical synpatosomes is also substantially inhibited by Leu-enkephalin (Table IV). The inhibition is dose dependent, and 50% inhibition is attained at an enkephalin concentration of about 2 μM. As can be seen

Table IV: Effects of Enkephalins on the Uptake of Pipecolic Acid by Rat Brain Cortical Synaptosomes

addition	concn (μM)	% inhibn of uptake of [^{3}H]pipecolic acid[a]
Leu-enkephalin	1	31 ± 3
	2	45 ± 5
	10	58 ± 5
Met-enkephalin	10	50 ± 8
des-Tyr-Leu-enkephalin	10	56 ± 1
des-Tyr-Met-enkephalin	10	51 ± 7
DSLET	10	24 ± 3
naloxone	50	1 ± 3
Leu-enkephalin + naloxone	10 + 50	56 ± 5
morphine	10	0
dynorphin 1-13	10	0
β_h-endorphin	10	0
[Leu^5]-β-endorphin	10	0

[a] Values represent means ± SEM of three independent determinations; control value for the uptake of pipecolic acid was 29.3 ± 5.3 pmol/mg of protein.

from Table IV, both Leu- and Met-enkephalins are effective inhibitors of pipecolic acid uptake as are the des-tyrosyl derivatives of the enkephalins. Morphine, dynorphin 1-13, β_h-endorphin, and [Leu^5]-β-endorphin have no effect within the concentration range studied. The inhibitory effects of enkepahlins on pipecolic acid uptake are not reversed by naloxone. The modified enkephalin [D-Ser^2]-Leu-enkephalin-Thr (DSLET), which is a selective agonist for the δ subclass of enkephalin receptors, also inhibits pipecolic acid uptake. However, as with proline (Rhoads et al., 1983c), the magnitude of the inhibition is less with DSLET than that obtained with either Met- or Leu-enkephalin.

The results presented in Table V show that Leu-enkephalin has no effect on the Na^+-independent uptake of glycine but significantly inhibits the Na^3-dependent uptake of glycine. The Na^+-dependent uptake is inhibited by Met-enkephalin as well as by the des-tyrosyl derivatives of Leu- and Met-enkephalins. This Na^+-dependent glycine uptake is not inhibited by morphine, dynorphin 1-13, or β-endorphins. This pattern of inhibition by the opioid peptides is similar to those observed for pipecolic acid (Table IV) and proline (Rhoads et al., 1983c) except that less inhibition of glycine uptake is obtained at a given peptide concentration.

Discussion

The present study extends our previous work on the interaction of enkephalins with the synaptosomal proline transport system. In that study (Rhoads et al., 1983), we showed that enkephalins and des-tyrosyl enkephalins strongly inhibited synaptosomal proline uptake and that this inhibition was not reversed by naloxone. Furthermore, the inhibition was attenuated by 1-butanol, a compound shown specifically to antagonize the binding of enkephalins to the δ receptors in brain (Simon et al., 1982). Inhibition was obtained with a δ agonist, DSLET, although higher concentrations were needed to obtain a similar extent of inhibition. On the basis of these results, we suggested that enkephalins interact with a subclass of enkephalin receptors distinct from the opioid receptors but which share some characteristics with the δ receptors. In the present study, we show that the inhibition by enkephalins also occurs with the transport of pipecolic acid and glycine. The results of mutual inhibition studies (Table II) strongly suggest that proline and pipecolic acid uptake and the strictly Na^+-dependent component of glycine uptake share a common transport system, similar to the iminoglycine system proposed

Table V: Effects of Enkephalins on Na^+-Independent and Na^+-Dependent Synaptosomal Glycine Uptake

addition	concn (μM)	[^{14}C]glycine uptake[a] Na^+-independent pmol/mg of protein	% inhibn	Na^+-dependent pmol/mg of protein	% inhibn
none		64.3 ± 5.5		69.7 ± 6.1	
Leu-enkephalin	10	64.9 ± 13.3	0	40.8 ± 3.0	41 ± 6
Met-enkephalin	10	70.1 ± 1.1	0	45.3 ± 5.8	35 ± 12
des-Tyr-Leu-enkephalin	10	68.6 ± 2.3	0	37.6 ± 2.9	46 ± 4
des-Tyr-Met-enkephalin	10	63.7 ± 1.4	1 ± 3	40.4 ± 3.1	42 ± 5
DSLET	30	63.4 ± 6.6	1 ± 3	43.9 ± 7.4	37 ± 6
naloxone	50	63.1 ± 2.1	1 ± 2	71.2 ± 4.9	0
Leu-enkephalin + naloxone	10 + 50	61.4 ± 8.7	5 ± 6	39.5 ± 8.3	43 ± 9
morphine	10	63.2 ± 3.2	2 ± 3	74.1 ± 6.2	0
dynorphin 1-13	10	64.9 ± 4.8	0	71.3 ± 4.3	0
β_h-endorphin	10	66.3 ± 2.0	0	70.4 ± 2.8	0
[Leu^5]-β-endorphin	10	64.8 ± 5.2	0	69.9 ± 4.5	0

[a] Means ± SEM of three independent determinations.

in renal tubules (Scriver, 1983). Proline and pipecolic acid strongly inhibit the uptake of one another, and glycine also inhibits the uptake of proline and pipecolic acid (Table II; Raghupathy & Peterson, 1977). The Na^+-independent glycine uptake in cortical synaptosomes is unaffected by proline and pipecolic acid but is strongly inhibited by several neutral amino acids (Raghupathy & Peterson, 1977). On the other hand, the strictly Na^+-dependent uptake is substantially inhibited by both imino acids (Table II) but not by the neutral amino acids which inhibit the Na^+-independent glycine uptake (Raghupathy & Peterson, 1977). We have observed (Peterson & Raghupathy, 1977) in adult brain cortical synaptosomes that proline has no effect on the uptake of leucine, phenylalanine, isoleucine, tyrosine, histidine, valine, threonine, serine, and aspartic and glutamic acids. Thus, mutual inhibition is restricted to proline, pipecolic acid, and the strictly Na^+-dependent component of glycine uptake, and these observations are in line with the hypothesis that cortical synaptosomes possess a Na^+-dependent, iminoglycine transport system which is selectively inhibited by enkephalins.

The present data support the contention that the iminoglycine transport system is present in synaptosomal plasma membrane vesicles as well (Table III). It is of interest to note, however, that proline and pipecolic acid are strong inhibitors of each other and inhibit glycine uptake to a much weaker extent. Also, glycine is a much weaker inhibitor of imino acid transport (Raghupathy & Peterson, 1977; Nomura et al., 1980; Hwang et al., 1983). One possible explanation for the weaker inhibition of glycine could lie in the observations of Mayor et al. (1981), who have demonstrated the presence of two Na^+-dependent glycine transport systems in synaptic vesicles. Only one of these systems may function via the iminoglycine transport system, which would then explain the lower inhibition of proline and pipecolic acid transport by glycine since only a portion of the glycine is available for interaction at the iminoglycine site. Such an explanation is also consistent with data obtained in the renal tubule system, where it has been shown that the membrane transport sites (carrier systems) can be delineated into (1) a site with high capacity which is common to the three substrates (the iminoglycine system), (2) a site with preference for glycine and which does not transport imino acids, and (3) a site with preference to the imino acids, although the capacity of this site is only about one-tenth that of the shared or iminoglycine sites (Scriver, 1983). More recently, we have shown that the vesicular iminoglycine transport system is also sensitive to inhibition by enkephalins (D. E. Rhoads, N. A. Peterson, and E. Raghupathy, unpublished results).

The physiological significance of the inhibition of the iminoglycine transport system by enkephalins is as yet unclear. It is significant, however, that all of the three amino/imino acids have been implicated in neurotransmission or neuromodulation (Felix & Kunzle, 1974; Zarzecki et al., 1975; aprison & Daly, 1978; Giacobini, 1983). Of additional interest are the facts that elevated levels of pipecolic acid are associated with severe mental retardation in both hyperpipecolic acidemia (Gatfield et al., 1968; Thomas et al., 1975; Burton et al., 1981) and Zellweger syndrome (Danks et al., 1975). Furthermore, it has been shown that in the mammalian brain pipecolic acid is actively synthesized from lysine whereas in other tissues lysine is metabolized principally via the saccharopine pathway (Chang, 1976, 1978a,b) and the pipecolic acid pathway is only a minor metabolic route.

In a previous paper (Rhoads et al., 1983c), we concluded that the inhibition of proline uptake by enkephalins is due to the possibility that the proline carrier is coupled to a nonopioid receptor, which shares some characteristics of the putative δ receptors for enkephalins. The present demonstration of an iminoglycine transport system in synaptosomes now suggests the possibility that it is the iminoglycine transport carrier which is coupled to an enkephalin receptor. The present study further raises the question of whether the iminoglycine transport systems of other tissues are also subject to modulation by enkephalins. In preliminary experiments, we have observed (V. Ganapathy, F. H. Leibach, D. E. Rhoads, and E. Raghupathy, unpublished results) that Leu-enkephalin at concentrations of 10 and 100 μM inhibits the uptake of proline and pipecolic acid by renal brush border membranes.

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Registry No. Pro, 147-85-3; pipecolic acid, 535-75-1; Gly, 56-40-6; Leu-enkephalin, 58822-25-6; Met-enkephalin, 58569-55-4; des-Tyr-Met-enkephalin, 61370-88-5; des-Tyr-Leu-enkephalin, 60254-83-3; DSLET, 75644-90-5; Na, 7440-23-5.

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