Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 262-268

BINDING OF THE BIDENTATE INHIBITOR [^{3}H]HACBO-GLY TO THE RAT BRAIN NEUTRAL ENDOPEPTIDASE "ENKEPHALINASE"

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Received July 9, 1985

The synthesis and binding properties to rat brain tissue of the enkephalinase inhibitor [^{3}H] N-[(R,S)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine ([^{3}H]HACBO-Gly, 45 Ci/mmole) is reported. [^{3}H]HACBO-Gly binding to membranes from various rat brain tissue is saturable (K_D=0.4 ± 0.05 nM) and linearly related to the amount of tissue. Non specific binding is less than 15% of total binding at the K_D concentration. The regional distribution of [^{3}H]HACBO-Gly binding and enkephalinase activity are closely correlated with highest levels in striatum and substantia nigra. The efficiency of inhibitors of various peptidases (thiorphan, captopril, bestatin ...) to inhibit [^{3}H]-HACBO-Gly binding or enkephalinase activity are similar. These results indicate that [^{3}H]HACBO-Gly binds selectively to enkephalinase. This compound should help to clarify the localization of the enzyme in the CNS.

The neutral endopeptidase (EC 3.4.24.11) first isolated from rabbit kidney (1) was afterwards discovered in the rat central nervous system (2). This enzyme is responsible for the cleavage of the Gly^3-Phe^4 bond of the opioid peptides enkephalins and has therefore been designated as enkephalinase (2). The physiological relevance of enkephalinase has been demonstrated by the use of highly potent inhibitors such as thiorphan (3), retrothiorphan (4) or kelatorphan (5), which induce antinociceptive responses after i.c.v. injection. Moreover, co-administered with the peptidase-sensitive Met-enkephalin, kelatorphan is able to decrease 50,000 times the efficient dose (E. D. 50) of the opioid peptide required to produce an analgesic response (5). These effects can be reversed by naloxone, demonstrating the interaction of enkephalins with the opioid receptors. Finally, enkephalinase inhibitors are

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0006-291X/85 $1.50

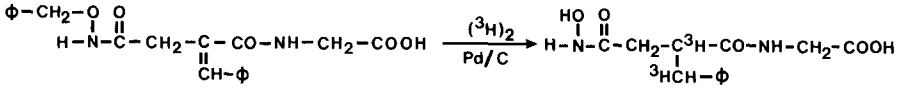
able to protect the K^+ evoked Met-enkephalin released from rat striatal slices (6). Nevertheless enkephalinase displays a wide substrate specificity, since in vitro the enzyme cleaves a variety of neuropeptides such as substance P (7) neurotensin (8) or CCK_{26-33} (9). This raises the question of the selective involvement of enkephalinase in enkephalin metabolism. If the enkephalinase has a selective involvement in enkephalin degradation, one would expect the enzyme to be in close proximity to enkephalin receptors. To further investigate this assumption a precise localization of enkephalinase by autoradiographic measurement, as recently shown in the case of brain ACE (10), is required.

Therefore we report in this paper the synthesis of a tritiated highly potent enkephalinase inhibitor : [^{3}H] N-[(R,S)-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine ([^{3}H]HACBO-Gly) (K_I = 1.4 ± 0.2 nM). This compound, belonging to a new series of bidentate inhibitors is poorly recognized by ACE (11), and behaves as a useful probe for the visualization of enkephalinase in the central nervous system (12).

METHODS

Synthesis of [^{3}H]HACBO-Gly

[^{3}H] N-[(R,S)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine was obtained by tritiation of the unsaturated precursor N-[3-(benzyloxyamino) carbonyl]-2-benzylidene-1-oxopropyl]-glycine (13) (scheme 1). 6.5 µM of the latter compound was dissolved in 1 ml pure methanol and the catalyst (1.6 mg of 10% palladium on charcoal) was added. The reaction was stirred at room temperature under tritium gas atmosphere (25 Ci) for 1 hour. The catalyst was then removed by filtration and labile tritium atoms eliminated by two successive evaporations with 20 ml ethanol. The product was purified by preparative TLC with butanol/acetic acid/water solvent system 4/1/1 (v/v/v). The silica band corresponding to final product (Rf = 0.52) was scraped and the product was extracted with methanol on a sep-pak C_{18} column (Waters Associates). The purity of [^{3}H]HACBO-Gly (45 Ci/mmole) was checked on a Zorbax O.D.S. column by isocratic elution in methanol/water (45/55) over 4.1 min with a flow rate of 1 ml/min.



Scheme 1

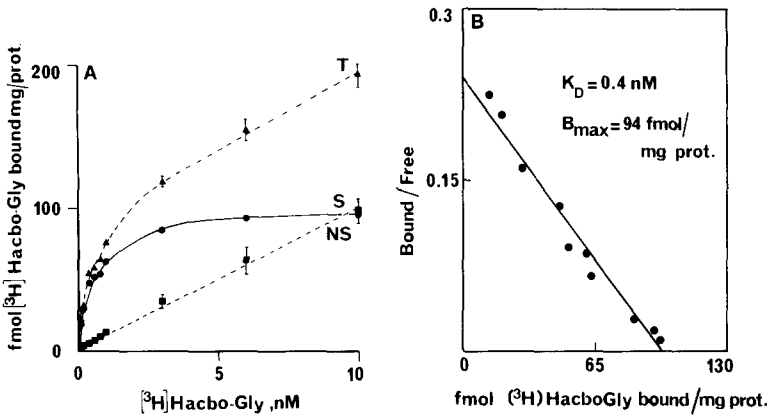
Binding experiments

Fresh brain tissue was obtained from male Sprague Dawley rats (150-200 g) and homogenized in 12 volumes of 0.32 M sucrose 5 mM Tris HCl pH 7.4 with 10 strokes of a potter Elvehjem tissue grinder using a teflon pestle. After centrifuging the homogenate at 100,000 g for 35 min, the pellet was suspended in 50 mM Tris-HCl pH 7.4 and centrifuged as before. The resulting pellet was resuspended in the original volume of Tris-HCl buffer to yield the membrane fraction. Binding assays were carried out at 25°C in 50 mM Tris HCl pH 7.4 and included the tissue suspension (0.6-0.7 mg/ml) and [^{3}H]HACBO-Gly in a volume of 1 ml. After 45 min the suspension was filtered over Whatman GF/B filters under vacuum with two 5 ml washes of the same buffer. The amount of radioactivity remaining on the filter was determined by liquid scintillation spectrometry with 5 ml of Beckman Ready Solv EP cocktail. Specific binding of [^{3}H]HACBO-Gly was determined after substracting the non specific binding assessed in the presence of 1 µM thiorphan. Neutral endopeptidase activity was measured with 20 nM [tyrosyl-3,5-^{3}H]-D-Ala^2-Leu^5-enkephalin and various concentrations of non radioactive D-Ala^2-Leu^5-enkephalin as already described in detail (14). Protein determinations were performed following the method described by Lowry et al. (15) using B.S.A. as standard.

RESULTS

The specific binding of [^{3}H]HACBO-Gly to a homogenate of rat striatum, assessed in the presence of 1 µM thiorphan is saturable (Fig. 1A), leading to the following parameters derived from Scatchard analysis : K_D value 0.4 $\pm$ 0.05 nM and B_{max} value 94 $\pm$ 12 fmol/mg protein (Fig. 1B). Under these experimental conditions the specific binding corresponds to 88% of the total binding at the K_D concentration of [^{3}H]HACBO-Gly, and the computer analysis of the saturation isotherms shows that the tritiated probe interacts with a single class of binding sites. The binding of [^{3}H]HACBO-Gly at 25°C is time dependent with a plateau at 30 min. Specific binding of [^{3}H]HACBO-Gly is linearly related to the amount of tissue in the assay (data not shown) allowing quantitative measurements of the binding sites capacity in various brain areas (Table 1). Relative levels of enkephalinase activity in some brain regions closely parallel [^{3}H]HACBO-Gly binding. Highest specific binding occurs in the caudate putamen and substantia nigra (Table 2).

Inhibition experiments, performed with various peptidase inhibitors, show a good correlation between the efficiency of these compounds to inhibit [^{3}H]HACBO-Gly binding and their inhibitory potency towards enkephalinase in



Binding of [^{3}H]HACBO-Gly to rat striatal membranes.

A Saturation curve of [^{3}H]HACBO-Gly binding to rat striatal membranes was performed using a suspension of 0.6-0.7 mg/ml of P_2 fraction in an assay volume of 1 ml as described in methods. The non specific binding NS (■) was evaluated with thiorphan 1 µM. Specific binding (●) is the difference between total T (▲) and non specific binding (■). These data represent the results of four independant experiments performed in triplicate.

B Scatchard plot of saturation of specific [^{3}H]HACBO-Gly binding. Data from figure 1A were replotted and linear regression analyses were done on a Hewlett-Packard HP85A desk calculator.

the same preparation (Table 2). Thus, by contrast to thiorphan, captopril, a highly potent and specific inhibitor of angiotensin converting enzyme (16) exhibits a very weak inhibitory potency of [^{3}H]HACBO-Gly binding. Likewise

Table 1. Rat brain distribution of [^{3}H]HACBO-Gly binding and neutral endopeptidase

	[^{3}H] HACBO-Gly binding B_{max} (fmol/mg protein)	Neutral endopeptidase V_{max} (pmol/min/mg prot)	V_{max}/B_{max}
Striatum	94±12	21.0±1.2	0.22
Substantia nigra	106±19	20.6±0.6	0.19
Cortex	15±1	5.6±0.3	0.37
Cerebellum	19±2	4.0±0.2	0.21

Rat tissues were homogenized and membrane fractions assayed for [^{3}H]HACBO-Gly binding and neutral endopeptidase activity as described in methods. Data are the means of 4 determinations performed in triplicate.

Table 2. Drug specificity of [^{3}H]HACBO-Gly binding and neutral endopeptidase

	K_i (nM) [^{3}H]HACBO-Gly binding	K_i (nM) Neutral endopeptidase activity
HACBO-Gly	0.40 ± 0.05	1.4 ± 0.20
Thiorphan	0.30 ± 0.04	1.8 ± 0.30
Phosphoramidon	0.35 ± 0.05	1.1 ± 0.25
Kelatorphan	0.50 ± 0.10	1.7 ± 0.20
Captopril	>100.000	>100.000
Bestatin	90.000	>100.000

Eight concentrations of each inhibitor were assayed for effects on specific binding of 3 nM [^{3}H]HACBO-Gly binding and neutral endopeptidase activity as described in methods. Results are the means of 4 experiments performed in triplicate.

the non specific aminopeptidase inhibitor bestatin (17) is poorly recognized by enkephalinase.

DISCUSSION

There is good correlation between the regional distribution of [^{3}H]HACBO-Gly binding and neutral endopeptidase activity. This is illustrated by the similar values of the ratio V_{max}/B_{max} in some rat brain regions (Table 1) indicating that the [^{3}H]HACBO-Gly binding corresponds to the membrane bound enkephalinase. This feature is strongly supported by the specificity of various peptidases inhibitors to inhibit the [^{3}H]HACBO-Gly binding with K_I values which parallel those found in enzymatic studies. This preliminary study confirms the heterogenous distribution of enkephalinase labelled by [^{3}H]HACBO-Gly in rat brain and shows that the striatum and the substantia nigra contain high levels of this enzyme (18).

The tritiated enkephalinase inhibitor [^{3}H]HACBO-Gly was synthesized : i) to study the characteristics of the peptidase active site at the molecular level ; ii) to determine the level of enkephalinase in different brain

regions after various pharmacological treatments; iii) to perform an acute localization of the enzyme in the CNS of different species including human.

Owing to a possible use of enkephalinase inhibitors as new analgesics it is of major interest to determine whether or not the brain neutral endopeptidase is exlusively involved in the in vivo metabolism of enkephalin. Indeed according to its poor substrate selectivity the neutral endopeptidase (E.C. 3.4.24.11) is able to cleave numerous neuropeptides (7-9). Furthermore, the cellular localization of this peptidase is somewhat striking since its levels are higher on cultured glial cells than on neuronal cells in primary culture (19). In addition, the NG 108-15 hybrid cells which bear only δ opioid receptors are devoid of enkephalinase (20). The use of [^{3}H]HACBO-Gly should help to clarify these apparent discrepancies. These studies are now in progress in our laboratory.

ACKNOWLEDGMENTS

We thank Doctor J.M. Zajac for helpful discussion and A. Bouju for typing the manuscript. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Recherche Médicale and the Université René Descartes.

REFERENCES

1. Kerr, M.A. and Kenny, A.J. (1974) Biochem. J., 137, 477-478.
2. Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) Nature 276, 523-526.
3. Roques, B.P., Fournié-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.C. (1980) Nature 288, 286-288.
4. Roques, B.P., Lucas-Soroca, E., Chaillet, P., Costentin, J. and Fournié-Zaluski, M.C. (1983) Proc. Natl. Acad. Sci. USA, 80, 3178-3182.
5. Fournié-Zaluski, M.C., Chaillet, P., Bouboutou, R., Coulaud, A., Chérot, P., Waksman, G., Costentin, J. and Roques, B.P. (1984) Eur. J. Pharmacol. 102, 525-528.
6. Patey, G., De La Baume, S., Schwartz, J.C., Gros, C., Roques, B.P., Fournié-Zaluski, M.C. and Soroca-Lucas, E. (1981) Science 212, 1153-1155.
7. Matsas, R., Fulcher, I.S., Kenny, A.J. and Turner, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 3111-3115.
8. Checler, F., Emson, P.C., Vincent, J.P. and Kitabgi, P. (1984) Neurochem. 43, 1295-1301.
9. Deschodt-Lanckman, M. and Strosberg, A.D. (1983) FEBS Lett. 152, 109-113.
10. Strittmatter, J.M., Lo, M.M.S., Javitch, J.A. and Snyder, S.H. (1984) Proc. Natl. Acad. Sci. USA, 81, 1599-1603.
11. Bouboutou, R., Waksman, G., Devin, J., Fournié-Zaluski, M.C. and Roques, B.P. (1984) Life Sci. 35, 1023-1030.
12. Waksman, G., Hamel, E., Bouboutou, R., Besselièvre, R., Fournié-Zaluski, M.C. and Roques, B.P. (1984) C.R. Acad. Sci. Paris, 299(III), 613-615.

13. Fournié-Zaluski, M.C., Coulaud, A., Bouboutou, R., Chaillet, P., Devin, J., Waksman, G., Costentin, J. and Roques, B.P. (1985) J. Med. Chem., in press.
14. Llorens, C., Malfroy, B., Schwartz, J.C., Gacel, G., Roques, B.P., Roy, J., Morgat, J.L., Javoy-Agid, F. and Agid, Y. (1982) J. Neurochem. 39, 1081-1089.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
16. Cushman, D.W., Cheung, H.S., Sabo, E.F. and Ondetti, M.A. (1977) Biochemistry 16, 5484-5491.
17. Wagner, G.W. and Dixon, J.E. (1981) J. Neurochem. 37, 709-713.
18. Schwartz, J.C., De La Baume, S., Malfroy, B., Patey, G., Perdrisot, R., Swerts, J.P., Fournié-Zaluski, M.C., Gacel, G. and Roques, B.P. (1980) Adv. Biochem. Psychopharmacol. 22, 219-235.
19. Horsthemke, B., Hamprecht, B. and Bauer, K. (1983) Biochem. Biophys. Res. Commun. 115, 423-428.
20. Palenker, J., Lentzen, H. and Brandt, U. (1984) Naunyn-Schmiedeberg's Arch. Pharmacol. 325, 214-217.