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[^{35}S]LANTHIONINE KETIMINE BINDING TO BOVINE BRAIN MEMBRANES

M. Fontana, G. Ricci, S. P. Solinas*, A. Antonucci+, I. Serao¶, S. Duprè and D. Cavallini

Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza", Italy

*Istituto di Scienze Biochimiche, Università di Chieti "G. D'Annunzio", Italy

+Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, Roma, Italy

¶ Sigma Tau Foundation

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2H-1,4-Thiazine-5,6-dihydro-3,5-dicarboxylic acid (trivial name: lanthionine ketimine) is a cyclic sulfur-containing imino acid detected in bovine brain extracts. This compound has been synthesized in a heavily labeled form starting from L-[^{35}S]cysteine and purified by high performance liquid chromatography. We demonstrate the existence of a saturable and reversible binding of [^{35}S]lanthionine ketimine to bovine brain membranes. A single population of binding sites with a concentration of 260 ± 12 fmol/mg protein and a dissociation constant of 58 ± 14 nM is present. Specific binding is competitively inhibited by other structurally similar imino acids, namely S-aminoethyl-L-cysteine ketimine and cystathionine ketimine. These results suggest a possible functional role for these ketimines in nervous system.

Abbreviations

LK, 2H-1,4-thiazine-5,6-dihydro-3,5-dicarboxylic acid (lanthionine ketimine); CK, 2,5,6,7-tetrahydro-1,4-thiazepine-3,5-dicarboxylic acid (cystathionine ketimine); AECK, 2H-1,4-thiazine-5,6-dihydro-3-carboxylic acid (S-aminoethyl-L-cysteine ketimine).

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A natural class of sulfur, nitrogen-containing cyclic compounds, named ketimines, has been recently identified in some mammalian tissues. Lanthionine ketimine (LK , fig. 1), cystathionine ketimine (CK) and S-aminoethyl-L-cysteine ketimine (AECK) have been chemically synthesized and found in detectable amounts in bovine brain and human urine (1 and references therein). These imino acids are probably formed *in vivo* by a transaminative step of the parent aminoacids catalyzed by a specific transaminase occurring in various mammalian tissues (2 and references therein). The biochemical and physiological relevance of this class of ketimines has not yet been clarified. The presence of these compounds in the central nervous system led us to investigate for specific functions. In this paper we describe the synthesis of [^{35}S]lanthionine ketimine starting from L-[^{35}S]cysteine and we demonstrate the existence of a high affinity binding site for [^{35}S]LK in bovine brain membranes. Efficient displacement of the labeled compound by the other two chemically related ketimines suggests a general role for ketimines in nervous system.

MATERIALS AND METHODS

Materials and chemicals. L-[^{35}S]Cysteine (specific activity > 600 Ci/mmol) was obtained from Amersham International (catalog n. SJ 232). Cysteine hydrochloride, dithiothreitol and bromopyruvic acid were from Sigma (U.S.A.). Syntheses of LK , AECK and CK are reported in (3, 4). All other chemicals were of best available commercial quality.
Bovine brain was obtained from the local slaughterhouse; cerebral cortex was removed on ice, frozen within 1 hour and stored at -20 °C until use.

Synthesis of [^{35}S]LK. Synthesis of [^{35}S]LK was performed following the previously reported synthesis of unlabeled LK (3), with minor modifications. To 0.08 nmol [^{35}S]cysteine (0.05 mCi) are added in the

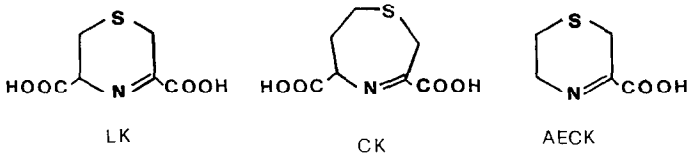

Fig. 1. **Structures of lanthionine ketimine (LK), cystathionine ketimine (CK) and S-aminoethyl-L-cysteine ketimine (AECK).**

order L-cysteine hydrochloride (0.8 nmol) and bromopyruvic acid (200 nmol) in a final volume of 0.066 ml 0.3 M K-phosphate buffer, pH 8.4. After 10 min at room temperature 30µl of 10 mM dithiothreitol were added. The solution was left 5 min at room temperature, then acidified up to pH 2 with 4 µl 4 N HCl and citric acid (O.1 M final concentration) and immediately submitted to chromatography on a Waters Assoc. (Millford, MA, U.S.A.) HPLC chromatograph equipped with two model 501 pumps and a U6K sample injector. The column was a 50 x 4 mm I.D. 5 µm Spherisorb ODS 2, equilibrated and eluted with ice-cold 0.01 M citric acid containing 5 µM dithiothreitol as LK stabilizer. The flow-rate was 1.0 ml/min. [^{35}S]LK is eluted after 8 min and collected in test tubes containing 0.4 ml 1 M Tris-citrate buffer, pH 7.1. This radioactive fraction coelutes with authentic LK on silica gel thin-layer chromatography (ethanol/water, 1:1). Final specific activity was 20-60 Ci/mmol. Binding experiments have been performed within 3 h from the synthesis.

Membrane preparation. Enriched membrane fractions were prepared from bovine brain cortex essentially as described in (5), with minor modifications as follows: washing procedures were performed with ice-cold water instead of Tris-HCl buffer and membranes were lyophilized and stored at -20 °C until use. Suspension of membranes for incubation was by rapid homogenization in ice-cold 50 mM Tris-citrate buffer, pH 7.1 (20 ml for 20 mg membranes). All procedures were performed at 0-4 °C. Protein concentration was determined by the method of Lowry *et al.* (6), using bovine serum albumin as standard.

Binding assay. Total incubation volume was 1.2 ml, and the incubation temperature was 25 °C. Unless otherwise indicated, the concentrations of [^{35}S]LK and protein used in these experiments were 10 nM and 0.4 mg, respectively, and the incubation time was 10 minutes. 0.1 mM LK was used as the competing ligand to determine nonspecific binding. Assays were terminated by a 10 min centrifugation at 48000 x g. The resulting pellet was rinsed rapidly and superficially with 1.5 ml of ice-cold deionized water, solubilized in 0.1 ml Soluene (Packard S.A., Zürich, Suisse) and radioactivity was determined by liquid scintillation spectrometry.

RESULTS

Binding of [^{35}S]LK to bovine brain cortex membranes is rapid and reaches a steady-state after a few minutes at 25 °C. Specific binding (about 50 % of the total) remains rather constant between 10 and 30 min incubation, and total binding increases no more than 20% during this time. Specific binding is not affected by temperature between 4 °C and 37 °C. Maximal specific binding is attained between pH 6.0 and 7.5.

It has been verified that total and specific binding are related to membrane concentration within a protein concentration range of 0.15 - 0.4 mg/assay. Radioligand is dissociated by addition of 0.1 mM unlabeled LK to the sample after the incubation time, or by suspension of membrane pellet (obtained by centrifugation after the binding experiment) in 1.2 ml of buffer. In both conditions about 60-70% of the specifically bound labeled LK was dissociated from membranes after 90 min incubation at room temperature.

Specific binding of [^{35}S]LK to bovine brain membranes is saturable, as shown in fig. 2, where average data of various experiments are shown, in which labeled LK at constant specific activity varies between 1.5 and 100 nM. These data submitted to a computerized Scatchard analysis are shown in the inset of fig. 2. A straight line is observed, indicating that LK

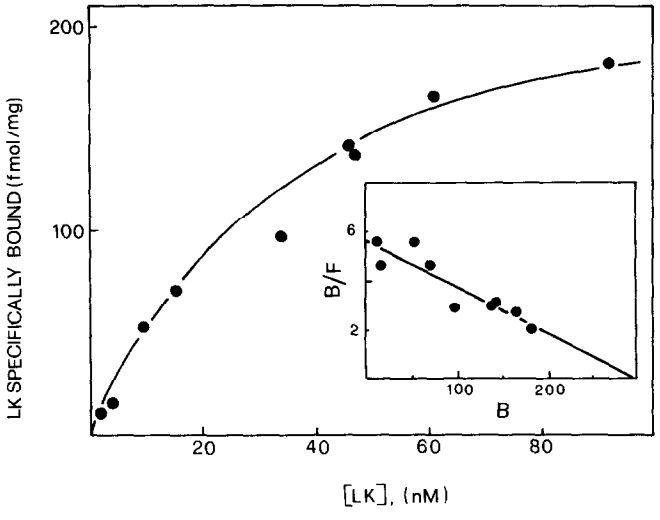

Fig. 2. **Saturability of [^{35}S]LK binding to bovine brain membranes.**
Bovine brain membranes (0.4 mg protein/assay) are incubated for 10 min with variable [^{35}S]LK (20 Ci/mmol, 1.5 - 100 nM). Bound radiolabel is estimated by centrifugation and nonspecific binding is obtained in the presence of 0.1 mM LK. Data are means of duplicate determinations of four different experiments. *Inset* , data of saturation experiments plotted as Scatchard plot. B is given as fmol/mg protein, B/F as fmol/mg protein · nM. K_d is calculated from the slope and the number of binding sites from the intercept on the abscissa of the straight line traced by computerized linear regression of experimental data.

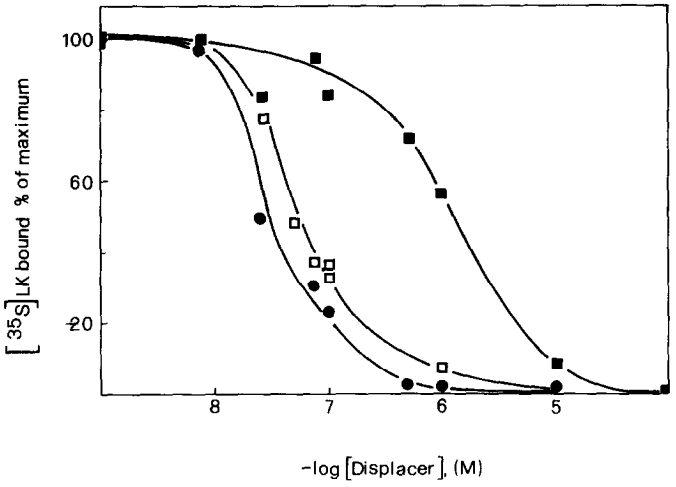

Fig. 3. **Effect of unlabeled LK (□), AECK (●), and CK (■) on the binding of [^{35}S]LK to bovine brain membranes.**
Membranes (0.4 mg protein/assay) are incubated with 10 nM [^{35}S]LK and various concentrations of the other ligands at 25°C. After 10 min bound radiolabel is estimated by centrifugation. Data shown are duplicate values of representative experiments. K_i values are calculated from three separate experiments according to the relationship: $K_i = IC_{50}/(1 + [L]/K_d)$ (7), where IC_{50} is the mean value of the concentration of ligand that displays 50% of the radioactive ligand bound under the conditions used, [L] is the concentration of [^{35}S]LK and K_d the dissociation constant of the radioactive ligand.

binds to a single population of binding sites. From four experiments, the dissociation constant K_d is estimated at 58 ± 14 nM (mean ± S.E.M.) and the concentration of binding sites (B_{max}) at 260 ± 12 fmoles/mg protein. The value of the Hill coefficient (n_H = 0.98) shows that no cooperative binding occurs.

As shown in fig. 3, unlabeled synthetic LK in the concentration range between 10^{-5} and 10^{-8} M competitively inhibits the binding of [^{35}S]LK to brain membranes. For this experiment half maximal inhibition is obtained at 45 nM LK. An average K_i value of 77 ± 32 nM is calculated, which is of the same order as K_d obtained from saturation experiments. The Hill coefficient of displacement data (n_H = 0.85) confirms the involvement of a single population of binding sites. Of interest is also the displacement effect of other naturally occurring ketimines, structurally related to LK.

Figure 3 reports also representative displacement curves by AECK and CK; K_i values of 72 nM and 1.3 μM (mean of two separate experiments) respectively may be calculated. Hill coefficients are close to unit.

DISCUSSION

The present paper deals with the first demonstration of LK binding in brain. The interaction of LK with bovine brain membranes fulfills the criteria of ligand receptor, i.e. reversibility, saturability and high affinity.

The calculated value of K_d of 58 nM is of the same order of dissociation constants of other known neuroeffectors and, compared with the physiological micromolar concentration of LK in the brain (8), points to a possible role for LK in the central nervous system. Scatchard analysis indicates that interaction of LK with its binding sites fits with a bimolecular association of LK and a population of non interacting receptor sites. Experiments with variable amounts of labeled LK at constant specific activity clearly show the saturability of binding sites. The relatively narrow concentration range used, due to the conditions of the synthesis, does not allow however to saturate fully the receptor sites. The strong displacing activity of the very similar six membered monocarboxylic analog AECK and of the seven membered ketimine CK suggests a similar binding of these iminoacids in the central nervous system.

Preliminary results with other tissues indicate that specific binding of labeled LK is found also with membranes prepared from bovine heart and kidney. Work is in progress to elucidate if this binding process is due to the presence of receptors specific only for ketimines.

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