OPIOID DETECTION IN PUPAL EXTRACT OF THE CHINESE OAK SILKWORM *Antheraea pernyi* GUERIN

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Pharmacologically active preparations obtained from silkworms have been known for centuries, and are still being used [2]. The character of some effects of such preparations, and data on the presence of opioids in insects [4, 5] suggest that ligands of opioid receptors (OR) are involved in the mechanism of the biological action of preparations obtained from silkworm tissues. Accordingly, the aim of this investigation was to test the hypothesis that opioids are present in an extract of silkworm pupae,

EXPERIMENTAL METHOD

Pupae of the Chinese oak silkworm were extracted with 50% ethanol solution. The extract was evaporated on a rotary vaporizer to remove most of the ethanol, and then freeze-dried. The freeze-dried product was dissolved in the original volume of water and lipids were extracted from the preparation by the addition of two volumes of ethyl acetate, after which the ethyl acetate fraction was removed in a separating funnel, The procedure was repeated 5 times. The aqueous fraction was freed from traces of ethyl acetate on a rotary vaporizer, freeze-dried, and used in the investigation.

To detect the presence of opioids in the preparation, radioreceptor analysis (RRA) and radioimmunoassay (RIA) were used. A membrane fraction of the Wistar rat brain for RRA is obtained and activity of the samples to displace ligands from OR was determined as described by the writers previously [3]. As labeled ligands we used a ligand of OR of μ-type ^{3}H-[D-Ala^2-MePhe^4-Gly-ol^5]-enkephalin (^{3}H-RX 783006 or ^{3}H-DAGO) – 40 Ci/mmole, and a ligand of OR of the δ-type D-Ala^2-D-Leu^5-enkephalin (^{3}H-DADLE) – 41.8 Ci/mmole (from Amersham, Great Britain),

During the study some samples of the preparation in a concentration at which the level of specific binding fell by about 50%, were dissolved in 50 mM Tris-HCl (pH 7.7 at 25°C) and incubated for 180 min at 37°C with pronase E (70 U/ml, from "Merck," Germany) in a concentration of 21 U/ml. The enzyme was then inactivated by transferring the specimens to a boiling water bath for 15 min. After cooling, the specimens were centrifuged (1100*g*, 15 min, 25°C). The residue was discarded and the displacing activity of the supernatant was determined by RRA In control series of experiments the specimens were transferred for 15 min to a boiling water bath or were exposed to the action of enzymes inactivated by the same method.

RIA of substances with the immunoreactivity of β-endorphin and Leu-enkephalin in the specimens was carried out with the aid of kits of reagents and in accordance with the recommendations of the firm Incstar Corporation (USA).

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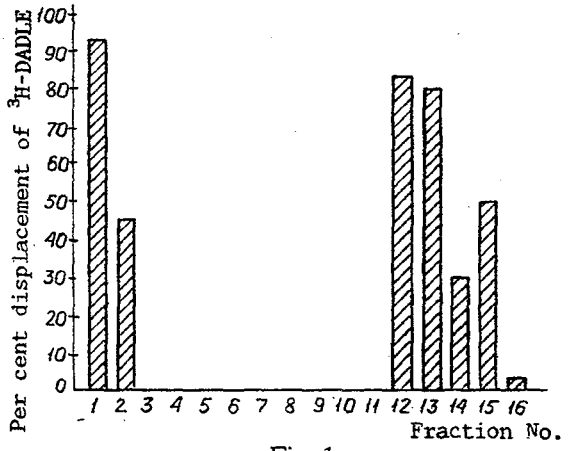

Fig. 1

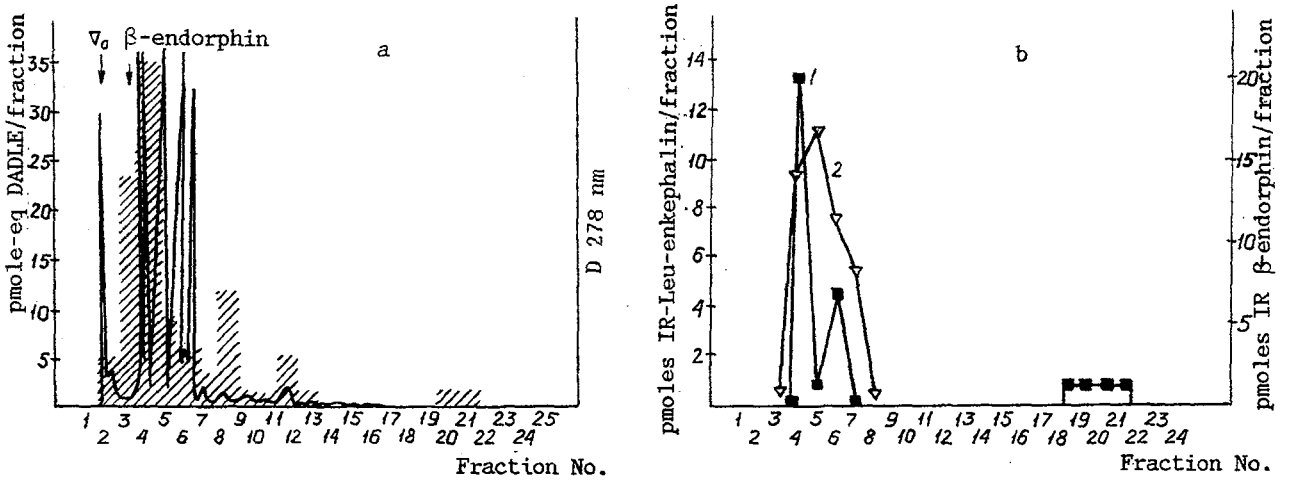

Fig. 2

Fig. 1. Activity of fractions of preparation obtained on S-sepharose during displacement of ^{3}H-DADLE from opioid receptors.

Fig. 2. Results of radioreceptor analysis and radioimmunoassay of fractions of preparation obtained by gel-filtration on TSK-gel HW-40F. a) Activity of fractions with respect to displacement of ^{3}H-DADLE from opioid receptors (shaded) and optical density (D) at 278 nm; b) immunoreactivity of Leu-enkephalin (1) and β-endorphin (2).

The preparation was fractionated on TSK-gel HW-40F ("Toyopearl," Japan) (1.6 × 90 cm). Chromatography was carried out in water at room temperature with a rate of elution of 40 ml/h · cm^2. Fractions were collected after 5-10 min and freeze-dried.

Ion-exchange chromatography was carried out on S-sepharose ("LKB-Pharmacia," Sweden). The S-sepharose (1 × 10 cm) was equilibrated with 5 mM acetic acid (pH 3.5), and material bound with the cation-exchange resin was eluted with a linear gradient (40 ml/40 ml) of 5 mM CH_3COOH (pH 3.5) – CH_3COONH_4 (pH 6.5) at the rate of 25 ml/h · cm^2.

The sodium concentration in the samples was determined by means of a flame photometer ("Turner," USA).

Electrophoretic verification of the purity of the fractions and efficacy of the proteolytic treatment, and also the rough determination of the molecular weights of the components of the preparation and its fractions, were carried out in a PAG gradient (8-25%) with SDS in standard plates measuring 4.5 × 5.0 cm, in the "Phase System" programmed apparatus from "Pharmacia" (Sweden), at 250 V and 10 mA. The gels were stained with silver in accordance with recommendations from the "Pharmacia" firm.

The optical density of the fractions of the preparation was determined at 278 nm using the "Uvicord S-2138" instrument, from LKB (Sweden).

EXPERIMENTAL RESULTS

It was shown by RRA that addition of the preparation to the incubation medium led to dose-dependent displacement of labeled opioids from OR. Curves of displacement OL the labeled ligands by the corresponding unlabeled preparations and the test preparation between log/logit coordinates were parallel. Displacing activity amounted to 0.41 ± 0.11 and 0.21 ± 0.09 pmole-equivalents of DADLE and DAGO per milligram of the preparation respectively.

To prove the absence of a nonspecific effect of proteins and Na^+ in the composition of the preparation on binding of the labeled ligands with OR, two series of control experiments were carried out. In the 1st series it was shown by flame photometry that the maximal Na^+ concentration in the incubation medium during RRA of the test preparation was 5.4 mM. Meanwhile, as the results show, in this concentration and under the conditions used Na^+ had no effect of receptor binding of DAGO and DADLE.

The 2nd series of control experiments was carried out to rule out the hypothetical possibility of a nonspecific effect of protein components of the preparation on receptor binding. The study of fractions obtained by fractionation of the preparation on S-sepharose yielded the following results. Substances responsible for the displacing activity of the preparation were determined by RRA either in fractions 1 and 2, in which the proteins did not stain on the electrophoretic gel, or in fractions 12-15 (Fig. 1), containing only low-molecular-weight proteins and peptides. It was thus shown that the ability of the preparation to interact with OR was not connected with any nonspecific effects of proteins and Na^+.

It was later found that treatment with pronase and boiling do not change the displacing activity of the preparation. It cannot be concluded from these data that the ligands of OR discovered are not peptides. It can be postulated that the absence of an effect of pronase was due either to the presence of endogenous inhibitors of proteolytic enzymes in the composition of the preparation or resistance of opioids in the "bound" state to enzymic treatment.

RRA of the displacing activity of fractions obtained by chromatography of the preparation on TSK-gel HW-40F demonstrated the heterogeneity of the OR ligands discovered. It was also shown that a large part of the displacing activity was due to ligands with mol. wt. of not more than 3-4 kD (Fig. 2a).

In the next stage of the investigation, for which RIA was used, the presence of substances with immunoreactivity of Leu-enkephalin and β-endorphin was demonstrated in the same fractions. Substances with immunoreactivity of Leu-enkephalin were found in fractions 4-6 and 18-21, in picomolar concentrations, with immunoreactivity of β-endorphin, and in fractions 4-7, in femtomolar concentrations (Fig. 2b).

Comparison of the results of RRA and RIA suggests that the displacing activity of OR ligands of the δ-type in fractions 4 and 6 was due to the presence of enkephalins in them. The displacing activity of the other fractions of the preparations was evidently linked with the presence of other opioids in their composition.

An alcoholic extract of the pupae of the Chinese oak silkworm thus has in its composition a heterogeneous group of OR ligands of δ- and μ-types, and in particular, Leu-enkephalin and β-endorphin. It can be tentatively suggested that a further study will prove that these ligands are involved in the mechanism of the biological effects of the preparation under investigation The possibility of certain opioids and preparations containing OR ligands in their composition, when applied locally to induce analgesic, antiinflammatory, antiviral, and various immunomodulating effects, and of stimulating wound healing, is now being discussed [1]. In this connection the development of the present investigation is interesting from the standpoint of creating a new pharmacological preparation.

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