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OPIATE BINDING SITES AND ENDOGENOUS OPIOIDS IN *Bufo viridis* OOCYTES

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SUMMARY: Binding sites with high affinity for [^{3}H]naloxone, but not for [^{3}H]morphine and [^{3}H](D-Ala^2, D-Leu^5)enkephalin, have been found in membranes of *Bufo viridis* oocytes. The binding is reversible and saturable. Bound [^{3}H]naloxone is easily displaced both by unlabeled naloxone and bremazocine, much worse by morphine and SKF 10,047; (D-Ala^2, D-Leu^5)enkephalin and ß-endorphin practically fail to displace [^{3}H]naloxone. Scatchard analysis is consistent with the existence of two classes of binding sites with K_d 15 nM and 10^3 nM. The number of binding sites with high affinity for naloxone is 16 pmol/mg protein of homogenized oocytes which is 20-50-fold higher than in toad or rat brain. Oocyte extract displaces [^{3}H]naloxone bound with oocytes' membranes and inhibits electrically evoked contractions of the rabbit vas deferens. This inhibition is reversed by naloxone. It is suggested that compounds similar to opiate kappa-agonists exist in oocytes. It cannot be ruled out that they participate via specific receptors in the regulation of oocyte maturation and egg development.

Physiological and biochemical studies have suggested that opiates, opioid peptides, and benzomorphans may have five distinct sites of action in the central and peripheral nervous system: the so-called mu-, delta-, kappa-, sigma- and epsilon-opiate receptors. Preferable agonists of these receptors are morphine, (D-Ala^2, D-Leu^5)enkephalin (DADL), ketocyclazocine (ethylketocyclazocine, bremazocine), SKF 10,047 and ß-endorphin, respectively.

Opioid binding sites were also found in adrenal medulla (1), leukocytes (2), and erythrocytes (3). There is evidence for the existence of opiate receptors in amoeba (4). Ligands of opiate

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receptors were isolated, besides the brain and hypophysis, from the bovine adrenal medulla (5) and amphibian skin (6).

This paper presents the data on the existence of [^{3}H]naloxone binding sites and endogenous compounds interacted with them in Bufo viridis oocytes.

MATERIALS AND METHODS

Full-grown oocytes untreated or defolliculated by collagenase (Sigma, type III) treatement were homogenized in 10 vols Tris-HCl buffer, 50 mM (pH 7.4) at 0°C. The homogenate was centrifuged at 700 x g for 10 min and the resulting supernatant was used in binding experiments. Opiate binding assays were carried out on the oocyte homogenate according to the method reported in ref. (7). Concentration of [^{3}H]naloxone (NEN), [^{3}H]morphine, and [^{3}H]DADL (Amersham) was 1 nM. Homogenate protein concentration was 100 µg/ml probe. All determinations were performed in triplicate at 25°C. Non-specific binding was measured using 10^{-6}M unlabeled naloxone.

A series of special experiments was carried out to study the effect of trypsin and p-Cl-HgBzO- on [^{3}H]naloxone binding sites. Trypsin (Worthington) was added into the tube at the beginning of incubation at 25°C. p-Cl-HgBzO- was preincubated with homogenized oocytes at 25°C for 30 min with subsequent addition of [^{3}H]naloxone or glutathione (1 mM).

To obtain an oocyte extract the oocytes were homogenized at 0°C in 10 vols 0.2 N HCl. The homogenate was centrifuged at 80,000 x g for 60 min,and the supernatant was lyophilized. The lyophilized extract was dissolved in 0.1 M acetic acid and chromatographed on a column of Sephadex G-25 (1x100 cm, 0.1 M acetic acid). The obtained fractions were lyophilized; the residues were dissolved in water, neutralized, and utilized for binding experiments.

Electrically stimulated rabbit vas deferens was used according to the procedure reported elsewhere (8), and opioid activity of the oocyte extract was determined and compared with those of bremazocine, morphine, SKF 10,047 and DADL in this bioassay.

Naloxone was obtained from "Endo Laboratories", bremazocine and SKF 10,047 from "Sandoz". DADL and ß-endorphin were synthesized by fragment condensation as described elsewhere. The purity of all the peptides was verified by TLC in four solvent systems and by aminoacid analysis of acid hydrolysates. The protein concentrations were estimated by the method of Lowry et al. (9), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The binding of labeled naloxone, morphine, and DADL with the homogenate of Bufo viridis oocytes is shown in Fig. 1. [^{3}H]naloxone exhibits the most active binding. [^{3}H]morphine was 5 to 10-fold less potent in binding, and [^{3}H]DADL practically didn't

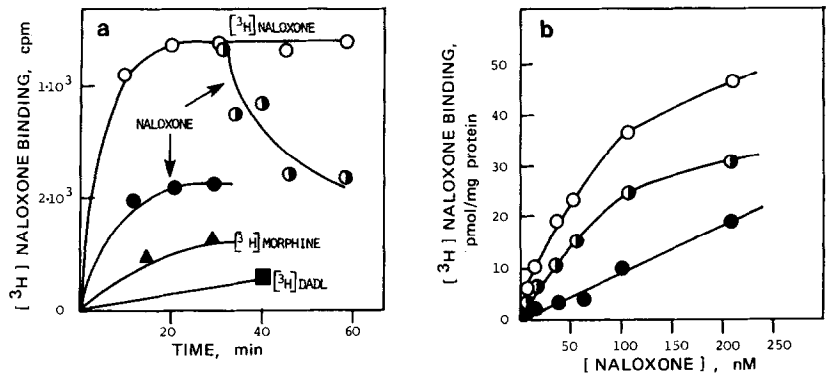

Fig. 1a. Kinetics of [^{3}H]naloxone (○ , ● , ◑), [^{3}H]morphine (▲), and [^{3}H]DADL (■) binding. [^{3}H]naloxone binding in the absence of additives (○), in the presence of 10^{-6} M naloxone (●), and with 10^{-6} M naloxone added 30 min after beginning of the incubation (◑). Binding of labeled ligands to the homogenate linearly depends on the homogenate protein content in the probe within 40 to 200 μg/ml. In some experiments with [^{3}H]DADL bacitracin (50 μg/ml) was added to the incubation media. [^{3}H]DADL binding does not grow in the presence of bacitracin. Special experiments using thin-layer chromatography showed that [^{3}H]DADL is not degraded during incubation with the oocyte homogenate. In a number of experiments, oocytes were defolliculated by collagenase treatment: 1 g oocytes was incubated for 30 min in 10 ml 75 mM K-phosphate buffer, pH 7.4, containing 0.15% collagenase for 30 min at 25°C; the oocytes were subsequently washed off collagenase with Tris-HCl buffer, pH 7.7. Experiments with untreated and defolliculated oocytes yielded the same results which indicates that opiate binding sites are localized in the oocytes, and not in the follicular cells.

Fig. 1b. Saturation curves for [^{3}H]naloxone binding with the oocyte homogenate in the presence (●) or in the absence (○) of unlabeled naloxone (10^{-6} M); "specific" binding (◑) corresponds to the difference between the binding in the presence and in the absence of unlabeled naloxone (10^{-6} M). Incubation time - 30 min.

bind at all. [^{3}H]naloxone and [^{3}H]morphine binding reaches a plateau in 10-20 min of incubation and then remains the same for 50 min. [^{3}H]naloxone reversibly interacts with oocyte binding

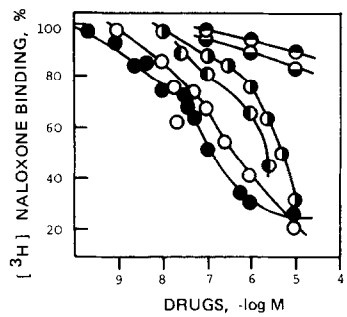

Fig. 2. Displacement of [^{3}H]naloxone binding by unlabeled naloxone (●), bremazocine (○), SKF 10,047 (◐), morphine (◑), ß-endorphin (◒), and DADL (◓). Incubation time - 30 min.

sites: the amount of bound [^{3}H]naloxone is 2 to 3-fold decreased when 10^{-6} M unlabeled naloxone is added to the incubation medium (Fig. 1a). As follows from Fig. 1b, specific binding of [^{3}H]naloxone is saturable. A study of the subcellular distribution revealed naloxone binding sites in the membrane fraction of oocytes precipitated during centrifugation at 105,000 x g for 1 h (no data are given).

Effect of a number of compounds on [^{3}H]naloxone binding with oocytes' membranes is shown in Fig. 2. As is seen, the membrane-bound [^{3}H]naloxone is equally well displaced both by unlabeled naloxone and bremazocine. SKF 10,047 and morphine are less effective, and DADL and ß-endorphin are completely ineffective. Thus, naloxone and kappa-agonist bremazocine have the highest affinity for these sites; the affinity of sigma-agonist SKF 10,047 and mu-agonist morphine is somewhat lower, while DADL and putative agonist of epsilon-receptors ß-endorphin were significantly less active. In most of the experiments (8 out of 10), [^{3}H]naloxone binding was displaced in a biphasic manner by unlabeled naloxone and bremazocine, i.e. there exists two or more types of [^{3}H]naloxone binding sites. The plot analysis of [^{3}H]-naloxor binding to the homogenate revealed the binding sites of

Table 1

Inhibition of [^{3}H]naloxone binding to oocyte membrane by trypsin and p-Cl-HgBzO-

	Bound [^{3}H]naloxone cpm	Specific binding / Non-specific binding
Control*	2,220	1.85
Trypsin, 5μg/ml	880	1.26
p-Cl-HgBzO-@, 10^{-4} M	1,030	0.87

Time of incubation with [^{3}H]naloxone - 30 min.
* Preincubation of the oocyte homogenate for 30 min at 25°C didn't affect [^{3}H]naloxone binding to oocyte membranes.
@ Glutathione added to the incubation media simultaneously with [^{3}H]naloxone, i.e. 30 min after p-Cl-HgBzO-, had no effect on a decrease of the labeled ligand binding with oocyte membranes facilitated by p-Cl-HgBzO-.

two types: with high affinity (K_d, 15 nM) and low affinity (K_d, 10^3 nM) for [^{3}H]naloxone. The number of high-affinity binding sites for naloxone was 15 pmol/mg protein which 20-50-fold exceeds the number of binding sites for naloxone (no data are given), bremazocine, and diprenorphine in toad brain (10, 11), etorphine, diprenorphine and other opiates in rat brain (12, 13). NaCl (100 mM) has practically no effect on [^{3}H]naloxone binding with oocyte membranes (no data presented).

As follows from Table 1, trypsin and p-Cl-HgBzO- substantially decrease [^{3}H]naloxone binding to oocyte membranes. These compounds mainly reduce the specific binding. These data indicate that polypeptide chains and SH-groups participate in formation of naloxone binding sites in oocyte membranes, just as in case of rat brain opiate receptors (14, 15).

Thus, [^{3}H]naloxone binding sites, which resemble in certain characteristics the opiate receptors in toad and mammalian brain, have been found in toad oocytes. It may be assumed that these sites are receptors via which the action of endogenous regulatory substances is mediated. According to another hypothesis, oocytes contain the proreceptors, which are transformed in the course of

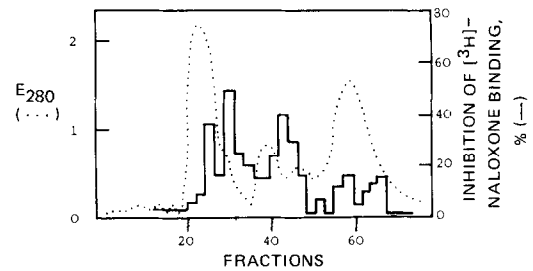

Fig. 3. Gel permeation chromatography of the oocyte extract. Utilization of extracts derived from untreated and defolliculated oocytes yielded the same results. 200 mg oocyte extract was applied to a column; the volume of each fraction - 1 ml. 0.1 part of the material from each fraction was added to the probe during binding studies.

the organism development into mature opiate receptors of the central and peripheral nervous system. To test the hypothesis of endogenous compounds effect of the oocyte extract on [^{3}H]naloxone binding with oocyte membranes and electrically evoked contractions of rabbit and mouse vas deferens has been studied. It was found that oocyte extract displaces the membrane-bound [^{3}H]naloxone: two peaks of compounds displacing [^{3}H]naloxone binding were observed following gel-filtration of the extract on a column of Sephadex G-25 (Fig. 3). The oocyte extract inhibits contraction of the rabbit vas deferens; the inhibitory effect is decreased in the presence of naloxone. The obtained extract also inhibits contraction of the mouse vas deferens (no data are given). According to the data of Oka et al. (8) and the data presented in Table 2, contraction of the rabbit vas deferens is inhibited by kappa-agonists, but not by the agonists of mu-, delta-, and sigma-receptors. The obtained data point to the presence of the kappa-type endogenous substances in the oocytes. Possibly, kappa-agonist-like compounds participate in the regulatory processes in oocytes mediating their action via the kappa-type receptors of these cells.

Table 2
Inhibition of electrically evoked contractions of the rabbit vas deferens by oocyte extract, bremazocine, morphine, DADL, and SKF 10,047

Treatment	Extract@	Inhibition (%)* Bremazocine $1.5 \cdot 10^{-8}$ M	Morphine 10^{-5} M	DADL 10^{-5} M	SKF 10,047 10^{-5} M
Control	50	75	<5	<5	<5
Naloxone#, 10^{-6} M	15	5			

The values represent the means of 4 determinations.
* Percentage of the inhibition observed 2-3 min following addition of the studied compound to the incubation medium.
@ Extract concentration - 6 μg protein/ml incubation medium.
\# Naloxone was added to the incubation medium 2-3 min prior to the introduction of the extract or bremazocine.

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