CHARACTERISTICS OF BRAIN AND SPINAL CORD OPIATE RECEPTORS IN MORPHINE-TOLERANT MICE

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An important step in the mechanism of tolerance to psychotropic drugs is the change in the concentration or affinity of their receptors which develops during subchronic administration of these compounds [9]. Investigation of the role of opiate receptors in the development of habituation to opiates by the radioligand binding method has given contradictory results. A change in the number of specific binding sites without any change in the dissociation constant of the labeled ligand [12], a selective reduction of affinity of opiate receptors for the agonist [4], an increase in affinity for the antagonist [5], and no change in the characteristics of receptors [7] have been demonstrated in animals tolerant to morphine. Because of the difficulty of functional identification of specific binding sites of psychotropic drugs there are advantages in combining an in vivo study with an in vitro study of light-receptor interaction in relation to the basic pharmacologic effect of the agonist (in this case, the analgesic effect). This problem can be resolved by determination of the apparent pA_2 of naloxone [13].

This paper describes a comparative study of characteristics of specific binding sites of ^{3}H-morphine by synaptic membranes of mouse brain and spinal cord and the apparent pA_2 of naloxone, in relation to its antagonism to the analgesic effect of morphine in intact and morphine-tolerant animals.

EXPERIMENTAL METHODS

Experiments were carried out on male mice weighing 20-25 g, divided into two groups (150 animals in each group). Physiological saline (0.1 ml/10 g body weight) was injected subcutaneously into the mice of group 1 twice in 24 h (at 9-10 a.m. and 4-6 p.m.) for 8 days. Animals of group 2 were given subcutaneous injection of morphine hydrochloride at the same times. On the first day the dose of morphine was 10 mg/kg, but later it was increased by 10 mg/kg daily up to 80 mg/kg. Fifty animals of each group were decapitated 24 h after the last injection and the brain and spinal cord were quickly removed and frozen in liquid nitrogen.

The nociceptive response of the remaining mice of each group was assessed, 24 h after the last injection also, as reflected in the increase in latent period of the reflex movement after immersion of the animal's tail in water at 56°C. Subgroups differing in the dose of naloxone received (0, 0.01, 0.03, and 0.1 mg/kg respectively) were distinguished in each group. Eight or nine animals receiving a particular dose of morphine were distinguished in each subgroup. The dose–effect relationship was calculated for morphine with respect to the half-effective dose (ED_{50}), against the background of assigned naloxone concentrations, and the value of pA_2 was determined [2, 3, 13].

Syanptic membranes of the unpurified synaptosome fraction (P_2) were obtained by disintegrating the tissue in 0.32 M sucrose, 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The P_2 fraction was suspended in 50 mM Tris-HCl and kept in liquid nitrogen. On the day of the experiment the membrane suspension was thawed and incubated for 30 min at 37°C to destroy endogenous opioid peptides, then washed three times in 50 mM TriS-HCl to remove the morphine and

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TABLE 1. Characteristics of Specific Binding of ^{3}H-Morphine with Spinal Cord and Brain Membranes of Mice during Development of Tolerance to Morphine (M ± m)

Structure	Group of animals	Characteristics of specific binding of ^{3}H-morphine			
		high-affinity		low-affinity	
		K_d, nM	B_{max}, fmoles/mg protein	K_d, nM	B_{max}, fmoles/mg protein
Brain	Control (n = 5)	9,2±0,8	250±37	89±11	560±49
	Tolerant to morphine (n = 5)	10,4±1,0	234±41	103±33	480±31
Spinal cord	Control (n = 2)	5,6	120	112	432
	Tolerant to morphine (n = 2)	7,1	159	141	394

Legend. K_d) dissociation constant, B_{max}) concentration of specific binding sites of ^{3}H-morphine, n) number of experiments.

remains of the opioid peptides. The specific binding of ^{3}H-morphine (Amersham Corporation, England) was determined in 1 ml of medium containing 200-300 μg membrane protein, 50 mM Tris-HCl, pH 7.4, and ^{3}H-morphine in a concentration of 0.5-25 nM. Altogether 25 concentrations of ^{3}H-morphine were used, with one repetition for each concentration. After incubation for 60 min at 25°C the samples were diluted with 5 ml of cold buffer and filtered under a vacuum through GF/B glass fiber filters (Whatman, England). The filters were washed twice with the same volume of buffer and extracted overnight in 5 ml of Bray's scintillator without preliminary drying. Radioactivity of the samples was determined with an Intertechnique (France) counter. The protein concentration in the samples was determined by the method in [10].

EXPERIMENTAL RESULTS

Subchronic administration of morphine caused the development of tolerance to the analgesic effect and the mean effective dose was increased by 3.6 times. Apparent dissociation constants of naloxone were determined by a modified method of pA_2 construction from experimental data on the basis of a model [15]. With alternative calculation of the analgesic effect of morphine (based on the relative number of animals with analgesia in the group), calculated values of I_oK_d of naloxone varied from −7.69 to −8.11, but in the case of stepwise estimation of the analgesic effect (based on the increase in latent period of the nociceptive effect) it varied from −7.67 to −8.05. Calculated values of the apparent I_oK_d in the troup of animals tolerant to morphine varied from −7.76 to −8.10 by the stepwise method of calculation and from −7.71 to −8.0 by the alternative calculation. Thus no differences were found between the values obtained in intact and morphine-tolerant animals.

Characteristics of specific binding of ^{3}H-morphine with brain and spinal cord membranes of control and morphine-tolerant mice showed no significant change on the development of tolerance to morphine (Table 1). To analyze the curvilinear Scatchard plots it was assumed that, under the conditions used, high-affinity binding sites of ^{3}H-morphine correspond to μ-receptors, low-affinity to Δ-receptors [6]. There is evidence in support of the presence of at least three types of specific ^{3}H-morphine binding sites in membranes: high-affinity (μ = 2), superhigh-affinity (μ = 1), and low-affinity (conjecturally, Δ) morphine receptors [1]. However, despite differences in the interpretation of data on types of specific ^{3}H-morphine binding sites corresponding to particular types of opiate receptors, it is justifiable to conclude on the basis of the virtually complete identity of adsorption isotherms on a Scatchard plot that no significant changes take place in opiate receptors of spinal cord and brain membranes of mice during the development of tolerance.

In response to subchronic administration of opiate, accompanied by moderate tolerance to the antinociceptive action of morphine, the effectiveness of the antagonistic action of naloxone was unchanged, indicating no change in interaction between agonist and antagonist at the receptor level. This is also confirmed by data on ^{3}H-morphine binding with spinal cord and brain membranes. Consequently, the cause of desensitization of the tissues to morphine may be connected with a change in translation of the signal from receptors to effector systems. Evidence that such a mechanism really exists is given by data on changes in activities of GTPases, adenylate cyclases, and calmodulin during opiate dependence and tolerance [8, 14, 16].

The adaptive reaction responsible for tolerance may be connected with a change in receptor and other parameters of those mediator systems for which the opioid system acts as modulator [11], and also with changes in the "ensemble" of opioid peptides [14].

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ADEQUATE CHOICE OF FREE LIGAND CONCENTRATIONS FOR DETERMINATION OF BENZODIAZEPINE BINDING

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Radioligand binding methods, which provide very valuable information about the character of interaction between substances and receptor, are now widely used for research in psychopharmacology [7, 9]. Two parameters are usually used to characterize drug-receptor interaction: the equilibrium dissociation constant (K_d) and the receptor density (B_{max}). K_d and B_{max} are obtained by analysis of the kinetics of saturation of receptors by the ligand under equilibrium conditions [1, 2]. The saturation curve is hyperbolic in shape between coordinates: abscissa – concentration of free ligand (F), ordinate – degree of specific binding (B). The dependence thus obtained is analyzed between Scatchard coordinates [8] in accordance with the equation:

$$B/F = \frac{B\max - B}{K}.$$

On a Scatchard plot intersection of the straight line with the abscissa, along which values of B are plotted, gives the maximal density of binding sites (B_{max}), and the slope of the straight line is equal to $1/K_d$. In the present investigation, using binding of ligands with benzodiazepine receptors [3] as the example, an attempt was made to demonstrate that, when determining binding parameters, errors are often introduced as a result of incorrect choice of free ligand concentrations. Analysis of the results of our own experiments and of data in the literature showed that the value of B_{max} for ^{3}H-diazepam or ^{3}H-flunitrazepam in the cerebral cortex varies within very wide limits: from 350 to 1900 fmoles/mg protein. If

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