EFFECT OF NUCLEOTIDES ON N-METHYLCYTISINE AND DIMETHYLTUBOCURARINE BINDING BY NICOTINIC ACETYLCHOLINE RECEPTORS OF THE SQUID OPTIC GANGLIA

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KEY WORDS: nicotinic acetylcholine receptor; ATP; cytisine; tubocurarine

There is now adequate experimental data to show that guanylic nucleotides can either modulate binding of ligands with receptors [8] or participate directly in the regulation of biochemical processes of an efferent cell in response to the action of mediators [6]. These effects are observed for a number of hormone receptors, muscarinic acetylcholine receptors, and adrenoreceptors. How widespread this phenomenon is among other receptor systems is not yet known [4, 5, 10].

The aim of this investigation was to study the effect of nucleoside mono-, di-, and triphosphates on binding parameters of agonists and antagonists with nicotinic acetylcholine receptors (NACHR) of squid optic ganglia in order to discover any regulatory activity they may possess. The writers showed previously that the squid optic ganglia contain numerous (~20 pmoles/g tissue) specific binding sites for ^{14}C-tubocurarine (^{14}C-TC) and ^{3}H-methylcytisine (^{3}H-MC). According to their pharmacologic properties, NACHR of squid optic ganglia differ from neuromuscular NACHR in the absence of sensitivity to snake α-neurotoxins and to decamethonium [2]. The NACHR in the composition of the membrane were tagged by photoaffinity modification by 2(3)-azido-N-^{3}H-methylcytisine (^{3}H-AMC). The receptor complex itself consists of a glycoprotein with mol. wt. of 400 kD and a subunit carrying the recognition site of cholinergic ligands and which, according to gel-chromatography, has mol. wt. of 45 kD [1].

EXPERIMENTAL METHOD

Reagents. The ^{3}H-methyl iodide (13 Ci/mmole) and ^{14}C-TC (94 Ci/mmole) were obtained from Amersham International (England). ^{3}H-MC and ^{3}H-AMC were obtained by alkylation of cytisine and 2(3)-azidocytisine with ^{3}H-methyl iodide by the method described previously [1]. Cholic acid and Tris-HCl were obtained from "Sigma" (USA), Sephacryl S-200 and sepharose CL-6B from "Pharmacia" (Sweden), and nucleoside mono-, di-, and triphosphates from "Reanal" (Hungary). The remaining reagents and solvents were of Soviet origin and of the chemically pure grade.

The membrane fraction enriched with NACHR was obtained from the optic ganglia of the squid Berryteuthis magister, generously provided by L. M. Epstein (Pacific Fisheries and Oceanographic Research Institute, Vladivostok), by the method in [12]. Photoaffinity modification was carried out in Ringer's solution containing 0.8 μM ^{3}H-AMC and 10^{-5} M ouabain, in the presence and absence of 0.1 mM nicotine and 1.0 mM ATP. The modification products were separated by gel-chromatography by the method described previously [1].

Specific binding of ^{3}H-MC and ^{14}C-TC with membrane preparation of NACHR was determined by centrifugation as the difference between radioactivity in the supernatants of samples containing and not containing 0.1 mM nicotine [2].

Radioactivity was measured on a RackBeta 1211 counter (LKB, Sweden) in Unisolv X-100 scintillator ("Koch Light," England). The counting efficiency was 40% in the channel for 3 h and 92% in the channel for ^{14}C.

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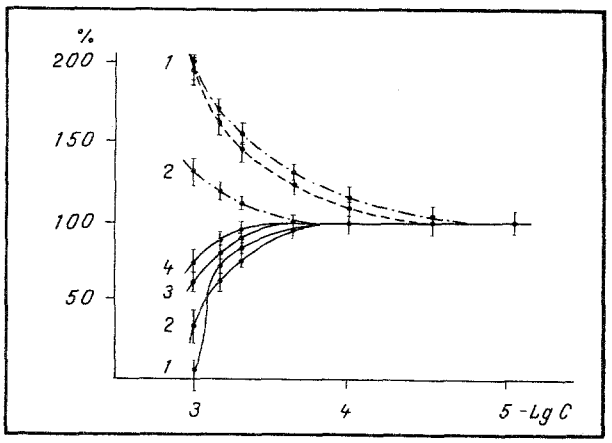

Fig. 1. Dependence of specific binding of ^{3}H-MC (line of dots and dashes), ^{3}H-AMC (broken line), and ^{14}C-TC (continuous line) on concentration of ATP (1), GTP (2), CTP (3), and UTP (4).

EXPERIMENTAL RESULTS

Cytisine, uridine, and their di- and triphosphates in concentrations of up to 1.0 mM had virtually no effect on specific binding of the agonist of nicotinic specificity ^{3}H-MC and the antagonist ^{14}C-TC with membrane preparations of NACHR of the squid optic ganglia. In the presence of ATP and GTP binding of ^{14}C-TC fell proportionally to the increase in triphosphate concentration; complete inhibition of binding was obtained, moreover, by a change in concentration of less than one order of magnitude (Fig. 1). Such a sharp reduction of binding cannot be explained in terms of classical models of competitive or noncompetitive inhibition or partial hydrolysis of the triphosphates during incubation, and the existence of a more complex mechanism must be suggested.

The fact that in the presence of ATP binding of ^{3}H-MC with membrane preparations of squid optic ganglia increased with an increase in the concentration of triphosphates, reaching 200 ± 20% of the initial level in the presence of ATP with a concentration of 1 mM, was very unexpected.

It must be pointed out that nonspecific binding of ^{3}H-MC by membrane preparations, measured in the presence of 0.1 mM nicotine, was virtually independent of the ATP concentration. Since the experiments were conducted with a concentration of ^{3}H-MC in the sample of 0.5 μM, which is 10 times higher than the dissociation constant of the ligand with NACHR of the optic ganglia of the squid B. magister (i.e., more than 95% of receptors are bound with the ligand), a twofold increase of specific binding can be explained only by the appearance of additional specific binding sites for ^{3}H-MC in the presence of ATP. GTP also, but to a lesser degree, potentiated binding of ^{3}H-MC; ADP did not affect, but AMP in a concentration of 1 mM inhibited the binding of ^{3}H-MC by only 20%.

Specific binding of the photoactivated cytisine derivative ^{3}H-AMC in darkness, in the presence of 1 mM ATP also was increased by 100 ± 20%. To obtain information about the molecular weight of the binding sites, appearing in the presence of ATP, photoactivated modification of the membrane preparation of the receptor with the aid of ^{3}H-AMC was carried out. For this purpose the membrane fraction of the squid optic ganglia was incubated in darkness in the presence of 1 mM ATP, 0.8 μM ^{3}H-AMC, and then irradiated for 10 min at 0°C with UV radiation [OI-18 source (λ_{max} > 300 nm)]. Preparations of NACHR irradiated after incubation with 0.8 μM ^{3}H-AMC in the presence and in the absence of 0.1 mM nicotine, were used as the control. Noncovalently-bound ^{3}H-AMC was separated by washing 3 times with Ringer's solution and solubilized by a modified membrane preparation under nondenaturing conditions with 2% sodium cholate. The undissolved components were removed by centrifugation and the supernatant was fractionated by gel-chromatography on sepharose CL-6B. Solubilized preparations of NACHR subjected to photoaffinity modification in the absence of ATP and in the presence of 1 mM ATP and 0.1 mM nicotine were used as the controls. In the course of chromatography the optical density at 280 nm and radioactivity in the fractions were measured. In the presence of 1 mM ATP, an approximately twofold increase, compared with the control, was observed in ^{3}H-AMC incorporation into the fraction containing the receptor complex with an apparent mol.

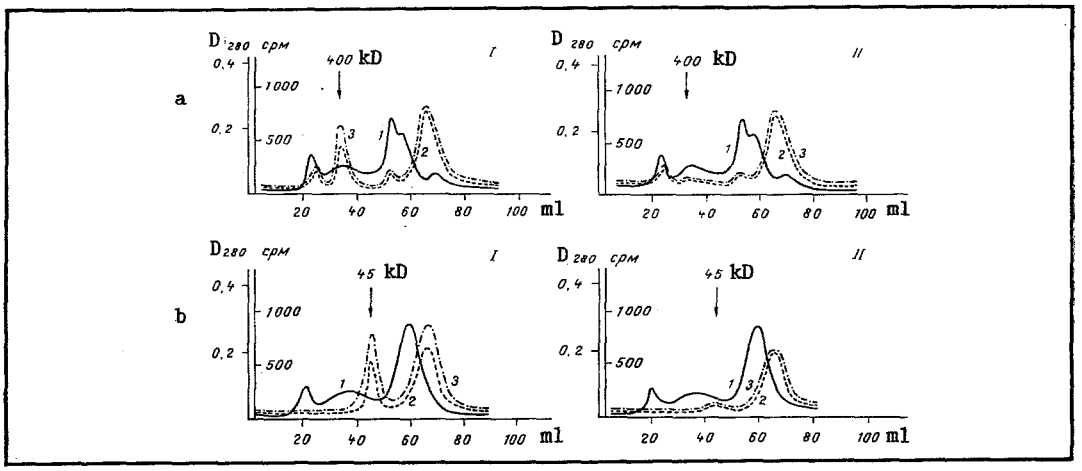

Fig. 2. Gel-chromatography of solubilized preparations of NACHR after photoaffinity modification of ^{3}H-AMC on sepharose CL-6B (a) and on Sephacryl S-200 (b) in absence (I) and presence (II) of 0.1 mM nicotine. 1) Optical density at 280 nm; 2) radioactivity in fractions; 3) radioactivity in fractions after photoaffinity modification in presence of 1 mM ATP.

wt. of 400 kD. Incorporation of the label into the low-molecular-weight fraction showed a very small increase (Fig. 2). Nicotine (0.1 mM) prevented modification of the receptor complex, as is confirmed by the specificity of incorporation of the label.

In another variant the membrane preparation, after photoaffinity modification, was solubilized in the presence of 2-chloroethanol, which destroys noncovalent protein–protein and protein–lipid interactions [12], and chromatographed on a column with Sephadex S-200. Under these circumstances increased incorporation of radioactivity into the fraction with apparent mol. wt. of 45 kD was observed, and was inhibited when photoaffinity modification was carried out in the presence of 0.1 mM nicotine.

On the basis of these results the following conclusions can be drawn. ATP and GTP have a modulating effect on interaction of cholinergic ligands with NACHR of the squid optic ganglia, inhibiting in a concentration of 1 mM binding of the bis-quaternary antagonist ^{14}C-TC and increasing the number of specific binding sites for ^{3}H-MC, an agonist of nicotine specificity. According to the mol. wt. of the subunitary complex and the subunit carrying the binding site of cholinergic ligands, the additional binding sites appearing in the presence of 1 mM ATP did not differ from the original ones.

The mechanism of the modulating effect of ATP and GTP is not yet clear. However, within the framework of Greengard's hypothesis that neurotransmitters induce changes in the degree of phosphorylation of membrane proteins [7], it can be tentatively suggested that the appearance of additional binding sites is due to a switch of the receptors from the inactive to the active state, and this switch itself takes place through the combined action of the agonists and ATP (or GTP), possibly on account of phosphorylation processes. This hypothesis is based on the work of Edelstain [3], who demonstrated the applicability of a model assuming the existence of receptors in inert (I) and reactive (R) states, and that in the presence of an agonist, moreover, equilibrium $R \rightleftarrows I$ shifts to the left. The possibility, in principle, of phosphorylation under the influence of the agonist also was discovered by the use of other receptor systems as the example. In particular, insulin stimulates phosphorylation of tyrosine residues of the insulin receptor in a cell-free system [9] and also at the level of solubilized preparations [11].

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COMBINED ACTION OF MELATONIN AND IMIPRAMINE ON THE STRUCTURE OF FORCED SWIMMING AND THE CIRCADIAN RHYTHM

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Mental depression is accompanied by disturbances of activity of the pineal gland. In particular, the character of secretion of its principal hormone, melatonin, is modified [4, 9]. Meanwhile, various antidepressants have been shown to interfere with melatonin synthesis [5, 10]. However, the connection between these changes and the specific activity of the drugs remains open.

The aim of this investigation was to study the character of interaction of the effects of imipramine, an antidepressant, and of exogenously administered melatonin. The forced swimming test was used as a favorite model for evaluation of antidepressant activity [6, 8]. The combined effect of the substances on the circadian rhythm of mobility, the organization of which depends on participation of the pineal gland [3], also was investigated.

EXPERIMENTAL METHOD

The character of forced swimming (FS) was studied in experiments on 40 male mice weighing 25-30 g. The animals were kept for 6 min in a cylindrical glass vessel filled with water (28-29°C). Movements of the mice were recorded by means of an ink writer throughout the experiment. The method used enables the time course of the components of FS to be assessed. Consideration was paid to the average (in 1 min) duration of periods of immobilization, its latent period (until appearance of the first episode of immobilization), the duration of active (intensive swimming movements) and passive (low amplitude of movements) swimming, the number of transitions from one state to another, and also the number of attempts to get out of the vessel.

The animals as a whole were divided into four equal groups: 1) control (injection of 0.14 M NaCl); 2) animals receiving melatonin (synthesized at the All-Union Pharmaceutical Chemical Research Institute, Moscow, under the direction of Professor N. N. Suvorov) 1 mg/kg, intraperitoneally 30 min before testing; 3) mice receiving imipramine (10 mg/kg) daily for 2 weeks; 4) animals receiving melatonin against the background of chronic administration of the antidepressant (on the day after the last injection). The animals were kept in natural daylight and under standard conditions (food, ambient temperature) and the experiments were carried out during the afternoon (1-3 p.m.).

The circadian rhythm was evaluated in 24 rats of both sexes, weighing 150-180 g. To study the circadian rhythm of motor activity, an actograph of our own design was used. It

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