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GUANINE NUCLEOTIDES PREFERENTIALLY INHIBIT BINDING OF ANTAGONISTS (MALE) AND AGONISTS (FEMALE) TO MUSCARINIC RECEPTORS OF RAT ADENOHYPOPHYSIS

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The effects of guanine nucleotides on the binding properties of muscarinic receptors for muscarinic ligands were studied in male and female rat adenohypophysis preparation, using direct ligand binding with [^{3}H]-N-methyl-4-piperidylbenzilate and by competition with oxotremorine. GTP and Gpp[NH]p (100 μM) decreased antagonist binding sites in the male, while those of the female were unaltered. Nucleotide effect on agonist binding was seen only in the female rat, where it stemmed mainly from conversion of high affinity to low affinity binding sites in the diestrous and proestrous stages, while at the estrous stage the opposite effect, i.e. conversion of low to high affinity state, was unexpectedly observed. Sex dimorphism as well as the physiological role of muscarinic receptors in the pituitary are discussed.

Introduction

In a recent study we demonstrated the presence of muscarinic receptors in the rat adenohypophysis and described their biochemical characteristics (1). Our results revealed that (i) in contrast to other brain regions, antagonist binding was heterogeneous in this area, with the existence of at least two subclasses of sites; (ii) agonist binding is characterized by a two-site model, specifying a high (H) and a low (L) affinity state; (iii) the female rat is characterized during the proestrous stage by a lower degree of agonist high affinity binding [K_H] and by an increase (by almost double) in the proportion of high affinity sites in comparison with female rats at other stages of the cycle as well as with male rats. Pituitary responsiveness to muscarinic binding therefore fluctuates during the estrous cycle. Recent reports have shown that guanine nucleotides selectively reduce the affinity for agonist binding but not for antagonist binding to muscarinic receptors (2-6). It was shown that the GTP effects are regionally

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specific to the heart (2)(3)(4), cerebellum and medulla pons (5), and do not occur to the same extent in the rest of the brain regions investigated; moreover, that they result in the interconversion of agonist binding sites from the high affinity into the low affinity state (5)(7). As an extension of our previous studies (1)(5)(6)(8) we now report on the regulatory effect of guanine nucleotides on agonist and antagonist binding in the female rat at the three stages of cycle as well as in male rats. While this work was in progress, a report of GTP effect on male rat adenohypophysis membrane was published (9).

Materials and Methods

[^{3}H]-N-methyl-4-piperidylbenzilate (4NMPB) (33 Ci/mmole) and unlabeled muscarinic ligands are those described and used previously (1). The nucleotides were purchased from Sigma.

Adult male and female rats of the CD strain were supplied by Levinstein's farm (Yokneam) and maintained in an airconditioned room at 24 ± 2° for 14 h under fluorescent illumination (0500 - 1900 h) and in darkness for 10 h daily. Food from Assia Maabarot Ltd. and water were supplied ad libitum. After an adjustment period of at least four weeks, daily vaginal smears were taken of all female rats and only those having a regular 4-day estrous cycle were used. The rats were then 3-4 months old and weighed 190-250 g. They were decapitated and their adenohypophyses were rapidly removed in a cold room.

Binding assays. Full details concerning homogenate preparation, as well as antagonist binding assay techniques carried out at 37° using the centrifugation method, are described elsewhere (1). The fact that ligand-muscarinic receptor complex dissociates much more rapidly in the adenohypophysis than in other brain regions creates difficulties with the filtration technique (1), therefore, the centrifugation method was used in this study. Binding of [^{3}H]-4NMPB that was inhibited by 5 μM of unlabeled atropine was considered to be specific.

Binding of agonists to homogenates of adenohypophyses (12-15 rats) in the absence and presence of guanine nucleotides was inferred by their ability to compete with specific binding of 2.0 nM of [^{3}H]-4NMPB (1)(10). Homogenates were incubated at 37° in modified Krebs-Hensleit solution, pH 7.3 (1) containing the labeled ligand together with various concentrations of unlabeled ligands (oxotremorine 5 x 10^{-8} - 1 x 10^{-4} M) and nucleotides. The reaction was terminated by centrifugation as described (1). Assays were carried out in triplicate. Protein was determined by the Lowry method.

Data analysis. The data obtained from direct binding assays with the antagonist were analyzed by a nonlinear least square curve fitting procedure using a generalized model for complex ligand receptor systems, as previously described (1). Computer analysis indicated that antagonist binding was best explained by assuming two affinity sites of the receptor (1). Theoretical competition curves were fitted to the experimental data points using the nonlinear least square regression computer program BMDPAR (November 1978 revision), as described in detail previously (8) (10)(11). (The program was developed at the Health Science Computing Facility of the University of California, Los Angeles; the Facility is sponsored by NIH Special Research Resources Grant RR-3).

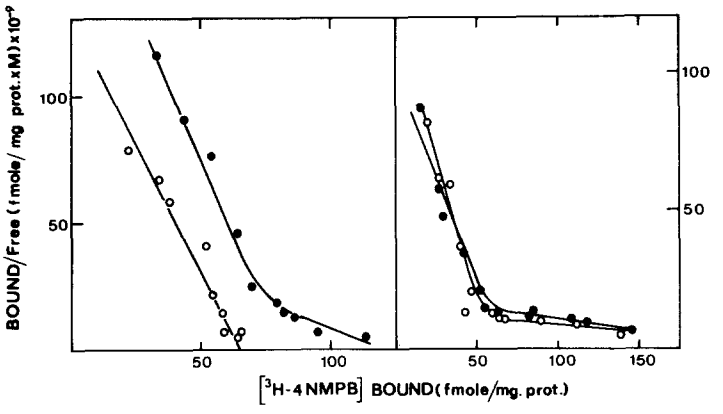

Fig. 1. Scatchard plot of binding of [^{3}H]-4NMPB to male (left) and female (right) adenohypophysis membranes in the absence (•) and presence of 100 μM GTP (o). Samples (0.05 ml) were incubated at 37° for 10 min in 0.5 ml modified Krebs-Hensleit solution containing various concentrations of [^{3}H]-4NMPB (0.25-20 nM).

Results

Effect of nucleotides on muscarinic antagonist binding sites

The binding of [^{3}H]-N-methyl-4-piperidylbenzilate ([^{3}H]-4NMPB) to male adenohypophyseal preparations is shown by Scatchard plot in Fig. 1. As discussed and described previously (1) computer analysis indicated that the binding characteristics of this ligand (as well as those of another antagonist, QNB) can be most simply accounted for by assuming the existence of two affinity sites of the receptor (although other explanations, e.g. negative cooperativity, cannot be excluded and are under investigation). Binding properties of these two sites (designated α for the higher affinity site and β for the lower affinity site) are given in Table I.

The effect of GTP on the binding of [^{3}H]-4NMPB is depicted in Fig 1 (left). As shown, for male rats the curvilinear Scatchard plot is converted into almost a straight line, indicating the presence of one population of sites, whose binding characteristics are given in Table I. Thus, antagonist subclass β disappears after treatment with 100 μM of GTP or its non-hydrolyzable analog Gpp(NH)p (not shown). The maximal effect of GTP was obtained at 100 μM, while ATP at this concentration was virtually inactive. The effects of GTP were persistent, lasting for at least 30 min. The effect of GTP on antagonist binding was completely reversible upon withdrawal of the reagent by washing the membranes and

Table I: Binding characteristics of the muscarinic antagonist [^{3}H]-4NMPB in male and female rat adenohypophysis in the absence and presence of 100 μM GTP

		K_α, nM	B^α_{max} fmol /mg protein	K_β, nM	B^β_{max} fmol/mg protein
Males	Control	0.41 ± 0.05	68 ± 5	2.1 ± 0.3	106 ± 10
	+ GTP	0.54 ± 0.04	75 ± 6	-	-
Female Proestrous	Control	0.84 ± 0.03	63 ± 5	10.8 ± 0.4	155 ± 10
	+ GTP	0.6 ± 0.1	65 ± 2	11.2 ± 1	160 ± 10
Female Estrous	Control	0.64 ± 0.06	65 ± 5	11.2 ± 0.9	157 ± 10
	+ GTP	0.6 ± 0.05	67 ± 3	10.5 ± 0.8	160 ± 10

α and β designate the higher and lower affinity antagonist binding sites, respectively. Average values of the binding characteristics were determined in 3-4 separate experiments.

subsequent assay with [^{3}H]-4NMPB. In the case of female rats, on the other hand, neither GTP nor Gpp(NH)p altered the binding characteristics of antagonist at any of the stages of the estrous cycle (Fig. 1 (right), Table I).

Effect of nucleotides on agonist binding sites

The effect of GTP on the binding of the agonists (oxotremorine and carbamylcholine) was investigated by means of agonist/[^{3}H]-antagonist competition experiments. As described previously (1), the inhibition curves for these agonists (in the absence of GTP) are flattened, and are characterized by a Hill slope smaller than unity. A possible explanation (1)(12) is that agonists bind with differing affinities to two or three distinct populations of binding sites. Our data could be best fitted by a two-site model specifying a high (H) and a low (L) affinity state. However, it should be pointed out that technical limitations (low receptor concentrations in the adenohypophysis as compared to brain regions, e.g. cortex) did not allow us to accumulate enough experimental data to investigate adequately the possible existence of a third population, i.e. super-high state (12). We therefore adopted the simpler model specifying two sites.

The binding data obtained from competition experiments using 2.0 nM [^{3}H]-4NMPB and oxotremorine (at 0.05 - 0.09 nM receptor concentrations)

Table II: Effects of GTP on oxotremorine binding to rat adenohypophysis, determined by competition with 2 nM [^{3}H]-4NMPB at 37°

	Male		Proestrous		Estrous		Diestrous	
	α, %	K_H(nM)	α, %	K_H(nM)	α, %	K_H(nM)	α, %	K_H(nM)
Control (- GTP)	14 ± 2	262 ± 40	83 ± 2	1370 ± 30	27 ± 2	37 ± 4	58 ± 2	47 ± 8
+ GTP (100 μM)	18 ± 1	220 ± 30	20 ± 3*	160 ± 9	47 ± 7**	74 ± 10	41 ± 3**	51 ± 8

Binding parameters ± S.D. at 37° were calculated by the nonlinear least square regression procedure for a two-site model as described in Methods. K_H is the affinity constant of oxotremorine to the high affinity binding sites; α denotes the proportion of high affinity binding sites. The average values of oxotremorine binding characteristics were determined in three separate experiments, each performed in triplicate. [^{3}H]-4NMPB binding site concentration was 0.05-0.09 nM. * $p < 0.001$. ** $p < 0.005$.

in the presence and absence of GTP at 37° are given in Table II. The following observations should be pointed out: (a) In male adenohypophysis the inhibition curves were shifted slightly to the right, indicating an apparent increase in the I_{50} value. The measurement of agonist binding indirectly, i.e. by means of competition experiments, precludes an absolute estimation of the number of agonist binding sites: the only observation possible is that all [^{3}H]-4NMPB binding sites can be blocked by oxotremorine, but the extent to which oxotremorine is blocked by [^{3}H]-4NMPB cannot yet be directly assessed. Therefore, the binding parameters given in Table II were based on the antagonist B_{max} value given in Table I for male preparations determined in the presence of GTP. Binding of oxotremorine to male preparation was not affected by GTP (Table II) or Gpp(NH)p (not shown). (b) For female rats in the proestrous stage, the GTP effect was characterized by a decrease in the proportion of high affinity sites with a concomitant significant decrease in the dissociation constant (K_H) to about 160 nM as compared to the control value of 1.3 M. (It should be noted in regard to this K_H value for the control that for the sake of simplicity we term the population "high affinity", even though in comparison with other brain regions its value is in the low rather than the high affinity range.) In low affinity sites no significant differences in K_L were found. (c) Surprisingly, the competition curves in the estrous stage were shifted in the presence of GTP or Gpp(NH)p about threefold to the left, i.e. the I_{50} value, which as shown in Table II stems mainly from an increase in proportion of high

affinity binding sites, from 27% (control) to about 47%; at the same time a twofold increase in the K_H value was observed. No significant differences in K_L were found. The GTP effect was maximal at 100 μM, but could also be detected at lower concentrations. For example, at 25 μM and 50 μM of GTP the corresponding values of α were 33 ± 5% and 42 ± 5% respectively. (d) In the diestrous stage the curves were shifted slightly to the right, an effect stemming mainly from the increase in the proportion of low affinity sites from 42% (control) to 60%. No substantial changes in either K_H or K_L were seen (Table II).

Discussion

The data presented here clearly indicate some unusual effects in the presence of guanine nucleotides. First, binding of antagonists to male adenohypophysis is dramatically altered. By contrast, binding of antagonists to female adenohypophysis, as well as to muscarinic receptors in the brain (at least in the areas examined so far) is much less subject (if at all) to nucleotide perturbation. These findings support and extend our previous suggestion of sex dimorphism in the binding characteristics of muscarinic receptors in the adenohypophysis (1). To the best of our knowledge, the reduction in muscarinic antagonist binding induced by GTP is the first report of such a phenomenon occurring in a membranal receptor system. It is somewhat reminiscent of the recent observations that (i) in heart homogenates, in the absence of inorganic ions, guanine nucleotides exert a strong effect on antagonist binding (7); and (ii) that guanine nucleotides inhibit the binding of antagonists to soluble opiate receptors but not to the membranal receptor (13). One possible explanation for the phenomenon reported here is that it reflects differences between post- and presynaptic receptors, since adenohypophysis is presumably enriched with presynaptic-like receptors (1). This seems unlikely, in view of the differing effects on male and female preparations. The physiological significance of the GTP effect on muscarinic antagonist binding in male adenohypophysis remains to be established. It should be noted that our results are at variance with those of Mukherjee and Snyder (9), who reported recently that in male pituitary GTP and Gpp(NH)p at a concentration of 100 μM markedly decreased the binding affinity of oxotremorine, but had no effect on the binding of the antagonist atropine. We cannot yet explain the discrepancy, although it could be at least partly caused by technical differences such as different buffers (Krebs-Tris vs. phosphate), the fact that [^{3}H]-antagonist concentration in Mukherjee and Snyder's competition experiments was much higher than K_D values, and

differences in binding assays (centrifugation vs. filtration) (see under Discussion in (1) and Materials and Methods in this report).

Secondly, the GTP effect on muscarinic agonist binding in the adenohypophysis of females at the estrous stage is unusual. In this organ at the proestrous and diestrous stages, as well as in muscarinic and other receptor systems in the various brain regions studied, the GTP effect is best explained in terms of an interconversion from high to low affinity states (5)(7)(14-19); but in the adenohypophysis at the estrous stage, quite the opposite effect is seen, i.e. an interconversion from low to high affinity states. The fact that in the estrous stage the changes in K_H are relatively small (twofold decrease in affinity) appears to exclude an explanation in terms of interconversion from super-high to high affinity state. On the other hand, this possibility cannot be dismissed altogether for the proestrous stage in which a one-order of magnitude increase in agonist affinity was observed after treatment with GTP. In the absence of GTP, the population, to which we refer as high affinity, was characterized during proestrous by a relatively high dissociation constant value, i.e. 1.3 μM as compared to 37 nM (at estrous) and 47 nM (at diestrous).

Turning back now to the estrous stage, in which guanine nucleotides induced interconversion of low to high affinity binding state, further elucidation of the "new" high affinity binding (which we regard in this report as the "normal" high affinity state) is clearly needed. It should be noted that we recently described a similar interconversion of agonist state induced by the transition metals Mn^{2+} and Co^{2+} in several brain regions, e.g. cortex and hippocampus (6). The fact that in these regions the GTP effect shows the "typical" pattern of high to low affinity interconversion suggests that mechanistically these two phenomena must be different. Nevertheless, the effects in both cases are in agreement with the requirement of microscopic reversibility.

In vivo endocrinic manipulations of the estrous cycle at the estrogenic level, such as ovariectomy of adult cyclic females or androgenization of newborn females, were reflected by alterations in the muscarinic system at the adenohypophysis (20). These results, taken together with the recently described effects of ß-estradiol and progesterone on the muscarinic system (8), strongly suggested that the muscarinic receptors play a part in the positive and/or negative regulation of estrogens on sex hormonal secretion. It is conceivable that the GTP modulation of the muscarinic receptors in the pituitary is part of that mechanism; this might explain fluctuations in muscarinic characteristics observed in

the pituitary during the estrous cycle, and lend support to the suggestion that the stimulation of pituitary hormone synthesis and secretion might be mediated through cyclic nucleotides (21).

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