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TEMPERATURE DEPENDENCE OF AGONIST BINDING TO MUSCARINIC RECEPTORS IN RAT HYPOTHALAMIC REGIONS

Ronit Gàlron, Sofia Avissar and
Mordechai Sokolovsky

Department of Biochemistry
George S. Wise Faculty of Life Sciences
Tel Aviv University, 69978 Tel Aviv, Israel.

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The temperature dependence of the muscarinic binding characteristics in various brain regions was investigated at 25° and 37°. An unusual degree of temperature dependence was observed for agonist binding parameters in the hypothalamus, especially in the preoptic area. We therefore chose this region for a more detailed study over a temperature range of 25°-42°. Changes due to temperature alterations in the proportion of high-affinity sites, as well as in the high-affinity state constant, were found in male rats, while in females at the proestrous stage only the high-affinity constant was affected. The possible involvement of the muscarinic system is discussed in light of the well known role of the hypothalamus in body thermoregulation.

Introduction

Recent in vitro binding studies of the muscarinic antagonist [^{3}H]-N-methyl-4-piperidylbenzilate ([^{3}H]-4NMPB) in rat and mouse hypothalamus, as well as competition experiments with agonists, have indicated differences in binding parameters between male and female rats, especially in the preoptic area (1). Similar studies in several other brain regions have revealed that agonist binding sites are more sensitive than antagonist sites to environmental restraints, e.g. they are more hydrophobic for low affinity sites and more hydrophilic for high affinity sites, and that interconversions between high and low affinity states can be induced (2 and ref. therein). It seemed reasonable to assume that conformational changes leading to such interconversion might be temperature dependent. In the present study we therefore scrutinized antagonist and agonist-binding characteristics at 25° and at 37° in several brain regions; the unusual degree of temperature dependence observed in the hypothalamus prompted us to conduct a more detailed study of membrane-bound receptors in the preoptic area over a temperature range of 25°-42° in male rats, as well as in female rats at the proestrous stage.

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Materials and Methods

The potent muscarinic antagonist [^{3}H]-N-methyl-4-piperidylbenzilate (4NMPB) (33 Ci/mmole) and unlabeled muscarinic ligands have been previously described (1).

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°C for 14 h under fluorescent illumination (0500 - 1900 h) and 10 h of darkness daily. Food from Ambar Ltd. (Hadera) and water were supplied ad libitum. After an adjustment period of at least 4 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 (between 1000 and 1200 hours) and their brains rapidly removed. After identification with the aid of a stereotaxic atlas (3), the various brain regions and the following hypothalamic areas were dissected out in a cold room: the preoptic area together with the suprachiasmatic nucleus (3.7-4.0 mg/animal); the median region, particularly the median eminence and the ventromedial, dorsomedial and arcuate hypothalamic nuclei (17-20 mg/animal); the posterior region, including the posterior hypothalamic nucleus and the mammillary bodies (7-9 mg/animal).

Binding Assay. Full details of the homogenates used and the binding assay employing the filtration method have been given elsewhere (1).

Homogenates prepared from the preoptic area (10-12 rats), the median region (5 rats) and the posterior region (8-10 rats) were used for binding assays. Binding of [^{3}H]-4NMPB that was inhibited by 1 μM unlabeled 4NMPB or atropine was considered to be specific. Binding of agonists was inferred from their ability to inhibit specific binding of 2 nM [^{3}H]-4NMPB, in triplicate experiments as described in detail in previous reports (1)(4)(5). Protein was determined by the Lowry method using bovine serum albumin as a standard.

Data Analysis. Theoretical binding curves were fitted to the experimental data points using the nonlinear least square regression computer program BMDPAR, revision date November 1978, as described in detail previously (1)(4)(5). (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 R-R-3). Statistical evaluations of agonist binding parameters (affinity constants and population of high affinity sites) were made using Student's t test.

Results

Specific binding of [^{3}H]-4NMPB to homogenates of various brain regions at 25° and 37° as a function of concentration was first studied. Scatchard plots of the specific binding in all brain regions of male rats and of female rats at the proestrous stage yielded a straight line, indicating a homogeneous population of high affinity sites. In all brain regions studied, the Bmax and the K_d values for antagonist binding were unaltered upon raising the temperature from 25° to 37°, as shown in Table I for female rats at the proestrous stage.

Binding of agonists (oxotremorine) was inferred from their ability to inhibit specific binding of 2 nM [^{3}H]-4NMPB. Data from inhibition curves (see, e.g., Fig. 1) were subsequently analyzed according to a two-site model specifying a high (H) and a low (L) affinity state (1)(5)(6). The results

Table I

Characteristics of ligand binding to muscarinic receptor to various brain regions of female rat (at proestrous stage) at 25° and 37°.

Brain Region	Temp (°C)	[^{3}H]-4NMPB[a]		oxotremorine[b]		
		Kd, nM	Bmax pmol/mg protein	α, %	K_H, nM	K_L, μM
Medulla-pons	25	0.82 ± 0.02	0.27 ± 0.02	63 ± 2	22 ± 2	1.4 ± 0.2
	37	0.78 ± 0.04	0.28 ± 0.02	64 ± 2	22 ± 2	1.1 ± 0.2
Cerebellum	25	0.62 ± 0.04	0.08 ± 0.02	76 ± 4	31 ± 3	0.8 ± 0.2
	37	0.66 ± 0.04	0.07 ± 0.02	76 ± 4	31 ± 3	0.7 ± 0.2
Caudate Putamen	25	0.36 ± 0.02	1.1 ± 0.06	26 ± 2	30 ± 1	1.0 ± 0.1
	37	0.38 ± 0.02	1.06 ±0.06	26 ± 2	32 ± 2	0.9 ± 0.1
Hypothalamus						
Preoptic area	25	0.62 ± 0.4	0.48 ± 0.05	56 ± 4	11 ± 2	1.5 ± 0.2
	37	0.66 ± 0.4	0.41 ± 0.04	61 ± 8	56 ± 8	1.4 ± 0.2
Anterior	25	0.6 ± 0.02	0.36 ± 0.04	39 ± 4	6 ± 1	1.3 ± 0.05
	37	0.7 ± 0.02	0.34 ± 0.04	42 ± 3	39 ± 3	1.2 ± 0.05
Posterior	25	0.6 ± 0.05	0.42 ± 0.03	43 ± 5	8 ± 1.5	1.5 ± 0.2
	37	0.7 ± 0.04	0.45 ± 0.04	46 ± 2	24 ± 2	1.3 ± 0.3

[a] Data taken from direct binding with [^{3}H]-4NMPB.

[b] The binding parameters K_H, K_L and α±SD were calculated by the nonlinear least squares regression procedure for a two-site model as described in "Methods". K_H and K_L are the affinity constants of oxotremorine to high and low affinity binding sites, respectively, and α denotes the proportion of high affinity binding sites. The average values of the binding characteristics for [^{3}H]-4NMPB and oxotremorine were determined in 3 separate experiments.

obtained by a nonlinear least square regression program are also given in Table I. As in the case of antagonist binding, agonist binding (as reflected by α,K_H and K_L) to muscarinic receptors in the medulla-pons, cerebellum, caudate putamen (Table I) and hippocampus and cortex (not shown) showed no significant differences at 25° and 37°.

On the other hand, the agonist high affinity dissociation constant (K_H) in the hypothalamus (the preoptic area, the anterior and the posterior regions) showed a marked increase upon increasing the temperature from 25° to 37°. To further establish the temperature dependence of the system, we repeated these experiments at four different temperatures, using homogenates prepared from the preoptic area, and compared the results obtained for pro-

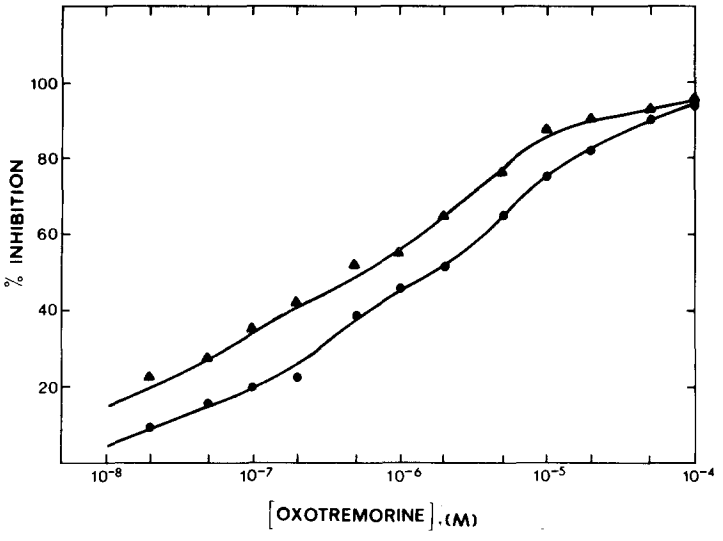

Fig. 1. Inhibition curves of [^{3}H]-4NMPB binding to the preoptic area of female rats at the proestrous stage by the muscarinic agonist oxotremorine at 25° (▲) and 37° (●). Each point represents the average of triplicate determinations.

estrous females and for males (Table II). While in the proestrous females only K_H increased with temperature, a significant increase in α (the proportion of high affinity sites) accompanied temperature increase in the case

Table II

Temperature dependence of the characteristics of oxotremorine binding to muscarinic receptors in the preoptic area of male and female (proestrous stage) rats.

Temp (°C)	Male				Female (proestrous)			
	α, %	K_H, nM	K_L, μM	K_L/K_H	α, %	K_H, nM	K_L, μM	K_L/K_H
25	34 ± 4	8 ± 1.5	1.2 ± 0.2	150	56 ± 4	11 ± 2	1.5 ± 0.2	136
30	39 ± 4	15 ± 1.5	1.3 ± 0.2	87	57 ± 4	15 ± 4	1.3 ± 0.1	93
37	57 ± 5*	54 ± 2.5*	1.3 ± 0.1	24	61 ± 8	56 ± 8**	1.4 ± 0.05	23
42	56 ± 5	84 ± 3	1.4 ± 0.06	16	57 ± 6	50 ± 9	1.4 ± 0.2	28

See Table I for definition of parameters. The average values of binding parameters were determined in 3 separate experiments. *= $p < 0.001$ vs. 25°. **= $p < 0.005$ vs. value at 25°.

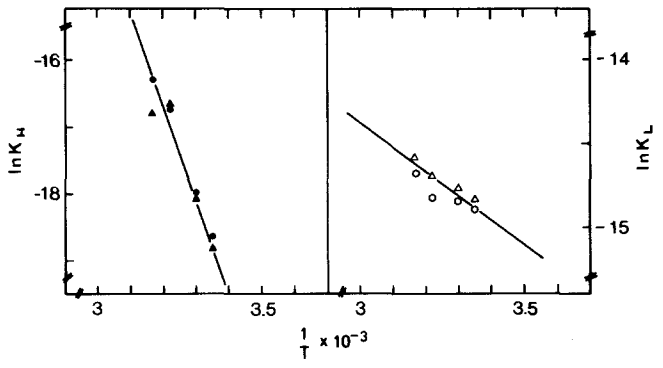

Fig. 2. Arrhenius plots for the data presented in Table II. Data are for proestrous females (Δ) and males (o).

of the male rats. K_L remained essentially unaltered in both cases. Both K_H and K_L exhibited linear Arrhenius plots (Fig. 2), yielding ΔG_o of 28 ± 2 Kcal/mol for K_H and ΔG_o of 2.9 ± 0.4 Kcal/mol for K_L. These values were identical for male and female rats (Fig. 2) and reflect the considerably stronger temperature dependence of K_H than of K_L. Moreover, the fact that the change with temperature exhibited by the ratio K_L/K_H is much greater than that exhibited by the fraction of agonist high affinity sites (α) indicates that the "cooperativity" (6) of agonist binding changes with temperature in the hypothalamic regions studied.

Discussion

The results of this investigation clearly indicate (i) sex dimorphism and (ii) temperature dependence of agonist binding characteristic of a specific brain region, the hypothalamus. By contrast, neither antagonist nor agonist binding was dependent on temperature in any of the other brain regions investigated under the experimental conditions described in this study. Some of the results reported here confirm previously published data concerning antagonist binding (4)(7), and extend them by characterizing both antagonist and agonist binding as a function of temperature in a variety of brain regions.

The conversion of high to low affinity state in male rat preoptic area is reminiscent of the GTP effect observed previously for the modulation of muscarinic receptors (2)(8)(9). Interestingly, GTP and Gpp(NH)p had no effect on either agonist or antagonist binding in any of the three hypothalamic areas studied here (unpublished results), indicating that the modulation of muscarinic receptors in the preoptic area by relatively small temperature changes constitutes a pathway of physiological regulation distinct from that

of the guanine nucleotides. This is not surprising since the hypothalamus, and especially the posterior and preoptic areas, are known to control the thermoregulation center, thus offering a possible biochemical process which is part of such system (10)(11). The temperature dependence of the cooperativity (6) in agonist binding is indicated by the fact that the changes in α and K_H values upon raising the temperature from 25° to 37° are of different magnitudes (i.e. in male rats, ∿2-fold increase in α vs. ∿7-fold increase in K_H; in female rats at proestrous, no substantial changes in α vs. 8-fold increase in K_H values.)

The markedly higher affinity of the agonist to its high-affinity sites at lower temperatures, and the almost non-existent increase in its affinity to the low-affinity sites, supports the notion that the low-affinity sites are more hydrophobic and the high affinity sites more hydrophilic (2 and Ref. therein).

Qualitatively similar observations have been reported recently (12) for β-adrenergic receptors in turkey erythrocyte membranes. In that study, however, complete conversion of high to low agonist affinity state was achieved by lowering the temperature from 37° to 20° (12). It should also be noted that alteration in cardiac muscarinic receptors with temperature has been reported (13). However, while the same pattern of agonist dependence was observed in the cardiac preparation [affinity of agonist (carbachol) was higher at 18° than at 37° (0.5 μM vs. 4.5 μM)], antagonist binding was similarly affected (3 nM vs. 30 nM). It seems reasonable to assume that the different temperature effect observed for antagonist binding in that study stems from differences in the experimental conditions employed; e.g., their findings may have been strongly affected by the isomerization of the antagonist-receptor complex resulting from incubation for a 3-hour period (1)(4).

What, then, is the physiological significance of the above findings? The much stronger dependence of K_H on temperature indicates that the relative occupancy of the high- and low-affinity agonist binding sites will change with temperature. The considerably greater affinity of agonist to the high-affinity sites at 25° than at 37° indicates that at the lower temperature most of the agonist will be bound at the high-affinity sites, and less will be therefore available for the low-affinity sites. It is therefore possible that, while the low-affinity sites are the physiologically functional ones, the high-affinity sites regulate the agonist occupancy of the low-affinity sites under various conditions. In the male rat, an additional regulatory mechanism may exist, namely, temperature-dependent changes in the ratio of high- to low-affinity sites (Table II). Since at lower temperature the population of high-affinity sites in the male rat preoptic area is smaller,

this interconversion will tend to moderate the effect of the increase in affinity to the high-affinity sites. It is noteworthy that changes in body temperature accompany the estrous cycle in female rodents as well as in humans. It should be noted that Birdsall et al. (6)(14) have suggested that the low-affinity binding sites are the important ones for neurotransmission. Our present results may be relevant to previous in vivo studies on cholinergic ligands conducted in our laboratory (15)(16), in which hypothermia induced in mice by oxotremorine injection was manifested by $\sim 6°$ decrease in the rectal temperature. The ligand concentration in the brain was about 10^{-7}M; assuming an even distribution of the ligand, i.e. oxotremorine, in the brain and taking into account the K_L values for male and female rats at 37°, i.e. 1.3 and 1.4 μM respectively, it is clear that under such conditions low-affinity sites would hardly be occupied. It therefore follows that the high-affinity and not the low-affinity sites are the ones involved in the process of hypothermia.

No obvious explanation has yet emerged as to why and how different agonist affinity sites are involved in the process of thermoregulation. The results presented here point to the need for a more detailed exploration, especially in conjuction with studies aimed at elucidating the interrelationships between the muscarinic system and other neurotransmitters known to be involved in the thermoregulation process, e.g. serotonin and dopamine.

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