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Rat Cardiac Muscarinic Receptors

II. Influence of Thyroid Status and Cardiac Hypertrophy

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SUMMARY

The effects of the thyroid state and of aortic stenosis on muscarinic cholinergic binding sites in heart membranes were compared (with proper controls) by simultaneously determining total and high-affinity binding sites and estimating low-affinity binding sites by difference. Hyper- and hypothyroidism induced decreased and increased concentration of high-affinity agonist binding sites, respectively, supporting the hypothesis that these sites were directly regulated by thyroid hormones. This was not the case for low-affinity binding sites, as they decreased in number in both hyper- and hypothyroidism. In hyperthyroid rats, this decreased number of low-affinity binding sites could be due to the rapidly developing cardiac hypertrophy. Indeed, cardiac hypertrophy provoked by aortic stenosis led also to a decreased concentration of low-affinity binding sites without affecting the concentration of high-affinity binding sites.

INTRODUCTION

In a recent study, Ehlert and co-workers (1) have shown that muscarinic binding sites represent a heterogeneous group of receptors in heart as well as in several other tissues. Two or three classes of muscarinic receptors can be distinguished on the basis of their relative ability to recognize agonist molecules, although they have identical affinities for antagonists (1). Whether high- and low-affinity agonist binding sites are distinct or reversibly interconvertible entities remains an open question. In a previous report (2) we described a method allowing the direct characterization of high-affinity agonist binding sites with [^{3}H]Oxo-M[2] and measurement of the total concentration of receptors with the antagonist L-[^{3}H]QNB. In this paper, we report experiments on the regulation of the number and affinity of high- and low-affinity binding sites in hyper- and hypothyroidism, which are known to decrease and to increase, respectively, the total number of muscarinic receptors, based on L-[^{3}H]QNB binding (3). Since the decreased total concentration of muscarinic receptors observed in hyperthyroidism might reflect either the action of thyroid hormones per se or be the indirect consequence of cardiac hypertrophy, the concentration of muscarinic receptors in rats with cardiac hypertrophy induced by surgical aortic stenosis served as a basis for comparison.

MATERIALS AND METHODS

Chemicals

L-[^{3}H]QNB (specific radioactivity 40 Ci/mmole) and [^{3}H]-Oxo-M (specific radioactivity 84 Ci/mmole) were obtained from New England Nuclear Corporation (Dreieich, Federal Republic of Germany). Atropine sulfate was obtained from Sigma Chemical Company (St. Louis, Mo.) Sodium L-thyroxine was obtained from Henning (Berlin, Federal Republic of Germany), and PTU from Koch-Light Laboratories (Coinbrook, Bucks., England). All other reagents were of the highest grade available.

Animals

Hyperthyroid rats. Male Wistar rats received s.c. injections of L-thyroxine (800 μg dissolved in 1 ml 0.01 N NaOH per kilogram of body weight) twice daily for 5 consecutive days, according to the method of McConnaughey *et al.* (4). The efficacy of the treatment was ascertained by a moderate decrease in body weight and by cardiac hypertrophy (Table 1). Control animals received injections of the same volume of 0.01 N NaOH.

Hypothyroid rats. Male Wistar albino rats were made hypothyroid by receiving for 7 weeks 0.5 g of PTU (dissolved in 0.8 ml of dimethyl sulfoxide and 0.8 ml of ethanol) per liter of drinking water. Control animals received the vehicle only. Hypothyroidism was documented by a decrease in growth rate and low serum T_3 levels at the time of sacrifice (150 ± 30 ng/liter in PTU-

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[2] The abbreviations used are: [^{3}H]Oxo-M, [*methyl*-^{3}H]oxotremorine acetate; L-[^{3}H]QNB, L-[*benzilic*-4,4′-^{3}H]quinuclidinyl benzilate; PTU, 6-*n*-propyl-2-thiouracil.

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treated rats as compared with 750 ± 50 ng/liter in control animals).

Mechanical hypertrophy of the heart induced by aortic stenosis. Fasting male Wistar albino rats were anesthetized with ether. Through a left side incision, the aorta was carefully dissected between the diaphragm and the origin of the mesenteric artery. A ligation was made near the diaphragm using a stainless steel needle (1.1-mm external diameter), and the needle was then removed.

The animals were allowed to recover for 12 days. Control animals were sham-operated. After surgery, increases in body weight were comparable in both groups. The efficacy of the aortic stenosis was verified at the time of sacrifice and its consequences were estimated by cardiac hypertrophy (Table 1).

Methods. Each animal was killed by decapitation and its heart was rapidly dissected out, rinsed at room temperature with physiological saline, weighed, and stored in liquid nitrogen. After thawing, heart membranes were prepared as detailed elsewhere (5) and stored in liquid nitrogen.

Muscarinic receptors were evaluated as previously described (2) by using the specific binding of the two labeled ligands L-[^{3}H]QNB and [^{3}H]Oxo-M at equilibrium. Briefly stated, 0.13 mg of heart membrane protein was incubated at 25° for 10 min in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$, 1% bovine serum albumin, and increasing concentrations of L-[^{3}H]QNB (0.2–4 nM) or [^{3}H]Oxo-M (0.5–8 nM). The samples were filtered on glass-fiber filters GF/C (Whatman, Maidstone, England) and rinsed four times with 2 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose, 2 mM β-mercaptoethanol, and 1% bovine serum albumin. The filters were dried and the radioactivity was counted by liquid scintillation. The nonspecific binding (including the radioactivity adsorbed on the filters) was determined in the presence of 1 μM atropine at each tracer concentration. This radioactivity represented no more than 10–20% of the total L-[^{3}H]QNB bound and 20–40% of the total [^{3}H]Oxo-M bound in the range of ligand concentrations tested and was subtracted from total bound radioactivity to give specific binding. The data were analyzed according to the method of Scatchard (6).

Protein was determined by the method of Lowry *et al.* (7) using bovine serum albumin as standard. Statistical evaluation of the data was performed using Student's *t*-test on unpaired values.

RESULTS

In all experimental situations tested, saturation curves for both L-[^{3}H]QNB and [^{3}H]Oxo-M yielded linear Scatchard plots compatible with the existence of binding sites with a single K_d value for the ligand. The maximal binding capacity of L-[^{3}H]QNB reflected the total number of muscarinic receptors, as this antagonist recognized all sites with equal efficacy (2) whereas the maximal binding capacity of the agonist [^{3}H]Oxo-M indicated the number of high-affinity agonist binding sites only. Therefore, the difference between both values yielded the number of low-affinity binding sites for agonists (whose K_d values were not determined in the present study).

TABLE 1

General characteristics of the male Wistar rats used

Results are the means ± standard error of the mean from six animals.

Treatment	No. of animals	Body wt	Heart wt	Heart wt to body wt ratio × 1000
		g	*g*	
Control	6	391 ± 15	1.076 ± 0.050	2.75
Hypothyroid	6	364 ± 13	0.931 ± 0.055	2.56
Control	6	205 ± 3	0.677 ± 0.030	3.30
Hyperthyroid	6	188 ± 8	0.886 ± 0.070	4.71[a]
Control	6	260 ± 15	0.823 ± 0.040	3.17
Aortic stenosis	6	260 ± 5	1.009 ± 0.022	3.88[a]

[a] Values significantly higher ($p < 0.01$) than for corresponding controls.

In hyperthyroid rats, the ratio of heart weight to body weight increased by 40% (Table 1). In cardiac membranes, the affinities for L-[^{3}H]QNB and [^{3}H]Oxo-M were not modified but the density of each type of binding sites was decreased: by 18% for high-affinity binding sites and by 36% for low- affinity binding sites (so that the total number of muscarinic receptors was decreased by 27%) (Fig. 1 and Table 2).

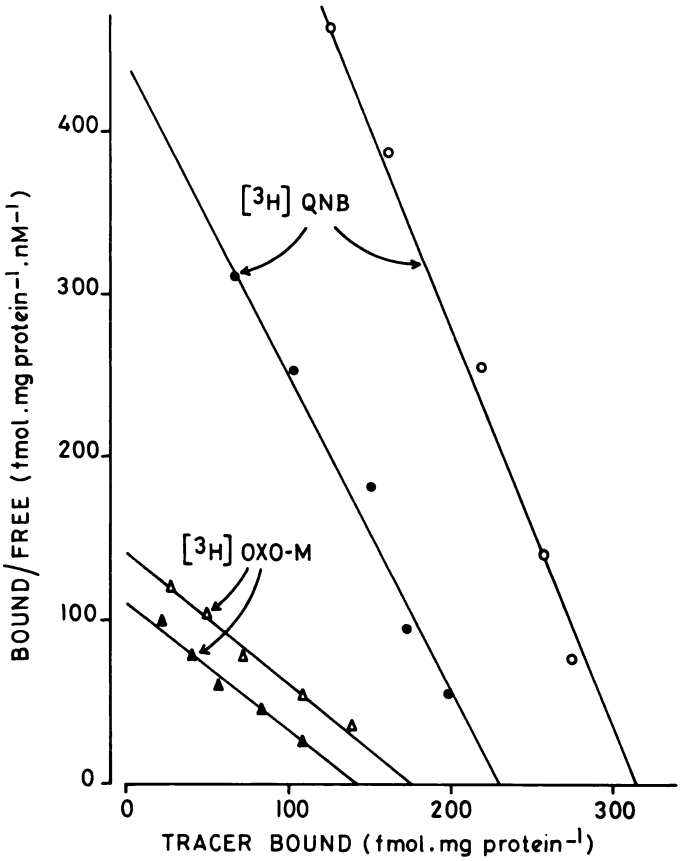

FIG. 1. *Scatchard analysis of L-[^{3}H]QNB and [^{3}H]Oxo-M binding to cardiac membranes from control (open symbols) and hyperthyroid (closed symbols) rats*

The binding data were obtained as described under Materials and Methods. Each point represents the mean of duplicate determinations from six animals.

TABLE 2

Effects of hypothyroidism, hyperthyroidism, and aortic stenosis on the dissociation constant (K_d) and receptor density (B_{max}) for L-[^{3}H]QNB and [^{3}H]Oxo-M in rat cardiac membranes

The K_d and B_{max} values were derived from Scatchard analysis as described in Figs. 1–3 and were the means ± standard error of the mean from six animals.

Treatment	L-[^{3}H]QNB		[^{3}H]Oxo-M	
	K_d	B_{max}	K_d	B_{max}
	nM	*fmoles/mg protein*	*nM*	*fmoles/mg protein*
Control	0.76 ± 0.02	250 ± 15	1.60 ± 0.12	100 ± 8
Hypothyroid	0.75 ± 0.03	260 ± 23	1.62 ± 0.13	160 ± 12[a]
Control	0.43 ± 0.02	312 ± 25	1.21 ± 0.08	170 ± 10
Hyperthyroid	0.48 ± 0.02	230 ± 15[a]	1.20 ± 0.10	140 ± 12[a]
Control	0.53 ± 0.03	315 ± 18	1.40 ± 0.10	162 ± 9
Aortic stenosis	0.55 ± 0.04	242 ± 13[a]	1.42 ± 0.12	150 ± 12

[a]Values significantly different ($p < 0.025$) from corresponding controls.

In cardiac membranes from PTU-treated hypothyroid rats, the total number of muscarinic receptors was unchanged but the number of high-affinity agonist binding sites increased by 60% and the number of low-affinity agonist binding sites was decreased by 30%. The affinity for L-[^{3}H]QNB and [^{3}H]Oxo-M was not modified (Fig. 2 and Table 2).

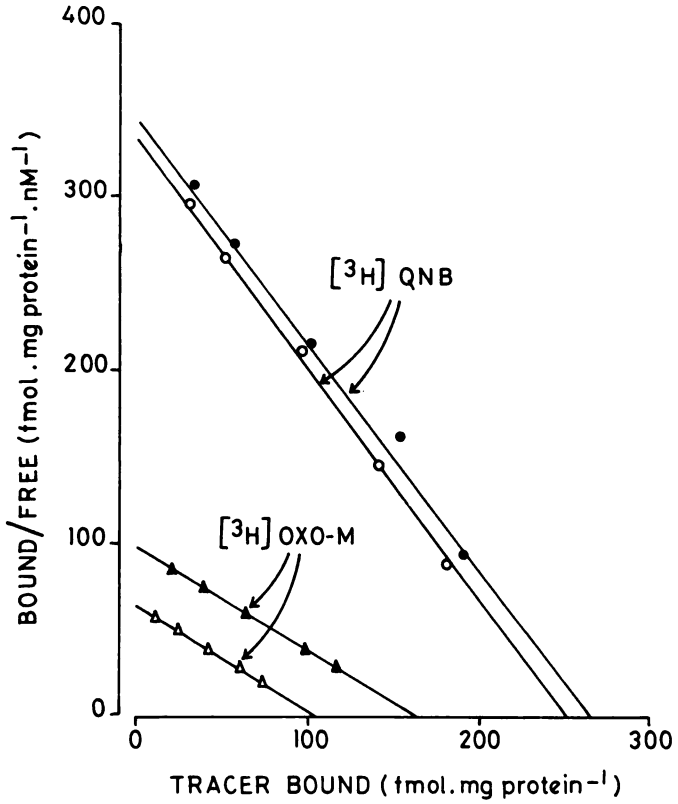

FIG. 2. *Scatchard analysis of L-[^{3}H]QNB and [^{3}H]Oxo-M binding to cardiac membranes from control (open symbols) and hypothyroid (closed symbols) rats*

The binding data were obtained as described under Materials and Methods. Each point represents the mean of duplicate determinations from six animals.

In rats with aortic stenosis and resulting heart hypertrophy (+22%; Table 1), the number of high-affinity agonist binding sites was unchanged but the number of low-affinity agonist binding sites and the total number of muscarinic receptors decreased by 40% and 23%, respectively, when compared with cardiac membranes from sham-operated rats. Tracer affinities were identical in both groups (Fig. 3 and Table 2).

Finally, when comparing cardiac membranes among control groups (Table 2), the number of high-affinity agonist binding sites was reduced by 65%, the number of low-affinity agonist binding sites was unchanged, and the total number of muscarinic receptors was reduced by 25% in control rats from hypothyroid animals as compared with the corresponding values in the two other control groups. The affinity for L-[^{3}H]QNB was also reduced in this control group. These differences were probably due to differences in the ages of the animals and/or to the prolonged intake of low amounts of dimethyl sulfoxide and ethanol; they emphasize the need for appropriate controls.

DISCUSSION

The total number of muscarinic receptors in rat heart membranes was decreased in hyperthyroidism and un-

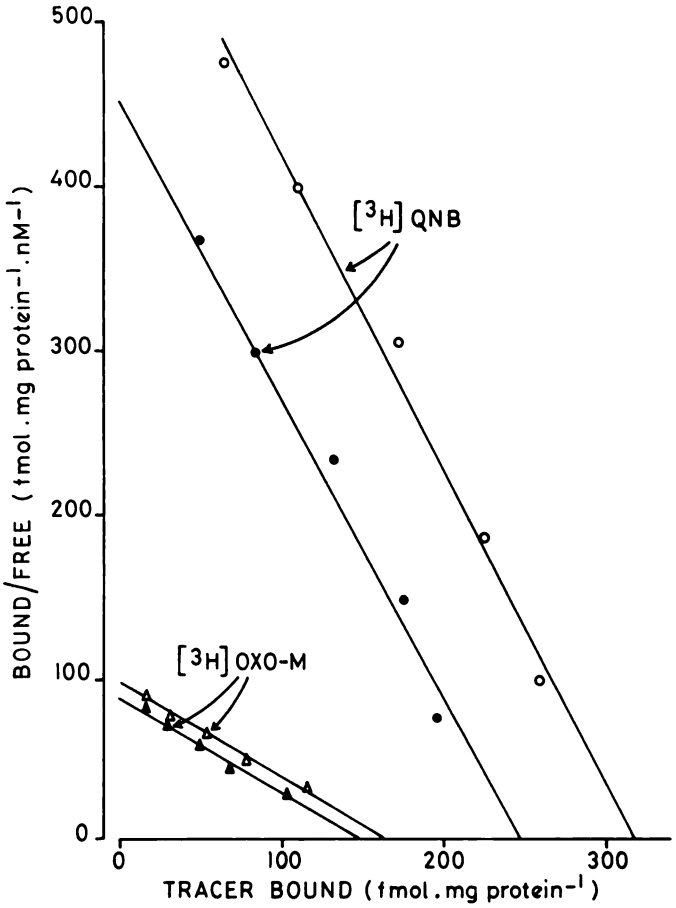

FIG. 3. *Scatchard analysis of L-[^{3}H]QNB and [^{3}H]Oxo-M binding to cardiac membranes from control sham-operated rats (open symbols) and rats with aortic stenosis (closed symbols)*

The binding data were obtained as described under Materials and Methods. Each point represents the mean of duplicate determinations from six animals.

changed in hypothyroidism. The reduced concentration of muscarinic receptors in cardiac membranes from hyperthyroid rats is paralleled by a reduced negative chronotropic response of the heart to vagal stimulation, which has been attributed to a desensitization toward acetylcholine (8, 9). Sharma and Banerjee (3) have previously observed that the total number of cardiac muscarinic receptors is decreased by 20% in hyperthyroid rats, in line with the present data, but they also reported an increased number of muscarinic receptors (+58%) in thyroidectomized rats. The discrepancy with our results in hypothyroid rats may be due to differences in the protocol used to induce hypothyroidism: the thyroidectomized rats of Sharma and Banerjee were killed 2 months after surgery and showed marked cardiac hypotrophy (−35%, based on heart weight to body weight ratio).

In this report, we present evidence that the number of high-affinity binding sites is decreased in hyperthyroidism and increased in hypothyroidism, whereas the number of low-affinity binding sites is decreased in both cases. One possible explanation for these data is that the thyroid status directly regulates the number of high-affinity but not that of low-affinity muscarinic binding sites.

Changes in the density of high-affinity muscarinic receptors are paralleled by reciprocal changes in the number of *beta*-adrenoceptors, which increases markedly in hyperthyroidism (4, 10–12) and decreases moderately in hypothyroidism (4, 10, 13).

The reduced number of low-affinity muscarinic binding sites in both hyper- and hypothyroidism, an adaptation parallel to that of *alpha*-adrenoceptors (4, 10, 14, 15), must obviously be attributed to several factors. In hyperthyroidism, one factor responsible for the decreased number of low-affinity muscarinic binding sites may be cardiac hypertrophy rather than the level of thyroid hormone per se. Indeed, the concentration of low-affinity muscarinic receptors was also decreased in cardiac hypertrophy induced by aortic stenosis (whereas the number of high-affinity receptors did not change significantly). Conceivably, there may be a relative decrease in the rate of synthesis of low-affinity muscarinic receptors when the myocardium develops rapidly in situations such as hyperthyroidism or aortic stenosis. However, these results with different models of heart hypertrophy should be interpreted with caution, as the mechanisms by which cardiac hypertrophy affects a variety of parameters is not known. For instance, in cardiac hypertrophy developing rapidly after aortic stenosis, the decreased number of low-affinity muscarinic receptors is accompanied by an increase in the number of *beta*-adrenoceptors [due probably to a reduced content in catecholamines (16)] and an increased number of $alpha_1$-adrenoceptors when chronic heart failure occurs [at variance with the situation prevailing in hyperthyroidism (17)]. By contrast, in congenitally hypertensive (SHR) rats, in which cardiac hypertrophy develops more progressively than in hyperthyroidism or after aortic stenosis, the total number of muscarinic receptors is unchanged (18) and the number of *beta*-adrenoceptors is decreased (18, 19).

Competition curves with muscarinic agonists indicate the existence of two or more classes of binding sites (20–28). It is still uncertain whether these sites are distinct entities or are reversibly interconvertible (1, 27, 29). In this respect, we cannot exclude the possibility of a conversion of low- to high-affinity binding sites in heart membranes from hypothyroid rats. However, the results obtained in hyperthyroidism and with aortic stenosis support the opposite hypothesis that the two classes of binding sites are likely to be distinct, independently regulated, entities.

If in rat heart, as in embryonic chick heart (30), the low-affinity receptors are coupled to the bradycardic effects of muscarinic agents, we would expect hypothyroidism and cardiac hypertrophy to induce a decreased sensitivity of the rats to muscarinic agents. Models such as those presented in this work should also allow identification of the class of receptors which inhibits the adenylate cyclase and possibly confirmation of the hypothesis that the different classes of muscarinic receptors are coupled to different effectors (27).

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