Biochemical Pharmacology, Vol. 35, No. 18, pp. 3151–3157, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

MUSCARINIC RECEPTOR SUBTYPES IN BOVINE ADRENAL MEDULLA

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(*Received* 26 *June* 1985; *accepted* 10 *February* 1986)

Abstract—Muscarinic receptors in bovine adrenal medullary microsomes were characterized by radioligand binding assay, using *l*-[^{3}H]quinuclidinyl benzilate (QNB), a muscarinic antagonist. Specific [^{3}H]QNB binding to microsomes was rapid, reversible, saturable and of high affinity. Saturation experiments revealed a single class of binding sites for the radioligand with a maximum number of binding sites and an apparent dissociation constant of 162.6 fmoles/mg protein and 40.3 pM respectively. According to computer-assisted nonlinear regression analysis, however, drug/[^{3}H]QNB competition curves indicated the presence of at least two affinity sites for muscarinic agonists (acetylcholine, carbamylcholine, oxotremorine), with a high (K_1) and a low (K_2) affinity (e.g. K_1 = 664.8 nM and K_2 = 36.5 μM for acetylcholine). The two affinity sites for acetylcholine showed only minimal regulation by magnesium and guanosine 5′-triphosphate. Furthermore, the presence of two affinity sites was suggested for the antagonists pirenzepine and gallamine, but not for atropine and pilocarpine. The K_1 and K_2 values for pirenzepine were 23.7 and 429 nM, respectively, with 54.5% of total sites having a high affinity. These results indicate that at least two distinct subtypes of muscarinic receptors exist in the bovine adrenal medulla and that they are distinguished by their relative binding affinity for muscarinic agonists and antagonists. The receptors are predominantly composed of the affinity state termed M_1, as described for the receptors of sympathetic ganglia.

The concept of heterogeneity of muscarinic receptors has been well established in receptor binding studies [1–5]. In many tissues, there are at least two affinity binding sites for muscarinic agonists, with a high and a low affinity [1, 3–7]. The distinct affinity subtypes were presumed to interact with different biochemical events within the cells [6–8].

Furthermore, muscarinic receptors are subclassified into M_1 and M_2 subtypes, according to the respective high and low affinity for a new muscarinic antagonist pirenzepine [9–11]. The M_1 receptors predominantly distribute in the sympathetic ganglia and cerebral cortex, whereas the M_2 receptors distribute in the heart, pons-medulla and ileum. Currently, the classification of muscarinic receptors is based on the affinity of agonists and the unique antagonist pirenzepine.

Recent studies have demonstrated the occurrence of functional muscarinic receptors, as well as nicotinic receptors, in the chromaffin cells of bovine adrenal medulla [12–16]. Muscarinic agonists enhance formation of cyclic GMP† [12–15] and phosphatidylinositol (PI) turnover [16] in the chromaffin cells, and these responses were specifically blocked by atropine. The muscarinic agonists failed to stimulate but rather reduced catecholamine release from the cells [15], suggesting that muscarinic receptors on the chromaffin cells may have an inhibitory role for stimulus–secretion coupling. This idea is supported by the evidence [17] that muscarinic agonists reduce catecholamine secretion induced by nicotine in perfused bovine adrenal glands.

However, characteristics of muscarinic receptors are poorly understood, although there is a report [18] of a small number of muscarinic receptors in the bovine adrenal medulla.

The current study was an attempt to clarify the properties of muscarinic receptors in the bovine adrenal medulla, based on the affinity of muscarinic agents. Our binding assays indicate the presence of relatively dense muscarinic receptors in the adrenal medullary microsomes and also the presence of distinct affinity subtypes of the receptors for several muscarinic agents.

METHODS

Tissue preparation. Fresh bovine adrenal glands obtained at a local slaughterhouse were kept on ice during transport and used within 1–2 hr postmortem. Subcellular fractionation of the bovine adrenal medulla was performed as described [19], with minor modifications. Briefly, the medullary tissue (5–10 g) was dissected free from the cortex and homogenized in 10 vol. of ice-cold 0.25 M sucrose containing 5 mM Tris–HCl (pH 7.5, at 25°), using a Potter–Elvehjem glass homogenizer with a loose-

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† Abbreviations: cyclic GMP, cyclic guanosine 3′,5′-monophosphate; PI, phosphatidylinositol; QNB, *l*-quinuclidinyl benzilate; B_{max}, maximum number of binding sites; K_D, apparent dissociation constant; n_H, Hill coefficient; K_a, association constant; K_d, dissociation constant; IC_{50}, effective dose producing half-maximal inhibition of [^{3}H]QNB binding; K_1, equilibrium dissociation constant at high-affinity site; K_2, equilibrium dissociation constant at low-affinity site; R_1, percentage of high-affinity site; pK_1 and pK_2, values of negative logarithm of K_1 and K_2 values respectively; and GTP, guanosine 5′-triphosphate.

fitting Teflon pestle. The homogenate was filtered through cheesecloth and centrifuged at 1,000 *g* for 10 min and at 22,600 *g* for 20 min, consecutively. The pellet contained exclusively mitochondria and chromaffin granules. The supernatant fraction was subjected to further centrifugation at 160,500 *g* for 30 min. The resultant pellet (which contained purified microsomes) was resuspended in original volumes of 50 mM Na^+/K^+ phosphate buffer (pH 7.4), by homogenization using a Kinematica Polytron PT-10 (setting at 6.0 for 10 sec), and recentrifuged at 160,500 *g* for 30 min. The final pellet was resuspended in 2.5 vol. (as tissue wet weight) of 50 mM Na^+/K^+ phosphate buffer and used for the receptor assays.

Binding assays. Muscarinic receptor binding assays were performed according to Yamamura and Snyder [20] with minor modifications [21]. For saturation studies, the microsomal fraction (80–180 μg protein) was incubated at 37° for 60 min with twelve different concentrations of *l*-[^{3}H]quinuclidinyl benzilate (QNB, 10–900 pM), in a final volume of 2 ml Na^+/K^+ phosphate buffer (pH 7.4). For drug competition studies, the microsomes (receptor concentration: 11.8 ± 0.4 fmoles in a final 2-ml volume) were incubated with a fixed concentration of the radioligand (73.0 ± 1.3 pM) and various concentrations (14–23 points) of a displacer. When acetylcholine was used as a displacer, the assay was done in the presence of physostigmine (1 μM) in order to inhibit acetylcholinesterase activity present in the microsomes. After 60 min of incubation, the binding was terminated by rapid filtration under reduced pressure through Whatman GF/B filters, followed by 4 × 4 ml rinses with ice-cold buffer. The filters were transferred into scintillation vials and dried overnight. The radioactivity retained on the filters was extracted with 8 ml of scintillation fluid and counted in a Tri-Carb scintillation spectrometer at efficiencies of 32–36%. The specific [^{3}H]QNB binding was taken as the difference between measurements in the absence and in the presence of 1 μM atropine. Nonspecific binding represented less than 10% of the total binding at concentrations up to 500 pM of the radioligand and 11–17% at higher concentrations examined. In the assays for estimating association rate (on-rate), the microsomal fraction (receptor concentration: 12.3 ± 1.1 fmoles in a final 2-ml volume) was incubated with a fixed concentration of [^{3}H]QNB (79.3 ± 2.9 pM) at 37° for various periods, and specific binding at each time was estimated. In the assays done to estimate the dissociation rate (off-rate), 1 μM atropine was added to the assay tube after the microsomes had been incubated with a single dose of [^{3}H]QNB for 60 min, and specific binding at various time intervals, thereafter, was measured. All assays were done in duplicate or triplicate. The determination of protein was performed by the method of Lowry *et al.* [22], using bovine serum albumin as a standard.

Data analysis. Saturation isotherms, and on-rate and off-rate curves were analyzed according to the methods described by Bennet [23]. Such analyses gave a maximum number of binding sites (B_{max}), the apparent dissociation constant (K_D), the Hill coefficient (n_H), and rate constants (K_a, K_d) respectively. The drug competition curves were analyzed according to the method of Weiland and Molinoff [24], and IC_{50} values (drug concentrations that produce 50% inhibition of specific ligand binding) and n_H values were estimated. Since competition curves of several agents exhibited heterogeneity (see Results) suggesting the presence of multiple binding sites (or states), the data were subjected to computerized nonlinear regression analysis to determine whether the curves would fit a one-site or two-site model. The program used for this analysis was the LIGAND system developed by Munson and Rodbard [25]. The data from three separate experiments for one agent were combined and analyzed in one simultaneous fit. The affinity of [^{3}H]QNB for the receptors was set constant to the mean value determined from four saturation experiments. This analysis gave equilibrium association constants for drug binding and populations of different affinity sites. The results, therefore, were expressed as transformed equilibrium dissociation constants (K_1, K_2) for each agent and percentage of the high-affinity site (R_1). Statistical evaluation of the fit between the one-site and two-site model was performed by a partial F-test, as described [25], using residual variances for each model between the predicted and actuai data points.

Materials. [^{3}H]QNB (33.2 Ci/mmole) was purchased from New England Nuclear (Boston, MA, U.S.A.), and the following agents used were obtained from commercial sources: atropine sulfate, hexamethonium chloride, nicotine bitartrate, and pilocarpine hydrochloride (Nakarai Chemicals Ltd., Kyoto, Japan); carbamylcholine chloride, gallamine triethiodide, and oxotremorine sesquifumarate (Sigma Chemical Co., St. Louis, MO, U.S.A.); and acetylcholine chloride (Daiichi Seiyaku, Co. Ltd., Tokyo, Japan). Pirenzepine dihydrochloride was donated by the Japan Boehringer Ingelheim Co. Ltd. (Hyogo, Japan).

RESULTS

Comparison of [^{3}H]QNB binding between granule and microsomal fractions. In a report by Kayaalp and Neff [18], specific [^{3}H]QNB binding was assayed using a membrane pellet obtained by centrifugation of the bovine adrenal medullary homogenate at 13,000 *g*. According to biochemical studies [19, 26, 27], this membrane fraction contains exclusively chromaffin granules and mitochondria (granule fraction). Plasma membranes of chromaffin cells were concentrated in the microsomal fraction obtained by centrifugation at a higher g-force [19, 26]. We first compared the specific binding of [^{3}H]QNB between granule and microsomal fractions and found that the specific [^{3}H]QNB (82.1 ± 0.8 pM) binding to microsomes was about 7-fold (69.8 ± 14.9 fmoles/mg protein, N = 3) over that in the granule fraction (10.8 ± 3.3 fmoles/mg protein, N = 3). Accordingly, adrenal medullary microsomes were used for the following receptor assays.

Kinetics of specific [^{3}H]QNB binding to microsomal fraction. Figure 1 shows representative on- and off-rate curves of [^{3}H]QNB binding to the microsomal fraction. The ligand binding rapidly increased,

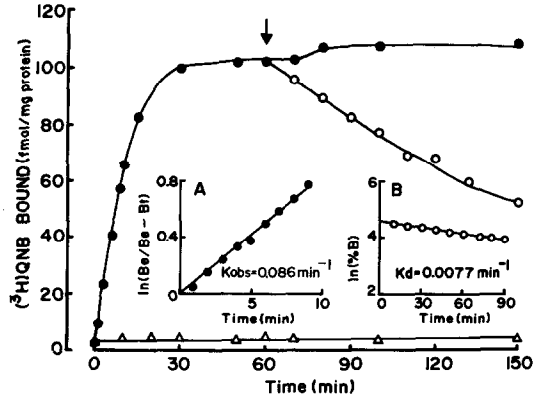

Fig. 1. Representative on- and off-rate curves of [^{3}H]QNB binding to bovine adrenal medullary microsomes. The kinetic experiments were performed as described in Methods. The final concentration of [^{3}H]QNB was 79.3 pM. The ordinate shows the amount of total binding, and the abscissa shows the time after initiation of incubation. The arrow represents the addition of 1 μM atropine to the assay medium following 60 min of incubation. Key: (●) total binding; (△) nonspecific binding; and (○) total binding after the addition of atropine. Insets A and B show the transformed on-rate and off-rate plots in detail as a function of time respectively. Since the K_a value was calculated to be $9.49 \times 10^8\ M^{-1}\ min^{-1}$, according to the equation $K_a = (K_{obs} - K_d)/[^3H]QNB$, the apparent K_D value was 8.13 pM by the ratio of K_d/K_a.

time-dependently, and reached a steady state by 30–40 min. The plateau level was maintained for an additional 30 min, but the binding, thereafter, increased slightly with no change in the nonspecific binding (defined by the presence of 1 μM atropine). The mean on-rate constant (K_a) estimated from three separate experiments was 8.98 ± 0.77 ($\times 10^8$) $M^{-1}\ min^{-1}$. After the achievement of equilibrium binding following 60 min of incubation, addition of 1 μM atropine to the assay medium dissociated the bound radioligand, time-dependently. The mean off-rate constant (K_d) estimated from three separate experiments was $0.0077 \pm 0.0006\ min^{-1}$ and the time ($T_{\frac{1}{2}}$) required for 50% dissociation was 90.9 ± 7.3 min. Accordingly, the K_D value was calculated to be 8.4 ± 0.5 pM, by the ratio of K_d/K_a.

Figure 2 shows a saturation isotherm and transformed Scatchard plot of specific [^{3}H]QNB binding from a representative experiment. [^{3}H]QNB bound to a single population of sites, as evidenced by an n_H value of unity. The mean K_D, B_{max} and n_H values from four separate experiments were 40.3 ± 4.6 pM, 162.6 ± 25.9 fmoles/mg protein and 1.02 ± 0.01 respectively.

Inhibition by muscarinic agents of specific [^{3}H]QNB binding to microsomal fraction. The specific ligand binding (73 pM) was inhibited by the presence of muscarinic agents, in a dose-dependent manner (Fig. 3). Most of the agents produced complete inhibition whereas the inhibition by gallamine was incomplete. The rank order of agonist potency based on their IC_{50} values (Table 1) was oxotremorine (956 nM) > acetylcholine (6.94 μM) > pilocarpine (7.33 μM) > carbamylcholine (73.4 μM), and the order of antagonist potency was atropine (1.3 nM) > pirenzepine (152 nM) > gallamine (4.63 μM). Nicotine and hexamethonium also competed with [^{3}H]QNB but the IC_{50} values were 31.7- (219.0 μM) and 36.4- (251.1 μM) fold greater than the value of acetylcholine, respectively, thereby indicating the muscarinic nature of the [^{3}H]QNB binding sites. The following agents (300 μM), which act on other types of receptors, produced only 10–30% reduction of the ligand binding: epinephrine, *l*-norepinephrine, histamine, isoproterenol, dopamine, γ-amino-*n*-butyric acid, phenylephrine and 5-hydroxytryptamine.

As seen in Fig. 3A, all competition curves of muscarinic agonists (except pilocarpine) were shallow with n_H values of less than one (Table 1). The curves of pirenzepine and gallamine also exhibited a heterogeneous manner deviating from a law of simple mass action, and n_H values were 0.65 and 0.52 respectively (Fig. 3B, Table 1).

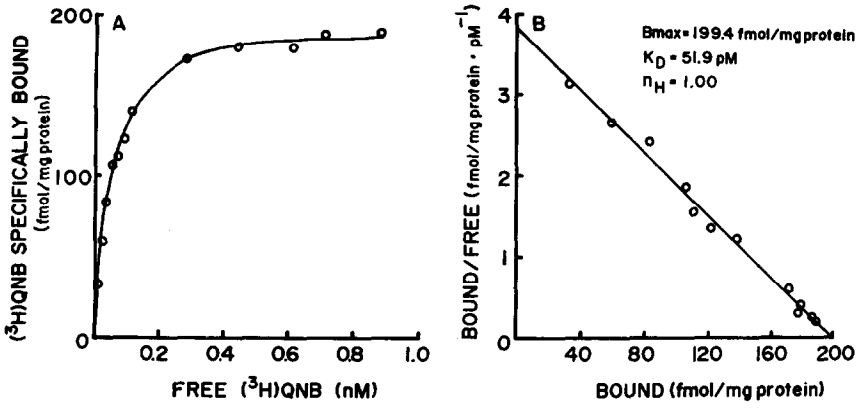

Fig. 2. Representative saturation isotherm (A) and transformed Scatchard plot (B) of specific [^{3}H]QNB binding to bovine adrenal medullary microsomes. The saturation experiment was performed as described in Methods. The B_{max} and K_D values were 199.4 fmoles/mg protein and 51.9 pM, respectively. The Hill analysis of the data gave a coefficient (n_H) of unity.

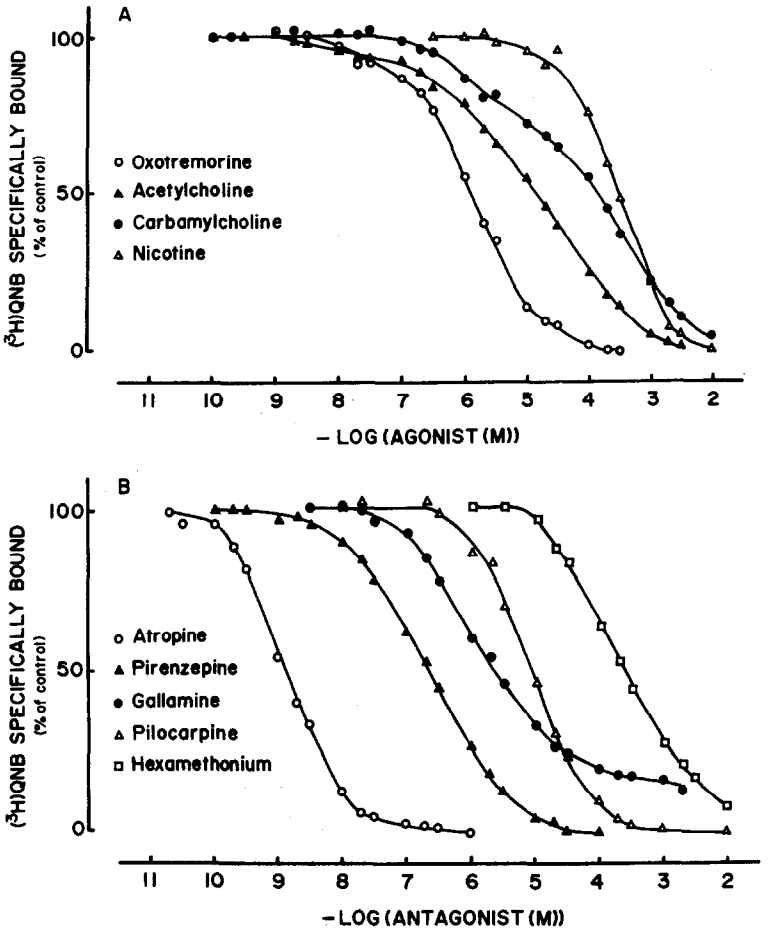

Fig. 3. Competition curves of cholinergic agonists (A) and antagonists (B) against specific [^{3}H]QNB binding to bovine adrenal medullary microsomes. The drug competition experiments were performed as described in Methods. The final concentration of [^{3}H]QNB was fixed at 73.0 ± 1.3 pM and the receptor concentration was 11.8 ± 0.4 fmoles in a 2-ml assay volume. Pilocarpine was included in antagonists in order to avoid the overlap between agonist curves. Each point represents the mean value from three separate experiments.

Existence of two affinity sites for muscarinic agonists, pirenzepine and gallamine. In general, shallow competition curves are indicative of the presence of more than one binding site (or states) for a displacer at radioligand binding sites [1, 3–5]. The curves, therefore, were analyzed by computer-aided curve fitting to determine whether the data fit a one-site or two-site model. Table 2 shows the equilibrium dissociation constants (K_1 and K_2 values) and populations of the binding sites (R_1 value) for agonists and antagonists. The values were estimated from the data in Fig. 3 and Table 1.

Table 1. IC_{50} Values and Hill coefficients for the inhibition of cholinergic agonists and antagonists of specific [^{3}H]QNB binding to bovine adrenal medullary microsomes

Drug	IC_{50} μM	n_H
Atropine	0.0013 ± 0.00015	0.99 ± 0.02
Pirenzepine	0.152 ± 0.044	0.65 ± 0.03
Oxotremorine	0.956 ± 0.273	0.79 ± 0.01
Gallamine	4.63 ± 1.33	0.52 ± 0.01
Acetylcholine	6.94 ± 1.98	0.48 ± 0.01
Pilocarpine	7.33 ± 0.43	0.92 ± 0.05
Carbamylcholine	73.4 ± 7.1	0.48 ± 0.04
Nicotine	219.0 ± 30.0	0.95 ± 0.03
Hexamethonium	251.1 ± 1.3	0.71 ± 0.03

Values represent the mean value ±S.E. from three separate experiments. The experiments with acetylcholine were performed in the presence of 1 μM physostigmine.

According to a partial F-test, the curves of the agonists (except pilocarpine) fit a two-site model ($P < 0.005$) better than a one-site model. As a result, the data were compatible with two populations of binding sites for agonists, a high- and a low-affinity site (Table 2). The K_1 (pK_1) value at the high-affinity site for acetylcholine was 664.8 nM (6.18) and the corresponding K_2 (pK_2) value at the low-affinity site was 36.5 μM (4.44), indicating that the ratio of K_2/K_1 was 54.9. Oxotremorine and carbamylcholine gave K_1 values of 49.2 and 611.9 nM, respectively, and these values were 21.6- and 289.6-fold lower than their K_2 values (1.06 and 177.2 μM) respectively. The number of high-affinity sites (R_1) was 49.7%,

Table 2. Equilibrium dissociation constants for muscarinic agonists and antagonists binding at high- and low-affinity sites, and percentage of the high-affinity site estimated from analysis of the competition curves

Drug	K_1 (nM)	R_1 (%)	K_2 (μM)	Ratio K_2/K_1
Oxotremorine	49.2	30.2	1.06	21.6
Acetylcholine	664.8	49.7	36.5	54.9
Carbamylcholine	611.9	35.8	177.2	289.6
Pirenzepine	23.7	54.5	0.429	18.1
Gallamine	221.1	71.9	5.94	26.9
Pilocarpine	3387.2	100		
Atropine	0.47	100		

The competition curves shown in Fig. 3 were analyzed by a computer-aided nonlinear regression technique, as described in Methods. The curves of muscarinic agents except atropine and pilocarpine significantly ($P < 0.005$) fit the two-site model better than the one-site model, according to a partial F-test [25]. The curves of atropine and pilocarpine best fit a one-site model. The binding constants at the high- and the low-affinity sites and percentage of the high-affinity site were expressed as K_1, K_2 and R_1 respectively.

30.2% and 35.8% of total sites for acetylcholine, oxotremorine and carbamylcholine respectively.

Furthermore, the curve of pirenzepine fit a two-site model ($P < 0.005$). The estimated K_1 and K_2 values were 23.7 and 429 nM, respectively, indicating an 18.1-fold difference between the two affinities. The high-affinity sites occupied 54.5% of the total sites. Gallamine also exhibited two affinity sites with 221.1 nM (K_1) and 5.94 μM (K_2), respectively, and 71.9% of the receptors represented high affinity.

In contrast, the curves of pilocarpine and atropine best fit a one-site model, and the K_1 (pK_1) values were 3.38 μM (5.47) and 0.47 nM (9.33) respectively.

Influence of magnesium and GTP on the inhibition by acetylcholine of specific [^{3}H]QNB binding to microsomal fraction. Usually, muscarinic receptors in various tissues can be regulated by magnesium and guanine nucleotides, which respectively produce an increase and a decrease of the agonist affinity [4, 5, 28]. Such regulation was therefore tested in the present assays.

There were no significant changes in the acetylcholine/[^{3}H]QNB competition curve in the presence of magnesium (5 mM), as compared with the curve shown in Fig. 3A. The IC_{50} and n_H values gave 5.22 ± 1.34 μM (N = 3) and 0.47 ± 0.02 in the presence of magnesium respectively. Further addition of guanosine 5′-triphosphate (GTP, 300 μM) to the assay medium produced only a minimal shift of the curve to the right. The IC_{50} and n_H values were 13.6 ± 9.5 μM (N = 3) and 0.47 ± 0.05 respectively. Magnesium and GTP had no effects on the specific binding of [^{3}H]QNB (78.0 ± 1.2 pM).

DISCUSSION

The present study demonstrates that [^{3}H]QNB binding to adrenal medullary microsomes was rapid, reversible, saturable and of high affinity. Saturation experiments revealed the presence of a single population of binding sites for [^{3}H]QNB with high affinity, in accordance with a report by Kayaalp and Neff [18]. However, we found about a 50-fold larger number of binding sites (B_{max} = 162.6 fmoles/mg protein), as compared with the number (B_{max} = 3.1 fmoles/mg protein) shown by those authors. This difference is probably due to the different membrane fractions used, that is, granule [18] and microsomal fractions (present study).

The K_D value of [^{3}H]QNB binding estimated from on- and off-rate constants was about five times smaller than the value estimated from saturation experiments. This seems to be the result of an underestimation of the off-rate constant, since incubation for more than 90 min increased the [^{3}H]QNB binding. The reason for the increased binding is unclear, but it may reflect an uptake of the radioligand by plasma membrane vesicle. Alternatively, the muscarinic receptor may undergo an increase in affinity for [^{3}H]QNB, on equilibration [29]. Nevertheless, [^{3}H]QNB binding sites were characteristic of muscarinic receptors, as evidenced by the low potency of different receptor agents in displacing [^{3}H]QNB binding.

In drug/[^{3}H]QNB competition experiments, muscarinic agonists (except pilocarpine) exhibited competition curves deviating from the simple mass action law (Hill coefficients of less than one), indicating heterogeneous interactions with [^{3}H]QNB binding sites. The computer-aided analysis revealed the presence of at least two affinity sites for agonists with a high and a low affinity. The K_1 and K_2 values for acetylcholine were 664.8 nM and 36.5 μM respectively. Similarly, oxotremorine and carbamylcholine exhibited K_1 values less than micromoles at the high-affinity site and K_2 values exceeding micromoles at the low-affinity site. Interestingly, there are at least two muscarinic responses in the adrenal chromaffin cells; that is, enhancement of cyclic GMP formation [12–15] and of PI turnover [16] respectively. The effective concentration (EC_{50}) of acetylcholine producing half-maximal cyclic GMP response is in the range of 100–200 nM [12, 13, 15]. On the other hand, PI turnover seems to be induced by higher concentrations of agonists, such as 300 μM [16]. Thus, the two binding affinities are roughly comparable to the effective doses for the two muscarinic responses. This suggests that the high- and the low-affinity sites may correspond to the distinct receptors linked to cyclic GMP formation and PI turnover respectively. The coupling of the low-affinity site of muscarinic receptors with PI turnover was suggested in guinea pig and rat synaptosomes [6]. Nevertheless, it is still unclear whether the two muscarinic responses are the result of occupation by the agonists of each affinity site or of both affinity sites, since many of the same muscarinic receptors that elicit cyclic GMP formation also cause PI turnover. The two responses may be interrelated.

There is a proposal [3, 6] that partial agonists exhibit lower ratios of K_2/K_1, as compared with the ratio of full agonists. In our study, oxotremorine showed a ratio of 21.6, which is much smaller than the ratios of acetylcholine (54.9) and carbamylcholine (289.6). This indicates that oxotremorine may be a partial agonist in the bovine adrenal

medulla. The partial agonist activity of oxotremorine in eliciting cyclic GMP formation and PI turnover has been noted in guinea pig and rat brain synaptosomes [6], murine neuroblastoma clone [8] and human astrocytoma (1321N1) cells [30].

By contrast, pilocarpine failed to discriminate between two affinity sites and exhibited a homogeneous competition curve (Hill coefficient close to unity). This agent is inactive in eliciting the cyclic GMP response in isolated chromaffin cells [15]. Interestingly, the antagonistic nature of pilocarpine was noted in a murine neuroblastoma clone [8], where pilocarpine antagonized cyclic GMP formation and PI turnover in response to muscarinic agonists, and it exhibited a homogeneous competition curve against [^{3}H]*N*-methylscopolamine binding. Based on such evidence, pilocarpine may be a specific antagonist against muscarinic receptors coupled to the cyclic GMP formation and PI turnover, even though it is an agonist toward the receptors inversely coupled to the adenylate cyclase [8, 31]. However, further studies are necessary to clarify the possibility in the bovine adrenal medulla.

Muscarinic receptors are classified into M_1 and M_2 subtypes based on the affinity of pirenzepine [9–11]. This antagonist exhibits higher affinity ($K_D \sim 20$ nM) toward M_1 receptors in the sympathetic ganglia and cerebral cortex, but exhibits a lower affinity ($K_D \sim 1\ \mu$M) toward M_2 receptors in the heart and ileum. The agonist affinity toward the M_2 receptor is increased by magnesium, and the enhanced agonist affinity is decreased by guanine nucleotides, whereas the affinity toward the M_1 receptor is minimally affected by these modulators [28]. Our findings of the presence of high-affinity sites for pirenzepine with a K_I value of 23.7 nM and of minimal guanine regulation of the receptors in the presence of magnesium indicate that the bovine adrenal medulla contains the M_1 receptor. The low-affinity site with a K_2 value of 429 nM in the adrenal medulla is comparable to the second site (K_2 280 nM) in the sympathetic ganglia [10]. Therefore, muscarinic receptors in the bovine adrenal medulla may have properties resembling receptors of the sympathetic ganglia.

The heterogeneity of M_1 and M_2 subtypes is suggested to reflect different conformational states of receptors restrained by coupling with other membrane components (or effectors) [32]. Therefore, muscarinic receptors in the bovine adrenal medulla may be composed predominantly of a high-affinity state for pirenzepine termed M_1, since the high-affinity site represented 54.5% of the total sites.

Gallamine may act on an allosteric site distinct from the recognition site for muscarinic agents and prevent the binding of the agonists and antagonists in the heart [33]. The allosteric inhibition by gallamine of [^{3}H]QNB binding was also observed in our assay, as seen by an incomplete inhibition of the specific ligand binding. Nevertheless, two affinity sites for gallamine were suggested by the nonlinear regression analysis. Two affinity sites for this antagonist have been reported in the rat forebrain and brain stem [34]. The high-affinity site of the adrenal medulla exhibited a similar affinity (K_1 221.1 nM) to the high-affinity site in the rat forebrain (K_H 150 nM) where M_1 receptors are predominant. This evidence may be consistent with the idea that the adrenal medulla belongs to the tissues of M_1-subtype, although the physiological significance of the gallamine site is unknown.

In conclusion, we have demonstrated that: (1) relatively dense muscarinic receptors are present in the bovine adrenal medulla; (2) the receptors consist of at least two distinct affinity subtypes for muscarinic agonists and antagonists; and (3) the receptors are predominantly composed of M_1-subtype resembling receptors of the sympathetic ganglia. Further studies will clarify localization of the receptor subtypes in the adrenal medulla and relations between agonist and antagonist affinity subtypes. Muscarinic receptors in the bovine adrenal medulla appear to be inhibitory for catecholamine secretion [15, 17] while the receptors in smaller animals are stimulatory [35–39]. A comparison of the muscarinic receptor properties among different species may provide further insight into relations between receptor heterogeneity and species differences in the role of the receptor for adrenal catecholamine secretion.

Acknowledgements—This work was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. We thank Dr. Peter J. Munson for the supply of the computer program, LIGAND, for the Apple II microcomputer, M. Ohara of Kyushu University for comments on the manuscript, and Ms. Naomi Yamada for secretarial services.

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