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CHOLINERGIC MUSCARINIC RECEPTORS IN BOVINE ADRENAL CORTEX

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SUMMARY

Crude membrane preparations from bovine adrenocortical tissue were shown to exhibit high affinity (Kd = 1.2 ± 0.25 nM) and limited capacity (Bsp = 67.2 ± 5.7 pmole ligand/g protein) binding sites for the muscarinic antagonist L-quinuclidinyl benzylate. Competitive binding experiments confirmed that the binding activity had the characteristic of a cholinergic receptor of the muscarinic type. These findings which cannot be explained by a contamination from adrenal medullary tissue suggest that a cholinergic mechanism (muscarinic type) may be considered in the modulation of adrenocortical functions.

Acetylcholine (Ach) released from the terminals of the splanchnic nerve is a well established positive modulator of catecholamine production by adrenal medulla (1-3). On the other hand, possible cholinergic influence on adrenal cortex secretory functions has been suggested from perfusion experiments (4) but clear cut evidence is lacking. More recently, nicotine has been shown to stimulate steroidogenesis when added to adrenocortical cell suspensions of felin origin (5). Similar observation has been made in this laboratory with acetylcholine and zona fasciculata cell suspensions from bovine adrenal cortex. This steroidogenic effect appeared to be of a muscarinic type, suggesting the existence of such receptors in the tissue (unpublished).

This report demonstrates the presence of specific muscarinic binding sites in bovine adrenal cortex and characterizes their interaction with cholinergic agents.

EXPERIMENTAL PROCEDURES

Drugs and chemicals. Tritium labeled muscarinic antagonist 1-Quinuclidinyl [phenyl 4-^{3}H] benzilate ([^{3}H] QNB) (43 Ci/mmol) was obtained from the Radio-

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chemical Centre, AMERSHAM; its purity was checked by thin layer chromatography (6). Unlabeled dl-QNB was a gift from Dr. H. Gutman and Dr. V. Solms (Hoffmann-La Roche - Basel - Switzerland). Dexetimide and levetimide were a gift from E. Richelson (Mayo Clinic - Rochester - USA) and P. Laduron (Janssen Pharmaceutica - Beerse - Belgium). Choline, acetylcholine, acetyl β methylcholine, dl-muscarine, pilocarpine, nicotine, hexamethonium, d-tubocurarine, scopolamine, dl-atropine, histamine, 5-hydroxytryptamine (serotonin) and eserine (physostigmine) were obtained from Serva Feinbiochemica. Carbamylcholine, procaine, tetracaine, epinephrine, norepinephrine and mecamylamine were purchased from Sigma Chemical Co. Oxotremorine and dimethylpiperazinium were from Aldrich. Sodium acetate, toluene, Triton X100 were obtained from Merck and permablend from Packard Co.

Crude membrane preparations. Adrenals from adult animals were obtained within 20 minutes after slaughter; the glands were freed of extraneous tissue and processed between 0-4°C. Each gland was bisected longitudinally and the medulla carefully removed. Scraped adrenal cortex tissue was extensively washed and homogenized (Potter homogenizer with a teflon pestle) in 10 volumes (W/V) of ice cold 50 mM, pH 7.4 sodium-potassium phosphate buffer containing 0.32 M sucrose. The homogenate was centrifuged at 1,000 x g for 10 min, the pellet discarded and the supernatant centrifuged at 17,500 x g for 20 min. The second pellet (crude membrane fraction) was resuspended in ice cold 50 mM, pH 7.4 sodium-potassium phosphate buffer then homogenized with a Dounce homogenizer and diluted at appropriate protein concentration for binding assay in the same buffer.

Binding assay. Incubations were carried out in a total volume of 1 ml in plastic tubes at 37°C, under continuous agitation for 45 min. Each incubation tube contained 1 nM of [^{3}H] QNB and 0.3 to 0.6 mg membrane protein/ml in 50 mM, pH 7.4 Na/K-phosphate buffer. An identical series of tubes received a 100 fold excess of unlabeled QNB. Radioactivity bound to membranes was retained on glass fiber GF/F filters (Whatman) by filtration under vacuum. The filters were washed four times with 5 ml of ice cold 50 mM Na/K-phosphate buffer, pH 7.4 and extracted by overnight agitation in 15 ml of Triton X100-Toluene-permablend mixture (333 ml, 667 ml and 5.5 g per liter). Radioactivity was assayed in a liquid scintillation spectrometer (Intertechnique SL30) at a counting efficiency of 40%. Specific [^{3}H] QNB binding was calculated by substracting the radioactivity bound in the presence of unlabeled QNB from that bound when [^{3}H] QNB only was present. In preliminary experiments specific binding was found to be linear from 0.3 to 0.9 mg protein per ml, and reached an equilibrium after 15 min of incubation to remain stable for at least the next 45 min. All experiments were performed at least three times in tripli-

cate. Protein concentration was determined by the method of Lowry et al (7) using bovine serum albumin as the standard. Fifty per cent inhibitory doses (IC50) were determined using dose-response displacement curve of [^{3}H QNB] binding (6).

RESULTS

1) [^{3}H] QNB binding to bovine adrenal cortex crude membrane preparations.

Concentration dependence of [^{3}H] QNB binding was studied in order to assess the existence of high affinity and saturable binding sites (Fig. 1). [^{3}H] QNB total binding curve exhibited the characteristics of a combination of high and low affinity binding sites (8). In the presence of a 100 fold excess of unlabeled QNB the binding was linear as a function of the concentration.

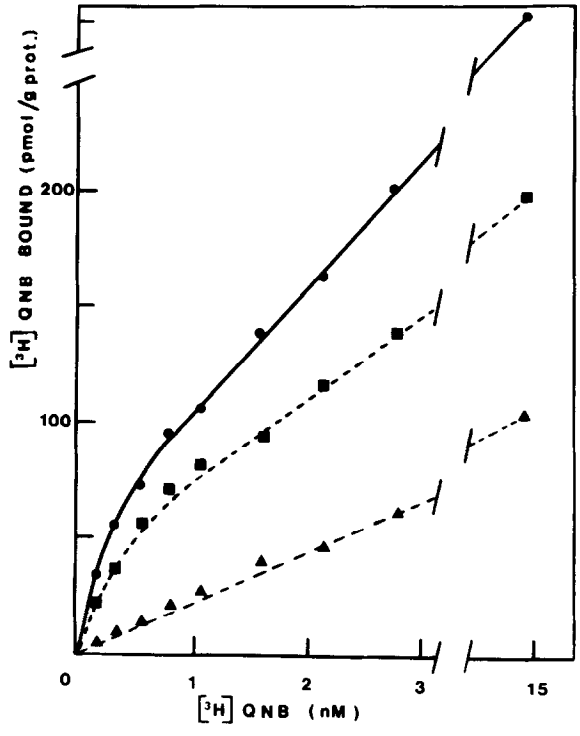

Fig. 1 - [^{3}H] QNB binding to bovine adrenocortical membrane preparation as a function of ligand concentration.
The upper curve (●——●) represents [^{3}H] QNB total binding; the lower line (▲---▲) is the non specific binding determined in the presence of a 100 fold excess of unlabeled QNB. The intermediate curve (■---■) is obtained by difference between total and non specific binding. The assay medium contained 0.6 mg/ml protein.

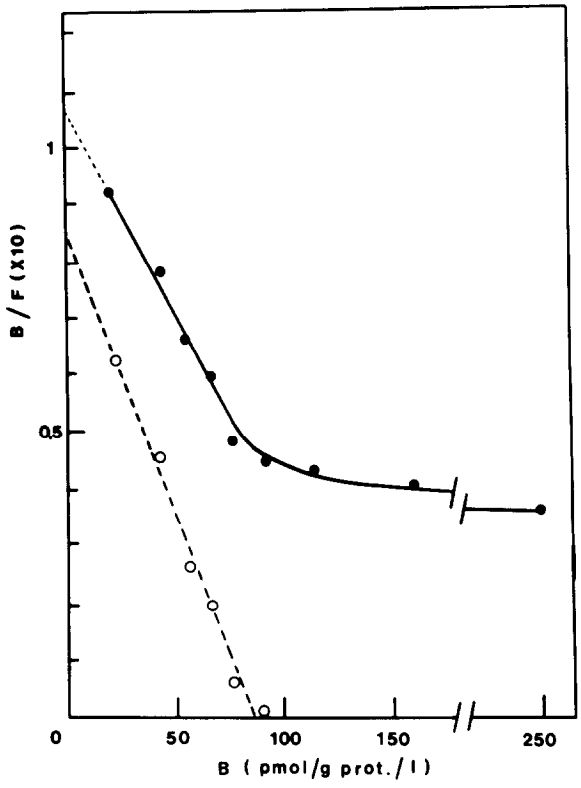

Fig. 2 - Scatchard plot derived from a saturation curve of [^{3}H] QNB binding to adrenocortical membrane preparation.
The graph was constructed from the total binding data (●—●) and using values of high affinity binding, obtained after substraction of non specific from total binding (o---o).

However, the curve obtained by calculating the difference between the total and the non specific binding did not show a plateau as expected for a saturable binding system. Scatchard plot analysis of the binding data confirmed the presence of a large low affinity binding site population (Fig. 2). After correction for low affinity binding (9), the graph permitted the determination of the specific binding capacity (Bsp = 67.2 ± 5.7 pmol QNB/g protein) and the dissociation constant (Kd = 1.2 ± 0.24 nM) of the high affinity binding sites (results expressed as mean ± S.E.M. from 9 experiments).

2) Specificity of high affinity [^{3}H] QNB binding.

The specificity of [^{3}H] QNB binding was first studied with various concentrations of typical cholinergic antagonists. Muscarinic antagonists at very

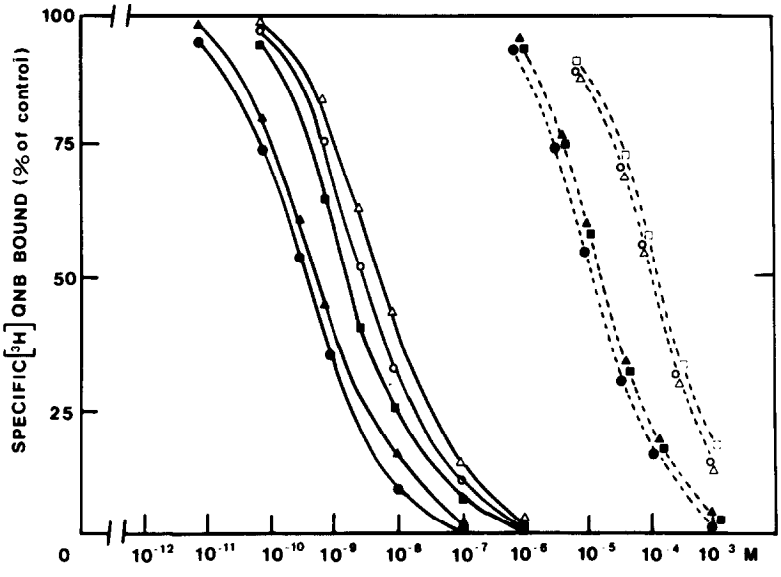

Fig. 3 - Inhibition of specific [^{3}H] QNB binding to bovine adrenocortical membrane preparation by cholinergic agents.
Muscarinic antagonists: QNB (●—●), dexetimide (▲—▲), scopolamine (■—■), atropine (o—o), and isopropamide (Δ---Δ). Muscarinic agonists: Ach (▲---▲), pilocarpine (■---■), muscarine (o---o), acetyl β methylcholine (Δ---Δ), and carbamylcholine (□---□). Membrane suspensions (0.6 mg protein) were incubated with 0.6 nM [^{3}H] QNB, as described under methods.

low concentration appeared to inhibit QNB binding (Fig. 3); this competitive effect was stereospecific since d-benzetimide (dexetimide) was as potent as QNB while l-benzetimide (levetimide), the biologically inactive enantiomer, failed to alter [^{3}H] QNB binding at 10^{-5} M. Typical muscarinic agonists also markedly decreased [^{3}H] QNB binding (Fig. 3). As shown in table I, nicotinic agonists, nicotine and dimethylphenyl piperazinium as well as nicotinic antagonists did not influence QNB binding. In addition, various non cholinergic drugs were tested; local anesthetics, which are potent inhibitors of the steroidogenic effect of Ach (unpublished) appeared to be as efficient as active muscarinic agonists in reducing [^{3}H] QNB binding (Table I). The products of acetylcholine hydrolysis namely sodium acetate and choline, had no effect at 10^{-5} M, as well as histamine which has been reported as a steroidogenic agent on dog adrenal cortex (10). In addition, catecholamines, ACTH

Table I. Effect of various cholinergic-interacting drugs and adrenocortical effectors on [^{3}H] QNB specific binding to bovine adrenocortical membrane preparations.

DRUG	IC50*
Muscarinic antagonists	
Quinuclidinyl benzilate	5×10^{-10} M
Dexetimide	6×10^{-10} M
Scopolamine	2.5×10^{-9} M
Atropine	4×10^{-9} M
Isopropamide	7.5×10^{-9} M
Muscarinic agonists	
Oxotremorine	1.5×10^{-5} M
Acetylcholine**	2.1×10^{-5} M
Pilocarpine	2.2×10^{-5} M
Muscarine	1.2×10^{-4} M
Acetyl β methylcholine	1.2×10^{-4} M
Carbamylcholine	1.5×10^{-4} M
Local anesthetics	
Tetracaine	2.5×10^{-5} M
Procaine	3.6×10^{-5} M

No detectable effect at 10^{-5} M : levetimide, sodium acetate, choline, d-tubocurarine, hexamethonium, mecamylamine, nicotine, dimethylphenylpiperazinium, serotonine, histamine, epinephrine, norepinephrine.

No detectable effect at 0.33×10^{-6} M : adrenocorticotropic hormone (ACTH), angiotensin II.

Values are the mean from three independent experiments whose results varied less than 15%.

* Concentration of drug which reduced specific [^{3}H] QNB binding by 50%.

** Physostigmine (1 μM) was added to the incubation medium to prevent possible enzymatic hydrolysis of Ach by endogenous cholinesterase.

and angiotensin II (a potent steroidogenic effector of zona fasciculata cells from bovine adrenal cortex (11)) did not interfere with the muscarinic binding activity of our preparations.

3) [^{3}H] QNB specific binding to adrenocortical zona fasciculata membrane preparations

In view of the aforementioned results, an additional control was introduced to confirm the attribution of the muscarinic binding site to adreno-

cortical cells since, despite careful dissection, a possible contamination with medullary cells could not be ruled out. Membrane preparations were obtained from the internal part (zona fasciculata) of the adrenal cortex, after 1 mm thick slicing and discarding of the 2 inner slices. This latter preparation may be considered as maximally avoiding contamination from medullary tissue. The specific [^{3}H] QNB binding capacity (Bsp = 63 ± 4.2 pmol/g protein) and dissociation constant (Kd = 1.0 ± 0.18 nM) (mean ± S.E.M.) obtained from three separate experiments with these membrane preparations were not different from those obtained from whole adrenal cortex membrane preparations.

DISCUSSION

The present data demonstrate the presence of specific, saturable and high affinity muscarinic cholinergic binding sites in bovine adrenal cortex. This muscarinic binding system is not likely due to medullary tissue contamination since Ach receptor of adrenal medulla from the same animal has been first characterized as of the nicotinic type (12). However, the presence of muscarinic receptors has been recently reported in bovine adrenal medulla (13); the absence of [^{3}H] QNB binding sites in bovine adrenal cortex was also mentioned in the same study (13). The reason of such a discrepancy may be found in different methodology especially with regard to the filtration technique used to separate bound and free ligand. It may be mentioned that the presence of muscarinic receptor in bovine adrenal cortex is in agreement with the specific muscarinic steroidogenic effect of Ach on zona fasciculata cells suspension from the same tissue (unpublished). On the contrary, the biological effect of Ach on bovine adrenal medulla has been shown to be entirely nicotinic (3,13). The similarity of [^{3}H] QNB binding characteristics obtained when crude membrane preparations from whole adrenal cortex or zona fasciculata were used supports the conclusion that the presence of muscarinic receptors in bovine adrenal cortex is not an artifact due to a contamination from medullary tissue.

The apparent dissociation constant found for bovine adrenocortical muscarinic receptor is close to the Kd determined in rat cerebellum (0.54 nM) (14) or chicken retina (0.4 nM) (15) at 37°C, but higher than the Kd determined in rat brain (0.06 nM) (6), or rabbit heart (0.0418 nM) (16) at 25°C. The amount of adrenocortical muscarinic receptor is 6.5 times smaller than found in similar preparations from rat brain (6). This difference may have been underestimated since our results were obtained with filters which retain smaller particles thant GF/B filters (14). The IC50 obtained in this study for potent antagonists and agonists are generally higher than those observed in brain homogenates (7); this may be related to the large non specific binding capacity which contributes to yield flattened binding curves and prevents an accurate calculation of the Hill coefficient (17). A purified membrane preparation would probably permit a better determination of [^{3}H] QNB specific binding parameters.

Nevertheless the presence of muscarinic binding sites in bovine adrenal cortex is in agreement with the steroidogenic activity of Ach on zona fasciculata cells from the same tissue. The biochemical processes involved in the biological activity remain to be established but a possible role of muscarinic receptors in bovine adrenal cortex physiology may already be considered.

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