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Adenylate Cyclase Activity of Synaptic Membranes from Rat Striatum

Inhibition by Muscarinic Receptor Agonists

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SUMMARY

Acetylcholine inhibits, by 30–40%, the basal adenylate cyclase activity of purified synaptic plasma membranes prepared from rat striatum (EC_{50} = 3 μM). Cholinergic receptor agonists inhibit this cyclase activity with the following rank order of potency: oxtremorine > acetylcholine > arecoline > methacholine ≥ muscarine ≥ carbachol > bethanechol. Nicotine fails to inhibit the cyclase, and *d*-tubocurarine fails to inhibit the action of cholinergic drugs. In contrast, atropine and scopolamine antagonize the effect of acetylcholine. The enzyme inhibition elicited by acetylcholine requires the presence of GTP, and disappears after intrastriatal injection of kainic acid. From these results, we infer that striatal adenylate cyclase can be modulated by muscarinic receptors.

INTRODUCTION

Activation of muscarinic receptors often increases guanylate cyclase activity and the tissue content of cyclic GMP (1). Cholinergic agonists however, can also affect tissue content of cyclic AMP. After exposure to acetylcholine, cyclic GMP accumulates in several intact cell systems, and the increase is associated with an influx of calcium into the cells (1). In contrast, muscarinic agonists decrease basal and hormonally stimulated cyclic AMP formation in intact cells and in broken-cell preparations (2–7); the decrease may be due to a direct action on adenylate cyclase activity (8–10).

In the central nervous system, there is not clear evidence that muscarinic receptors are coupled to adenylate cyclase. There are reports, however, that guanine nucleotides are capable of modulating the binding of cholinergic agonists to muscarinic recognition sites (11, 12), an indication that receptor-cyclase coupling may exist. The present report provides direct evidence that the occupancy of muscarinic receptors of striatum is associated with inhibition of adenylate cyclase activity.

MATERIALS AND METHODS

Isolation of synaptic plasma membranes. Striata from 15–20 male Sprague-Dawley rats (Zivic-Miller, 200–300 g) killed by decapitation were homogenized (1:10, w/v) in 5 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 1 mM EGTA[1] (TDE buffer), and 10% sucrose (w/w) with a motor-driven Teflon-glass tissue grinder (5-ml capacity; clearance 0.25 mm; 400 rpm). Synaptic plasma membranes were isolated as described by Jones and Matus (13). All steps were carried out at 0–4°.

The homogenate was centrifuged at 800 × *g* for 10 min. The supernatant was maintained on ice, while the pellet was resuspended in TDE buffer (11–15 ml) and centrifuged again. The combined supernatants were centrifuged at 9,000 × *g* for 20 min. The pellet (P_2) was washed once with TDE buffer by gentle resuspension and centrifugation at 9,000 × *g* for 20 min. The washed P_2 fraction was resuspended in 3–5 ml of cold TDE buffer (pH 8.0) and incubated at 0° for 30 min. The membranes were resuspended by five to eight strokes by hand in a Teflon-glass tissue grinder. This preparation of membranes was adjusted to 34% (w/w) sucrose by adding an appropriate volume of 48% (w/w) sucrose in TDE buffer (pH 7.4) and placed at the bottom of nitrocellulose tubes (2–2.5 ml/tube). A discontinuous sucrose gradient was formed by adding 28.5 (w/w) and then 10% sucrose (w/w) in TDE buffer (pH 7.4) to the centrifuge tubes (1.5–1.7 and 0.7–0.4 ml, respectively). The sucrose gradient was centrifuged at 60,000 × *g* for 110 min at 4° in an SW 56 rotor (Beckman, Palo Alto, Calif.). The material layered at the interface between the 34% and 28.5% sucrose was aspirated with a 19-gauge needle. This fraction, which contained approximately 85% of the total adenylate cyclase activity recovered from the gradient, was diluted 3-fold with TDE buffer (pH 7.4) containing 10% (w/w) sucrose and centrifuged at 150,000 × *g* for 60 min. The pellet was resuspended in TDE buffer (pH 7.4) to give a protein concentration of approximately 1 mg/ml. The suspension was divided into aliquots, slowly frozen to −70°, and stored for no longer than 10 days. An aliquot

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine.

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was quickly thawed before the beginning of an enzyme assay; the unused portion was discarded.

The protein recovered in the final membrane fraction was approximately 0.5% of the original tissue (wet weight). The specific activity of adenylate cyclase was about 2–3 times higher than the initial homogenate.

Adenylate cyclase assay. Enzyme activity was routinely assayed in a 150-μl reaction mixture containing 75 mM Tris-HCl (pH 7.4), 0.5 mM [α-^{32}P]ATP (30–40 cpm/pmole), 2 mM $MgCl_2$, 1 mM cyclic AMP, 0.5 mM IBMX, 5 mM phosphocreatine, creatine phosphokinase (50 units/ml), 0.1 mM GTP, 100 mM NaCl, bovine serum albumin (50 μg), 0.33 mM EGTA, 0.33 mM dithiothreitol, 10 μM eserine, and 10–15 μg of membrane protein. The inclusion of NaCl in the reaction mixture optimized the inhibition of adenylate cyclase by acetylcholine, although it was not absolutely required for the detection of the inhibition.

The reaction was initiated by adding the membrane fraction and continued for 5 min at 37°. The incubation was stopped by adding 200 μl of a solution containing 2% sodium dodecyl sulfate (w/v), 45 mM ATP, and 1.3 mM cyclic AMP (pH 7.5). Following the addition of [2–8-^{3}H] cyclic AMP (15–20 × 10^3 cpm), to monitor cyclic AMP recovery, the samples were placed in a boiling water bath for 3 min. Cyclic AMP was isolated as described by Salomon *et al.* (14).

Cyclic nucleotide phosphodiesterase assay. Cyclic AMP phosphodiesterase activity of the striatal synaptic membrane fraction was assayed in the same reaction mixture employed for the adenylate cyclase assay with 1 mM [2–8-^{3}H]cyclic AMP (4 × 10^3 cpm/nmole) as the substrate. Samples were incubated for 5 min at 37°, the reaction was terminated by boiling for 45 sec, and the samples were assayed according to the procedure described by Thompson *et al.* (15).

Striatal lesions with kainic acid. Male rats (200–250 g) were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and placed in a stereotaxic frame. Kainic acid was dissolved in 10 mM sodium phosphate buffer (pH 7.4) and injected (1 μg in 0.5 μl over a 5-min period) into the head (*A* +3; *L* ±2.8; *V* 5.0) and body (*A* +1; *L* ±3.5; *V* 4.5) of the right caudate nucleus. These coordinates were obtained from the atlas of Pellegrino and Cushman (16). The contralateral caudate nucleus was injected with an equivalent volume of vehicle. Ten days after surgery, animals were decapitated and the adenylate cyclase activity was assayed in a synaptosomal preparation (see legend to Table 2) from the control and lesioned caudate nucleus.

Protein content was determined by the method of Bradford (17), using bovine serum albumin as a standard.

Materials. [α-^{32}P]ATP (30–40 Ci/mmole) was obtained from Amersham (Arlington Heights, Ill.). [2–8-^{3}H] cyclic AMP (25 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). All other compounds were purchased from Sigma Chemical Company (St. Louis, Mo.) and were of the highest purity available. ATP (Sigma A-2383) was prepared by phosphorylation of adenosine.

RESULTS

Adenylate cyclase activity of striatal synaptic plasma membranes appeared linear with time of incubation for up to 10 min. In the presence of acetylcholine (100 μM), enzyme activity was markedly attenuated (Fig. 1).

To rule out the possibility that the reduced formation of cyclic AMP was the consequence of cyclic AMP phosphodiesterase activation, we assayed this enzyme under the same experimental conditions used to measure the adenylate cyclase activity. In the presence of 0.5 mM IBMX, phosphodiesterase activity was inhibited by about 50% [69 ± 4 versus 138 ± 5 nmoles of cyclic AMP hydrolyzed per minute per milligram of protein (± standard deviation)], and acetylcholine (100 μM) failed to change this activity [69 ± 4 nmoles of cyclic AMP hydrolyzed per minute per milligram of protein (± standard deviation)].

Inhibition of adenylate cyclase activity by acetylcholine was concentration-dependent (Fig. 2). The lowest concentration of acetylcholine tested which produced significant inhibition was 0.1 μM (basal, 1.66 versus acetylcholine, 1.58 nmoles of cyclic AMP per minute per milligram of protein; $n = 7$, $p < 0.005$, paired *t*-test). Maximal inhibition was achieved at a concentration of approximately 0.1 mM and corresponded to a 30–40% decrease in the adenylate cyclase activity.

A number of acetylcholine receptor agonists reduced the activity of striatal adenylate cyclase (Fig. 3). All of the compounds had similar efficacy with the exception of bethanechol, which was the least active. Estimation of the concentrations which elicited a half-maximal effect (EC_{50}) (Table 1) revealed the following rank order of potency: oxotremorine > acetylcholine > arecoline > methacholine ≥ muscarine ≥ carbachol > bethanechol. The Hill coefficients were less than unity for all of the agonists. Nicotine failed to inhibit striatal adenylate cyclase activity even at concentrations up to 1 mM.

Two muscarinic receptor antagonists, atropine and scopolamine, prevented adenylate cyclase inhibition elic-

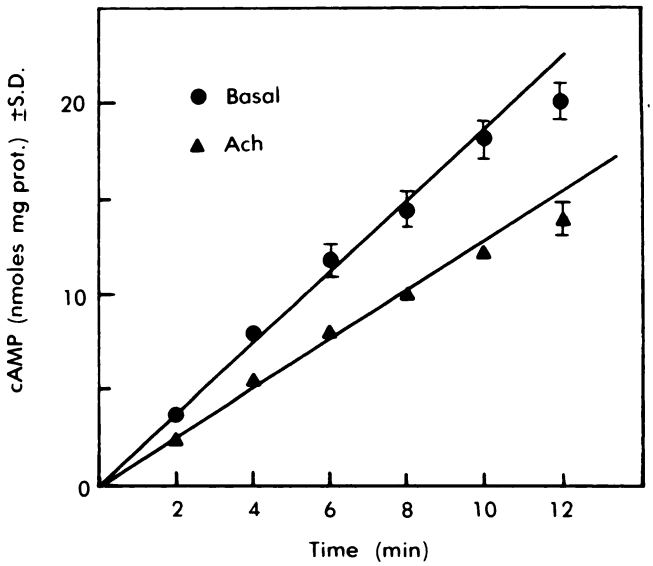

FIG. 1. *Striatal adenylate cyclase activity versus time of incubation in the absence and presence of acetylcholine (Ach)*

The concentration of acetylcholine was 100 μM. Eserine (10 μM) was present in the incubation mixture and did not affect basal adenylate cyclase activity. Each point represents the mean ± standard deviation of triplicate determinations. The data are representative of four experiments with a range of variability of 10%.

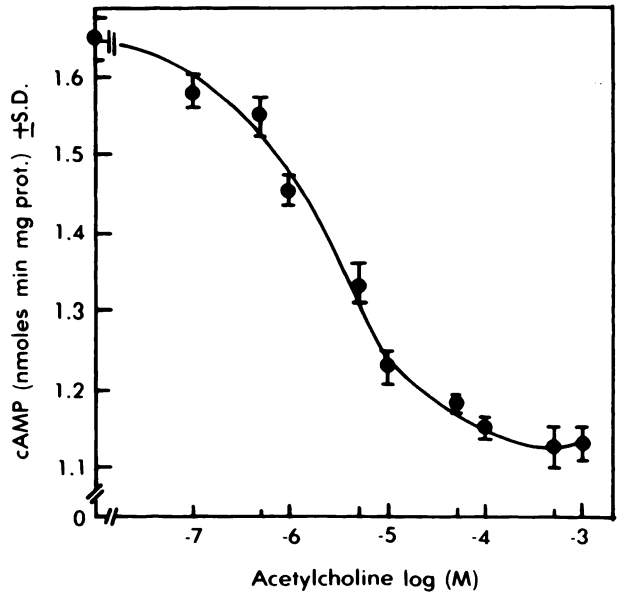

FIG. 2. *Concentration-dependent inhibition of striatal adenylate cyclase by acetylcholine*

The enzyme activity was assayed as described under Materials and Methods in the presence of the indicated concentration of acetylcholine. The values reported are means ± standard deviation of triplicate determinations and are representative of six experiments. Interassay variability was < 12%.

TABLE 1

Potency of cholinergic agonists for inhibiting striatal adenylate cyclase

EC_{50} values were obtained from log-probit plots of the inhibition of enzyme activity, expressed as percentage of the maximal effect, at different concentrations of each agonist.

Cholinergic agonist	EC_{50}[a]	*N*	Relative potency[b]	Hill coefficient
	μM		%	
Acetylcholine	3.5 ± 0.7	6	100	0.63
Oxotremorine	2.2 ± 0.1	3	159	0.72
Arecoline	6.6 ± 2	3	53	0.52
Methacholine	10 ± 1	4	35	0.79
Muscarine	14 ± 4	3	26	0.81
Carbachol	15 ± 0.1	4	23	0.76
Bethanechol	>100	4	ND[c]	ND[c]
Nicotine	Inactive	3		

[a] Values presented are the means ± standard error of the mean obtained from the indicated number of experiments (*N*) performed in triplicate.

[b] Relative to acetylcholine.

[c] ND, Not determined.

ited by acetylcholine (Fig. 4) without changing basal enzyme activity. From the dose-response curves we calculated apparent IC_{50} values (concentration which antagonized by 50% the effect of acetylcholine) of 16 nM for atropine and 40 nM for scopolamine, and Hill coefficients of 1.08 and 0.96, respectively. In contrast to the muscarinic antagonists, *d*-tubocurarine, a nicotinic receptor antagonist, did not prevent the inhibition by acetylcholine (Fig. 4).

The inhibition of adenylate cyclase elicited by acetylcholine could be reversed by adding atropine after the inhibition was operative (Fig. 5). Atropine acted rapidly and brought about a full recovery of the enzyme activity.

In the absence of added GTP, acetylcholine did not affect adenylate cyclase activity (Fig. 6). A dose-response curve of basal enzyme activity in the presence of increasing concentrations of GTP showed maximal stimulation at a concentration of about 1 μM. There was a slight decline of activity at higher concentrations of the nucleotide. In the presence of 100 μM acetylcholine, the maximal activation induced by 1 μM GTP alone was significantly decreased, and at higher concentrations of GTP the falloff of the response curve became more pronounced. Thus, the inhibition of adenylate cyclase by acetylcholine requires the presence of GTP. The concentration of GTP that permits acetylcholine to inhibit adenylate cyclase by one-half of the maximal is about 0.6 μM.

Muscarinic receptors are located on different cellular structures within the striatum. A large portion of the receptor population resides on neuronal elements intrinsic to the striatum or originating in this area (18). However, muscarinic receptors are also present on dopaminergic afferents to the corpus striatum (19) and on glial cells (20). To gain information on the cellular localization

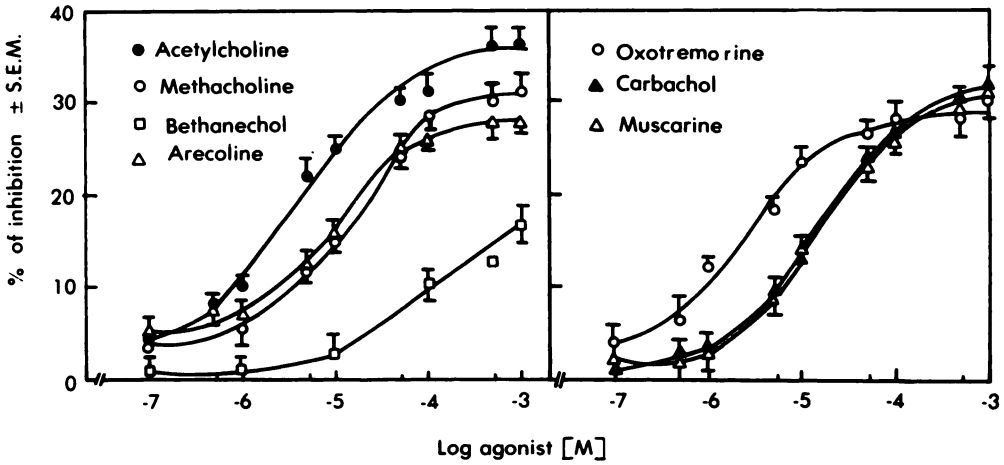

FIG. 3. *Concentration-response inhibition by several cholinergic agonists of striatal adenylate cyclase activity*

Inhibition of adenylate cyclase is expressed as the mean percentage inhibition ± standard error of the mean of basal enzyme activity [1.8 ± 0.05 nmoles of cyclic AMP per minute per milligram of protein (± standard error of the mean)]. The number of experiments for each agonist is reported in Table 1.

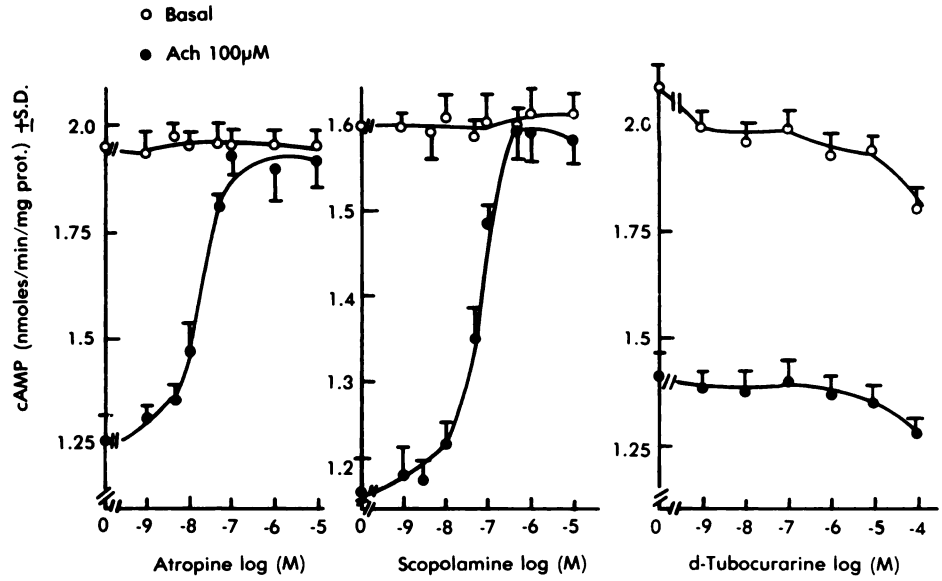

FIG. 4. *Influence of cholinergic antagonists on the inhibition of striatal adenylate cyclase by acetylcholine (Ach)*
The effect of increasing concentrations of each antagonist was evaluated on the enzyme activity assayed in the absence (○) and in the presence (●) of 100 μM acetylcholine. Values are mean ± standard deviation of triplicate determinations and are representative of four experiments with a range of variability < 15%.

of the muscarinic receptors mediating the inhibition of adenylate cyclase, we examined the response of this enzyme to acetylcholine following the intrastriatal injection of kainic acid. This compound destroys the neuronal cell bodies located in the striatum, but causes minimal damage to the axons and nerve endings projecting to this region (21). Ten days after the injection of the neurotoxin, basal adenylate cyclase was decreased by about 70% in the lesioned striatum as compared with the contralateral control striatum (Table 2). Acetylcholine inhibited adenylate cyclase in the intact but not in the striatum lesioned by kainic acid.

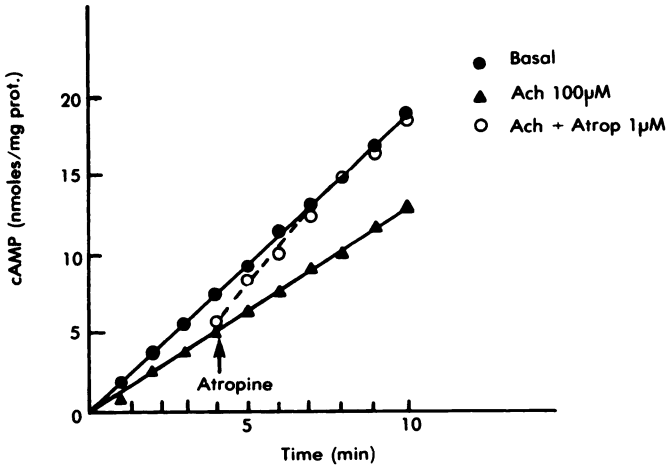

FIG. 5. *Reversal of acetylcholine (Ach) inhibition of striatal adenylate cyclase activity by atropine (Atrop)*
Atropine sulfate was added at the time indicated by the *arrow* to membranes preincubated with acetylcholine. The formation of cyclic AMP was determined in 150-μl aliquots withdrawn from each reaction mixture. The results were confirmed by three additional experiments. The range of variability between experiments was 10%.

DISCUSSION

Little is known about the regulation of adenylate cyclase activity by cholinergic receptors in the central nervous system. Walker and Walker (22) observed that acetylcholine inhibited adenylate cyclase activity in homogenates of rat striatum, but the inhibition was not characterized. Tang and Cotzias (23) reported that several cholinergic agonists as well as antagonists competi-

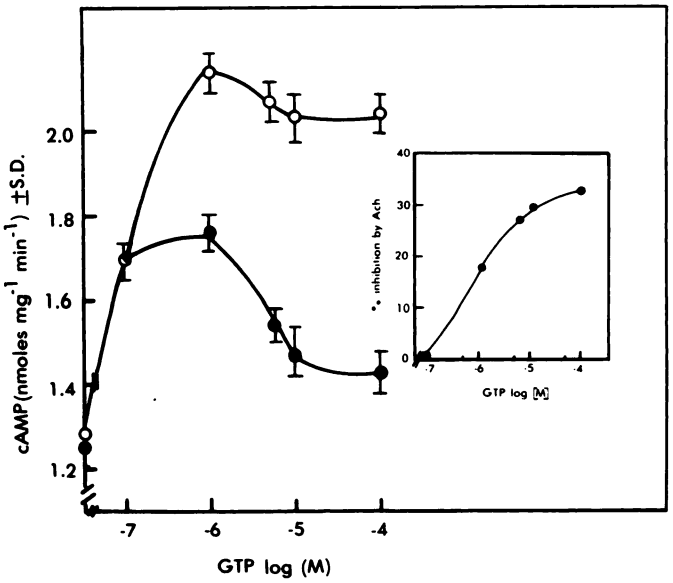

FIG. 6. *GTP dependency of the inhibition of adenylate cyclase by acetylcholine (Ach)*
The enzyme activity was assayed in the absence (○) and in the presence (●) of 100 μM acetylcholine. In the *inset*, data are plotted as percentage inhibition of enzyme activity by acetylcholine at different concentrations of GTP. Each value represents the mean ± standard deviation of triplicate determinations. The data are representative of four similar experiments.

TABLE 2

Intrastriatal injection of kainic acid abolishes the ability of acetylcholine to inhibit adenylate cyclase

The control and lesioned striatum from each rat were homogenized by hand with 2 ml of TDE buffer containing 10% (w/w) sucrose. The homogenate was centrifuged at 800 × *g* for 10 min and the resulting supernatant was centrifuged at 9,000 × *g* for 20 min. The pellet was resuspended in 5 ml of ice-cold water and kept at 0° for 5–10 min. Thereafter, the tissue preparation was centrifuged at 30,000 × *g* for 20 min. The pellet was resuspended in TDE buffer to give a final protein concentration of 400–600 μg/ml and immediately assayed for adenylate cyclase activity as described under Materials and Methods.

Striatum	Cyclic AMP ± SEM	
	Basal	Acetylcholine (100 μM)
	pmoles/mg/min	
Control side	729 ± 70	554 ± 50[a]
Lesioned side	215 ± 30	199 ± 30[b]

[a] $p < 0.001$ (paired *t*-test).
[b] Not significant; $n = 5$.

tively inhibited the stimulation of adenylate cyclase by dopamine, although both classes of drugs had no effect on basal activity. We now show that (a) acetylcholine and other cholinergic agonists inhibit striatal adenylate cyclase by acting on specific recognition sites; (b) these sites possess pharmacological properties of muscarinic receptors; (c) the inhibition requires GTP; and (d) the acetylcholine-inhibited adenylate cyclase is located on intrinsic striatal neurons that are destroyed by kainic acid.

In the dog thyroid, stimulation of cholinergic receptors, which increases cyclic GMP formation, may decrease the concentration of cyclic AMP via stimulation of cyclic AMP phosphodiesterase(s) (3). Such a mechanism cannot be invoked to explain our results. In fact, we used a purified preparation of synaptic plasma membranes, and our assay system contained EGTA. Under these conditions the cyclic GMP content cannot increase in response to stimulation of muscarinic receptors (1). Moreover, the activity of cyclic AMP phosphodiesterase of our tissue preparation was measured and found not to change following the addition of acetylcholine. Therefore, we postulate that muscarinic agonists act directly by reducing the efficiency of the membrane-bound adenylate cyclase.

Muscarinic recognition sites may be negatively coupled to membrane adenylate cyclase; hence activation of these receptors reduces enzyme activity. This suggestion would imply that the striatal cyclic AMP content is decreased by stimulation of acetylcholine receptors. However, there is not a clear correlation between the content of cyclic AMP in striatum and activation of muscarinic receptors. Many investigators have studied the cyclic AMP content in either brain slices or brain tissue after treatment *in vivo*. Cholinergic agonists were found not to change (24) or to decrease the cyclic AMP content (25). Hanley and Iversen (26) reported that oxotremorine increased the cyclic AMP content of rat striatum, but this response was blocked by α-flupentixol, a dopaminergic antagonist. Apparently the increase in cyclic AMP induced by oxotremorine was mediated by a release of endogenous dopamine. In the membrane preparation used in our studies we can exclude the consequence of an interaction due to trans-synaptic events.

The inhibitory effect of acetylcholine on adenylate cyclase was mimicked by muscarinic agonists and blocked by muscarinic antagonists. Nicotinic receptor agonists were ineffective. The rank order of potency of the muscarinic agonists and their EC_{50} and Hill coefficient values correlated well with the capacity of these compounds to displace radiolabeled antagonists bound to muscarinic recognition sites (27). Thus, occupancy of the receptors by agonists parallels their ability to promote inhibitory coupling of the receptors to adenylate cyclase located in striatal synaptic membranes. Enzyme inhibition required the continuous occupancy of the receptor site by the agonist, as the addition of supramaximal concentrations of an antagonist was followed by a rapid return of the agonist-inhibited enzyme activity to control levels. These results indicate that the inhibition of adenylate cyclase was a receptor-mediated process and occurred without an irreversible change in the functional properties of the enzyme system. The dependency of the inhibitory effect on GTP supports the idea that muscarinic receptors may interact with a regulatory component(s) rather than with the catalytic subunit of adenylate cyclase. GTP may be involved in the activation of an inhibitory regulatory protein which is part of the receptor system and couples the cyclase to the muscarinic receptor (28). Alternatively, GTP may serve as substrate for a specific GTPase which controls the rate of turn-off of the adenylate cyclase activity and which can be activated by inhibitory transmitters (29, 30).

The physiological role of muscarinic attenuation of striatal adenylate cyclase remains to be determined. The location of a muscarinic-sensitive enzyme in subcellular fractions enriched in synaptic plasma membranes and on cellular elements destroyed by kainic acid indicate that the complex may be part of the neuronal membrane rather than of glial cells. The inhibition of adenylate cyclase by muscarinic agonists may be a complementary event in the regulation of cyclic GMP production in the neurons and/or may represent a specific mechanism to modulate the sensitivity of the adenylate cyclase to stimulatory neurotransmitters, such as dopamine. The latter view would be in keeping with the widely accepted concept that dopaminergic and cholinergic inputs to the striatum are in a functional balance. On a biochemical level, this balance could operate via an interaction of the two transmitters at the complex between dopamine-adenylate cyclase and cholinergic recognition sites.

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