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MUSCARINIC CHOLINERGIC INHIBITION OF CYCLIC AMP FORMATION AND ADRENOCORTICOTROPIN SECRETION IN MOUSE PITUITARY TUMOR CELLS

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SUMMARY: Cholinergic muscarinic receptors were identified in AtT-20/D16-16 (AtT-20) cell membranes by receptor binding techniques and the effect of carbachol on basal and stimulated cyclic AMP formation and ACTH release was investigated. Carbachol markedly decreased the stimulatory effect of the adenylate cyclase activator, forskolin, on both cyclic AMP formation and ACTH secretion. Carbachol also reduced forskolin-stimulated adenylate cyclase activity. The stimulatory effects of (-)isoproterenol on cyclic nucleotide formation and ACTH secretion were also blocked by carbachol. The inhibitory effects of carbachol on (-)isoproterenol-stimulated cyclic AMP synthesis and ACTH secretion were reversed by the muscarinic antagonist, atropine, and not by the nicotinic antagonist, gallamine. These data suggest that in AtT-20 cells, inhibition of ACTH secretion may be regulated by activation of muscarinic receptors coupled negatively to adenylate cyclase.

The regulation of adrenocorticotropin (ACTH) synthesis, storage, and secretion has been extensively studied in the AtT-20 mouse pituitary tumor cell line (1-11). Secretion appears to be a cyclic AMP dependent process since several secretagogues, including CRF, β_2-adrenergic agonists and VIP stimulate the formation of cyclic AMP and secretion of immunoreactive ACTH from AtT-20 cells; direct activators of adenylate cyclase, such as forskolin or cholera toxin, also stimulate ACTH release (7, 8). Somatostatin (SRIF), on the other hand, was recently reported to inhibit ACTH secretion activated by CRF, (-)isoproterenol, VIP, and forskolin by exerting an inhibitory action on adenylate cyclase (7).

Abbreviations: ACTH, adrenocorticotropin hormone; CRF, corticotropin releasing factor; IBMX, 3-isobutyl-1-methylxanthine; QNB, quinuclidinyl benzilate; SEM, standard error of the mean; SRIF, somatostatin; VIP, vasoactive intestinal peptide.

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Muscarinic cholinergic receptors are believed to be negatively coupled to adenylate cyclase since muscarinic agonists reduce adenylate cyclase activity (see 12) and decrease cyclic AMP accumulation in some tissues (13, 14). Binding sites for the muscarinic antagonist QNB were identified recently in crude membrane pellets of anterior pituitary (15), however, carbachol, tested in primary cultures of dispersed anterior pituitary cells, did not alter basal ACTH secretion (16); it was not tested for antagonistic properties. In this study, we report that carbachol, acting at a muscarinic receptor, is capable of inhibiting ACTH secretion from AtT-20 tumor cells stimulated by cyclic AMP-dependent secretagogues; the mechanism involved appears to be associated with the inhibition of cyclic AMP synthesis.

METHODS

Mouse AtT-20 tumor cells were grown and subcultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (6-10). For receptor binding studies, membrane fractions from AtT-20 cells were prepared (7, 9, 10) and the specific muscarinic antagonist, L-[^{3}H]QNB (35 Ci/mmol) was used to label receptors (18). For ACTH release and cyclic AMP synthesis studies, cells were plated in 2 cm^2 culture dishes at an initial density of 5 X 10^4 cells/well and were used 5-6 days after subculturing (60-80% confluency). Adenylate cyclase was measured in buffer containing 40 mM Tris-HCl (pH 8.0), 1 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5 mM EGTA, 5 mM $MgCl_2$, and 10^{-5} M GTP (17). Cyclic AMP formation and ACTH release were studied and quantitated by radioimmunoassay (6-11).

RESULTS

The muscarinic receptor on AtT-20 cell was characterized in membrane fractions by receptor ligand technique using the specific muscarinic antagonist [^{3}H]QNB. Specific [^{3}H]QNB binding (displaceable by 10 μM atropine) accounted for about 75% of total tissue binding at 2 X 10^{-10} M [^{3}H]QNB. Analysis of [^{3}H]QNB binding by the Scatchard plot (Fig. 1) showed a straight line; the density of muscarinic receptors (B_{max} value) was 116 fmol/mg protein and the apparent affinity of these sites (K_D) for [^{3}H]QNB was 0.2 nM. [^{3}H]QNB binding was inhibited in a dose-dependent manner by atropine with an apparent inhibitory constant of 1.5 nM (not shown).

Forskolin, which bypasses membrane receptors (see 19) and activates adenylate cyclase directly, has agonistic effects on cyclic AMP synthesis and ACTH secretion in AtT-20 cells (7-9). The effect of carbachol on basal and

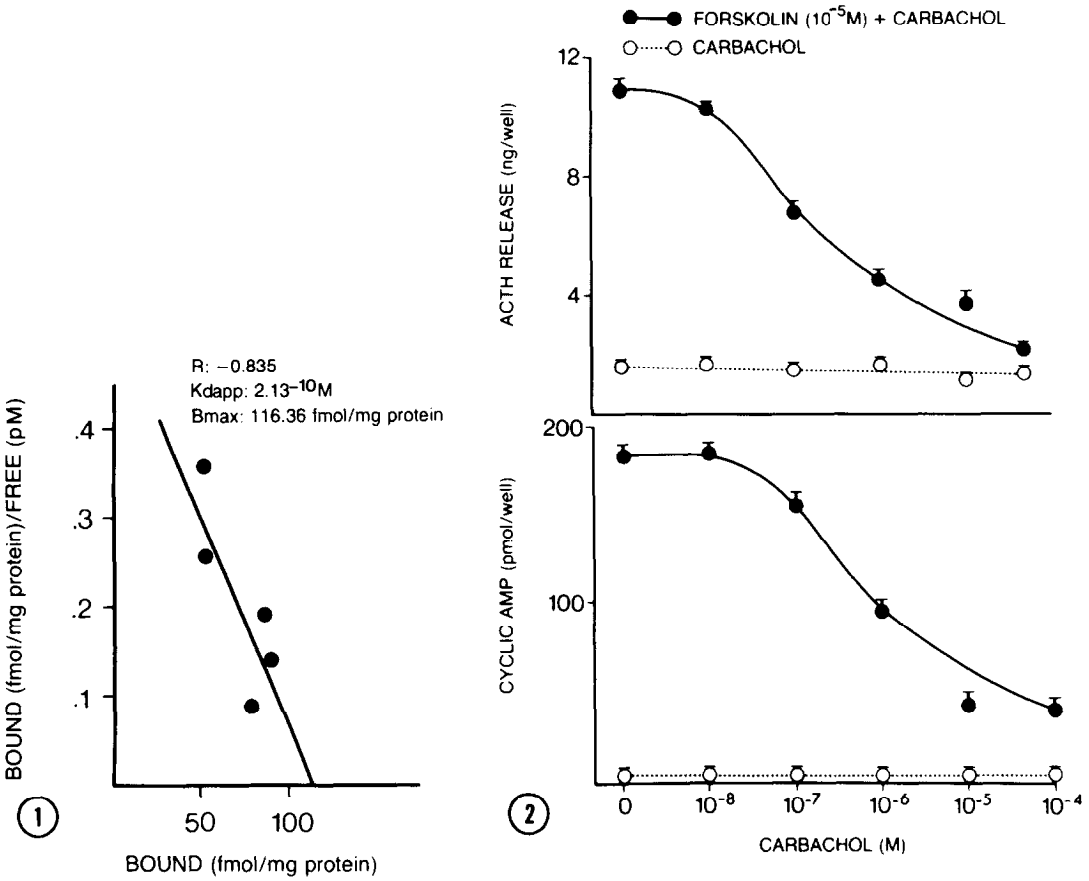

Figure 1. Scatchard plot of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to AtT-20 cells. The analysis represents a saturation isotherm of experiments in which 4 X 10^{-11} to 10^{-9} M of [^{3}H]QNB were incubated with 30-50 µg of AtT-20 membrane protein for 120 min at 37°C. Specific binding is defined as that displaced by 10 µM of atropine. A plot representative of 3 different experiments is depicted in the figure.

Figure 2. Carbachol-induced inhibition of forskolin-stimulated cyclic AMP synthesis and ACTH secretion. Immunoreactive ACTH release was measured after 60 min incubation with or without test agents. Cyclic AMP levels were quantitated after 15 min incubation. Values are means ± SEM.

forskolin-stimulated cyclic AMP formation and release of ACTH is illustrated in Fig. 2. Though not seen in this study, carbachol, in concentrations greater than 10^{-5} M, generally reduced basal cyclic AMP formation and ACTH secretion by about 25%. Forskolin markedly increased both cyclic AMP synthesis and ACTH release; carbachol (within the same concentration range) had a strong inhibitory effect of both of these forskolin-stimulated responses.

The effect of carbachol on forskolin-stimulated adenylate cyclase activity is described in Table 1. Carbachol at the highest concentration used inhibited about 50% of the increase in cyclase activity stimulated by forskolin.

Table 1. The effect of carbachol on forskolin-stimulated adenylate cyclase activity in AtT-20 cells

	Cyclic AMP (nmol/mg protein/5 min)
Control	0.3 ± 0.01
Forskolin (10^{-5} M)	3.4 ± 0.1
Carbachol (10^{-6} M)	0.3 ± 0.02
Carbachol (10^{-4} M)	0.4 ± 0.02
Forskolin (10^{-5} M) + Carbachol (10^{-6} M)	2.5 ± 0.1
+ Carbachol (10^{-4} M)	1.8 ± 0.2

Values represent mean ± SEM of three separate determinations. Experiments were done as described in methods. Protein content was 6.8 µg/tube.

Stimulation of AtT-20 cells with β_2-adrenergic agonists results in an increase in both cyclic AMP synthesis and secretion of ACTH (7-10). The inhibitory effect of carbachol on (-)isoproterenol-stimulated cyclic AMP synthesis and ACTH release is shown in Fig. 3 and is non-competitive as suggested by the reduction in the maximal responses to (-)isoproterenol.

ACTH secretion stimulated by CRF or VIP in AtT-20 cells is also associated with cyclic AMP formation (7, 8, 11). Carbachol (10^{-5} M) was equally effective in blocking both cyclic AMP formation and ACTH secretion stimulated by CRF and VIP (not shown).

The inhibitory effect of carbachol on the secretory process in AtT-20 cells is associated with activation of cholinergic muscarinic receptors since the muscarinic antagonist atropine reversed the ability of carbachol to inhibit (-)isoproterenol-stimulated cyclic AMP formation and ACTH secretion (Fig. 4); the nicotinic antagonist gallamine did not alter the inhibitory effects of carbachol.

DISCUSSION

Like activation of the SRIF receptor in AtT-20 cells (7, 17), activation of cholinergic receptors results in a reduction in cyclic AMP levels and inhibition of the ACTH secretory process stimulated by a variety of secretagogues (forskolin, (-)isoproterenol, CRF, VIP) whose activity appears mediated by an increase in the formation of the cyclic nucleotide.

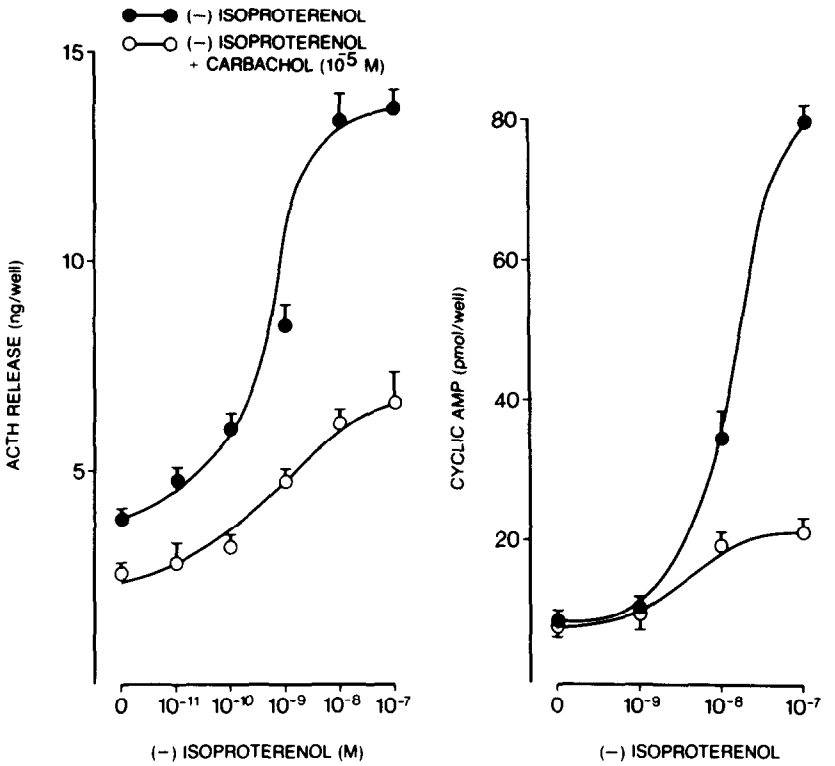

Figure 3. Effect of carbachol on the (-)isoproterenol dose-response curve for cyclic AMP synthesis and ACTH secretion. Immunoreactive ACTH release was measured after 60 min incubation with or without test agents. Cyclic AMP levels were quantitated after 15 min incubation. Values are means + SEM.

Consistent with the belief that the cholinergic receptor on AtT-20 cells is muscarinic in nature are the observations that a) these sites bind the reversible muscarinic antagonist [^{3}H]QNB with high affinity, b) bound [^{3}H]QNB can be displaced by another muscarinic antagonist, atropine, and c) inhibitory effects of carbachol on cyclic nucleotide synthesis and ACTH secretion can be reversed by atropine, and not by a nicotinic antagonist such as gallamine.

The mechanism by which carbachol inhibits cyclic AMP formation in AtT-20 cells is unknown. It is unlikely, however, that carbachol interferes with the binding of secretory agonists, thereby reducing cyclic AMP formation and ACTH secretion, since carbachol acts as a non-competitive antagonist of (-)isoproterenol action on AtT-20 cells. Furthermore, carbachol also blocks the receptor-independent (see 19) cyclic AMP response to forskolin. Muscarinic agonists have been reported to activate phosphodiesterase (20), but it is

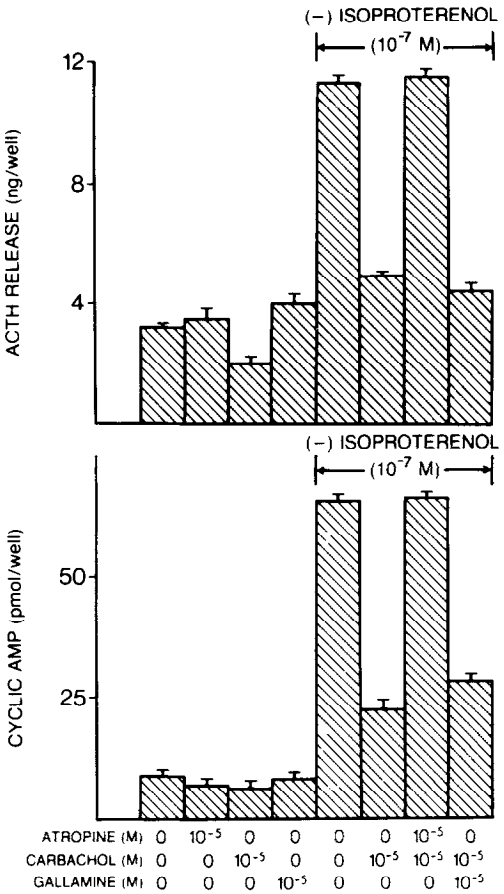

Figure 4. Reversal of the carbachol inhibitory effect on (-)isoproterenol-stimulated cyclic AMP synthesis and ACTH secretion. Immunoreactive ACTH release was measured after 60 min incubation with or without test agents. Cyclic AMP levels were quantitated after 15 min incubation. Values are means ± SEM.

also unlikely that a similar effect can account for the carbachol-induced reduction in cyclic AMP levels in AtT-20 cells since IBMX, a potent phosphodiesterase inhibitor, was present in all incubation media in excess. Additionally, while IBMX abolished the inhibitory effect of the muscarinic agent, oxotremorine, on (-)isoproterenol-stimulated cyclic AMP accumulation in astrocytoma cells (20), it did not have a similar effect on carbachol action in AtT-20 cells. Muscarinic agonists directly reduce adenylate cyclase activity in cell-free preparations (see 12), a finding confirmed in this study by the observation that carbachol reduces forskolin-activated cyclase activity. The muscarinic cholinergic receptor in AtT-20 cells appears, therefore, like that of the SRIF receptor (7, 17) to be negatively coupled to adenylate cyclase.

Preliminary results suggest that both the SRIF and carbachol receptor may share a common, post-receptor mechanism in regulating adenylate cyclase activity.

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