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Muscarinic Cholinergic Receptors on Cultured Thyroid Cells

II. Carbachol-Induced Desensitization

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SUMMARY

Muscarinic cholinergic receptors were previously characterized on cultured porcine thyroid cells. The receptor number was increased by chronic thyrotropin or prostaglandin E_2 treatments [Champion, S., and J. Mauchamp. *Mol. Pharmacol.* 21:66–72 (1982)]. The long-term effect of carbachol was studied. After chronic treatment with carbachol, cells were completely desensitized to acute carbachol stimulation. This process was blocked by muscarinic antagonists. A complete desensitization was obtained after 6 hr of treatment with 100 μM agonist. Under these conditions the quinuclidinyl benzilate binding capacity of cell homogenates was reduced by 50%. Withdrawal of carbachol allowed the complete restoration of the sensitivity of cells within 6 hr with only a partial recovery of the binding capacity (25%). The complete complement of receptors was obtained after 24 hr of recovery. Desensitization and agonist-induced decrease in receptor number were not affected by cycloheximide, whereas the recovery of both effect and binding sites required active protein synthesis.

INTRODUCTION

As shown in the accompanying paper (1), cholinergic agonists acutely decreased the cyclic AMP response of cultured thyroid cells to acute thyrotropin stimulation. This effect was the result of an interaction between the agonists and a muscarinic receptor which has properties, as defined by [^{3}H]QNB[3] binding, close to those described for other muscarinic receptors. The thyroid stimulators TSH and PGE_2, which enhance the sensitivity of cultured thyroid cells to acute TSH stimulation, increased the number of QNB binding sites.

We know from previous work that TSH, PGE_2, and isoproterenol, which are stimulators of thyroid cell adenylate cyclase (EC 4.6.1.1) activity, can modulate the response of cultured cells to acute stimulation (2–5). Both positive and negative regulations have been observed. Each agonist is able to induce a homologous desensitization. We therefore studied the long-term effect of carbachol on the cholinergic sensitivity of cultured thyroid cells and on the QNB binding capacity of cell homogenates. We report here that the chronic presence of carbachol induced a complete desensitization to acute stimulation and a partial decrease in the number of QNB binding sites. The desensitization process was independent of protein synthesis, whereas recovery upon agonist withdrawal required active protein synthesis. Moreover, after desensitization a complete recovery was obtained after 6 hr with only a limited increase in the number of binding sites.

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[3] The abbreviations used are: QNB, quinuclidinyl benzilate; TSH, thyroid-stimulating hormone; PGE_2, prostaglandin E_2.

MATERIALS AND METHODS

The methods for cell isolation, culture, and acute stimulation have been described in the preceding paper (1). In the present work, cells were cultured in the presence of TSH (100 μU/ml) (TSH-treated cells) or without effector (control cells) for 4 days.

Chronic incubation with carbachol. Desensitization studies were performed by incubation in the presence of carbachol at different concentrations (1–100 μM). Carbachol was added to the culture medium at various times before conclusion of the culture as indicated, and then the cells were washed three times with 50 ml of phosphate-buffered saline (pH 7.4) (NaCl/Pi) of the following composition (milligrams per liter): NaCl, 8000; KCl, 200; $Na_2HPO_4 \cdot 2H_2O$, 2890; KH_2PO_4, 200; $CaCl_2 \cdot 2H_2O$, 66.6; and $MgCl_2 \cdot 6H_2O$, 100. Washed cells were resuspended in NaCl/Pi containing TSH and other effectors to be

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tested, and then incubated for 5–10 min at 37°. Each assay contained 2×10^6 cells/0.5 ml of NaCl/Pi. Cyclic AMP was measured by radioimmunoassay (6).

Receptor number determination. For the determination of [^{3}H]QNB binding sites, washed thyroid cells were homogenized in NaCl/Pi (20×10^6 cells/ml), and aliquots of the homogenate were incubated in the same medium in the presence of [^{3}H]QNB (0.1–1.5 nM) for 60 min at 26°. Each assay contained homogenate derived from about 2×10^6 cells. Bound radioactivity was measured after filtration on glass-fiber discs (Whatman GF/C) as described in the accompanying paper (1).

RESULTS

Chronic effects of carbachol. All studies on the chronic effects of carbachol were performed using cells cultured in the presence of TSH (100 μU/ml) which display a maximal acute cyclic AMP response to TSH (5). After a culture period of 4 days, carbachol was added. Cells were recovered after various periods of time, washed, and acutely challenged with TSH (10 mU/ml) in the presence or absence of carbachol (100 μM). Simultaneously the [^{3}H]QNB binding capacity was measured on cell homogenates.

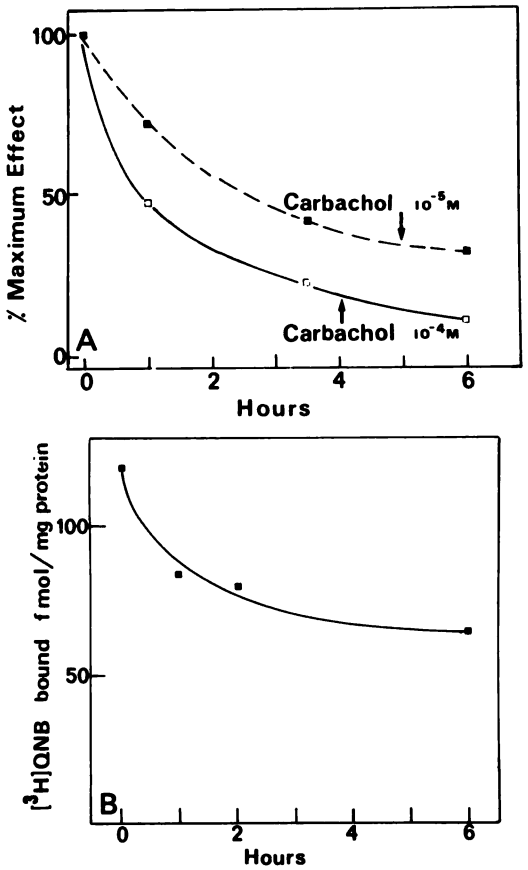

FIG. 1. *Time course of carbachol-induced desensitization and decrease of [^{3}H]QNB binding capacity*

Thyroid cells were cultured for 4 days in the presence of TSH (100 μU/ml). Carbachol (10 or 100 μM) was then added. Cells were recovered after indicated times of carbachol treatment, washed, and acutely stimulated with TSH (10 mU/ml) in the presence of methyl isobutylxanthine (1 mM) with or without carbachol (100 μM). The inhibition by carbachol of the cyclic AMP response to TSH was calculated and plotted as percentage of the initial effect (A). Simultaneously the [^{3}H]QNB binding capacity of the corresponding homogenates was measured (B).

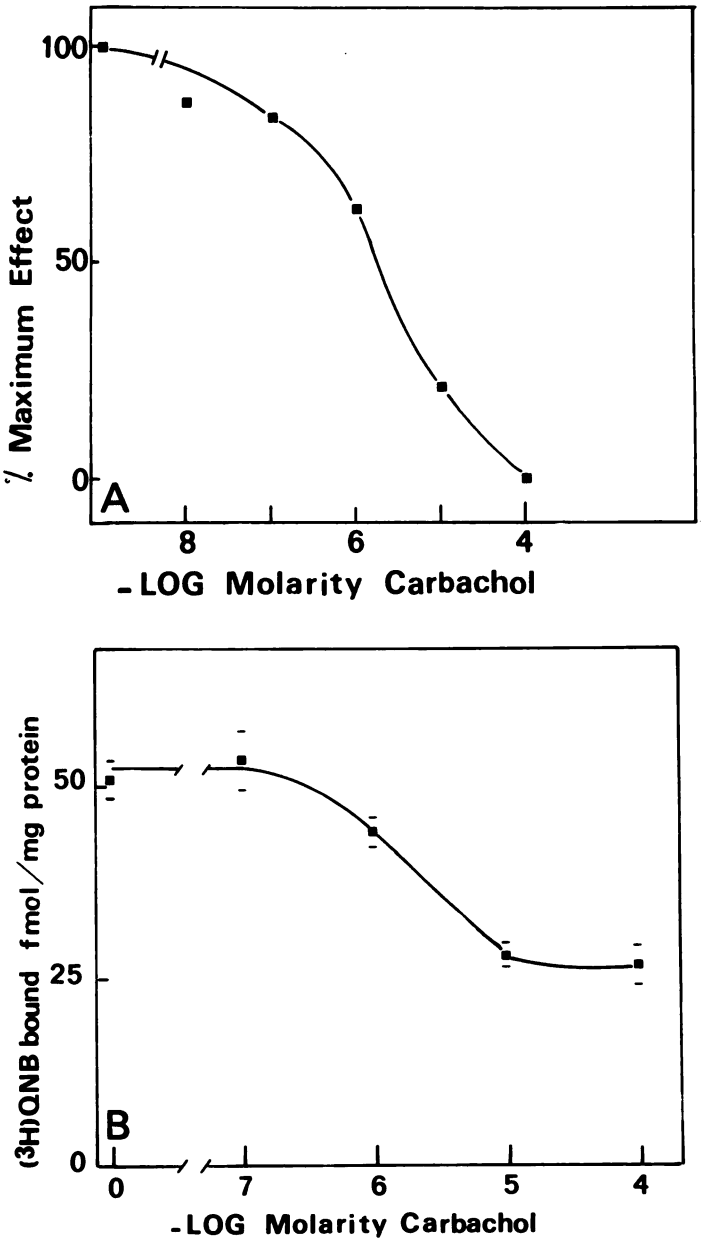

FIG. 2. *Chronic effects of graded concentrations of carbachol on acute carbachol effect and on [^{3}H]QNB binding capacity*

The experiment was conducted as in Fig. 1 except that the cells were treated for 6 hr with graded concentrations of carbachol before being acutely challenged with TSH (A) and before the [^{3}H]QNB binding capacity of the corresponding homogenates was measured (B).

As shown in Fig. 1A the inhibitory effect of carbachol gradually decreased with time in the presence of 100 μM agonist. After 6 hr in the presence of 100 μM carbachol, an almost complete disappearance of the effect of carbachol on TSH-stimulated cyclic AMP accumulation was observed. Simultaneously, QNB binding capacity was decreased but after 6 hr it was only reduced to one-half its initial value (Fig. 1B). The $t_{1/2}$ for both desensitization and decrease in receptor number were around 45 min.

When cells were exposed for 6 hr to graded concentra-

TABLE 1

Carbachol-induced desensitization and decrease of [^{3}H]QNB binding: effect of atropine

Cells were cultured for 4 days in the presence of TSH (100 μU/ml) and during the last 6 hr carbachol (100 μM), atropine (0.1 μM), or carbachol + atropine were added. Cells were washed and challenged with TSH (10 mU/ml) with or without carbachol (100 μM). Simultaneously the QNB binding capacity of the corresponding homogenates was measured. Values in parentheses represent percentages of inhibition produced by carbachol.

	Compound present during 6-hr incubation			
	Control	Carbachol (100 μM)	Atropine (0.1 μM)	Carbachol + atropine
Cyclic AMP (pmoles/5 min/mg protein) after acute stimulation with				
TSH (10 mU/ml)	469 ± 10	335 ± 16	—	383 ± 16
TSH (10 mU/ml) + carbachol (100 μM)	241 ± 17 (48%)[a]	328 ± 28 (2%)[b]	—	274 ± 19 (28%)[c]
[^{3}H]QNB binding capacity (fmoles/mg protein)	49.6 ± 1.2	29.5 ± 1.3[d]	49.2 ± 1.6[e]	46.0 ± 1.5[e]

[a] Versus TSH alone, $p < 0.001$.
[b] Versus TSH alone, not significant.
[c] Versus TSH alone, $p < 0.01$.
[d] Versus control, $p < 0.001$.
[e] Versus control, not significant.

tions of carbachol, a dose-dependent desensitization was observed in the 0.1–100 μM concentration range (Fig. 2A). The decrease in QNB binding capacity was observed in the 0.1–10 μM concentration range, and no further decrease was obtained after 6 hr of treatment with 100 μM carbachol (Fig. 2B). A half-maximal effect was observed after 6 hr in the presence of about 2 μM carbachol.

Atropine when present in the culture medium had no effect per se on either effect or QNB binding but inhibited carbachol-induced densensitization and decrease in [^{3}H] QNB binding capacity (Table 1). Scatchard analysis of QNB binding showed that no change in equilibrium constant was observed upon chronic carbachol treatment. The number of receptors per cell was decreased from 4300 to 2300 after 6 hr of treatment with carbachol (Fig. 3).

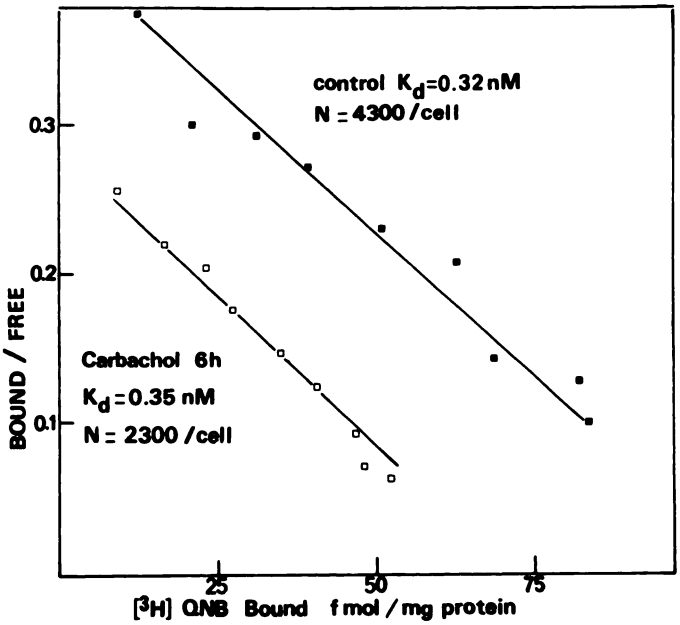

FIG. 3. *Scatchard plot analysis of [^{3}H]QNB binding after chronic treatment with carbachol*

Cells were cultured for 4 days in the presence of TSH (100 μU/ml) and treated for 6 hr with carbachol (100 μM). The specific binding of graded concentrations of [^{3}H]QNB was measured on homogenates derived from cells treated or not with carbachol. Nonspecific binding, measured in the presence of 10 μM scopolamine, was subtracted from total binding.

The QNB binding capacity of cells not treated with carbachol was stable for the last 30 hr of incubation.

Recovery from carbachol-induced desensitization. Cells treated with 100 μM carbachol for 24 hr were washed and further incubated in the absence or presence of agonist. After 6 hr, cells were washed and challenged with TSH, together or not with carbachol. Simultaneously, QNB binding was measured on the corresponding homogenates. As shown in Table 2, cells treated for 30 hr with 100 μM carbachol were no longer responsive to carbachol and had lost two-thirds of their QNB binding sites. Cells which were incubated for 6 hr in the absence

TABLE 2

Recovery from carbachol-induced desensitization

Cells were cultured for 4 days with TSH (100 μU/ml). During the last 24 hr carbachol (100 μM) was added. Cells were washed and further cultured for 6 hr in the presence or absence of carbachol. Cells were then recovered, washed, and challenged with TSH (10 mU/ml) with or without carbachol (100 μM). Simultaneously the QNB binding capacity of the corresponding homogenates was measured. Values in parentheses represent percentages of inhibition induced by carbachol.

	Conditions of chronic carbachol treatment		
	Control	Carbachol (100 μM) for 30 hr	Carbachol for 24 hr, then 6 hr without
Cyclic AMP (pmoles/5 min/mg protein) after acute stimulation with			
TSH (10 mU/ml)	1455 ± 108	1709 ± 99	1428 ± 86
TSH (10 mU/ml) + carbachol (100 μM)	1083 ± 69 (25%)[a]	1785 ± 124 (4%)[b]	1108 ± 89 (23%)[a]
[^{3}H]QNB binding capacity (fmoles/mg protein)	120 ± 6.2	37.0 ± 4.7[c]	59.5 ± 2.2[d]

[a] Versus TSH alone, $p < 0.005$.
[b] Versus TSH alone, not significant.
[c] Versus control, $p < 0.001$.
[d] Versus 30-hr carbachol treatment, $p < 0.02$.

TABLE 3

Effect of cycloheximide on carbachol-induced desensitization

Cells were cultured for 4 days in the presence of TSH (100 μU/ml). During the last 6 hr cycloheximide (100 μM), carbachol (100 μM), or cycloheximide + carbachol were added. Cells were recovered, washed, and challenged with TSH (10 mU/ml) in the presence or absence of carbachol (100 μM). Simultaneously the QNB binding capacity was measured on the corresponding homogenates. Values in parentheses represent percentages of inhibition induced by carbachol.

	Conditions of chronic treatment for 6 hr			
	Control	Cycloheximide (100 μM)	Carbachol (100 μM)	Carbachol + cycloheximide
Cyclic AMP (pmoles/5 min/mg protein) after acute stimulation with				
TSH (10 mU/ml)	752 ± 30	595 ± 35	786 ± 34	586 ± 21
TSH (10 mU/ml) + carbachol (100 μM)	467 ± 38 (38%)[a]	379 ± 11 (36%)[a]	652 ± 40 (17%)[b]	531 ± 41 (9%)[c]
[^{3}H]QNB binding capacity (fmoles/mg protein)	47 ± 2.3	39 ± 1	27 ± 1.5[d]	31 ± 0.6[e]

[a] Versus TSH alone, $p < 0.01$.
[b] Versus TSH alone, $p < 0.05$.
[c] Versus TSH alone, not significant.
[d] Versus control, $p < 0.01$.
[e] Versus cycloheximide alone, $p < 0.01$.

of carbachol after 24 hr of treatment recovered their full sensitivity to acute carbachol stimulation. In contrast, the recovery of binding capacity was partial (Table 2). Only 25% of the lost receptors reappeared. The full complement of receptors was therefore not required for the expression of the acute sensitivity to carbachol. After 24 hr of incubation in the absence of agonist the initial QNB binding capacity was restored (not shown).

Effect of cycloheximide on carbachol-induced desensitization and recovery. Previous results showed that chronic treatment with carbachol induced a complete desensitization and a partial reduction of QNB binding capacity. Sensitivity could be restored without complete recovery of initial QNB binding levels. This phenomenon suggested either receptor-adenylate cyclase coupling or an altered renewal of the receptor protein. We therefore tested the effect of an inhibitor of protein synthesis on both desensitization and recovery. Under the conditions used, 99% inhibition of protein synthesis was observed during 6 hr without change in cell viability.

As shown in Table 3, cycloheximide, which reduced the cyclic AMP response to TSH, did not impair the acute carbachol response. When cells were treated with both cycloheximide and carbachol, their acute sensitivity to carbachol was abolished as when cells were treated with carbachol alone.

Simultaneously, the QNB binding capacity of the corresponding homogenates was measured. Cycloheximide alone reduced the number of QNB binding sites during 6 hr of incubation, but if carbachol was also present a larger decrease was observed. Agonist-induced desensitization and the concomitant decrease in QNB binding were therefore not dependent on active protein synthesis.

Similar experiments were performed on the effect of cycloheximide on the recovery process following carbachol-induced desensitization. Cells were treated with carbachol during 24 hr and allowed to recover during 6 hr. As shown in Table 4, the sensitivity was not restored when cycloheximide was present during the recovery process. The QNB binding was further decreased when,

TABLE 4

Effect of cycloheximide on recovery process after carbachol-induced desensitization

Cells were cultured for 4 days in the presence of TSH (100 μU/ml). During the last 24 hr, carbachol (100 μM) was added. Cells were washed and further cultured without carbachol in the presence or absence of cycloheximide (100 μM). Cells were then recovered and challenged with TSH (10 mU/ml) with or without carbachol (100 μM). Simultaneously the QNB binding capacity of the corresponding homogenates was measured. We checked that QNB binding did not change significantly in control cells during the desensitization and recovery period.

	Conditions of chronic treatment			
	Control	Carbachol for 24 hr then recovery for 6 hr	Carbachol for 30 hr	Carbachol for 24 hr + cycloheximide for 6 hr
Cyclic AMP (pmoles/5 min/mg protein) after acute stimulation with				
TSH (10 mU/ml)	—	978 ± 59	—	688 ± 90
TSH (10 mU/ml) + carbachol (100 μM)	—	767 ± 59 (22%)[a]	—	784 ± 53[b]
[^{3}H]QNB binding capacity (fmoles/mg protein)	47 ± 5	32 ± 2.7	12 ± 1.5[c, d]	13.2 ± 1.6[e]

[a] Versus TSH alone, $p < 0.05$.
[b] Versus TSH alone, not significant.
[c] Versus TSH alone, $p < 0.001$.
[d] Versus carbachol for 24 hr then recovery for 6 hr, $p < 0.001$.
[e] Versus carbachol for 30 hr, not significant.

after 6 hr of incubation in the presence of carbachol, cells were incubated without carbachol and with cycloheximide. It therefore appears that recovery of both the effect and QNB binding required active protein synthesis. The QNB receptors which were present after chronic carbachol treatment were not susceptible to activation in the absence of new protein synthesis.

DISCUSSION

Our results show that the cholinergic muscarinic sensitivity of cultured thyroid cells is susceptible to agonist-induced desensitization. This phenomenon has the following characteristics. (a) Complete desensitization was obtained within 6 hr in the presence of 100 μM carbachol. (b) The process was completely reversible within 6 hr after removing the agonist. (c) Desensitization did not require protein synthesis, whereas recovery was dependent on active protein synthesis. These properties of the desensitization process are shared with other muscarinic systems recently described (7–10). In the same cell culture system we showed that thyrotropin- and prostaglandin-induced homologous desensitization was inhibited by puromycin and cycloheximide (4). This suggests that the various desensitization phenomena do not proceed by similar mechanisms.

The disappearance of the carbachol effect could be dependent on receptor loss or on uncoupling between the receptor and the adenylate cyclase system. We observed that the decrease in receptor number was partial (50%) under conditions in which sensitivity was completely lost. Since binding studies were performed using cell homogenates, the possibility exists that the residual binding may reflect an intracellular pool of receptors. This possibility cannot be ruled out and is supported by the rapid recovery of the effect (6 hr) without a simultaneous increase in binding capacity. The effect of cycloheximide on the recovery of sensitivity weighs against the existence of this intracellular pool of receptors unless we suppose that the coupling between newly exposed receptors and adenylate cyclase is dependent on *de novo* protein synthesis.

We have defined the receptors only with the binding of [^{3}H]QNB, a ligand which had a single population of binding sites (1, 9, 11). It has been recently shown that the use of muscarinic agonists reveals receptors with various affinities (12–14). Studies using these ligands might be necessary to discriminate between receptors found on cells cultured under various conditions.

The role of guanyl nucleotides on the properties of muscarinic receptors might also be relevant to our observations, since we have shown that TSH, when present chronically, increases the intracellular guanosine triphosphate levels of thyroid cells 5–10 times (15); on the other hand, a dramatic effect of guanosine triphosphate on muscarinic receptors binding properties has been reported in other systems (16, 17). Multiple binding states for muscarinic receptors have recently been described (14, 18, 19) and a model has been proposed to account for these observations. This hypothesis suggests that modification of binding properties occurred at two sites, one being the muscarinic receptor proper and the other a regulatory site. TSH, PGE_2, and carbachol might have effects at various levels on the thyroid cell muscarinic receptor-adenylate cyclase complex.

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