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Muscarinic Cholinergic Receptor-Mediated Control of Cyclic AMP Metabolism

Agonist-Induced Changes in Nucleotide Synthesis and Degradation

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SUMMARY

Activation of muscarinic cholinergic receptors on 1321N1 human astrocytoma cells results in a 40–70% inhibition of isoproterenol- or prostaglandin E_1 (PGE_1)-stimulated accumulation of cyclic AMP. Previous investigations have demonstrated that this effect is due to a Ca^{2+}-dependent activation of phosphodiesterase in the presence of muscarinic receptor agonists. However, during prolonged exposure of 1321N1 cells to a cholinergic agonist, a series of adaptive changes occurs which culminates in a complete loss of the muscarinic receptor-mediated inhibition of cyclic AMP accumulation. These alterations include: (a) A 50–100% increase in the capacity of isoproterenol and PGE_1 to stimulate cyclic AMP accumulation. This phenomenon was rapid in onset, reached a maximum in 15–20 min, and disappeared over the next 2 hr even in the continued presence of carbachol. (b) A loss of the effects of muscarinic receptor stimulation on cyclic AMP accumulation. This phenomenon was apparent within 15 min after addition of carbachol, and complete desensitization was observed after 75 min. The loss of muscarinic receptor-mediated effects on cyclic AMP levels was due to a loss of the Ca^{2+}-dependent stimulation of phosphodiesterase activity by muscarinic receptor agonists. (c) A loss of muscarinic receptors as assessed by [^{3}H]quinuclidinyl benzilate binding. This effect was apparent after 90 min in the presence of carbachol. More than 80% of the receptors were lost after 24 hr, with no change occurring in the K_D of [^{3}H]quinuclidinyl benzilate. The concentration-effect curve for carbachol-induced changes in agonist responsiveness of the cyclic AMP system was similar to that for carbachol-induced reductions in cyclic AMP levels. Coincubation of carbachol with a saturating concentration of atropine prevented these adaptive changes from occurring. Although incubation of cells in Ca^{2+}-free buffer or in the presence of 20 mM Co^{2+} prevented the inhibitory effects of muscarinic receptor stimulation on cyclic AMP accumulation, carbachol preincubations under these conditions still produced the adaptive changes in agonist responsiveness. The divalent cation ionophore, A23187, mimics the effects of muscarinic receptor stimulation on cyclic AMP levels by activating phosphodiesterase. Following complete carbachol-induced loss of responsiveness to muscarinic receptor agonists, A23187 was still capable of inhibiting cyclic AMP accumulation.

INTRODUCTION

Adaptive changes in the muscarinic cholinergic receptor system have been shown to result from prolonged exposure of a variety of target cells to muscarinic receptor agonists (1–8). These agonist-induced alterations have included both decreases in cellular responsiveness to homologous agonists (1–3, 5–8) and decrements in the number of muscarinic cholinergic receptors (2, 4–7). In N1E-115 neuroblastoma cells the loss of responsiveness to muscarinic agonists occurred prior to the loss of receptors, leading to the suggestion that in these cells receptor loss is not primarily responsible for agonist-induced desensitization (2).

Although a number of studies of agonist-induced desensitization of muscarinic receptor systems have focused on the accumulation of cyclic GMP (1, 2) or on muscle contraction (5, 6) as the primary measure of muscarinic receptor activation, recent experiments have begun to explore the relationship between muscarinic receptor activation and regulatory changes in cyclic AMP metab-

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olism (7, 8). In this regard, we recently have confirmed (9) the initial observations of Gross and Clark (10) demonstrating that stimulation of muscarinic receptors on 1321N1 human astrocytoma cells results in a decrease in cyclic AMP accumulation. This effect is due to a muscarinic receptor-mediated activation of phosphodiesterase with little or no direct effect of muscarinic receptors on adenylate cyclase activity (9). During the characterization of this novel mechanism of hormonal control we have observed that incubation of 1321N1 cells with muscarinic receptor agonists results in adaptive alterations in the cyclic AMP system of these cells. Data are reported in the present study that suggest that this adaptive response consists of a complex series of events reflecting modifications of both synthesis and degradation of cyclic AMP.

EXPERIMENTAL PROCEDURES

Materials. DMEM,[2] trypsin, and fetal calf serum were purchased from GIBCO (Grand Island, N. Y.). OXO, ISO, atropine sulfate, carbachol, Hepes, Tris, and Dowex 50-X8-400 H^+-form were obtained from Sigma Chemical Company (St. Louis, Mo.). EDTA, EGTA, and neutral alumina (Brockman activity 1, 80–200 mesh) were obtained from Fisher Scientific Company (Pittsburgh, Pa.). All chemicals and drugs were of reagent grade or the highest quality available.

[^{3}H]QNB (40.2 Ci/mmole) and [α-^{32}P]ATP (15–33 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Adenine (26–29 Ci/mmole) was obtained from Amersham (Arlington Heights, Ill.), and cyclic [^{3}H]AMP (15 Ci/mmole) was purchased from ICN (Irvine, Calif.).

Cell culture and incubation with agonists. Conditions for maintenance of 1321N1 human astrocytoma cells were as previously described (9, 11). Briefly stated, cells from postconfluent stock cultures were subcultured into 24-mm glass scintillation vials (4.5 cm^2) or 150-mm dishes at a density of 5000–8000 cells/cm^2. The cells were allowed to grow for 7 days in bicarbonate-buffered DMEM + 5% fetal calf serum at 37° in an atmosphere of 8% CO_2/92% air.

Cholinergic agonist-induced changes in the hormone responsiveness of the cyclic AMP system were studied following incubation of 1321N1 cells with carbachol for various periods of time.[3] For long-term incubations, carbachol (usually 100 μM final concentration) was dissolved in DMEM buffered with 25 mM Hepes (bicarbonate-free, pH 7.4) and was filtered under sterile conditions directly into 150-mm culture dishes or glass scintillation vials containing 1321N1 cells and the growth medium described above. In experiments involving carbachol pretreatment periods of less than 90 min, the normal growth medium was replaced with DMEM buffered with 25 mM Hepes (bicarbonate-free, pH 7.4), and incubations were carried out in a shaking water bath at 37°. Incubations with carbachol were terminated by three rapid washes with DMEM-Hepes (whole-cell cyclic AMP assays) or 10 mM Tris + 1 mM EDTA (binding assays).

Membrane preparation. Membranes were prepared by aspirating the growth medium and swelling the cells by the addition of 10 ml of 1 mM Tris buffer (pH 7.5 at 0°). After 15 min on ice in the lysing buffer the cells were scraped from the plate with a rubber spatula. Washed membranes were prepared by three centrifugations at 30,000 × g for 10 min in 10 mM Tris containing 1 mM EDTA (pH 7.5 at 0°). The final pellet was resuspended in 10 mM Tris (pH 7.5 at 37°). Membranes not used on the day of preparation were frozen at −80° in 250 mM sucrose/5 mM $MgCl_2$/50 mM Tris (pH 7.5 at 0°). Tissue stored in this manner showed no changes in [^{3}H]QNB binding over a 2-month period.

Measurement of cyclic AMP accumulation in intact cells. The accumulation of cyclic AMP was measured utilizing 1321N1 cells usually grown for 7 days in glass scintillation vials. At the time of assay, each vial contained approximately 0.8–1.0 × 10^6 cells or 100–150 μg of protein. In most experiments cyclic AMP accumulation was monitored by a modification (12) of the method of Shimizu *et al.* (13). The growth medium was aspirated and replaced with 1.0 ml of DMEM (bicarbonate-free) + 25 mM Hepes (pH 7.4) containing [^{3}H]adenine (2.0 μCi/ml). After 1 hr in a shaking water bath at 37°, the medium was aspirated and replaced with fresh DMEM-Hepes, and hormonal challenge was carried out as described below. The [^{3}H]adenine prelabeling step was always carried out in the 1-hr period preceding measurement of agonist-induced effects on cyclic AMP accumulation. Thus, in experiments requiring preincubations with carbachol, both muscarinic agonist and [^{3}H]adenine were present; carbachol had no effect on the extent of labeling of ATP pools. In some long-term incubations, [^{3}H]adenine was added directly to the serum-containing growth medium in a volume of 50 μl rather than changing the medium to DMEM-Hepes.

In most experiments, the synthesis of [^{3}H]cyclic AMP was induced by the addition of ISO in a volume of 50 μl (10 μM final concentration). The inhibition of [^{3}H]cyclic AMP accumulation was measured by challenging the cells with a muscarinic receptor agonist (usually OXO, 100 μM final concentration) added simultaneously with ISO. Unless otherwise indicated, cyclic AMP accumulation was measured over a 5-min period. The reaction was terminated by aspiration followed immediately by the addition of 1 ml of 5% trichloroacetic acid (w/v). [^{3}H]Cyclic AMP was extracted into 5% trichloroacetic acid overnight at 4°.

[2] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium with glucose (1 g/liter), L-glutamine, and sodium pyruvate, without sodium bicarbonate; OXO, oxotremorine sesquifumarate; ISO, (−)-isoproterenol (+)-bitartrate; Hepes, 4-(2-hydroxyethyl) 1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)- $N,N,N'N'$-tetraacetic acid; QNB, (−)-quinuclidinyl benzilate; $K_{0.5}$, the concentration of drug that produces 50% of the maximal effect; PGE_1, prostaglandin E_1.

[3] Carbachol was more effective than OXO for the induction of adaptive changes in agonist responsiveness. This difference was at least in part due to the greater stability of carbachol during long-term incubation with cells. However, carbachol was routinely more effective for inducing these changes even at short times of incubation, suggesting that, although these agents are equally effective in activating phosphodiesterase and decreasing cyclic AMP levels, carbachol is more efficacious in inducing desensitization.

Chromatographic separation of [^{3}H]cyclic AMP from [^{3}H]ATP was carried out as previously described (9) by a modification of the method of Salomon *et al.* (14). The [^{3}H]cyclic AMP formed during hormonal challenge is expressed as the percentage conversion of [^{3}H]ATP to [^{3}H]cyclic AMP, i.e., (dpm of [^{3}H]cyclic AMP/dpm of [^{3}H]ATP + dpm of [^{3}H]cyclic AMP) × 100. In some experiments endogenous cyclic AMP also was determined by radioimmunoassay using the method of Steiner *et al.* (15). Tricholoroacetic acid extracts containing [^{3}H] cyclic AMP were processed as described above except that 100-μl samples were taken from each extract for radioimmunoassay after the Dowex column step. Results obtained with the radioimmunoassay of cyclic AMP were always similar to those obtained with the prelabeling assay. The [^{3}H]adenine prelabeling technique was routinely used because of the speed and relative reproducibility of this method.

[^{3}H]QNB binding assay. The number of muscarinic cholinergic receptors in membranes from 1321N1 cells was assessed using [^{3}H]QNB as we have previously described (9, 16).

Protein determination. Tissue protein concentration was determined by the method of Lowry *et al.* (17) or by the method of Bradford (18) when Hepes buffer was present in the samples.

RESULTS

As we (9) and others (10) have reported, incubation of 1321N1 cells with muscarinic receptor agonists results in a decrease in ISO-stimulated cyclic AMP levels (Table 1). However, after a 24-hr exposure of cells to 100 μM carbachol,[3] the inhibitory effect of OXO was lost, with no consistent change occurring in the level of ISO-stimulated activity (Table 1). As is illustrated below, this adaptive change in the responsiveness of the cyclic AMP system to a muscarinic receptor agonist consists of several components.

The most straightforward alteration to quantify following extended muscarinic receptor stimulation was a loss of muscarinic receptors as assessed by [^{3}H]QNB binding.

TABLE 1

Accumulation of cyclic AMP in intact 1321N1 cells after long-term preincubation with carbachol

1321N1 Cells were incubated for 24 hr at 37° in DMEM + 5% fetal calf serum in the absence or presence of 100 μM carbachol. Cyclic AMP accumulation was then measured as described under Experimental Procedures after a 5-min exposure of cells to ISO (10 μM) or ISO (10 μM) + OXO (100 μM). Results are expressed as the mean ± standard error of the mean of the percentage conversion of [^{3}H]ATP to [^{3}H] cyclic AMP and as the percentage inhibition by OXO of ISO-stimulated cyclic AMP accumulation. The data are representative of five experiments.

Treatment	Drug	% Conversion [^{3}H]ATP to [^{3}H]cyclic AMP	% Inhibition
Control	ISO	2.12 ± 0.06	
	ISO + OXO	0.60 ± 0.03	71.7
Carbachol, 100 μM	ISO	1.97 ± 0.06	
	ISO + OXO	2.53 ± 0.09	−22.1

Receptor loss in the presence of 100 μM carbachol followed a relatively slow time course, with a $t_{1/2}$ of approximately 5 hr (Fig. 1). No significant loss ($p > 0.05$) of receptors was observed until after 2 hr of incubation with 100 μM carbachol ($p < 0.05$); maximal loss was achieved by 24 hr ($p < 0.001$). Isotherms of [^{3}H]QNB binding with membranes from 1321N1 cells incubated for 24 hr in 100 μM carbachol are illustrated in Fig. 2. Receptor density decreased from 54.7 ± 9.0 fmoles/mg of protein in control tissue to 10.2 ± 3.3 fmoles/mg of protein in membranes from cells incubated with carbachol. No significant change ($p > 0.05$) occurred in the K_D (13.7 ± 2.0 pM and 12.2 ± 3.6 pM for membranes from control and desensitized cells, respectively; $n = 4$). The recovery of receptors following a 24-hr exposure to 100 μM carbachol was also examined (Fig. 3). After a lag of approximately 4 hr, reappearance of [^{3}H]QNB binding sites was observed, with full expression of receptors attained after 24 hr of incubation in the absence of carbachol.

The loss of capacity of OXO to inhibit cyclic AMP accumulation occurred much faster than the decrement in receptor number in cells incubated with carbachol (Fig. 1). After a lag of 15 min, desensitization to the inhibitory effects of OXO occurred with a $t_{1/2}$ of approximately 30 min; maximal effects were observed after 75–90 min (Fig. 1). The concentration-effect relationship for the induction of this apparent desensitization by carbachol is illustrated in Fig. 4. In control cells, OXO inhibited ISO-stimulated cyclic AMP accumulation by 40–75%. Essentially complete loss of inhibition occurred subsequent to incubation with carbachol (100 μM) for 75

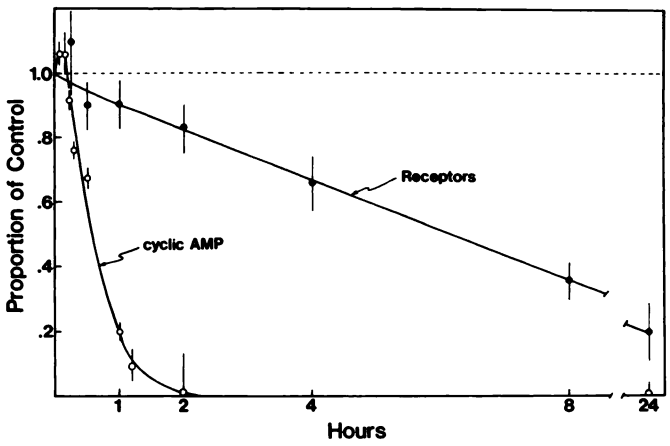

FIG. 1. *Carbachol-induced loss of muscarinic receptor-mediated inhibition of cyclic AMP accumulation and muscarinic receptors*

Cells were incubated in 100 μM carbachol for the indicated times and then washed free of the agent. The capacity of OXO (100 μM) to inhibit ISO-stimulated (10 μM) cyclic AMP accumulation in whole cells during a subsequent 5-min incubation and the number of muscarinic receptors in membranes prepared from carbachol-treated cells were determined as described under Experimental Procedures. Each curve represents the composite of 9–16 determinations in 3 or 4 similar experiments. The data are plotted as the percentage of activities determined with untreated cells; that is, the percentage inhibition by OXO of ISO-stimulated cyclic AMP accumulation at each time point is divided by the percentage inhibition (45–70%) observed in control cells, and the number of receptors measured at each time point is divided by the initial level of the receptors. The data are presented as means ± standard error of the mean.

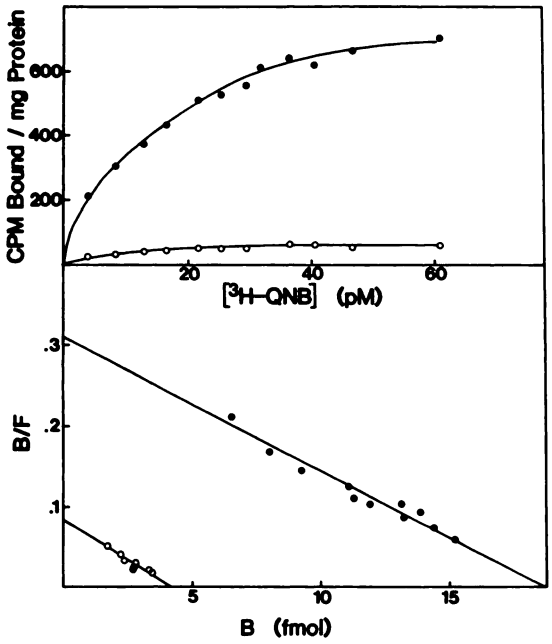

FIG. 2. *Carbachol-induced alteration in muscarinic receptor density*

Cells were incubated in the absence (●) or presence (○) of 100 μM carbachol for 24 hr. Membranes were prepared and [^{3}H]QNB binding assays carried out as described under Experimental Procedures. *Top panel.* Saturation binding isotherms for [^{3}H]QNB incubated with membranes of 1321N1 cells exposed to 100 μM carbachol (○) or vehicle (●) for 24 hr. *Bottom panel.* Scatchard plot of the same data. Results are representative of four similar experiments.

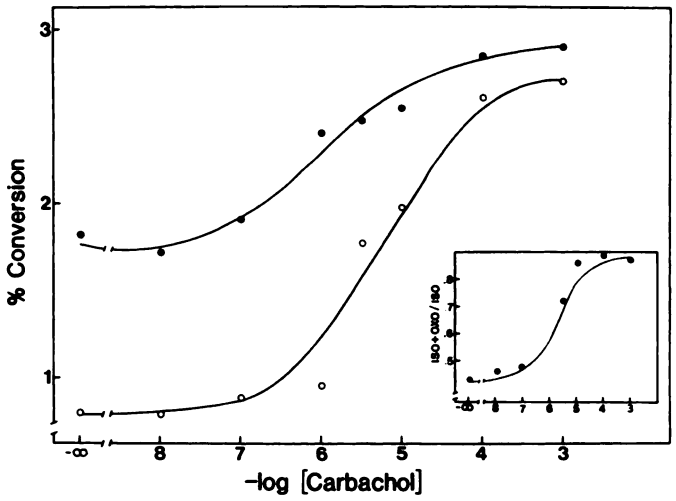

FIG. 4. *Concentration-effect curve for carbachol-induced changes in agonist responsiveness*

Cells were incubated for 75 min in the presence of various concentrations of carbachol, then washed free of agonist and rechallenged for 5 min with 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○). The data are averages of four experiments, each in triplicate.

Inset. The ratio of the activity in the presence of ISO + OXO to that with ISO alone is plotted as a function of the concentration of carbachol in the preincubation. The average standard error for the determination of the level of inhibition was ± 15% (range 11–26%).

min (Fig. 4). Preincubation with carbachol also resulted in an increase in the capacity of ISO to elevate cyclic AMP levels. This point is considered in more detail below. The level of cyclic AMP accumulation in ISO + OXO-stimulated cells relative to ISO-stimulated levels is depicted in the *inset* to Fig. 4 and illustrates the net desensitization to muscarinic receptor agonists that occurred as a function of carbachol concentration in the preincubation. The $K_{0.5}$ for carbachol-induced desensitization determined from this type of curve was 5.1 ± 1.1 μM (n = 4). This value agrees well with the $K_{0.5}$ (5.0 ± 1.0 μM) for carbachol-induced reduction in cyclic AMP levels in control cells (data not shown). As illustrated in Fig. 5,

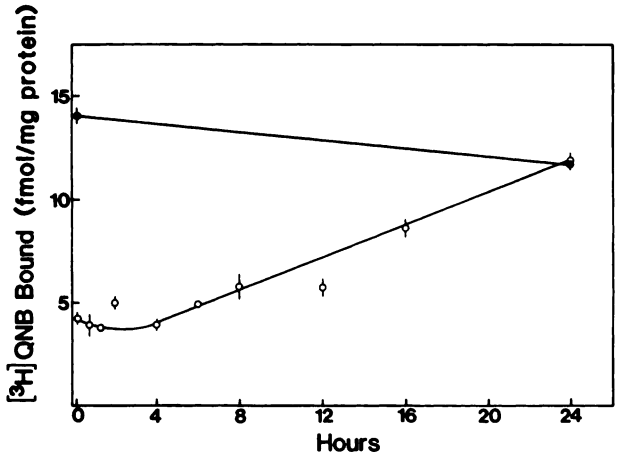

FIG. 3. *Recovery of [^{3}H]QNB binding after incubation with carbachol*

Cells were incubated with 100 μM carbachol (○) or vehicle (●) for 24 hr, then washed free of agonist and incubated at 37° in DMEM + 5% fetal calf serum. [^{3}H]QNB binding was carried out with membranes prepared at the indicated times as described under Experimental Procedures. The means ± standard error of the mean of four determinations are shown.

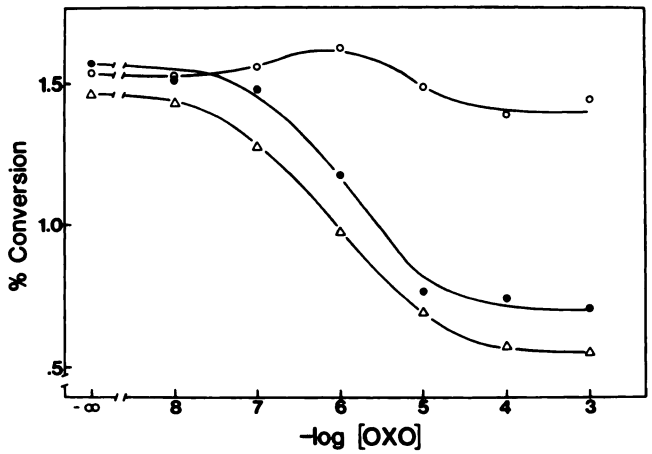

FIG. 5. *Concentration-effect relationship for muscarinic receptor-mediated inhibition of cyclic AMP accumulation in control and desensitized cells*

1321N1 cells were incubated for 75 min in the absence of added drug (●) or in the presence of 100 μM carbachol (○) or 100 μM carbachol + 1 μM atropine (△). The cells were then washed free of drug and rechallenged for 5 min with 10 μM ISO and the indicated concentrations of OXO. The data are means of triplicate determinations and are representative of four similar experiments. The error for the determination of cyclic AMP accumulation ranged from 6% to 20% and averaged 12% across all conditions.

the loss of responsiveness to OXO involves a decrease in efficacy of agonist, since concentrations of OXO as high as 1 mM failed to reduce cyclic AMP levels in desensitized cells. The presence of 1 μM atropine during incubation with carbachol essentially eliminated the carbachol-induced changes in muscarinic receptor responsiveness (Fig. 5).

Although demonstrating a loss of agonist-induced inhibition of cyclic AMP accumulation, these findings present an oversimplified view of the effects of extended activation of muscarinic receptors with carbachol. As has already been pointed out, the level of desensitization to the inhibitory effects of a muscarinic receptor agonist is the result of the contribution of two events (see Fig. 4). First, the accumulation of cyclic AMP during challenge with ISO + OXO was enhanced with increasing duration of pre-exposure of 1321N1 cells to carbachol (Fig. 6, *top panel*). This is the expected result if an effect of muscarinic receptor activation by carbachol is to desensitize the muscarinic receptor to subsequent activation. Second, preincubation of cells with carbachol resulted in an enhanced responsiveness to ISO alone (Fig. 6, *top panel*). The increased ISO responsiveness was transient, returning to control levels by 24 hr of incubation of cells with carbachol.

The data in Fig. 6 (*bottom panel*) represent the accumulation of cyclic AMP in the ISO + OXO condition relative to accumulation in the presence of ISO alone. When the data are considered in this manner, desensitization to the inhibitory effects of muscarinic receptor activation was not expressed until after 15 min of preincubation with carbachol. This time corresponds closely to the peak of enhancement of ISO responsiveness. Both the enhancement of ISO-stimulated activity and muscarinic receptor-mediated inhibition of cyclic AMP accumulation began to diminish at the same time (Fig. 6). Full desensitization to the inhibitory effects of a muscarinic receptor agonist was observed after 75 min and occurred under conditions where ISO-stimulated cyclic AMP accumulation was still 40% above control.

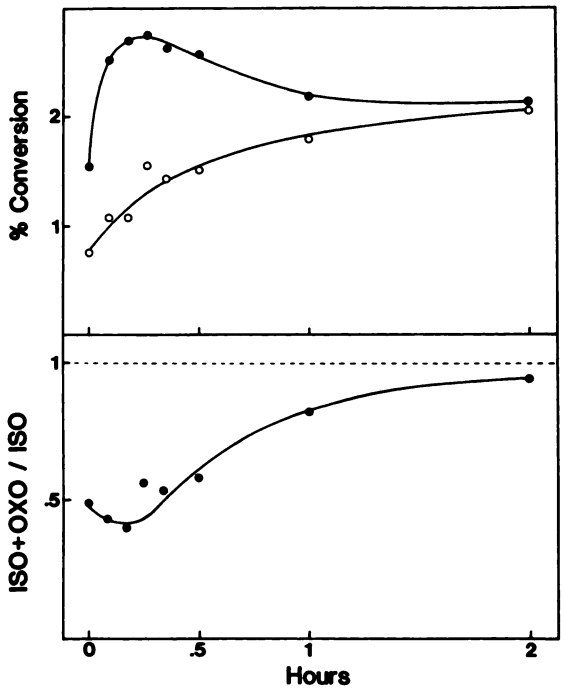

FIG. 6. *Time course of carbachol-induced changes in agonist responsiveness*

Top panel. 1321N1 cells were incubated in 100 μM carbachol for the indicated times, then washed free of agonist and re-challenged for 5 min with 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○). The accumulation of [^{3}H]cyclic AMP was measured as described under Experimental Procedures. The data are averages from 4–19 experiments with 3–6 replicates per experiment. Levels of cyclic AMP accumulation varied between experiments, yielding standard error values of 7–20% for each point. *Bottom panel.* The data are plotted as the ratio of cyclic AMP accumulation in the presence of ISO + OXO relative to the cyclic AMP accumulation in the presence of ISO alone.

The values summarized in Table 2 illustrate typical levels of ISO- or PGE_1-stimulated cyclic AMP accumulation in control cells and in cells previously incubated for 75 min with carbachol. A similar level of inhibition was observed with muscarinic receptor activation regardless of whether synthesis of cyclic AMP was stimulated with ISO or PGE_1. This inhibition was almost totally reversed after 75 min of preincubation with carbachol. A facilitation of both ISO-stimulated cyclic AMP accumulation and PGE_1-stimulated accumulation also was observed subsequent to preincubation with carbachol.

In an earlier report (9) we showed that the reduced accumulation of cyclic AMP in 1321N1 cells stimulated with a muscarinic receptor agonist could be attributed almost completely to a faster rate of degradation of cyclic AMP. Therefore, the rate of cyclic AMP degradation was compared in control cells and in cells previously incubated with carbachol. The degradation of cyclic AMP can be determined directly in intact cells by first elevating cyclic AMP levels by the addition of isoproterenol, then terminating the synthetic reaction by the addition of a saturating concentration of propranolol (9, 11; Fig. 7). Degradation of [^{3}H]cyclic AMP in control cells occurred in a single component with $k_{deg} = 0.21 \pm .05$ min^{-1} ($n = 3$). In accordance with earlier work (9), the initial rate of cyclic AMP degradation in the presence of 100 μM OXO (Fig. 7) was markedly enhanced ($k_{deg} = 1.13 \pm 0.13$ min^{-1}; $p < 0.01$, $n = 3$).[4] In contrast, following pretreatment of cells with carbachol (100 μM) for 75 min, the rate of degradation in the presence of OXO ($k_{deg} = 0.14 \pm 0.09$ min^{-1}; $n = 3$) was not different ($p > 0.05$) from the degradation rate in the absence of OXO. Thus, the loss of inhibitory effects of OXO on cyclic AMP accumulation after carbachol pretreatment can be correlated with a loss of muscarinic-receptor mediated activation of phosphodiesterase.

Following removal of carbachol from the medium, the inhibitory effect of OXO recovers with a much slower time course (Fig. 8) than was observed with the loss of inhibition (Fig. 1). The return of receptor responsiveness to levels measured prior to desensitization exhibited two components. After incubation with carbachol, both ISO-

[4] As we have previously reported (9), cyclic AMP degradation in the presence of OXO occurs by a predominant, rapid component and by a slower component that usually comprises less than 30% of the total degradation. The k_{deg} values that are provided in the text were calculated from data points obtained during the first 30 sec after the addition of propranolol.

TABLE 2

Inhibition of hormone-stimulated cyclic AMP accumulation after pretreatment with carbachol

Cells were incubated in 100 μM carbachol or vehicle (DMEM-Hepes, pH 7.4) for 75 min. The cells were washed free of carbachol and then rechallenged for 5 min with the indicated drugs. Cyclic AMP accumulation was measured as described under Experimental Procedures. The data are expressed as the mean ± standard error of the mean (n = 3–5) and are representative of five such experiments.

Pretreatment	Drug	% Conversion [^{3}H]ATP to [^{3}H]cyclic AMP	% Inhibition
Control	Basal	0.05 ± 0.002	
	ISO	1.28 ± 0.33	
	ISO + OXO	0.71 ± 0.19[a]	44.4
	PGE_1	1.04 ± 0.04	
	PGE_1 + OXO	0.60 ± 0.03[a]	42.8
Carbachol, 100 μM	Basal	0.03 ± 0.001	
	ISO	2.29 ± 0.28	
	ISO + OXO	1.91 ± 0.26	16.6
	PGE_1	1.63 ± 0.23	
	PGE_1 + OXO	1.45 ± 0.10	11.0

[a] Significantly different ($p < 0.05$) from cyclic AMP accumulation in the presence of stimulating agonist alone.

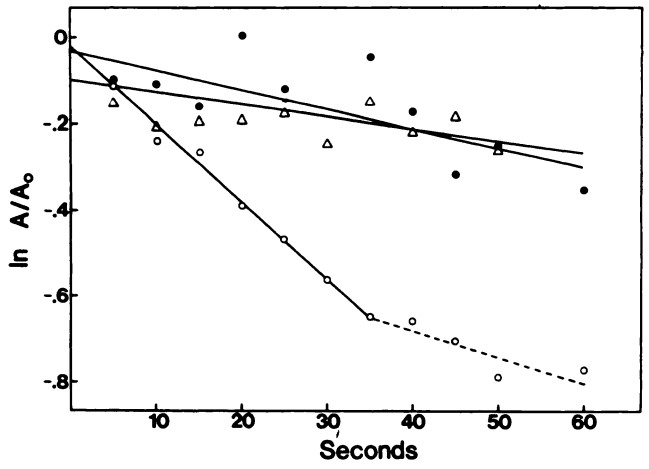

FIG. 7. *Effect of carbachol pretreatment on the rate of cyclic AMP degradation*

Cells were preincubated for 75 min in the absence (●, ○) or presence (△) of 100 μM carbachol. Following two washes with DMEM-Hepes the cells were rechallenged with 10 μM ISO. After 5 min the synthesis of cyclic AMP was blocked by the addition of propranolol (●, 10 μM final concentration) or propranolol + 100 μM OXO (○, △), and cyclic AMP levels were measured as a function of time after addition of propranolol. The results are expressed as the natural logarithm of the ratio of cyclic AMP accumulation at the indicated time (A) relative to the level prior to the addition of propranolol or propranolol + OXO (A_o). Each point represents the average of three determinations. Mean variation was 7.4–8.5% of measured cyclic AMP. The data are representative of four similar experiments.[4]

and ISO + OXO-stimulated cells accumulated the same levels of cyclic AMP (fig. 8). This level was significantly higher than accumulation in control cells stimulated with ISO. The enhanced capacity to accumulate cyclic AMP decayed rapidly under both conditions following removal of carbachol from the medium. Both ISO- and ISO + OXO-stimulated cyclic AMP accumulation reached the level of nondesensitized, ISO- stimulated cells approximately 15 min after the cells were washed free of carbachol and remained at that level until after approximately 2–4 hr, when the inhibitory effects of muscarinic receptor stimulation reappeared. Full inhibitory activity of OXO was not restored until after 24 hr of incubation in the absence of carbachol.

The enhanced capacity of ISO to stimulate cyclic AMP accumulation after a brief exposure to carbachol was an unexpected finding. Nevertheless, as evidenced in Figs. 4, 6, and 8 and Table 2, this effect was consistently observed in a variety of experiments. Concentration-effect curves to ISO for cells pretreated with carbachol for 15 min indicated that the efficacy of ISO was increased with no alteration ($p > 0.1$) occurring in the affinity ($K_{0.5}$ for control = 34 ± 2 nM; $K_{0.5}$ for carbachol-pretreated cells = 26 ± 3 nM). The kinetics of ISO-stimulated cyclic AMP accumulation was examined at the peak of the enhancement of responsiveness (i.e., after 15 min of preincubation with carbachol). As illustrated in Fig. 9, the rate of cyclic AMP accumulation was increased in carbachol-pretreated cells irrespective of whether incubations were carried out in the presence or absence of 3-isobutyl-1-methylxanthine. No alteration in the time to maximal accumulation of cyclic AMP was observed. Direct analysis of cyclic AMP degradation after pretreatment with carbachol for 15 min revealed no significant difference in phosphodiesterase activity determined in

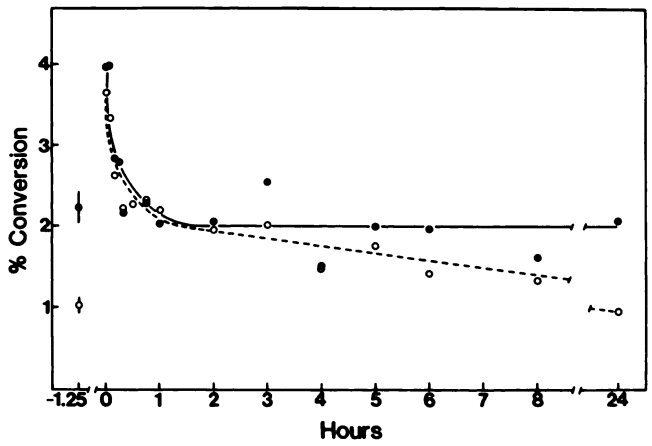

FIG. 8. *Recovery of muscarinic receptor-mediated inhibition of cyclic AMP accumulation after preincubation with carbachol*

Cells were incubated for 75 min in 100 μM carbachol, washed three times in sterile DMEM + 5% fetal calf serum, and then incubated in the absence of agonist. After the indicated recovery times, the cells were challenged with 10 μM ISO (●) or 10 μM ISO + 100 μM OXO (○) for 5 min. Control levels of cyclic AMP accumulation were measured prior to carbachol treatment (−1.25 hr). The data represent the average of five experiments with three to six replicates per experiment. Standard errors for each data point ranged from 3% to 20% of the mean. Control values are means ± standard error of the mean.

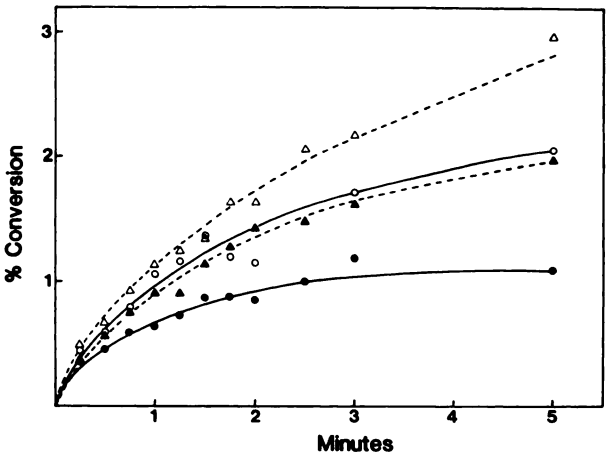

FIG. 9. *Time course of accumulation of cyclic AMP after preincubation with carbachol*

Cells were incubated in the presence (○, △) or absence (●, ▲) of carbachol for 15 min. The dishes were then rapidly washed free of agonist and rechallenged for 0.25–5 min with ISO (10 μM) in the absence (●, ○) or presence (▲, △) of 0.5 mM 3-isobutyl-1-methylxanthine. The results are averages of four determinations and are representative of four additional experiments. The average standard error was 6.0 ± 0.4% of measured cyclic AMP levels.

the absence of a cholinergic receptor agonist (k_{deg} = 0.42 ± 0.06 min^{-1} for control, k_{deg} = 0.50 ± 0.06 min^{-1} for carbachol-pretreated cells; n = 4).

We have previously demonstrated that the inhibitory effects of muscarinic receptor activation on cyclic AMP accumulation are dependent on the presence of Ca^{2+} in the medium (9). In the absence of Ca^{2+}, OXO does not reduce cyclic AMP levels, and no enhancement of phosphodiesterase activity is observed. In contrast, the enhancement of ISO-stimulated cyclic AMP accumulation that occurred subsequent to preincubation of 1321N1 cells with carbachol did not depend on the presence of Ca^{2+}. Preincubation with 100 μM carbachol for 15 min resulted in a 61% increase in ISO-stimulated cyclic AMP accumulation in cells that had been extensively washed with EGTA and maintained in Ca^{2+}-free medium (data not shown). The role of Ca^{2+} in the induction of desensitization by carbachol also was examined. Full desensitization to the inhibitory effects of OXO occurred when the carbachol preincubation was carried out under conditions that greatly reduced Ca^{2+} availability (i.e., Ca^{2+}-free buffer or 20 mM Co^{2+}; Table 3) and consequently prevented the inhibitory effects of OXO on cyclic AMP accumulation (ref. 9, and data not shown).

A dissociation between the requirements for inhibition of cyclic AMP accumulation versus desensitization to this response was further illustrated by the effects of the divalent cation ionophore, A23187, on cyclic AMP accumulation. We have previously shown (9) that A23187 accelerates the rate of cyclic AMP degradation and its effects can be completely reversed by the presence of a phosphodiesterase inhibitor. The presence of 1 μM A23187 inhibited ISO-stimulated cyclic AMP accumulation by 60–85% (Table 4). After incubation of 1321N1 cells in 100 μM carbachol for 75 min the response to A23187 was unaffected even though inhibition of cyclic AMP accumulation mediated by the muscarinic receptor was lost.

TABLE 3

Desensitization of muscarinic receptor-mediated inhibition of cyclic AMP accumulation in the absence of Ca^{2+}

Cells were washed with Hepes-buffered saline + 0.5 mM EGTA and then incubated at 37° for 60 min in Ca^{2+}-free buffer or in DMEM + 20 mM Co^{2+}, with or without 100 μM carbachol. After washing once, the medium was replaced with DMEM-Hepes (pH 7.4) and the cells were allowed to reequilibrate for 10 min. The cells were then challenged for 5 min with 10 μM ISO or 10 μM ISO + 100 μM OXO, and the percentage conversion from [^{3}H]ATP to [^{3}H]cyclic AMP was measured as described under Experimental Procedures. The results are expressed as the mean ± standard error of the mean (n = 5) and are representative of four similar experiments.

Desensitization medium	Pretreatment condition	Addition	% Conversion [^{3}H]ATP to [^{3}H]cyclic AMP
Ca^{2+}-free buffer	Control	ISO	1.45 ± 0.11
		ISO+ OXO	0.83 ± 0.06[a]
	Carbachol	ISO	1.96 ± 0.19
		ISO + OXO	2.04 ± 0.11
DMEM + 20 mM Co^{2+}	Control	ISO	1.13 ± 0.06
		ISO + OXO	0.66 ± 0.05[a]
	Carbachol	ISO	1.48 ± 0.05
		ISO + OXO	1.45 ± 0.12

[a] Significantly different ($p < 0.01$) from percentage conversion in the presence of ISO alone.

DISCUSSION

Muscarinic cholinergic receptors of 1321N1 astrocytoma cells are linked to the cyclic AMP-generating system by a novel mechanism. Rather than reducing cyclic AMP levels via an inhibition of adenylate cyclase, as is the case in a number of other tissues (16, 19–21), stimulation of the muscarinic receptor of these cells results in an accelerated rate of cyclic AMP degradation due to the activation of phosphodiesterase (9). This event is Ca^{2+}-dependent and appears to account for most of the muscarinic receptor-mediated reduction of cyclic AMP levels in 1321N1 cells (9, 10).

Similar to other tissues (7, 8), extended incubation of

TABLE 4

Inhibition of cyclic AMP accumulation by A23187 in control and desensitized 1321N1 cells

Cells were preincubated for 75 min at 37° in 100 μM carbachol or vehicle. After one wash with 10 ml of DMEM-Hepes, ISO (10 μM) alone or in combination with OXO (100 μM) or A23187 (1 μM) was added to the medium. The reaction was terminated after 5 min, and the percentage conversion from [^{3}H]ATP to [^{3}H]cyclic AMP was determined as described under Experimental Procedures and in Table 1. Values represent the mean ± SEM of four determinations.

Pretreatment	Addition	% Conversion [^{3}H]ATP to [^{3}H]cyclic AMP	% Inhibition
None	ISO	1.75 ± 0.09	
	ISO + OXO	1.10 ± 0.15[a]	37.5
	ISO + A23187	0.31 ± 0.05[a]	82.6
Carbachol, 100 μM	ISO	2.50 ± 0.06	
	ISO + OXO	2.43 ± 0.15	2.6
	ISO + A23187	0.41 ± 0.01[a]	83.7

[a] Significantly different ($p < 0.01$) from the percentage conversion in the presence of ISO alone.

1321N1 cells with a muscarinic receptor agonist results in refractoriness to the inhibitory effects of these receptors on cyclic AMP accumulation. The kinetics of appearance of desensitization is very similar to that observed previously, particularly for the inhibition of cyclic GMP accumulation in the N1E-115 neuroblastoma line (1, 2), where the phenomenon has been examined extensively. Thus, following a lag of several minutes in neuroblastoma cells and of 15 min in 1321N1 cells, responsiveness decreases rapidly until a complete loss occurs after 30–60 min. Desensitization occurs in both systems prior to a significant loss of receptors. However, these results do not rule out the possibility that the receptor-effector system is altered in a major way by short-term exposure of cells to a muscarinic receptor agonist. For example, desensitization of the *beta*-adrenergic receptor/adenylate cyclase system of 1321N1 cells involves a rapid loss of catecholamine-stimulated adenylate cyclase activity followed by a much slower loss of measurable receptors (22, 23). Although total receptor number is unchanged after short-term desensitization, a proportion of the receptors is lost in a functional sense since they are no longer associated with the plasma membrane and cannot couple with adenylate cyclase (24).

Although it is clear that at least short-term desensitization results from a loss of muscarinic receptor-mediated activation of phosphodiesterase, the present data do not allow direct conclusions concerning the mechanism of desensitization of the muscarinic receptor system of 1321N1 cells. However, results to date suggest that a modification(s) occurs at the level of the receptor rather than at subsequent points in the series of events whereby muscarinic receptors regulate cyclic AMP levels. Agonist stimulation of the muscarinic receptor under conditions where Ca^{2+} influx and effects on phosphodiesterase activity were blocked still resulted in desensitization to the inhibitory effects of muscarinic receptor stimulation measured subsequently in the presence of Ca^{2+}. Desensitization to the effects of muscarinic receptor stimulation on cyclic GMP accumulation in neuroblastoma cells also occurs in the absence of Ca^{2+} (1). The decrease in cyclic AMP levels that occurs subsequent to addition of the divalent cation ionophore, A23187, to 1321N1 cells was still observed after carbachol-induced desensitization of the muscarinic receptor system. These results suggest that the Ca^{2+}-phosphodiesterase system does not undergo reduction in responsiveness during stimulation for long periods of time. Thus, refractoriness to muscarinic receptor stimulation is likely to occur at the level of coupling of this receptor to other cellular components. Whether such an effect involves an alteration of the receptor per se or a change in its interface with another effector protein is not known.

In contrast to the loss of responsiveness, the recovery of muscarinic receptor function following either short- or long-term incubation of the cells with carbachol is very slow. This was not surprising in the case of long-term incubation with carbachol, since large decreases in muscarinic receptor number occurred. As has been shown with muscarinic cholinergic receptors of other tissues (2, 4, 7) and with *beta*-adrenergic receptors of 1321N1 cells (23, 25), recovery from agonist-induced down-regulation of receptor number usually occurs over a period of hours. Recovery of muscarinic receptors in several cell types has been shown to require protein synthesis (2, 4, 7). In contrast to the results obtained following long-term incubation of 1321N1 cells with carbachol, the slow recovery of responsiveness following short-term desensitization is markedly different from that previously observed by Taylor *et al.* (2) for muscarinic receptors on N1E-115 cells and for the short-term desensitization of the *beta*-adrenergic receptor-linked adenylate cyclase of 1321N1 cells (23). Although surprising, slow recovery of responsiveness after short-term desensitization is not without precedence. For example, recovery from agonist-induced desensitization of the *beta*-adrenergic receptor-linked adenylate cyclase of C6-2B glioma cells requires several days. Desensitization in this cell line occurs rapidly prior to receptor loss and involves the induction of the synthesis of a "refractoriness protein" (26, 27). Although this protein has a short half-life, the messenger RNA from which it is translated apparently has a very long half-life (27). Thus, even the desensitization that occurs following short-term incubation with catecholamines is very long-lived in C6 cells. Additional work clearly is required to delineate the significance of the slow time course for recovery from short-term desensitization of the muscarinic receptor of 1321N1 cells.

The enhancement of ISO- and PGE_1-stimulated cyclic AMP accumulation that occurs as a result of incubation of 1321N1 cells with carbachol could be the result of either an increase in adenylate cyclase activity or a reduction of phosphodiesterase activity. The increase in the rate of nucleotide accumulation suggests that the effect is on synthesis of nucleotide. Furthermore, the lack of change in the time to maximal cyclic AMP levels and the occurrence of this phenomenon in the presence of 3-isobutyl-1-methylxanthine reduces the likelihood that changes in phosphodiesterase activity make a significant contribution. Moreover, no change in the degradation rate constant determined in the absence of a muscarinic receptor agonist was observed in carbachol-pretreated cells. Although these results suggest that an increase in adenylate cyclase activity occurs during incubation of cells with a muscarinic receptor agonist, little increase of adenylate cyclase activity has been detected in broken-cell preparations from carbachol-pretreated cells.[5] Such a finding is not surprising, considering the rapid decay of the enhanced responsiveness even in the continued presence of carbachol and the possibility that the alteration responsible for the increase may reverse during membrane preparation. The increased responsiveness is not receptor-selective (increases in response to ISO or PGE_1 are observed) and does not appear to be restricted to hormone agonists, since preliminary experiments in intact cells have demonstrated a 2-fold increase in the ability of forskolin to activate adenylate cyclase after brief pretreatment of 1321N1 cells with carbachol.[5] Forskolin apparently directly activates the catalytic component of adenylate cyclase (28, 29).

An increase in adenylate cyclase activity during incubation of cells with a muscarinic receptor agonist has been reported previously for other systems. For example, long-term (24–30 hr) incubation of NG108-15 neuroblas-

[5] R. B. Meeker and T. K. Harden, unpublished observations.

toma × glioma cells with carbachol resulted in a 200–300% increase in adenylate cyclase activity (3). Possibly more germane to the present data, Green and Clark (8) have observed an increase in epinephrine-stimulated cyclic AMP accumulation after short-term (5–60 min) preincubation of WI-38 fibroblasts with carbachol. The locus of the modification of adenylate cyclase is yet to be determined for each of these systems.

The enhancement of responsiveness to ISO during incubation with carbachol disappears with a time course similar to that of desensitization to the inhibitory effects of muscarinic receptors. Such a result does not indicate that a common mechanism is involved in both the enhancement of hormone responsiveness and the effects of muscarinic receptor stimulation on phosphodiesterase activity. Indeed, such seems not to be the case. While the effects of muscarinic receptor stimulation on phosphodiesterase activity are immediate, carbachol-induced enhancement of hormone-stimulated cyclic AMP accumulation requires 10–15 min to reach a maximum. The former effect is Ca^{2+}-dependent whereas the latter effect also is observed when hormone challenge is carried out in Ca^{2+}-free medium. However, to the extent that both processes are dependent on the ability of agonists to effect functional coupling of muscarinic receptors to other cellular components, a loss of the effects on both phosphodiesterase activity and enhancement of ISO-stimulated cyclic AMP accumulation might be expected to occur in parallel.

In summary, these results with 1321N1 cells demonstrate that muscarinic receptor agonists are capable of simultaneously inhibiting and facilitating cyclic AMP accumulation via two different processes: the former is Ca^{2+}-dependent and is fully active at the earliest times measured and the latter is apparently Ca^{2+}-independent and requires 15–20 min to reach a maximum. During prolonged exposure to a muscarinic receptor agonist, both responses disappear with similar time courses. Future investigations will be directed toward the delineation of the molecular mechanisms whereby muscarinic receptors regulate both cyclic AMP synthesis and degradation in 1321N1 astrocytoma cells.

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