Muscarinic acetylcholine receptor down-regulation limits the extent of inhibition of cell cycle progression in Chinese hamster ovary cells

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ABSTRACT Cellular desensitization is believed to be important for growth control but direct evidence is lacking. In the current study we compared effects of wild-type and down-regulation-resistant mutant m3 muscarinic receptors on Chinese hamster ovary (CHO-K1) cell desensitization, proliferation, and transformation. We found that down-regulation of m3 muscarinic acetylcholine receptors was the principal mechanism of desensitization of receptor-activated inositol phosphate phospholipid hydrolysis in these cells. Activation of wild-type and mutant receptors inhibited anchorage-independent growth as assayed by colony formation in agar. However, the potency for inhibition of anchorage-independent growth was greater for cells expressing the mutant receptor. Activation of either receptor also initially inhibited anchorage-dependent cell proliferation in randomly growing populations. Rates of DNA synthesis and cell division were profoundly reduced by carbachol in cells expressing either receptor at early time points. Analysis of cell cycle parameters indicated that cell cycle progression was inhibited at transitions from G1 to S and G2/M to G1 phases. However, mutant receptor effects on anchorage-dependent growth were sustained, whereas wild-type receptor effects were transient. Thus, receptor down-regulation restored cell cycle progression. In contrast, activation of either receptor blocked entry into the cell cycle from quiescence, and this response was not reduced by receptor down-regulation. Therefore, activation of m3 muscarinic acetylcholine receptors inhibited CHO cell anchorage-dependent and -independent growth. In anchored cells carbachol inhibited the cell cycle at three distinct points. Inhibitions at two of these points were eliminated by wild-type receptor down-regulation while the other was not. These results directly demonstrate that desensitization mechanisms can act as principal determinants of cellular growth responses.

Whether or not activation of a specific guanine nucleotide-binding protein (G-protein)-linked receptor will influence growth or transformation of a particular cell is unpredictable. Clearly, many G-protein-linked receptors are able to influence the rate of cellular proliferation (1, 2). However, a specific receptor may influence growth in one cell type but not in another. Furthermore, two receptors that activate similar intracellular second messenger pathways may or may not influence growth in the same cell. In vitro studies have also shown that G-protein-linked receptors can act as agonist-dependent oncogenes in some cell models (3–6). Yet, transforming effects of these receptors must be rare as they function physiologically in normal tissues. One parameter that varies depending upon the specific cell and the specific receptor and that may help explain these disparate observations is desensitization.

Desensitization is the process whereby continuous or repeated exposure to a stimulus results in a waning of the cellular response. Physiologically desensitization is manifest in the processes of tachyphylaxis and adaptation and is of major consequence in pharmacologic therapy. Cellular desensitization occurs at the level of the receptor and the level of intracellular effectors. Receptor desensitization has been widely studied and involves several processes, including uncoupling, sequestration, and down-regulation (7, 8). Receptor down-regulation is the agonist-induced loss of receptors from the cell. In addition to down-regulation of receptors, prolonged agonist treatment also leads to down-regulation of G proteins (9–13) and intracellular effectors such as protein kinase C (14) and the inositol 1,4,5-trisphosphate receptor (15). Reductions in these downstream effectors may participate in overall cellular desensitization. However, the relative contributions of receptor and intracellular effector desensitization mechanisms are poorly understood and may vary for different cellular functions.

The time course of a specific desensitization mechanism is an important determinant of its impact on a particular biological response. While rapid desensitization mechanisms, such as receptor uncoupling, take place over time periods of seconds to minutes, other mechanisms, such as down-regulation of receptors or effectors, require several hours to develop. For responses of short duration, such as secretion, receptor activation for seconds to minutes results in full manifestation and only rapid desensitization processes are able to influence the response to an initial stimulation. For responses of long duration, such as growth responses (16, 17), all desensitization mechanisms could be involved in determining the ultimate biological response. Previously it has been suggested that receptor down-regulation or desensitization accounted for alterations in cell growth responses (18, 19); however, direct evidence supporting this hypothesis was lacking.

In this study we directly examined the importance of receptor down-regulation on the growth inhibitory effects of m3 muscarinic acetylcholine (m3 ACh) receptor activation on Chinese hamster ovary (CHO-K1) cells. m3 ACh receptors belong to the family of seven transmembrane-spanning G-protein-linked receptors. Activation of m3 ACh receptors has been reported to lead to stimulation of cell growth (6, 20) and transformation (5, 6) or to inhibition of growth (21, 22) and reversal of transformation (23) in different cell models. The mechanisms responsible for these differences in growth actions are not currently understood. However, in all cases, development of the full cellular growth effect requires a prolonged duration of agonist exposure. Therefore, desensitization mechanisms might be expected to diminish the growth response of cells to m3 ACh receptor activation. We utilized a recently characterized mutant

Abbreviations: CHO, Chinese hamster ovary; FBS, fetal bovine serum; ACh, acetylcholine; CCH, carbachol; TCA, trichloroacetic acid; PPI, inositol phosphate phospholipid.
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human m3 ACh receptor with three carboxyl-terminal threonines converted to alanines (Hm3ala). In previous studies we found that this mutant receptor bound ligand and coupled to intracellular effectors equivalently to the wild-type human m3 ACh receptor (Hm3) (24). However, the mutant receptors were severely impaired in their ability to down-regulate. In the current study we compared the effects of activation of these two receptors to directly determine the consequences of receptor down-regulation on various cellular growth parameters.

**EXPERIMENTAL PROCEDURES**

**Receptor Desensitization.** Desensitization of inositol phosphate phospholipid (PPI) hydrolysis was determined in CHO-K1 cells stably expressing wild-type Hm3 and mutant Hm3ala receptors essentially as described (24). However, in the current study the magnitude of PPI hydrolysis was corrected for differences in the pool of [3H]PPI and was reported as a percentage of that observed in untreated control cultures. To determine the unreleased pool of [3H]PPI, the trichloroacetic acid (TCA)-insoluble pellets were solubilized with 0.1 M NaOH and radioactivity was counted.

**Cell Growth Assays.** The cells utilized for the current study were the WT #14 (Hm3) and the Ala500,553,554 (Hm3ala) cell clones previously described that express equivalent numbers of ACh receptors (24). Results were confirmed using one additional CHO cell clone expressing either wild-type or mutant receptors (data not shown). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics and 5% fetal bovine serum (FBS) (growth medium) or without serum (serum-free medium) at 37°C in a humidified atmosphere of 5% CO2. For proliferation assays, cells were subcultured in 24-well dishes at a density of 25,000 cells per well unless otherwise stated and allowed to attach overnight in growth medium.

DNA synthesis was estimated by measurement of [3H]thymidine incorporation into TCA-precipitable material. For experiments on randomly cycling cells, cells cultured in growth medium were treated with various concentrations of carbachol (CCH) for 24 hr or for the time periods indicated. [3H]Thymidine was added to each well (final concentration, 0.1 µCi/ml; 1 Ci = 37 GBq) during the last hour of treatment to label newly synthesized DNA. For experiments examining entry into the cell cycle, cells were made quiescent by culturing in serum-free medium for either 24 or 48 hr. For time-course experiments, quiescent cells were then stimulated with 5% FBS in the presence or absence of CCH for the indicated time intervals and [3H]thymidine was added during the last hour of the treatment. For experiments examining the effects of receptor down-regulation prior to serum-stimulated entry into the cell cycle, cells were pretreated with or without 0.1 mM CCH during 24 hr of serum starvation and then were stimulated with 5% FBS in the presence or absence of CCH for the indicated time intervals and [3H]thymidine was added during the final 6 hr. At the end of the labeling period, cells were washed twice with ice-cold phosphate-buffered saline, washed twice with TCA (6% final concentration), and removed by dissolving in 0.1 M NaOH. Radioactivity was measured by liquid scintillation counting.

Cell numbers and colony formation in soft agar were determined as described (25).

**Analysis of Cell Cycle Parameters.** For cell cycle analysis a single-cell suspension was made and cells were fixed with ethanol (70%) for 30 min at 4°C. Cells were then incubated in ribonuclease (100 µg/ml) and propidium iodide (50 µg/ml) and incubated for 30 min at 37°C. Cell cycle analysis was conducted using a Coulter fluorescence-activated cell sorter and the MULTICYCLE computer program.

**Statistical Analysis.** Statistical analysis was carried out by using a commercial statistical program (INSTAT; Graphpad Software, San Diego). Differences between individual conditions and controls were tested with ANOVA and were considered significant when P < 0.05.

**RESULTS**

**Desensitization of Wild-Type and Down-Regulation-Resistant m3 ACh Receptors.** We previously reported that wild-type human m3 ACh receptors (Hm3) but not mutant receptors with three carboxyl-terminal threonines converted to alanines (Hm3ala) were down-regulated when expressed in CHO-K1 cells (24). However, in that study we noted an apparent desensitization of PPI hydrolysis in mutant and wild-type receptor-bearing cells. To account for effects on cell numbers, in the current study, desensitization of PPI hydrolysis was reanalyzed and was corrected for changes in total [3H]PPI content. Using this protocol, CCH pretreatment significantly reduced the subsequent ability of agonist to stimulate release of [3H]inositol phosphates in cultures of cells bearing wild-type but not mutant m3 ACh receptors (Fig. 1). Thus, in CHO cells, m3 ACh receptor down-regulation was the principal mechanism of desensitization and the mutant receptors were blocked in their abilities to down-regulate and desensitize.

**Effects of m3 ACh Receptor Activation on CHO Cell Growth.** To investigate the influence of receptor desensitization on cell growth we compared the effects of wild-type and mutant receptors. Initially, effects on DNA synthesis at short times were examined using a [3H]thymidine incorporation assay. After 24 hr of treatment with CCH, cells bearing either wild-type or mutant receptors showed reduced DNA synthesis (Fig. 2A). No differences were previously noted in the potency or efficacy of CCH activation of second messengers via either receptor (24). Atropine completely blocked the inhibitory effects of CCH (data not shown). CCH had no effect on DNA synthesis in serum-starved cells, indicating that the inhibition required the presence of a growth stimulus (data not shown). These observations agree with those previously reported for growth-inhibitory effects after activation of m1 ACh receptors in A9 L cells (21) and m3 ACh receptors on small lung cell carcinomas (22).

While there were no differences in the growth-inhibitory effects of either receptor in the short term, there was a significant difference in their effects over the longer time period (2 weeks) of an assay of colony formation in soft agar (Fig. 2B). CCH acting on either receptor inhibited colony formation of CHO cells expressing Hm3ala receptors with 80±4% inhibition compared with controls, but not that of CHO cells expressing Hm3 receptors (20±5% inhibition compared with controls). These experiments also demonstrated that the effects of CCH were not observed in untreated control cultures. Data are means ± SE of four experiments. *, P < 0.05.

![Fig. 1. Time course of CCH-induced desensitization of PPI hydrolysis in wild-type (●) or mutant (▲) ACh receptors. Cells stably expressing Hm3 or Hm3ala receptors were labeled with myo-[3H]inositol for 24 hr. During the labeling period, cells were pretreated with or without 1 mM CCH for the indicated times. Cells were then stimulated with or without 1 mM CCH for a further 30 min at 37°C and released [3H]inositol phosphates and unreleased [3H]PPI were quantified. The magnitude of PPI hydrolysis was corrected for differences in the pool of [3H]PPI and was reported as a percentage of that observed in untreated control cultures. Data are means ± SE of four experiments. *, P < 0.05.](image-url)
Fig. 2. (A) Effect of CCH on DNA synthesis in cells expressing wild-type (○) or mutant (■) ACh receptors. Cells were serum-starved for 24 hr and then incubated continuously in the presence of 5% FBS and the indicated concentrations of CCH for an additional 24 hr. Subsequently, the cells were pulsed for 1 hr with [3H]thymidine. TCA-precipitable radioactivity was determined and expressed as a percentage of the radioactivity in untreated samples. Data are means ± SE for three separate experiments conducted in quadruplicate. (B) Effects of CCH on colony formation in soft agar for cells expressing wild-type (○) or mutant (■) ACh receptors. Cells were plated at 1000 cells per dish into 35-mm dishes in agar and treated with the indicated concentrations of CCH. After 2 weeks the dishes were examined and all colonies over 0.5 mm in diameter were counted. Results are expressed as a percentage of colonies formed in control dishes and represent means ± SE for three separate experiments.

formation in a concentration-dependent manner. However, the effect of activation of the mutant receptor was more pronounced and occurred at a lower concentration of agonist. The ability to inhibit anchorage-independent growth has been previously reported for the m3 ACh receptor (23, 26). CCH did not affect colony formation in native CHO cells, which do not express endogenous ACh receptors (data not shown).

Since significant down-regulation of m3 ACh receptors required several hours (24), we examined the effect of agonist treatment on cell numbers for up to 4 days. Cells expressing wild-type receptors were grown with CCH for the first 24 hr of treatment but returned to control rates of growth at later times (Fig. 3A). In contrast, cells expressing the down-regulation-resistant receptors were severely growth inhibited at all times to at least 4 days of culture (Fig. 3B). The effects of activation of the mutant receptor were reversible upon removal of the agonist (data not shown). A similar pattern of inhibition was observed when incorporation of [3H]thymidine into DNA was followed over several days of culturing (Fig. 4).

Effects of m3 ACh Receptor Activation on CHO Cell Cycle Parameters. We next analyzed the effects of CCH on the growth of cells expressing wild-type and mutant receptors utilizing flow cytometry. Analysis of the DNA contents of asynchronously dividing populations of cells expressing either receptor indicated a similar cell cycle distribution in untreated cell populations and a similar pattern of redistribution at early time points after CCH treatment (Fig. 5). These observations supported the hypothesis that both receptors utilized the same mechanisms to inhibit cell growth. In cells expressing wild-type receptors, this redistribution was transient and after prolonged treatment the cells returned to the control pattern of cell cycle distribution (Fig. 5A; Table 1). In contrast, in cells expressing mutant receptors, this effect was sustained (Fig. 5B; Table 1). Treatment resulted in a decreased proportion of cells in S phase and an increased proportion of cells in G1 and G2/M phases. Reduction of the proportion of cells in S without a significant reduction of cells in G1 suggested that CCH decreased the rate of G1 to S transition. Because an inhibition of G1 to S transition would not account for the observed increase of cells in G2/M (Table 1), inhibition must have also occurred at some point during the G2/M to G1 transition.

FIG. 3. Effect of CCH on the proliferation of cells expressing wild-type (A) or mutant (B) ACh receptors. Cells were grown in the presence of 5% FBS and in the presence (○, ○) or absence (■, ■) of CCH (100 μM). Data are means of quadruplicate determinations from a representative of three experiments.

FIG. 4. Effect of CCH on DNA synthesis measured in cells expressing wild-type (○, ○) or mutant (■, ■) ACh receptors. Cells were grown in the presence of 5% FBS and in the presence (○, ○) or absence (■, ■) of CCH (100 μM). Separate cultures were pulsed with [3H]thymidine for 1 hr each day. Data are presented as the amount of [3H]thymidine incorporated as a percentage of that measured on day 1 in the absence of CCH. Data shown are means ± SE for three to five experiments.

FIG. 5. Effect of CCH treatment on the cell cycle distribution of cells expressing wild-type (A) or mutant (B) ACh receptors. Analysis of DNA content was performed using a Coulter flow cytometer and analyzed using the MULTICYCLE computer program. Results are representative of four separate experiments.
Table 1. Effects of CCH treatment on cell cycle distribution of CHO-K1 cells bearing Hm3 or Hm3ala receptors

<table>
<thead>
<tr>
<th>Phase</th>
<th>Hm3</th>
<th>Hm3ala</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>48 hr</td>
</tr>
<tr>
<td>G1</td>
<td>43 ± 3</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>S</td>
<td>48 ± 5</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>G2/M</td>
<td>9 ± 3</td>
<td>17 ± 1</td>
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</tbody>
</table>

Cell cycle analysis was performed as described in the legend to Fig. 5. Results are reported as the percentage of cells in each phase of the cell cycle and are the mean ± SE for three or four separate experiments. * P < 0.05 compared to untreated controls.

To further define the inhibitory actions of m3 ACh receptor activation, effects on early events in the cell cycle were examined. Asynchronously dividing cells treated with CCH for various times showed a rapid inhibition of DNA synthesis (Fig. 6A). This observation indicated that the previously noted inhibition of G1 to S transition occurred in late G1 or early S phase. In a different assay, utilizing serum-starved quiescent cells, CCH treatment prevented serum-stimulated reentry of cells into the cell cycle (Fig. 6B). Unlike in randomly cycling, in quiescent cells, activation of either wild-type or mutant receptors caused a persistent inhibitory effect. Receptor down-regulation was identical in serum-fed and serum-starved cells (data not shown). Thus, a clear difference in sensitivity to receptor down-regulation was observed between inhibition of G1 to S and G2 to S transition.

The cellular basis of the difference between the impact of m3 ACh receptor down-regulation on inhibition of cell entry into and progression through the cell cycle is unclear. One possible explanation would involve the existence of a temporally sensitive step in serum-induced progression toward S phase. An alternative explanation would involve differences in sensitivity to m3 ACh receptor activation. To discriminate between these two models, cells were pretreated with CCH during the serum deprivation period to induce receptor down-regulation before serum stimulation. When this protocol was utilized, CCH pretreatment sufficient for maximal receptor down-regulation did not prevent the inhibitory effects of CCH on serum-stimulated DNA synthesis (Fig. 7). This was not due to a nonreversible effect as CCH pretreatment itself had only a small effect on serum-induced increases in DNA synthesis. Thus, the m3 ACh receptor-activated mechanisms that prevented cells from entering the cell cycle most likely were more sensitive than those involved in the inhibition of cycling cells. These observations indicate that in quiescent cells, activation of m3 ACh receptors blocks G2 to S transition, and this inhibition is not relieved by receptor desensitization. In contrast, in cycling CHO cells, activation of m3 ACh receptors inhibits transition from G1 to S and from G2/M to G1 phases, and receptor desensitization alleviates these inhibitory effects. Further investigations will be necessary to determine the cell cycle-specific mechanisms involved in these growth-inhibitory effects.

**DISCUSSION**

In the current study, activation of m3 ACh receptors led to a profound inhibition of CHO cell growth and transformation. However, in normally growing populations of CHO cells, activation of wild-type Hm3 receptors had only a transient effect on growth, whereas Hm3ala, a mutant receptor deficient in the ability to down-regulate and desensitize, had a persistent effect. These data clearly demonstrate that alteration of the extent of receptor desensitization has a profound influence on cellular growth regulation by G-protein-linked receptors. Thus, characteristics of receptor desensitization are likely as important as the duration of stimulant exposure in determining the ultimate cellular response. The balance between the rate of signal attenuation due to desensitization and the magnitude and duration of signal required for a growth effect determines whether the effect is observed.

Desensitization characteristics depend upon the receptor, the biological response being investigated, and the cell model. The current data indicate that, of the many processes that are potentially involved in cellular desensitization, Hm3 receptor down-regulation was the principal mechanism responsible for desensitization of PPI hydrolysis and cell growth inhibition in CHO cells. In contrast, we have found that in HEK293 cells, rapid desensitization of Hm3 receptors occurs in the absence of receptor down-regulation (27). It is unclear at the present time what accounts for these cell-specific differences. As wild-type and desensitization-resistant receptors expressed in CHO cells couple to the same extent and with the same concentration dependency to intracellular mechanisms, they would both be expected to down-regulate downstream effectors to a similar extent. In support of this we have found that treatment of CHO cells with CCH for 24 hr led to a down-regulation of Gq α-subunits that was not different for cells.

![Fig. 6](#)

![Fig. 7](#)

**Fig. 6.** (A) Effect of CCH treatment on DNA synthesis in cells proliferating asynchronously in serum. Cells bearing wild-type (●) and mutant (■) receptors growing in the presence of 5% serum were treated with 100 μM CCH for the indicated times and then pulsed for 1 hr with [3H]thymidine. The incorporation of [3H]thymidine into DNA was analyzed and results are shown as a percentage of DNA synthesis in untreated cells. (B) Effect of CCH treatment on entry of cells bearing wild-type (●, ○) and mutant (■, □) receptors into the cell cycle. Cells were serum-starved for 48 hr and then stimulated with 5% FBS in the presence (○, □) or absence (●, ■) of 100 μM CCH. DNA synthesis was analyzed at the times indicated by measuring the incorporation of [3H]thymidine into DNA during a 1-hr pulse. Results are shown as fold basal level for triplicate determinations from a representative of four experiments. Basal values were 496 ± 33 cpm for Hm3 and 648 ± 72 cpm for Hm3ala.

**Fig. 7.** Effect of CCH pretreatment on entry of quiescent cells into S phase of the cell cycle. Cells bearing Hm3 and Hm3ala receptors were serum-starved for 24 hr to induce quiescence. To down-regulate receptors, some cells were treated with 100 μM CCH during serum starvation. Cells were then treated with 5% FBS in the presence or absence of 100 μM CCH. DNA synthesis was analyzed by measuring incorporation of [3H]thymidine during a 6-hr pulse. Results are shown as a percentage of DNA synthesis compared to that observed in serum-starved cells stimulated with serum alone and are means ± SE of three experiments.
bearing wild-type Hm3 or desensitization-resistant mutant Hm3ala receptors (28). Therefore, in the current model, downstream desensitization mechanisms, such as effects of G-proteins or other effectors, were not sufficient to cause a significant decrease in PPI hydrolysis or to overcome the growth-inhibitory effects of receptor activation. Thus, receptor down-regulation was necessary for relieving CHO cell growth inhibition. Whether receptor down-regulation itself was sufficient for functional desensitization or whether additional mechanisms were required could not be discerned in the current model.

Separate specific effects of m3 ACh receptor activation were observed at three positions in the cell cycle. In randomly cycling cells, inhibition of G₁ to S and G₂/M to G₁ transitions was observed. These inhibitory effects were sensitive to receptor down-regulation as concluded from the transient nature of the effects of wild-type but not mutant receptor activation. Activation of either receptor also blocked entry of quiescent cells into the cell cycle. This inhibition was not sensitive to receptor down-regulation. Thus, the block of G₀ to G₁ transition must have occurred at a point separate from the G₁ to S inhibition observed in cycling cells.

Activation of wild-type and mutant receptors also led to an inhibition of anchorage-independent cell growth. This effect has been referred to as “reverse transformation” and has previously been reported as a consequence of activation of m1 or m3 ACh receptors in CHO cells (23, 26). It was suggested that Ca²⁺ entry accounted for the reverse transforming effects of m3 ACh receptor activation (23). In the current study anchorage-independent growth was inhibited by wild-type and desensitization-resistant receptors. A difference in concentration dependence between the effects mediated by these two receptors likely represented the influence of wild-type receptor desensitization. Currently it is unclear whether inhibition of colony formation requires sustained receptor function or whether inhibition during a critical early period results in a prolonged effect. Thus, it is difficult to determine whether this effect on cellular transformation represents a separate inhibitory mechanism or is the consequence of the inhibitory effects noted on the cell cycle.

In the current model, receptor desensitization relieved an initial growth effect. In other cases, receptor desensitization may prevent the occurrence of a growth response. Physiologically, many receptors may desensitize too rapidly for growth effects to be manifest, which may explain the lack of cell growth response often observed after activation of G-protein-coupled receptors. In this way desensitization may serve as a protective mechanism that prevents undesirable growth effects. Thus, receptor desensitization is likely to be an essential component of normal cell growth control. In the current study, desensitization limited an inhibitory effect of receptor activation on proliferation and transformation. Since growth effects generally require prolonged signals, by analogy, in different cell models desensitization would also be expected to limit other growth effects including mitogenesis (6, 20) and transformation (3–6). Thus, mutations that result in the loss of normal receptor desensitization, similar to mutations that constitutively activate receptors (4), may lead toward cellular transformation. In view of the important role of receptor desensitization as a limit of growth-inhibitory effects shown in the current study, the relevance of receptor desensitization in cellular transformation clearly needs to be examined.

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