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Activation of muscarinic acetylcholine receptors inhibits cell cycle progression of small cell lung carcinoma

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We previously reported that activation of muscarinic acetylcholine receptors (mAChR) of M_3 subtype causes hydrolysis of phosphoinositides and inhibits voltage-gated Ca^{2+} channel activity in small cell lung carcinoma (SCLC) cells. We now report that mAChR activation causes exponentially growing SCLC cells to arrest in S and G_2/M phases of the cell cycle, concomitant with a decrease in DNA synthesis. Cell cycle progression and DNA synthesis resume when mAChR are down-regulated. In serum-starved SCLC cells, mAChR activation inhibits DNA synthesis induced by serum, bombesin, insulin, or insulin-like growth factor-I. The finding that DNA synthesis is inhibited even when mAChR are activated after exposure of cells to growth factors indicates that decreased signal transduction by growth factor receptors is not the mechanism of mAChR-mediated growth inhibition. Our data suggest that mAChR activation disrupts a common event that is induced by different growth factors and is fundamental for cell cycle progression.

Introduction

In normal cells, the transition from proliferation to terminal differentiation is marked by withdrawal from the cell cycle and an inability to reenter the cycle in response to growth factors. Transformed cells do not undergo this transition, but constitutively remain in the cell cycle; the regulation of cell cycle progression is disrupted (Heldin and Westermark, 1984; Weinstein, 1987; Kraus *et al.*, 1988). Small cell lung carcinoma (SCLC)[1] provides a clinically relevant model for studying the dysregulation of cell cycle progression. This highly metastatic tumor accounts for 25–30% of all lung cancers and causes >25 000 deaths annually in the United States (Birrer and Minna, 1989). SCLC cells express receptors for several autocrine growth factors, including gastrin-releasing peptide (GRP, homologous to amphibian bombesin) (Cuttitta *et al.*, 1985; Carney *et al.*, 1987), insulin-like growth factor-I (IGF-I) (Macauly *et al.*, 1988; Nakanishi *et al.*, 1988b), and a transferrin-like molecule (Nakanishi *et al.*, 1988a; Vostrejs *et al.*, 1988). Activation of these receptors generates different intracellular second messengers, which must converge on a common point to induce SCLC proliferation.

In addition to receptors for peptide growth factors, some SCLC cell lines express muscarinic (Sorenson *et al.*, 1983; Cunningham *et al.*, 1985; Williams and Lennon, 1990a) and nicotinic acetylcholine receptors (Maneckjee and Minna, 1990). The ability of acetylcholine receptors to regulate cell proliferation has recently been recognized (Conklin *et al.*, 1988; Ashkenazi *et al.*, 1989; Maneckjee and Minna, 1990). Based on our finding that activation of M_3 muscarinic acetylcholine receptors (mAChR) causes hydrolysis of phosphoinositides (PI) and inhibits voltage-gated Ca^{2+} channel activity in SCLC cells, we proposed that mAChR activation may affect SCLC proliferation (Williams and Lennon, 1990a). We report here that mAChR activation inhibits DNA synthesis induced by serum, bombesin, insulin, or IGF-I in SCLC cells. mAChR agonists arrest SCLC cells in both S and G_2/M phases of the cell cycle. mAChR activation appears to disrupt a common event induced by different growth factors, which follows signal transduction by growth factor receptors.

Results

mAChR activation decreases DNA synthesis by cells growing exponentially in serum

Carbachol decreases ^{3}H-thymidine uptake by cells cultured in serum (Figure 1). This inhibition

[1] Abbreviations: GRP, gastrin-releasing peptide; IC_{50}, half-maximal inhibitory concentration; IGF-I, insulin-like growth factor-I; mAChR, muscarinic acetylcholine receptors; PBS, phosphate-buffered saline; PI, phosphoinositides; SCLC, small cell lung carcinoma.

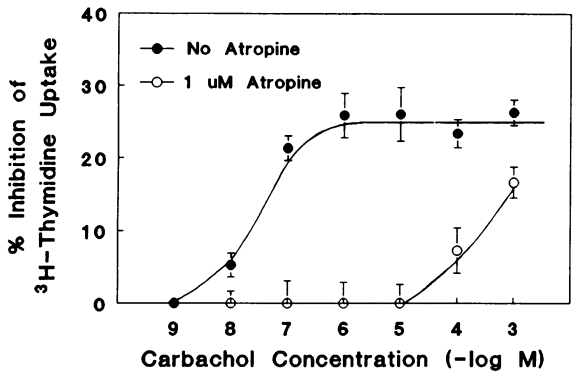

Figure 1. **Carbachol (●) inhibits ^{3}H-thymidine uptake over a 24-h period by exponentially growing SCC-9 cells.** This effect is suppressed by the mAChR antagonist atropine (1 μM) (○). Results presented are the mean (±1 SE) of nine determinations from three independent experiments. There was no significant difference in ^{3}H-thymidine uptake by untreated cells and cells treated only with atropine (32 681 ± 607 cpm/2 × 10^4 cells vs. 33 102 ± 1002 cpm/2 × 10^4 cells, respectively).

is due to mAChR activation because the mAChR antagonist atropine abrogates the effect of carbachol (Figure 1). The half-maximal inhibitory concentration (IC_{50}) of carbachol is 35.6 ± 9.9 (SE) nM. Inhibition of DNA synthesis is not due to carbachol-mediated cytotoxicity because the percentage of viable cells (determined by trypan blue-exclusion) is the same in carbachol-treated and untreated cultures.

The rate of DNA synthesis decreases within 1 h after adding carbachol and remains low during the first 12 h of treatment (Figure 2A). After exposure to carbachol for >12 h, the rate of DNA synthesis returns to normal (Figure 2A) and remains so even if the cells are cultured several days with carbachol (Figure 2B).

mAChR are down-regulated in cells continuously exposed to carbachol for 12 h

The rate of DNA synthesis may return to normal after 12 h of carbachol exposure because mAChR are down-regulated. To investigate mAChR down-regulation, we measured intracellular Ca^{2+} mobilization in cells exposed to carbachol for various times. Cells no longer mobilize Ca^{2+} in response to carbachol (1 μM) after they have been exposed to the agonist for 12 h (Figure 3). This suggests that mAChR are no longer transmitting intracellular signals in these cells. In support of this, we also found that voltage-gated Ca^{2+} channel activity is normal in cells treated with carbachol for 12 h, even though it is decreased in cells exposed to carbachol for shorter times (Williams and Lennon, 1990a). Cells exposed to carbachol (1 μM) for 12 h also have decreased surface binding of the mAChR antagonist ^{3}H-*N*-methylscopolamine (Figure 4). These results agree with our interpretation that mAChR are down-regulated in cells exposed to carbachol for 12 h and are consistent with reports of mAChR internalization after agonist exposure in other systems (Liles *et al.*, 1986; Andre *et al.*, 1988).

mAChR activation arrests cells in S and G_2/M phases of the cell cycle

Exponentially growing SCLC cells accumulate in S phase during the first 12 h of carbachol exposure (Figure 5B). This is consistent with the

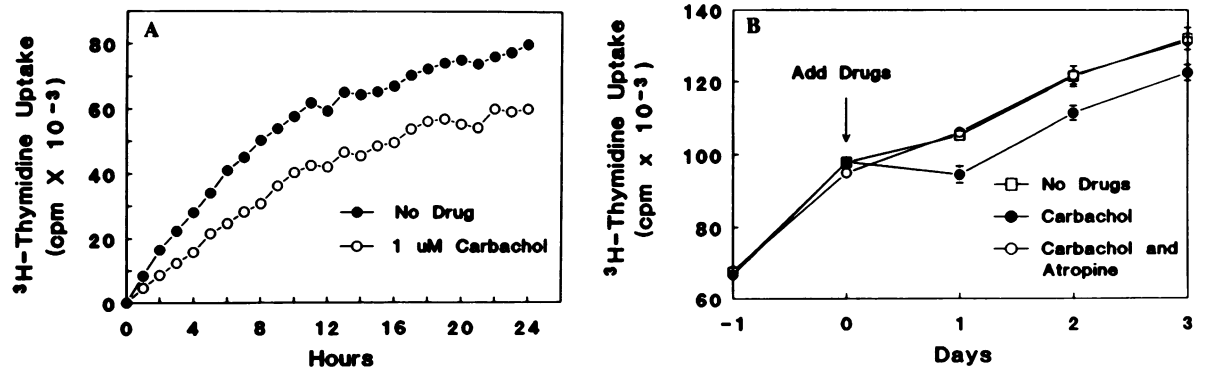

Figure 2. **The inhibitory effects of mAChR activation on ^{3}H-thymidine uptake are transient.** (A) Exponentially growing cells treated with carbachol (1 μM) (○) take up less ^{3}H-thymidine than untreated cells (●) during the first 12 h, but thereafter have a normal rate of uptake. Results shown are the mean of 12 determinations from one experiment. SEs are <10% of the means. A duplicate experiment produced similar results. (B) Carbachol (1 μM) added at day 0 (●) decreases ^{3}H-thymidine uptake for the first 24 h relative to untreated cells (□) but has no effect after this time. Atropine (1 μM) (○) suppresses the effects of carbachol. Results are the mean (±1 SE) of 6 determinations from one experiment. Three other experiments produced similar results.

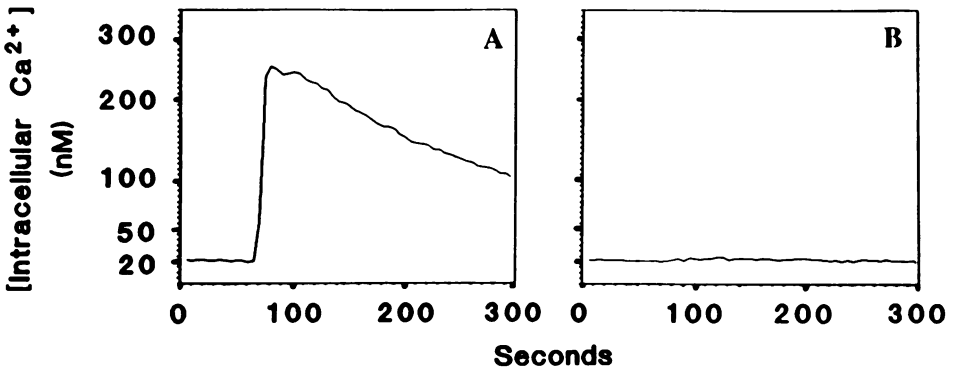

Figure 3. **Carbachol (1 μM, added at 60 s) induces Ca^{2+} mobilization in previously unexposed cells (A), but has no effect on cells preincubated with carbachol (1 μM) for 12 h (B).** Results are representative of three experiments.

finding that ^{3}H-thymidine uptake is maximally inhibited during this time (Figure 2A). After 12 h in the presence of carbachol, cells are released from S-phase arrest (Figure 5B). This corresponds with the return to a normal rate of ^{3}H-thymidine uptake (Figure 2A) and may reflect down-regulation of mAChR, as discussed above.

Carbachol-treated cells also progressively accumulate in G_2/M phase (Figure 5C). An arrest in G_2/M should decrease the number of cells entering G_0/G_1. Thus, the decrease in G_0/G_1 cells during the first 12 h of carbachol exposure (Figure 5A) supports our interpretation that cells are arrested in G_2/M during this time. The percentage of cells entering G_0/G_1 begins to increase after 12 h with carbachol (Figure 5A), suggesting that the cells are no longer arrested in G_2/M. If this is true, the continued accumulation of cells in G_2/M after 12 h exposure to carbachol (Figure 5C) may reflect cells entering G_2/M as they are released from S phase-arrest.

mAChR activation decreases DNA synthesis induced by serum

Having found that mAChR activation decreases ^{3}H-thymidine uptake by exponentially growing cells, we next determined the effects of carbachol on DNA synthesis in cells previously cultured without serum. DNA synthesis is increased when serum-starved cells are exposed to serum, and carbachol reduces this response (Figure 6A). mAChR activation decreases maximal DNA synthesis but does not delay the initiation of synthesis (Figure 6B).

If mAChR activation decreases DNA synthesis because it inhibits signal transduction by SCLC growth factor receptors, DNA synthesis should be inhibited only if mAChR are activated before cells are exposed to growth factors. However, we found that carbachol added even 16 h after serum decreases ^{3}H-thymidine uptake as effectively as carbachol added immediately before serum (Figure 7). These results suggest that inhibiting signal transduction by growth factor receptors is not the mechanism by which mAChR activation decreases DNA synthesis in SCLC. Instead, mAChR activation may inhibit late events induced by serum or reverse an early effect of serum.

DNA synthesis induced by bombesin, insulin, and IGF-I is decreased by mAChR activation

Bombesin, insulin, and IGF-I induce DNA synthesis in serum-starved SCC-9 cells (Figures 8 and 9). This agrees with reports by others using different SCLC cell lines (Cuttitta *et al.*, 1985; Carney *et al.*, 1987; Nakanishi *et al.*, 1988a,b). The report that insulin at high concentrations (>1 μg/ml) activates both insulin and IGF-I receptors (Czech, 1990) may explain the stepwise

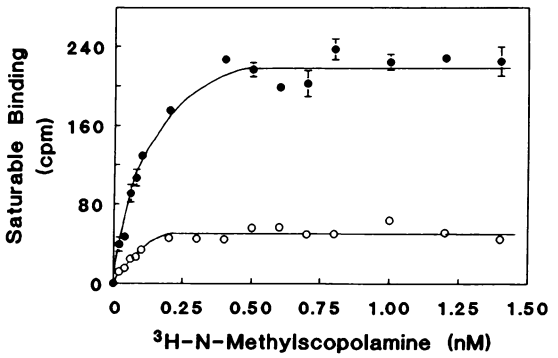

Figure 4. **The saturable surface binding of the mAChR antagonist ^{3}H-*N*-methylscopolamine in SCC-9 cells (●) is decreased by preexposure to carbachol (1 μM, 12 h) (○).** Results are the mean (±1 SE) of 24 determinations from two independent experiments.

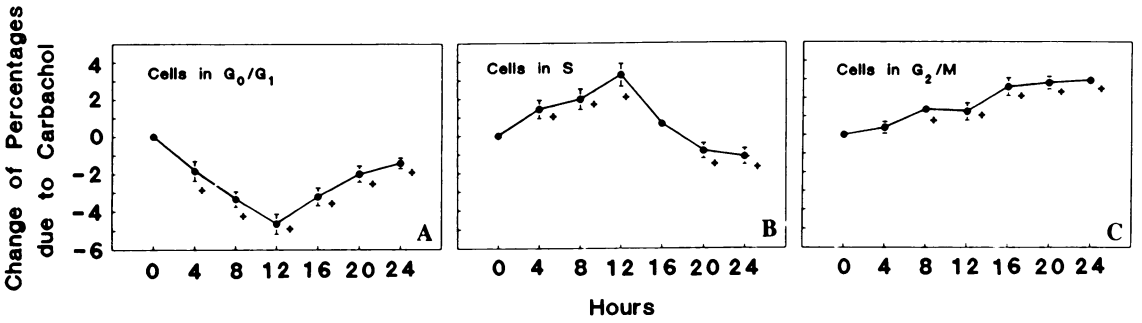

Figure 5. **Carbachol (1 μM) changes the percentage of exponentially growing cells that are in G_0/G_1 phase (A), S phase (B), and G_2/M phase (C).** Results are the mean (±1 SE) of 9–23 determinations from three to seven independent experiments. Crosses indicate means that are significantly different from 0 ($p < 0.05$ by matched-pairs Student's t test). Method: Asynchronous, exponentially growing cells were incubated with or without carbachol beginning at time 0 and stained with propidium iodide at the times indicated. The values expressed as the change of percentages due to carbachol were derived as explained in the Methods.

increase in ^{3}H-thymidine uptake shown in Figure 8C. Carbachol decreases DNA synthesis induced by bombesin, insulin and IGF-I with IC_{50} values of 23.4 ± 4.4, 32.9 ± 5.6, and 18.7 ± 6.6 nM, respectively (Figure 9). Atropine abrogates these effects (Figure 9), indicating the inhibitory effects of carbachol are due to mAChR activation. In some experiments, carbachol decreased ^{3}H-thymidine uptake by cells that were not exposed to exogenous growth factors (e.g., Figure 8, B and C). One explanation for this finding is that in these experiments, some of the cells continued to cycle in the absence of exogenous growth factors, possibly in response to autocrine growth factors such as GRP or IGF-I. An mAChR-mediated inhibition of this response could account for the reduced ^{3}H-thymidine uptake by these cells in the presence of carbachol.

Discussion

This study demonstrates that mAChR activation decreases DNA synthesis by SCLC cells. The phenomenon appears to be a common characteristic of SCLC cells expressing functional M_3 mAChR (similar data for a 2nd SCLC line not shown). The M_1 and M_3 subtypes of mAChR, which induce PI hydrolysis, have been found to regulate DNA synthesis in other cell lines more efficiently than the M_2 and M_4 subtypes, which activate adenylate cyclase (Ashkenazi *et al.*, 1989). This has led to the suggestion that PI hydrolysis may play a crucial role in the ability of mAChR to regulate proliferation (Ashkenazi *et al.*, 1989; Hanley, 1989). However, factors other than PI hydrolysis must be involved, because we found that concentrations of car-

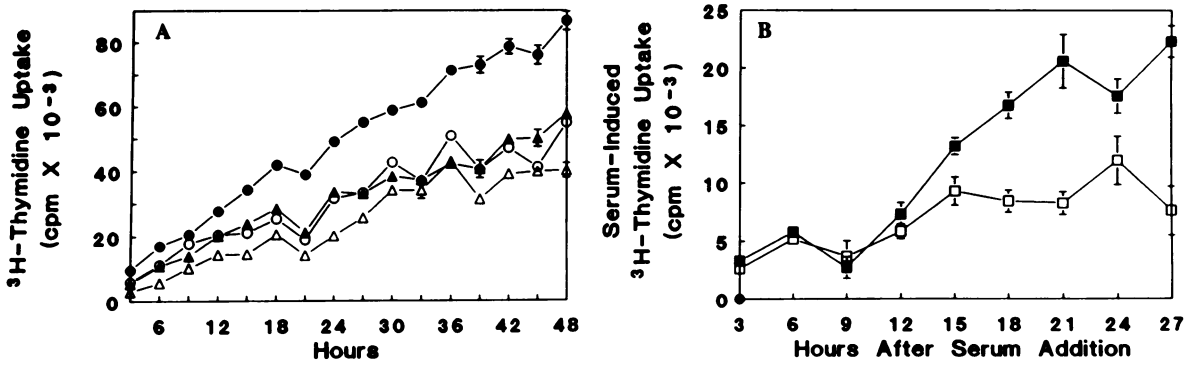

Figure 6. **Carbachol inhibits ^{3}H-thymidine uptake induced by serum.** (A) ^{3}H-thymidine uptake is less in serum-starved cells (○, △) than in cells exposed to 2% serum (●, ▲). Cells treated with carbachol (1 μM) (▲, △) have less ^{3}H-thymidine uptake than untreated cells (●, ○). Results shown are the mean (±1 SE) of 12 determinations from one experiment. A duplicate experiment produced similar results. (B) Carbachol-treated cells (□) initiate serum-induced DNA synthesis at the same time as untreated cells (■), but have a decreased maximal response. Serum-induced ^{3}H-thymidine uptake was calculated by subtracting the amount of ^{3}H-thymidine taken up by serum-starved cells from the amount taken up by cells exposed to serum, using the values presented in Figure 6A. Method: Cells were incubated in serum-free media for 3 d before addition of RPMI 1640 media containing ^{3}H-thymidine with or without 2% calf bovine serum. When present, carbachol was added 10 min before ^{3}H-thymidine. Cells were harvested at the times indicated.

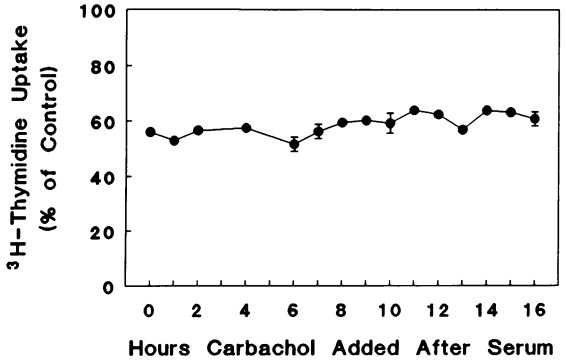

Figure 7. **^{3}H-thymidine uptake induced by serum in serum-starved cells is inhibited by carbachol (1 μM) to the same extent whether carbachol is added with serum or 16 h after serum.** Results shown are the mean (±1 SE) of 12 determinations from two independent experiments. Method: Cells were incubated in serum-free media for 3 d before adding calf bovine serum (2%). At the times indicated after serum addition, experimental cells received carbachol in RPMI 1640 media, whereas control cells received only RPMI 1640. ^{3}H-thymidine was added 16 h after serum, and cells were harvested 8 h later.

bachol and bombesin, which elicit the same level of PI hydrolysis in SCLC cells, produce opposite effects on DNA synthesis.

Although we previously found that signal transduction by GRP receptors is inhibited by mAChR activation in SCLC cells (Williams and Lennon, 1990b), our present studies indicate that decreased signal transduction by growth factor receptors is not the mechanism of mAChR-mediated growth inhibition. First, carbachol is equally effective in inhibiting DNA synthesis induced by bombesin, insulin, and IGF-I. These growth factors activate different signaling pathways. Bombesin activates GRP receptors, causing PI hydrolysis and intracellular Ca^{2+} mobilization (Bunn *et al.*, 1990), whereas insulin and IGF-I do not elicit these responses in most cells but increase protein tyrosine kinase activity (Czech *et al.*, 1988; Czech, 1990). It is unlikely that these different signals would be inhibited to the same extent by carbachol. Second, carbachol inhibits serum-induced DNA synthesis even when it is added to the cells 16 h after adding serum. This indicates mAChR activation affects an event occurring many hours after growth factor receptors have transduced intracellular signals. Our earlier finding that bombesin-induced Ca^{2+} mobilization is decreased after mAChR activation (Williams and Lennon, 1990b) may be explained if GRP receptors and mAChR are functionally coupled to mobilize the same intracellular pool of Ca^{2+} in SCLC cells. Functional coupling of different receptors to the same intracellular pool of Ca^{2+} occurs in other SCLC cell lines (Bunn *et al.*, 1990). The anti-proliferative signals generated during mAChR activation apparently override any mitogenic effects of Ca^{2+} mobilization induced by activating mAChR or growth factor receptors.

Several of our results suggest that mAChR activation inhibits the progression of DNA synthesis rather than impairing its initiation. First, mAChR activation causes cells to arrest in S phase (Figure 5B), indicating an inability to complete DNA synthesis once it is started. The cells would arrest in G_0/G_1 if initiation of DNA synthesis were impaired. Second, carbachol inhibits DNA synthesis equally well whether it is added before or soon after the onset of synthesis (Figure 7). This indicates that mAChR activation can inhibit DNA synthesis after it has been initiated. Finally, the finding that carbachol-treated cells begin serum-induced DNA

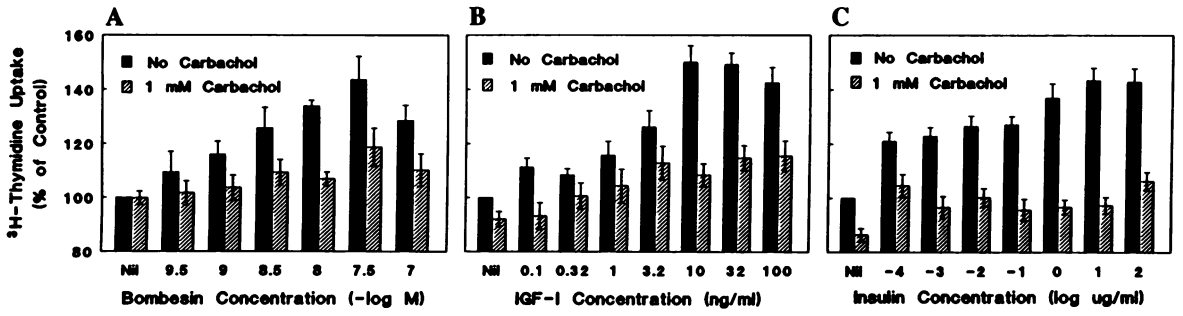

Figure 8. **Carbachol (1 mM) inhibits ^{3}H-thymidine uptake induced by bombesin (A), IGF-I (B), and insulin (C) in serum-starved cells.** Results shown are the mean (±1 SE) of 18–24 determinations from three or four independent experiments. Similar results were obtained with 1 μM carbachol. Method: Cells were incubated in serum-free media for 24 h before addition of ^{3}H-thymidine and the indicated concentration of growth factor. When present, carbachol was added 10 min before ^{3}H-thymidine. Control cells received no growth factor or carbachol. Cells were harvested 24 h later.

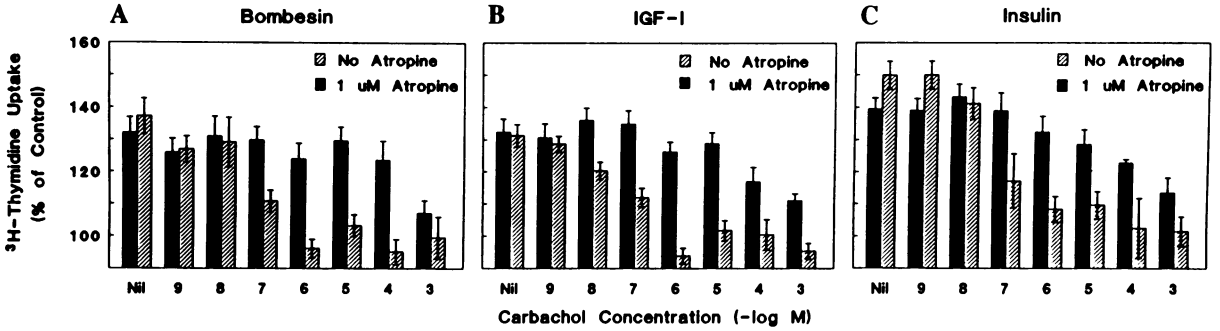

Figure 9. **Atropine (1 μM) suppresses the inhibitory effects of carbachol on ^{3}H-thymidine uptake induced by bombesin (10 nM) (A), IGF-I (10 ng/ml) (B), and insulin (10 ng/ml) (C).** Results shown are the mean (±1 SE) of 18–30 determinations from three to five independent experiments. Method: Cells were incubated in serum-free media for 24 h before addition of ^{3}H-thymidine and the indicated concentration of carbachol, with or without atropine. Growth factor was added 10 min later. Control cells received no growth factor or carbachol. Cells were harvested 24 h later.

synthesis at the same time as untreated cells, but have a lower maximal response (Figure 6B), further suggests that initiation is normal, but progression is impaired.

We found that carbachol maximally inhibits DNA synthesis by 25% in exponentially growing cells (Figure 1). Several lines of reasoning indicate that this is an underestimate of the maximal inhibitory effect of mAChR activation on DNA synthesis. First, this value was determined by measuring ^{3}H-thymidine uptake in cells continuously exposed to carbachol for a 24-h period. Our data indicate that normal DNA synthesis resumes after 12 h of carbachol treatment, when mAChR are down-regulated. This resumption of normal DNA synthesis will reduce the calculated difference between the ^{3}H-thymidine uptake by untreated and treated cells measured at 24 h, leading to a low estimate of inhibitory activity. In support of this, we found that the calculated inhibitory effect of carbachol (1 μM) is greater at 2 h, when mAChR are still signaling (48% inhibition, Figure 2A), than it is at 24 h, when mAChR are down-regulated (25% inhibition, Figures 1 and 2A). Second, the use of exponentially growing cells may cause a low estimate of maximal inhibitory activity. mAChR activation may inhibit the progression of DNA synthesis because a protein that is required at a certain point in S phase is inactivated. In an asynchronous population, cells in S phase that do not yet need (or no longer require) this protein would continue synthesizing DNA even during mAChR activation. Continued DNA synthesis by these cells would lower the calculated maximal inhibition by carbachol. Support for this is the finding that carbachol inhibits DNA synthesis to a greater extent in serum-starved cells (Figures 6 and 7) than in exponentially growing cells. Serum starvation increases the number of cells in G_0/G_1, increasing the number of cells that would synchronously enter S phase on serum stimulation, making them susceptible to the inhibitory effects of mAChR activation.

The finding that mAChR activation arrests cells in both S and G_2/M phases suggests that mAChR activation may disrupt the expression or activation of a protein that controls progression through these phases of the cell cycle. Consistent with the hypothesis that mAChR activation alters the expression of a cell cycle-regulatory protein is the recent finding that mAChR activation alters c-*fos* and c-*jun* mRNA and protein levels in the glial cell line 1321N1 (Trejo *et al.*, 1990). In SCLC cells, candidates for proteins affected by mAChR activation are those implicated in regulating cell cycle progression and DNA synthesis, including p34^{cdc2} (Lee *et al.*, 1988), c-*myc* protein (Penn *et al.*, 1990), thymidine kinase (Jaskulski *et al.*, 1988; Sherley and Kelly, 1988), proliferating cell nuclear antigen (Jaskulski *et al.*, 1988; Morris and Mathews, 1989), and tumor suppressor proteins such as p53 (Reich and Levine, 1984) and pRB (the retinoblastoma-associated protein) (Laiho *et al.*, 1990; Pietenpol *et al.*, 1990). It is conceivable that the expression or activation of one or more of these proteins may be altered by mAChR activation, resulting in decreased DNA synthesis and cell cycle arrest. A precedent for this suggestion is the correlation of the antiproliferative effect of transforming growth factor-β1 with decreased pRB phosphorylation in late G_1 phase in keratinocytes (Pietenpol *et al.*, 1990) and lung epithelial cells (Laiho *et al.*, 1990).

The finding that DNA synthesis returns to normal when mAChR are down-regulated in exponentially growing cells suggests that DNA

synthesis is inhibited only when mAChR are signaling. In exponentially growing cells, DNA synthesis may be initially decreased because signals transmitted by mAChR reduce the expression or activity of a cell cycle-regulatory protein, as discussed above. After mAChR down-regulation, levels of the activated protein may increase, causing the resumption of normal DNA synthesis. In apparent contrast to the results obtained with exponentially growing cells, when serum-starved cells are exposed simultaneously to both serum and carbachol, DNA synthesis is decreased many hours after mAChR are down-regulated (Figure 6). This apparent paradox could be explained if the putative regulatory protein affected by mAChR activation is expressed or activated soon after the cells are exposed to serum, when mAChR are signaling. Thus, these cells would have decreased DNA synthesis even after mAChR are down-regulated because they begin DNA synthesis with reduced levels of the activated regulatory protein. Many of the proposed proteins that may be affected by mAChR activation undergo changes in expression or activity soon after quiescent cells are stimulated with growth factors (Reich and Levine, 1984; Jaskulski *et al.,* 1988; Lee *et al.,* 1988; Sherley and Kelly, 1988; Morris and Mathews, 1989; Laiho *et al.,* 1990; Penn *et al.,* 1990; Pietenpol *et al.,* 1990).

The effect of M_3 mAChR activation on DNA synthesis depends on the cellular environment in which the receptor is expressed. For example, activation of M_3 mAChR decreases DNA synthesis in mouse A9 L cells transfected with the receptor (Conklin *et al.,* 1988) but increases DNA synthesis in transfected chinese hamster ovary cells (Ashkenazi *et al.,* 1989). Activating mAChR in SCLC produces a state similar to terminal differentiation in that the cells do not respond to growth factors. The inhibition of SCLC proliferation by mAChR activation may reflect a reduction in the aberrant expression or activation of proteins that contribute to SCLC transformation. Thus, mAChR activation in SCLC cells provides a novel system to investigate regulatory steps in growth factor-induced DNA synthesis that are common to all cell types and others that may be unique to SCLC cells. The mAChR on SCLC cells warrant further investigation as potential therapeutic targets for this highly lethal form of cancer.

Methods

Reagents

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

SCLC cells

The SCC-9 cell line used in this study was established from a patient's skin metastasis and is maintained in tissue culture. Extensively characterized (Cunningham *et al.,* 1985; DeAizpurua *et al.,* 1988; Williams and Lennon, 1990a), it has a human karyotype and classic SCLC morphology, forms tumors in athymic nude mice, and secretes GRP. It expresses only one class of ^{3}H-quinuclidinyl benzilate binding sites (Cunningham *et al.,* 1985), which has been defined pharmacologically as mAChR of M_3 subtype (Williams and Lennon, 1990a). SCC-9 cells do not express detectable binding sites for ^{125}I-bungarotoxin (Cunningham *et al.,* 1985), indicating they do not express detectable nicotinic acetylcholine receptors of the type found in skeletal muscle (Lindstrom *et al.,* 1987; Luetje *et al.,* 1990). Karyotype analysis and subcloning indicate that the SCC-9 cell line is of clonal origin. The stability of this cell line is evidenced by the fact that the IC_{50} of carbachol for decreasing Ca^{2+} channel activity in these cells (Williams and Lennon, 1990a) has remained constant for >1 y in continuous tissue culture.

Cell culture

For exponential growth, cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% calf bovine serum (Hyclone Labs, Logan, UT) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Serum-free medium consisted of RPMI 1640 supplemented with selenium (30 nM) and human transferrin (10 μg/ml).

^{3}H-thymidine uptake studies

Cells plated in a 96-well microtiter plate at a density of 2 $\times$ 10^4/well were allowed to settle for $\geq$2 hr before adding drugs and ^{3}H-thymidine (130 Ci/mmol, Research Products International, Giv-sur-Yve, France). After incubating at 37°C, 5% CO_2/95% air, the cells were washed and lysed with distilled water and collected on filters with an automatic cell harvester (Skatron, Sterling, VA). The filters were placed in Ultima-Gold scintillation fluid (Packard, Downers Grove, IL) and counted with a LS-7000 β-counter (Beckman Instruments, Fullerton, CA).

Measurement of intracellular free Ca^{2+} concentration

Cells were incubated with Indo-1 AM (4 μM) and pluronic (0.1%) (Molecular Probes, Eugene, OR) for 30 min at 37°C, diluted 1:1 with culture medium, incubated another 30 min, and washed. The ratio of intracellular Indo-1 fluorescence at 390 and 500 nm was measured with a Facstar-Plus (Becton-Dickenson, Mountain View, CA). Ratios were converted to Ca^{2+} concentrations as previously described (Williams and Lennon, 1990a).

Receptor binding assays

The cells were washed three times by centrifugation (200 $\times$ *g*) in cold RPMI 1640 and plated at 5 $\times$ 10^5 cells/well in a 96-well microtiter plate. After incubating with ^{3}H-*N*-methylscopolamine (Amersham, Arlington Heights, IL) for 90 min at 20°C (to decrease reexpression of internalized mAChR) in the presence or absence of atropine (2 μM), the cells were washed with saline (0.9%) and collected on filters with an automatic cell harvester. Radioactivity was measured as described above. Saturable binding to surface mAChR was the difference between the amount of ^{3}H-*N*-methylscopolamine bound in the presence and absence of atropine.

Cell cycle analysis

A single-cell suspension was made by incubating the cells in trypsin (0.25%) at 37°C for 4 min, then passing them through a 27.5-gauge needle into phosphate buffered saline (PBS) containing lysolecithin (0.002%) and paraformaldehyde (1%). After incubation at 4°C for 30 min, the cells were washed twice in PBS by centrifugation (200 × *g*) and incubated in RNase (70 U/ml PBS) (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C. The cells were washed once, resuspended in propidium iodide (50 μg/ml PBS), and incubated at 4°C for ≥1 h. Fluorescence of propidium iodide was measured at 408 nm with a fluorescence-activated cell sorter (Becton-Dickenson). Cell cycle analysis of the data was done with the FMFCD computer program (Dean and Jett, 1974).

All data from cell cycle analyses are presented as the difference between the percentage of carbachol-treated cells and the percentage of untreated cells in a particular phase at each time point (Figure 5). Throughout the experiments, the percentage of untreated cells in G_0/G_1, S, and G_2/M phases was 64.6 ± 0.4, 28.1 ± 0.4, and 7.3 ± 0.2, respectively. Thus, our finding that the percentage of cells in S phase increased to 31.4% after 12 h of carbachol treatment is presented in Figure 5 as a value of 3.3% (determined by subtracting 28.1% from 31.4%). It should be noted that this means carbachol increased the number of cells in S phase by 11.7% compared with control cells (calculated from [31.4/28.1 − 1] × 100).

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