Growth inhibition by the muscarinic M₃ acetylcholine receptor: evidence for p21^{Cip1/Waf1} involvement in G₁ arrest

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We have assessed the growth response of Chinese-hamster ovary (CHO) cells to activation of recombinantly expressed G-protein-coupled muscarinic M₂ or M₃ acetylcholine receptors (AChRs). We show that activation of these receptors leads to divergent growth responses: M₂ AChR activation causes an increase in DNA synthesis, whereas M₃ AChR activation causes a dramatic decrease in DNA synthesis. We have characterized the M₃ AChR-mediated growth inhibition and show that it involves a G₁ phase cell-cycle arrest. Further analysis of this arrest indicates that it involves an increase in expression of the cyclin-dependent kinase (CDK) inhibitor, p21^{Cip1/Waf1} (where Cip1 is CDK-interacting protein 1 and Waf1 is wild-type p53-associated fragment 1), in response to M₃ AChR activation. This increase in protein expression leads to an increase in p21^{Cip1/Waf1} association with CDK2, a decrease in CDK2 activity and an accumulation

of hypophosphorylated retinoblastoma protein. The increased p21^{Cip1/Waf1} expression is due, at least in part, to an increase in p21^{Cip1/Waf1} mRNA, and receptor-mediated changes in phosphorylation of c-Jun provide a mechanism to account for this p21^{Cip1/Waf1} transcriptional regulation. Evaluation of the extracellular signal-regulated protein kinase and c-Jun N-terminal kinase activities has shown striking differences in the profiles of activation of these mitogen-activated protein kinases by the M₂ and M₃ AChRs, and their potential involvement in mediating growth arrest by the M₃ AChR is discussed.

Key words: c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK), G-protein-coupled receptor, growth regulation.

INTRODUCTION

The eukaryotic cell cycle is a spatially and temporally regulated process that allows cells to synthesize DNA and divide. Appropriate regulation of this cycle is critical for normal cell growth and mutations in many cell-cycle proteins are associated with cancers [1,2] and other diseases [3]. Cell-cycle progression through the G₁/S checkpoint precedes DNA synthesis and requires hyperphosphorylation of a tumour suppressor, retinoblastoma protein (pRb) [4]. This phosphorylation is mediated by cyclin-dependent kinases (CDKs) and allows the dissociation of transcription factors, such as E2 promoter-binding factor ('E2F'), from discrete binding pockets within the tumour suppressor [5]. Phosphorylation of pRb at the G₁/S checkpoint is primarily mediated by CDK4/6 and CDK2, which are activated by their interactions with cyclin D1 and cyclin E respectively [6]. G₁ to S-phase progression can also be negatively regulated by CDK inhibitors (CDKIs) that interfere with cyclin-CDK association. There are two families of CDKI, of which three proteins appear to be important for G₁/S progression: p21^{Cip1/Waf1} (where Cip1 is CDK-interacting protein 1 and Waf1 is wild-type p53-associated fragment 1) and p27^{Kip1} (where Kip1 is kinase-inhibitory protein 1), which belong to the Cip/Kip family, and the Ink4 family member p16^{Ink4a} (where Ink4 is inhibitor of CDK4) [7].

Mitogen-activated protein kinases (MAPKs) are a family of proline-directed serine/threonine kinases that regulate cell

growth as well as differentiation and apoptosis. MAPKs are activated by a variety of external stimuli as diverse as growth factors, hormones, neurotransmitters and cellular stresses, and can be grouped into three main subfamilies represented by extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 [8]. The first evidence for MAPK involvement in growth regulation came from experiments demonstrating that transient expression of either the complete ERK1 antisense RNA or a kinase-deficient form of ERK1 was sufficient to abrogate growth factor-induced fibroblast proliferation [9]. MAPKs have been implicated in CDK regulation by a number of mechanisms. Cyclin D1 protein expression can be regulated both positively and negatively by ERK and p38 respectively, at the level of the cyclin D1 promoter [10]. In addition, JNK can directly phosphorylate and activate c-Jun, a subunit of the transcription factor activator protein 1 (AP-1). c-Jun appears to have a critical role in G₁ progression through direct transcriptional control of cyclin D1 via an AP-1 site in the promoter region of the gene [11]. CDK regulation by MAPKs can also occur independently of cyclin expression. For example, inhibition of ERK activity has been shown to correlate with CDK2 inhibition in NIH3T3 cells via a mechanism involving CDK-activating kinase ('CAK'), the enzyme responsible for CDK phosphorylation at its activating phosphorylation site Thr¹⁶⁰ [12]. MAPKs may also influence the cell cycle via CDKIs. For example, the direct association between p21^{Cip1/Waf1}

Abbreviations used: AChR, acetylcholine receptor; AP-1, activator-protein 1; CCh, carbachol; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CHO, Chinese-hamster ovary; Cip1, CDK-interacting protein 1; ERK, extracellular signal-regulated protein kinase; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; Ink4, inhibitor of CDK4; JNK, c-Jun N-terminal kinase; Kip1, kinase-inhibitory protein 1; MAPK, mitogen-activated protein kinase; pRb, retinoblastoma protein; RT, reverse transcriptase; sst, somatostatin; TCA, trichloroacetic acid; Waf1, wild-type p53-activated fragment 1.

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and JNK may contribute to cell-cycle regulation by sequestering p21^{Cip1/Waf1} from CDKs [13]. Alternatively, ERK activation can increase the population of cells in S-phase by decreasing p27^{Kip1} expression [14]. ERK appears to affect p27^{Kip1} expression by altering its rate of degradation, possibly via a proteasomal pathway, rather than by affecting its synthesis [15].

In common with receptor tyrosine kinases, G-protein-coupled receptors (GPCRs) are also capable of regulating cell growth. Activation of the G_{i/o}-coupled somatostatin (sst) 2 receptor inhibits proliferation of Chinese-hamster ovary (CHO) cells through the induction of p27^{Kip1}, resulting in a G₁ cell-cycle arrest [16]. Further investigation showed that sustained ERK activation and prolonged p38 phosphorylation were essential for the growth inhibitory effects of the sst2 and sst4 receptor subtypes [17]. The sst1 receptor has also been shown to inhibit basic fibroblast growth factor ('bFGF')-induced proliferation [18]. This inhibition correlated with an increase in ERK activity, and concomitant addition of basic fibroblast growth factor and sst caused an increase in p21^{Cip1/Waf1} protein expression. Activation of the G_q-coupled 5-hydroxytryptamine 2B receptor stimulates cell-cycle progression through cyclin D1/CDK4 and cyclin E/CDK2 activation [19]. This activation was shown to be ERK-dependent and required the non-receptor tyrosine kinase c-Src. Other recent work [20] has shown that activation of the G_q-coupled muscarinic M₃ acetylcholine receptor (AChR), which can also couple to G_{i/o}-proteins, induces both growth stimulatory and inhibitory responses in NIH3T3 fibroblasts, depending on the growth conditions of the cells [21].

In the present study, we have examined the growth regulatory effects of the G_{i/o}-coupled M₂ AChR and the G_q-coupled M₃ AChR stably expressed in CHO cells. We demonstrate that activation of the M₃ AChR causes a G₁ phase arrest of the cell cycle. This arrest involves increased expression and association of the CDK inhibitor p21^{Cip1/Waf1} with CDK2, inhibition of CDK2 activity, accumulation of hypophosphorylated pRb and a dramatic decrease in DNA synthesis. By contrast, the M₂ AChR stimulates DNA synthesis, highlighting the potential diverse cellular responses regulated by a single subfamily of GPCRs. We have also evaluated the regulation of the ERK and JNK subfamilies of MAPKs by the M₂ and M₃ AChRs and their contribution to growth regulation by muscarinic receptors is discussed.

EXPERIMENTAL

Cell culture

CHO-K1 cells stably expressing either the human recombinant M₂ (CHO-m2) or M₃ (CHO-m3) AChR were maintained in minimal essential medium- α (Gibco-BRL) at 37 °C in a 5% CO₂/95% air mixture. The medium was supplemented with 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Gibco-BRL). Where indicated, cells were made quiescent by serum starvation for 24 h prior to agonist addition.

ERK immunoprecipitation and assay

ERK proteins were isolated by immunoprecipitation from CHO cell lysates and assayed for enzyme activity. Cells were solubilized with lysis buffer containing 20 mM Tris/HCl (pH 7.4), 1% (v/v) Triton X-100, 10% (v/v) glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF and 10 µg/ml leupeptin. Insoluble material was removed by centrifugation at 14000 g for 10 min at 4 °C. Lysates were incubated for 60 min at 4 °C with a 1:100 dilution of a rabbit polyclonal

antiserum (200 µg/ml) directed against ERK1 (C16: sc-93; Santa Cruz). Immune complexes were incubated for a further 60 min at 4 °C with 70 µl of a 15% (v/v) slurry of Protein A-Sepharose (Amersham Biosciences) and collected by centrifugation. Immunoprecipitates were washed twice in 200 µl of lysis buffer and twice in 200 µl of kinase buffer containing 20 mM Hepes (pH 7.2), 20 mM β -glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol and 50 µM sodium vanadate. Immune complex ERK assays were initiated by addition of 40 µl of kinase buffer containing 20 µM [γ -³²P]ATP (2.5 µCi/nmol) and 200 µM of a synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor ('EGF') receptor. Reactions were incubated for 20 min at 30 °C and terminated by addition of 10 µl of 25% (w/v) trichloroacetic acid (TCA). Mixtures were centrifuged at 14000 g for 2 min and the resulting supernatant was spotted onto P81 cation-exchange paper (Whatman). Papers were washed three times in 0.5% orthophosphoric acid, once in acetone, and allowed to dry before being counted in a Tri-Carb liquid-scintillation counter.

JNK immunoprecipitation and assay

CHO cell lysates, prepared exactly as for ERK immunoprecipitation, were incubated for 60 min at 4 °C with a 1:100 dilution of a rabbit polyclonal antiserum (200 µg/ml) directed against JNK1 (C17: sc-474; Santa Cruz). Immune complexes were incubated overnight at 4 °C with 70 µl of a 15% (v/v) slurry of Protein A-Sepharose (Amersham Biosciences) and collected by centrifugation. JNK was assayed under identical conditions with those described for ERK, except that 5 µg of purified glutathione S-transferase (GST)-c-Jun was used as a substrate. Reactions were terminated by addition of 40 µl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 12% (w/v) acrylamide in the presence of 0.1% SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into c-Jun was quantified by liquid-scintillation counting of the excised bands.

CDK and cyclin immunoprecipitation and assay

Rabbit polyclonal antibodies (all 200 µg/ml and from Santa Cruz Biotechnology) to CDK2 (M2: sc-163), CDK4 (C-22: sc-260), cyclin E (M-20: sc-481), and mouse monoclonal antibodies (all 200 µg/ml) cyclin D1 (R-124: sc-6281; Santa Cruz) and cyclin A (05-155; Upstate Biotechnologies) were used for immunoprecipitation from CHO cell lysates prepared as described for ERK immunoprecipitation, except that lysates were precleared with 70 µl of a 15% (v/v) slurry of Protein A-Sepharose prior to antibody addition. Immunoprecipitates were washed twice in 200 µl of a wash buffer containing 25 mM Hepes (pH 7.4), 150 mM NaCl and 0.1% (v/v) Triton X-100 and twice in 200 µl of an assay buffer containing 50 mM Hepes (pH 7.4), 1 mM dithiothreitol, 15 mM EGTA and 20 mM MgCl₂. Immune complex CDK2 reactions were performed at 30 °C for 20 min in this assay buffer containing 20 µM [γ -³²P]ATP (2.5 µCi/nmol) and 25 µg of histone H1 as a substrate. Reactions were terminated by addition of 40 µl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 15% (w/v) acrylamide in the presence of 0.1% SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Phosphorylated histone H1 was quantified by liquid-scintillation counting of the excised bands.

Figure 1 Effects of CCh on CHO-m2 and CHO-m3 cells

(A and B) Concentration-dependent effects of CCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated for 24 h with the indicated concentrations of CCh and incubated with 2 μ Ci of [³H]thymidine for the final 2 h. DNA synthesis was measured using the diethyl ether-extraction method as outlined in the Experimental section. Control values represent cells that were not treated with CCh. (A) CHO-m2 cells were serum-starved (–Serum) for 24 h prior to CCh addition. (B) A growing population of CHO-m3 cells (+Serum) was treated with CCh. (C and D) Time courses over 24 h showing the effect of 10 μ M CCh on DNA synthesis in CHO-m2 and CHO-m3 cells. Cells were stimulated with 10 μ M CCh (Treated) or an equivalent addition of water (Control) for the times indicated. (C) CHO-m2 cells were serum-starved (–Serum) 24 h before CCh addition. The inset shows the effect on DNA synthesis of stimulating growing CHO-m2 cells (+Serum) with 10 μ M CCh (+) or an equivalent volume of water (–) for 24 h. (D) Growing CHO-m3 cells (+Serum) were treated with CCh. The inset shows DNA synthesis in CHO-m3 cells that have been serum-starved (–Serum) for 24 h prior to addition of 10 μ M CCh or an equivalent volume of water for 24 h. All values represent the means $\pm$ S.E.M. of four independent determinations. Results shown are representative of 3–10 separate experiments.

Western-blot analysis

Proteins were separated by SDS/PAGE and transferred on to a nitrocellulose membrane for 30 min at 12 V in transfer buffer [48 mM Tris base, 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol] using a semi-dry transfer apparatus (Bio-Rad Laboratories). Membranes were blocked for 1 h at 18–22 °C with 5% (w/v) non-fat milk powder in TTBS [50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.1% Tween-20] and incubated overnight at 4 °C with primary antibody [anti-ERK1 (C16: sc-93), 1:500; anti-JNK1 (C17: sc-474), 1:500; anti-p21 (H-164: sc-756), 1:500; anti-p27 (M-197: sc-776), 1:500; anti-p16 (M-156: sc-1207), 1:500; anti-CDK2, (M2: sc-163), 1:500; anti-CDK4 (C-22: sc-260), 1:500; anti-cyclin D1 (R-124: sc-6281), 1:1000; anti-cyclin E (M-20: sc-481), 1:500; c-Jun (H-79: sc-1694), 1:500 (all from Santa Cruz Laboratories); anti-cyclin A (05-155), 1:1000, (Upstate Biotechnologies), and anti-pRb (M032700), 1:500, (Pharmingen)] in TTBS. After washing in TTBS, blots

were incubated for 1 h at 18–22 °C with a 1:1000 dilution in TTBS of anti(rabbit IgG) or anti(mouse IgG) antibodies coupled to peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham Biosciences). To detect p21^{Cip1/Waf1} associated with CDK2, immunoprecipitates of CDK2 were prepared (as described for CDK and cyclin immunoprecipitation and assay) and resuspended in 100 μ l of Laemmli sample buffer.

Thymidine incorporation

CHO cells were plated at 50000 cells/well and incubated overnight under normal culture conditions. Cells were then incubated for a further 24 h in medium in the absence or presence of 10% (v/v) newborn calf serum as indicated in the Figure legends. Carbachol (CCh) incubations were for 24 h unless otherwise stated. [³H]thymidine (2 μ Ci; 25 Ci/mmol; TRK120; Amersham

Figure 2 Activation of ERK and JNK by CCh in CHO-m2 and CHO-m3 cells

CHO-m2 (▲) and CHO-m3 (●) cells, either serum-starved for 24 h prior to addition of CCh (**A** and **C**) or growing cells (**B** and **D**), were incubated with 10 μ M CCh for the appropriate times as shown. (**A** and **B**) Following treatment, cells were lysed and ERK1 was immunoprecipitated using a selective antibody as described in the Experimental section. Western-blot (WB) analysis of total ERK protein (ERK1 and ERK2) was also performed on lysates prepared for ERK assay, and representative blots are shown. (**C** and **D**) Following treatment, cells were lysed and JNK1 was

Biosciences) was added to each well 2 h prior to lysis. Cells were washed three times with minimal essential medium- α and incubated with 2 ml of 5% (w/v) TCA for 1 h at 4 °C. TCA was removed and cells were washed once in fresh TCA solution. A 2 ml volume of ice-cold ethanol containing 200 μ M potassium acetate was placed in each well for 5 min, after which the cells were incubated twice for 15 min in 2 ml of an ethanol/diethyl ether mixture (3:1, v/v). Plates were allowed to air dry before the cellular precipitates were solubilized with 0.5 ml of 0.5 M NaOH. [³H]Thymidine incorporated into DNA was measured by adding samples to scintillation fluid and counting.

FACS analysis

Exponentially growing CHO cells were treated with 10 μ M CCh for 24 or 48 h, or left untreated. Cells were lifted using Cell Dissociation Agent™ (Sigma) and fixed in 70% (v/v) ethanol at -20 °C for 15 min. Fixed cells were then stained overnight with propidium iodide (200 μ g/ml) before being analysed using a Becton Dickinson FACScan.

RNA extraction and reverse transcription

Exponentially growing CHO-m3 cells were stimulated with 10 μ M CCh for the times indicated or left untreated as controls before total RNA was extracted using RNeasy™ B according to the manufacturer's instructions (Qiagen Ltd). After photometric quantification, 50 ng of RNA was used for a reverse transcriptase (RT) reaction (20 μ l total volume) in buffer containing 200 units of Superscript™ II RNase H⁺ RT (Invitrogen), random primers and dNTPs, as recommended by the manufacturer (Invitrogen).

PCR

PCRs were performed using 2 μ l of the transcribed RNA as a template. For the p21^{Cip1/Waf1} reaction, 200 nM each of the sense primer, 5'-GTG GCC TTG TCG CTG TCT TGC AC-3', and antisense primer, 5'-TTT CTC TTG CAG AAG ACC AAT C-3', were used in the presence of *Taq* DNA polymerase, dNTPs and MgCl₂ (Invitrogen). After a 2 min denaturing period at 94 °C, PCRs were performed over 40 cycles (94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min), with a final extension step at 72 °C for 7 min. For the β -actin reaction, 100 nM of the sense primer, 5'-TTC AAC TTC ATC ATG AAG TGT GAC GTG-3', and antisense primer, 5'-CTA AGT CAT AGT CCG CCT AGA AGC ATT-3', were used. After a 3 min denaturing period at 95 °C, reactions were performed over 25 cycles (95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min), with a final extension step at 72 °C for 5 min.

RESULTS

Effect of CCh on DNA synthesis in CHO-m2 and CHO-m3 cells

Activation of muscarinic M₃ AChRs has been shown to exert opposing effects on DNA synthesis in NIH3T3 fibroblasts, depending on the growth conditions of the cells [21]. Figure 1

shows the effect of the muscarinic agonist CCh on DNA synthesis, as measured by [³H]thymidine incorporation, in CHO cells expressing either the muscarinic M₂ or M₃ AChR at comparable levels (1.5 $\pm$ 0.1 and 2.2 $\pm$ 0.2 pmol/mg of total cellular protein respectively; means $\pm$ S.E.M., *n* = 6) [22]. Activation of the M₂ AChR under serum-starved conditions (Figure 1A) resulted in a 2.9-fold increase in DNA synthesis over the basal level at a maximally effective concentration of 10 μ M CCh. By contrast, M₃ AChR activation in growing CHO-m3 cells (Figure 1B) caused a decrease in DNA synthesis. At 10 μ M CCh, DNA synthesis was maximally inhibited by 77% compared with control levels. DNA synthesis was examined over a 24 h time course of receptor activation by 10 μ M CCh in the two cell lines (Figures 1C and 1D). In serum-starved CHO-m2 cells, an increase in DNA synthesis was detected after 12 h of CCh stimulation and was maximal by 24 h of agonist treatment. In growing CHO-m2 cells (Figure 1C, inset), 10 μ M CCh did not cause any change in DNA synthesis compared with control levels. The inhibitory effect of CCh on DNA synthesis in growing CHO-m3 cells (Figure 1D) was far more rapid than the opposing stimulation observed in the CHO-m2 cells (Figure 1C). For example, 6 h after CCh addition, DNA synthesis was inhibited by 48% in CHO-m3 cells, whereas no effect was seen after 6 h of agonist stimulation of CHO-m2 cells. By 12 h of agonist stimulation of CHO-m3 cells, inhibition was almost maximal (72%), and by 18 h the DNA synthesis was inhibited by 82% compared with control levels (Figure 1D). Under serum-starved conditions, a modest inhibition of DNA synthesis by CCh was still observed (38% inhibition compared with 76% inhibition in the presence of serum at 24 h; Figure 1D inset), suggesting that serum starvation is insufficient to drive CHO-m3 cells fully into quiescence. Indeed, cyclin D1 protein expression was still detected by Western blotting of serum-starved CHO-m3 cells (results not shown).

Effects of muscarinic receptor activation on ERK and JNK activity

Both ERK and JNK have been implicated in growth control responses to extracellular stimuli in many different cellular systems. Stimulation of the M₂ and M₃ AChRs in CHO cells has been shown to differentially activate ERK and JNK [22]. However, it was important to re-examine the extent of ERK and JNK activation by these muscarinic receptors under the growth conditions in which they perturb DNA synthesis. Figure 2(A) shows the ERK activation profiles in CHO-m2 and CHO-m3 cells over a 24 h period of CCh stimulation under serum-starved conditions. Peak ERK activation was observed at 5 min of agonist stimulation in both cell lines (specific ERK activities of 7.62 and 7.23 pmol of ATP/min per mg of total cellular protein in CHO-m2 and CHO-m3 cells respectively), although a lower basal ERK activity in the CHO-m2 cells resulted in a higher fold-over-basal value (20.9 compared with 12.1 for the CHO-m3 cells). However, ERK activation was more sustained in CHO-m3 cells. After 1 h of CCh treatment, ERK activity had returned towards basal levels in CHO-m2 cells, whereas it remained elevated in the CHO-m3 cells (4.5-fold over basal). This may represent a potential model for transient versus sustained ERK activation, which could account for the differential effects of CCh on the growth responses of CHO-m2 and

immunoprecipitated (IP) with a specific antibody as described in the Experimental section. Kinase activity was measured using GST-c-Jun as a substrate and representative autoradiograms of c-Jun phosphorylation detected after overnight (CHO-m2) or 2 h (CHO-m3) exposure to autoradiography film are shown. Representative Western blots (WB) of total JNK protein in cleared cell lysates prepared for JNK assay are also shown. Specific ERK and JNK activities (pmol of ATP/min per mg of total cellular protein) were calculated for each time point using a protein value obtained from a Bradford protein assay of the cleared cell lysates. Each data point represents the mean $\pm$ S.E.M. (*n* = 4–8).

Figure 3 FACS analysis of growing CHO-m2 and CHO-m3 cells

Growing CHO-m2 and CHO-m3 cells were treated with 10 μ M CCh for 24 and 48 h. Control cells were not treated with CCh. Cells were lifted, fixed in methanol at -20°C , and then stained overnight with propidium iodide for FACS analysis. 2n represents the normal diploid number and 4n the replicated DNA. **(A)** Representative profiles of control and 48 h CCh-treated CHO-m3 cells respectively. **(B)** Tabulated data showing the percentage of CHO-m3 and CHO-m2 cells in G₁, S- and G₂/M phases of the cell cycle. Values are means $\pm$ S.E.M. **(C)** Growing CHO-m3 cells were left untreated (zero time point) or stimulated with 10 μ M CCh for 24 h. The cells were lysed and Western-blot analysis of pRb performed. The hypo- (pRb) and hyperphosphorylated (ppRb) forms of the protein are shown. Three independent cell lysates were prepared per condition as shown.

CHO-m3 cells under serum-starved conditions. In the presence of serum, basal ERK activity was high, resulting in small fold-over-basal increases in activity at 5 min in both cell lines in response to CCh (2.3- and 1.7-fold in CHO-m2 and CHO-m3 cells respectively) (Figure 2B). The peak specific activities in both cell lines in the presence of serum (7.80 and 5.35 pmol of ATP/min per mg of total cellular protein in CHO-m2 and CHO-m3 cells respectively) were similar to those obtained under serum-starved conditions, suggesting that the main difference with respect to ERK activation between the growth conditions is the dramatically increased basal activity seen in growing cells. However, a key difference between the two cell lines was apparent over the longer ERK time course in the presence of serum. Whereas ERK activity returned to basal values by 2 h of CCh stimulation of CHO-m2 cells, ERK activity dropped dramatically below basal levels in CHO-m3 cells at this time point (0.38-fold

Figure 4 Effect of CCh on CDK activities in growing CHO-m3 cells

CHO-m3 cells were left untreated or stimulated with 10 μ M CCh. Cyclins and CDKs were immunoprecipitated from lysed cells using the appropriate antisera and kinase activities were measured using histone H1 as a substrate. Specific activity was calculated after determination of the protein content of cleared cell lysates using a Bradford protein assay. **(A)** CDK2 was assayed from CHO-m3 cell lysates from untreated (zero time point) and after addition of CCh at the time points shown. Values are means $\pm$ S.E.M. ($n = 4$). **(B)** CDK2, CDK4, cyclin A, cyclin D1 and cyclin E were immunoprecipitated and associated kinase activity was assayed from CHO-m3 cells lysates stimulated for 24 h with 10 μ M CCh (+) or left untreated as controls (—). Results shown represent the percentage kinase activity of each protein with respect to its individual control and are means $\pm$ S.E.M. ($n = 4$). A one-tailed paired Student's *t* test was used as a measure of significance. * $P < 0.05$ compared with control.

compared with basal). At 6 h of agonist incubation, specific activity in the growing CHO-m3 cells was 0.85 pmol of ATP/min per mg of total cellular protein, representing 0.27-fold of the basal value, and approaching the basal specific activity obtained under serum-starved conditions in this cell line (0.60 pmol of ATP/min per mg of total cellular protein). Thus the high ERK activity remains elevated in growing CHO-m2 cells following CCh stimulation, whereas ERK activity in growing CHO-m3 cells is reduced by CCh to levels approaching those observed under serum-starved conditions over the long term. Western-blot analysis of ERK1 and ERK2 proteins indicated that the total

Figure 5 Effect of CCh on cell-cycle protein expression in growing CHO-m3 cells

Growing CHO-m3 cells were stimulated for 24 h with 10 μ M CCh or left untreated as controls (zero time point). Three independent cell lysates were generated per condition as shown. (A) Western-blot analysis of cell-cycle proteins. Cell lysates were prepared and proteins were detected by using the appropriate antibodies as described in the Experimental section. (B) Analysis of p21^{Cip1/Waf1} association with CDK2. CDK2 was immunoprecipitated from control (0) and 24 h CCh-stimulated cells. p21^{Cip1/Waf1} and CDK2 were detected in these immunoprecipitates by Western blotting with appropriate antisera. Total cell lysate from a growing population of CHO-m3 cells stimulated with CCh was also blotted for p21^{Cip1/Waf1} and CDK2. Three independent cell lysates were generated per condition as shown.

amount of ERK protein was not affected by CCh stimulation of CHO-m2 or CHO-m3 cells under either growth condition (Figures 2A and 2B, lower panels).

The time course of JNK activity was also investigated in CHO-m2 and CHO-m3 cells. Under serum-starved conditions, CHO-m3 cells displayed a robust JNK response to CCh, peaking at 45 min and returning close to basal levels by 4 h of agonist addition (Figure 2C). The peak activity of 7.49 pmol of ATP/min per mg of total cellular protein represented a 23.9-fold JNK activation over the basal level. CCh did not elicit a large JNK response in CHO-m2 cells, giving a peak value at

15 min of 0.99 pmol of ATP/min per mg of total cellular protein. Figure 2(C) (middle panel) shows representative autoradiograms of GST-c-Jun phosphorylation by JNK1 immunoprecipitates. Figure 2(C) (bottom panel) shows Western blots using an antibody to JNK1, indicating that the total JNK1 protein did not change during the time course of the experiments. In the presence of serum, CCh produced a large JNK activation in CHO-m3 cells compared with CHO-m2 cells (Figure 2D), confirming that the M₃ AChR couples relatively poorly to JNK activation [22]. The main difference between the growth conditions was observed with respect to basal JNK activities, which were higher in growing cells, giving lower fold responses to CCh in the presence of serum (Figure 2D) compared with its absence (Figure 2C). Thus the M₃ AChR is more effectively coupled to JNK activation than the M₂ AChR, regardless of the growth condition.

Nature of the inhibition of DNA synthesis in CHO-m3

Having ascertained that activation of the M₃ AChR caused a dramatic decrease in DNA synthesis, it was important to determine the nature of this inhibition. It could result from the induction of a pro-apoptotic response, a general decrease in protein synthesis or a specific block of the cell cycle. Figure 3(A) shows the results of FACS analysis performed on exponentially growing CHO-m3 cells stimulated with CCh for 48 h. The profiles indicated an increase in the proportion of cells in G₁ with a resulting decrease of cells in S-phase and in G₂/M. Figure 3(B) summarizes in tabular form data from a number of independent experiments in which CHO-m3 and CHO-m2 cells were stimulated with CCh for 24 or 48 h in the presence of serum. In the control CHO-m3 cells, approx. 56% of cells are in G₁, with 25% in S-phase and 18% in G₂/M. After 48 h of CCh stimulation, 80% of cells were in G₁, an increase of 23.6% above the control cells. Only 10.6% and 9.2% of cells were in S-phase and G₂/M respectively, after the incubation with CCh for 48 h. This profile is indicative of a population of cells that have been growth arrested at the G₁ phase of the cell cycle. Data is also included in Figure 3(B) for the CHO-m2 cells, which show no corresponding changes in cell-cycle phase in the presence of CCh and thus serve as a control. Confirmation of G₁ phase arrest of CHO-m3 cells by CCh was obtained by Western blotting of pRb (Figure 3C). pRb is a tumour-suppressor protein that must be phosphorylated for G₁/S progression. When cells are cycling, pRb exists exclusively in its hyperphosphorylated form, releasing transcription factors that promote growth. pRb was hyperphosphorylated in the growing CHO-m3 cells under control conditions, where it displayed a retarded electrophoretic mobility (Figure 3C, lanes 1–3). After incubation with CCh for 24 h, pRb became hypophosphorylated, as shown by the appearance of the lower-molecular-mass form (Figure 3C, lanes 4–6), indicative of a population of cells becoming growth arrested in G₁.

CCh inhibits CDK2 and cyclin-associated kinase activity in CHO-m3 cells

Because growth inhibition of CHO-m3 cells by CCh is due to a G₁ phase block of the cell cycle, proteins known to be involved in the regulation of G₁ phase progression were investigated. We therefore assessed the activation of CDKs following CCh stimulation of CHO-m3 cells, because they exert their regulatory effects on the cell cycle by direct phosphorylation of pRb. Figure 4(A) shows a time course of CDK2 activity following immuno-

Figure 6 Regulation of p21^{Cip1/Waf1} protein expression by CCh in growing CHO-m3 cells

(A and B) The involvement of protein stability in p21^{Cip1/Waf1} protein expression. Growing CHO-m3 cells were stimulated with 10 μ M CCh for 24 h or left untreated as control cells. After 24 h cells were incubated further with 0.25 mg/ml cycloheximide and lysed at the times indicated (at the top of the gel). The zero time point represents cells with no cycloheximide addition. (A) Western blots of p21^{Cip1/Waf1} were analysed densitometrically for the times indicated after cycloheximide addition. Values are means $\pm$ S.E.M. ($n = 3-4$). (B) Representative Western blots of p21^{Cip1/Waf1} in the control and CCh-treated cell populations following cycloheximide treatment. (C) RT-PCR of p21^{Cip1/Waf1} and β -actin. Growing CHO-m3 cells were stimulated with 10 μ M CCh for 1 or 2 h, or left untreated as controls. Reverse transcription was performed on the total RNA extracted from the cells, and p21^{Cip1/Waf1} mRNA was detected by PCR using the transcribed RNA as a template. Control reactions were also performed using primers specific for β -actin. Each individual lane for duplicate conditions was generated from a separate RNA extraction of two independent wells of cells. Also shown is the pUC19 DNA/Msp1 marker (MBI Fermentas) that contains 13 DNA fragments ranging from 501 to 26 bp. (D) Western-blot analysis of c-Jun in lysates derived from CHO-m3 cells stimulated with CCh for the indicated times (shown at the top of the gel).

precipitation from CHO-m3 cells stimulated with CCh. CCh inhibited CDK2 with a time course remarkably similar to that observed for DNA synthesis (Figure 1D). By 6 h of CCh incubation, CDK2 activity was inhibited by 38 % compared with control, and at 24 h the activity was inhibited by 68 %. This observation indicates that activation of the M₃ AChR inhibits CDK2 activity, leading to an inhibition of DNA synthesis and the accumulation of cells in G₁. Because other CDKs are known to be involved in this transitional point, the effects of CCh on CDK2 and CDK4 activity and on cyclin D1-, cyclin E- and cyclin A-associated kinase activities were also examined in parallel (Figure 4B). CCh had a significant inhibitory effect ($P < 0.05$ as assessed by Student's t test) on CDK2 activity as well as on both cyclin E- and cyclin A-associated kinase activities. CDK2 activity was inhibited by 66 %, whereas cyclin A- and cyclin E-associated kinase activities were inhibited by 42 % and 40 % respectively. Cyclin E and cyclin A are known to associate with, and activate, CDK2 during G₁/S-phase and therefore the CDK activity associated with these cyclins is likely to reflect CDK2 activity. However, it is also likely that the inhibition of cyclin A- and cyclin E-associated kinase activities by CCh is not as great as that observed in CDK2 immunoprecipitates, because additional CDK proteins may associate with these

cyclins. CCh did not have a significant effect ($P > 0.05$) on CDK4 activity or on the kinase activity associated with cyclin D1, which specifically activates CDK4 and CDK6 at the G₁/S checkpoint. Thus CDK2 activity is inhibited by M₃ AChR activation as assessed by direct kinase assays of either immunoprecipitated CDK2 or CDK2 associated with immunoprecipitated cyclins A and E. CCh had no significant effect on CDK4 activity in CHO-m3 cells, and the agonist is also unlikely to inhibit CDK6 based on the lack of inhibition of cyclin D1-associated kinase activity.

CCh selectively induces p21^{Cip1/Waf1} expression in CHO-m3 cells

Changes in cyclin expression can regulate CDK activity. Therefore Western-blot analysis was performed to detect changes in expression of cyclin D1, cyclin E and cyclin A following agonist stimulation of CHO-m3 cells. No changes in protein levels of these cyclins were detected after 24 h of CCh treatment (Figure 5A). In addition, the levels of CDK2 or CDK4 protein did not change following agonist stimulation (Figure 5A). Thus the M₃ AChR-mediated CDK2 inhibition does not appear to involve changes in expression of CDK2 or the cyclins with which it associates. CDKIs are another class of cell-cycle proteins capable of regulating CDKs. Figure 5(A) (lower three panels) shows

three CDKI proteins that were investigated by Western-blot analysis. Although no changes in expression of p27^{Kip1} or p16^{Ink4a} were observed following CCh treatment, the agonist clearly increased the expression of p21^{Cip1/Waf1}. Stimulation of CHO-m3 cells with CCh for 24 h led to a 4.5-fold increase in expression of p21^{Cip1/Waf1}, as measured by densitometry. p21^{Cip1/Waf1} can inhibit both CDK2 and CDK4/6 by direct association and this may contribute to the inhibition of DNA synthesis. Western-blot analysis of p21^{Cip1/Waf1} was performed on CDK2 immunoprecipitates to test whether CCh stimulates p21^{Cip1/Waf1} association with CDK2 in CHO-m3 cells. Agonist treatment increased the association of p21^{Cip1/Waf1} with CDK2 without affecting the amount of CDK2 immunoprecipitated (Figure 5B), suggesting that the mechanism of CDK2 inhibition involves the binding of p21^{Cip1/Waf1}.

Mechanisms underlying p21^{Cip1/Waf1} protein induction

G₁ arrest induced by the M₃ AChR involved an increase in p21^{Cip1/Waf1} protein expression and association with CDK2. An increase in p21^{Cip1/Waf1} protein levels can be mediated by an increase in protein stability [23]. To investigate this as a possible mechanism, protein synthesis in control and CCh-treated CHO-m3 cells was disrupted by cycloheximide and the rate of p21^{Cip1/Waf1} protein degradation was then measured in both cell populations by Western-blot analysis of p21^{Cip1/Waf1} (Figures 6A and B). Little difference in the rate of degradation between control and CCh-treated cells was observed, demonstrating that protein stability was not affected by M₃ AChR activation. A second mechanism that could account for the receptor-mediated increase in p21^{Cip1/Waf1} protein expression is transcriptional activation of the p21^{Cip1/Waf1} promoter. To address this possibility, RT-PCR was performed to measure receptor-mediated changes in p21^{Cip1/Waf1} mRNA. Following 2 h of CCh stimulation of CHO-m3 cells, p21^{Cip1/Waf1} mRNA was increased by 2.6-fold relative to the control (Figure 6C), as measured by densitometry, and returned to basal levels by 24 h (results not shown). As the data in the present study have shown that JNK is strongly activated by the M₃ AChR, and previous studies [24,25] have shown that c-Jun is involved in transcriptional regulation of cell-cycle proteins, the phosphorylation of c-Jun was assessed in CHO-m3 cells in response to CCh. As shown by Western blotting (Figure 6D), activation of the M₃ AChR caused a clear shift corresponding to phosphorylation of c-Jun that appeared to be maximal at 2 h of agonist stimulation, returning to basal levels by 24 h. This time course correlates well with the induction of p21^{Cip1/Waf1} mRNA and is consistent with c-Jun involvement in the transcriptional regulation of p21^{Cip1/Waf1}.

DISCUSSION

The present study has examined the growth regulatory effects of the M₃ AChR in CHO cells. The data presented indicate that the activation of this GPCR leads to growth arrest. This arrest occurs during the G₁ phase of the cell cycle and is accompanied by an increase in p21^{Cip1/Waf1} mRNA and protein levels, inhibition of CDK2 activity and hypophosphorylation of pRb. These effects are consistent with the reported role of the M₃ AChR as a tumour suppressor [26]. The finding that activation of the M₂ AChR, which preferentially couples to G_{i/o} rather than G_q, is proliferative may have important physiological implications and shows the diverse cellular functions mediated by a single sub-family of GPCR. In addition to the classical second messenger pathways regulated by muscarinic GPCRs, the M₂ and M₃ AChRs are also capable of activating intracellular signalling

cascades leading to MAPK activation. Previous studies from this laboratory [22] have characterized the acute phases of MAPK activation by the M₂ and M₃ AChRs in CHO cells. However, since the temporal pattern of MAPK activation can determine the growth response of cells to various agonists, more extensive time courses of ERK and JNK activation were examined under the conditions in which growth regulatory effects were observed. The most apparent difference was seen with respect to JNK activation, where M₃ AChR stimulation caused a dramatic activation relative to that induced by the M₂ AChR. JNK is classically involved in the induction of apoptosis [27], but it has also been implicated in G₁ arrest [28]. If JNK activation is responsible for the growth inhibition observed in CHO-m3 cells, this would account for the failure of the M₂ AChR to cause growth arrest of the cells. However, the absence of commercially available specific JNK inhibitors prevented a direct test of this hypothesis. Analysis of the ERK-activation profiles in the CHO-m2 and CHO-m3 cells suggests an alternative hypothesis to account for the growth arrest. The proliferative effect of ERK is well established and blocking ERK activity has been shown to arrest growth in many cell systems [29,30]. Whereas the high basal ERK activity in the presence of serum was maintained following agonist stimulation of CHO-m2 cells, activation of the M₃ AChR appeared to suppress this high basal ERK activity after the initial peak of activation. Indeed, the activity of ERK at 2–6 h of agonist stimulation of exponentially growing CHO-m3 cells was equivalent to that of serum-starved cells. This receptor-mediated inhibition of basal ERK activity could be responsible for the growth arrest of CHO-m3 cells by suppressing the normal proliferative pathways induced in the presence of serum. This is consistent with our observation that treatment of growing CHO cells with the MAPK/ERK kinase 1 ('MEK1') inhibitors U0126 or PD98059 essentially abolished DNA synthesis in growing CHO cells (results not shown), suggesting that the high basal level of ERK activity is essential to maintain cell-cycle progression. Because pharmacological inhibition of the MAPK pathway has this dramatic effect on the basal level of DNA synthesis, independent of muscarinic receptor activation, our experiments have focused on characterizing the cell-cycle events associated with the G₁ phase growth arrest induced by the M₃ AChR.

Of the cell-cycle proteins examined, only the expression of p21^{Cip1/Waf1} was affected by M₃ AChR activation. M₃ AChR activation led to an increase in expression and association of p21^{Cip1/Waf1} with CDK2 by 24 h of agonist stimulation, suggesting that p21^{Cip1/Waf1} contributes to maximal CDK2 inhibition and the G₁ arrest. An increase in p21^{Cip1/Waf1} mRNA was detected at 2 h of agonist stimulation, implying that a mechanism to enhance p21^{Cip1/Waf1} protein expression is stimulated by M₃ AChR activation at early time points. However, Western-blot analysis failed to detect an increase in p21^{Cip1/Waf1} protein expression at 6 h of agonist stimulation (results not shown), a time point at which approximately half-maximal inhibition of DNA synthesis and CDK2 activity was observed, suggesting that additional mechanisms may contribute to the early phase of CDK2 inhibition. For example, covalent modification of CDK2 may be involved in the mechanism by which the M₃ AChR inhibits CDK2 activity, although preliminary experiments suggest that expression of the CDK phosphatase Cdc25A is not affected by M₃ AChR activation (results not shown). Although an increase in total p21^{Cip1/Waf1} protein could not be detected during the early phase of receptor activation and was relatively modest at 24 h of agonist stimulation, when maximal CDK2 inhibition was observed, the activity of CDKs is dependent on small stoichiometric changes within the CDKI/CDK complexes, rather

than large changes in total cellular protein expression [31]. For example, a 1.8-fold increase in p21^{Cip1/Waf1} protein expression is sufficient to arrest IEC-6 cells in G₁ after polyamine depletion [28]. Similarly, a 2.5-fold increase in p21^{Cip1/Waf1} association with CDK2 causes G₁ arrest of bladder carcinoma cells [32]. Experiments using p21^{Cip1/Waf1} antisense RNA to block a transforming-growth-factor- β ('TGF- β ')-mediated G₁ arrest indicated that expression of p21^{Cip1/Waf1} was sufficient to cause G₁ arrest of gastric carcinoma cells [33]. p21^{Cip1/Waf1} has been shown to have a role in G₁ arrest in many cell systems and it can bind to and inhibit the kinase activity of cyclin D1/CDK4 and cyclin E/CDK2 complexes [25]. Although M₃ AChR activation caused a marked inhibition of CDK2 activity, it had little effect on CDK4 activity. In the asynchronous population of growing CHO-m3 cells, the activity of CDK4 was low compared with that of CDK2 and may be insufficient to detect any potential inhibitory effects mediated by p21^{Cip1/Waf1}. However, our current studies suggest that CDK2 activity is more important than CDK4 in regulating G₁ progression and growth arrest in this cell line.

The increased p21^{Cip1/Waf1} protein levels induced by the M₃ AChR appear to be mediated by an increase in p21^{Cip1/Waf1} mRNA rather than by increasing protein stability, because the rate of p21^{Cip1/Waf1} degradation in the presence of the protein synthesis inhibitor cycloheximide was unaffected by the agonist. However, other regulatory mechanisms, such as protein translation, may also contribute to the increased p21^{Cip1/Waf1} expression. There are many potential sites for transcriptional regulation of the p21^{Cip1/Waf1} promoter, and factors such as p53, AP-1 and SP-1 can influence p21^{Cip1/Waf1} transcription [34]. The tumour suppressor p53 is induced by DNA-damaging agents that cause growth arrest and is an important upstream transcriptional activator of p21^{Cip1/Waf1} [35]. Both ERK and JNK have been implicated in p53 phosphorylation [36,37], and JNK has been shown to specifically phosphorylate Thr⁸¹ of p53 and to cause a 10-fold increase in p21^{Cip1/Waf1} promoter activity [38]. Although the M₃ AChR could induce MAPK-dependent changes in p53 phosphorylation, it is unlikely that the p53 pathway regulates p21^{Cip1/Waf1} expression in CHO-m3 cells for the following reasons: (i) p53 is difficult to detect by Western-blot analysis of CHO-m3 cell lysates using commercially available antisera (results not shown); (ii) the DNA-damaging drug cisplatin does not affect p21^{Cip1/Waf1} protein expression in CHO-m3 cells (results not shown); (iii) reporter gene assays reveal no detectable p53 activation by the muscarinic agonist CCh in CHO-m3 cells (results not shown); and (iv) a point mutation in the Chinese hamster p53 gene that changes Thr²¹¹ to lysine and abrogates the ability of DNA-damaging agents to induce the protein or activate the G₁ checkpoint has been identified in various CHO cell lines, including the parental CHO-K1 used in the current study [39].

The finding that the M₃ AChR preferentially activates JNK and causes c-Jun phosphorylation provides an alternative potential mechanism to enhance p21^{Cip1/Waf1} transcription, independent of p53. The transcription factor SP-1 can be activated by increased association with c-Jun as well as JunB, JunD and activating transcription factor 2 ('ATF2'), leading to transactivation of the p21^{Cip1/Waf1} promoter [24]. Western-blot analysis has shown that SP-1 expression in CHO-m3 cells is not changed after agonist addition (results not shown). However, preliminary experiments suggest that CCh enhances the association of SP-1 and c-Jun in CHO-m3 cells (results not shown) and this may contribute to activation of the p21^{Cip1/Waf1} promoter. These observations provide an attractive mechanism linking M₃ AChR-mediated activation of JNK with cell-cycle machinery and growth

arrest. Future work will involve the use of reporter gene assays to assess whether activation of the M₃ AChR increases SP-1 transcriptional activity in CHO cells.

The ability of GPCRs to influence tyrosine kinase-encoded growth factor receptors has been well documented [40]. For example, MAPK activation by G_q- and G_i-coupled receptors can be mediated through agonist-independent transactivation of the epidermal growth factor receptor [41]. Similarly, the G_q-coupled 5-hydroxytryptamine 2B receptor has been shown to cause cell proliferation through activation of the platelet-derived growth factor ('PDGF') receptor via a mechanism involving c-Src [19]. It is possible that M₃ AChR activation could induce G₁ arrest by disrupting a growth factor signalling pathway that acts to suppress p21^{Cip1/Waf1} promoter activity in the presence of serum factors. The AP-1 complex has been implicated in cell proliferation by interaction with the cyclin D1 promoter, and expression of c-Jun or another component of AP-1, c-Fos, has been shown to induce cyclin D1 mRNA expression [42,43]. However, recent reports [25,44] suggest that AP-1 can also stimulate growth by repressing p21^{Cip1/Waf1} expression. A conditionally active component of AP-1, c-Fos, was shown to down-regulate p21^{Cip1/Waf1} promoter activity leading to cell-cycle progression [44]. Furthermore, dominant-negative c-Jun has been proposed to cause a G₁ arrest by affecting AP-1 interaction with the promoter of the p21^{Cip1/Waf1} gene rather than by altering the expression of cyclin or CDK proteins [25]. Therefore activation of the M₃ AChR may be alleviating an AP-1-mediated suppression of the p21^{Cip1/Waf1} promoter under the tonic control of a growth factor signalling pathway. However, the simplest hypothesis would be that p21^{Cip1/Waf1} regulation is due to the activation of a transcription factor enhancing p21^{Cip1/Waf1} promoter activity. Results obtained to date would support this model, and the link between M₃ AChR, JNK, c-Jun/SP-1 and p21^{Cip1/Waf1} is a pathway that can be investigated experimentally.

In summary, in the present study, we have shown that activation of the M₃ AChR causes a rapid inhibition of DNA synthesis in growing CHO-m3 cells. An induction of p21^{Cip1/Waf1} protein expression in response to M₃ AChR activation involves an increase in p21^{Cip1/Waf1} mRNA and results in an increased association of the CDK1 with CDK2. Enhanced formation of the CDK2/p21^{Cip1/Waf1} complex in response to M₃ AChR activation is likely to contribute to maximal CDK2 inhibition, accumulation of hypophosphorylated pRb and the G₁ phase arrest. Our data have also shown significant differences in the MAPK activity profiles downstream of the proliferative M₂ AChR and the growth inhibitory M₃ AChR, suggesting that JNK may contribute to growth arrest of agonist-stimulated CHO-m3 cells. Future studies, involving the expression of dominant-negative MAPK components, will identify the intracellular pathways regulating p21^{Cip1/Waf1} expression and G₁ phase arrest by the M₃ AChR.

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