Regulation of extracellular-signal regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors

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Extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs, or stress-activated protein kinases) are activated by diverse extracellular signals and mediate a variety of cellular responses, including mitogenesis, differentiation, hypertrophy, inflammatory reactions and apoptosis. We have examined the involvement of Ca^{2+} and protein kinase C (PKC) in ERK and JNK activation by the human G-protein-coupled m2 and m3 muscarinic acetylcholine receptors (mAChR) expressed in Chinese hamster ovary (CHO) cells. We show that the Ca^{2+}-mobilizing m3 AChR is efficiently coupled to JNK and ERK activation, whereas the m2 AChR activates ERK but not JNK. Activation of JNK in CHO-m3 cells by the agonist methacholine (MCh) was delayed in onset and more sustained relative to that of ERK in either CHO-m2 or CHO-m3 cells. The EC_{50} values for MCh-induced ERK activation in both cell types were essentially identical and similar to that for JNK activation in CHO-m3 cells, suggesting little amplification of the response. Agonist-stimulated Ins(1,4,5)P_3 accumulation in CHO-m3 cells was insensitive to pertussis toxin (PTX), consistent with a G_q/phosphoinositide-specific phospholipase C-β mediated pathway, whereas a significant component of ERK and JNK activation in CHO-m3 cells was PTX-sensitive, indicating $G_{i/o}$ involvement. Using manipulations that prevent receptor-mediated extracellular Ca^{2+} influx and intracellular Ca^{2+}-store release, we also show that ERK activation by m2 and m3 receptors is Ca^{2+}-independent. In contrast, a significant component ($> 50\,\%$) of JNK activation mediated by the m3 AChR was dependent on Ca^{2+}, mainly derived from extracellular influx. PKC inhibition and down-regulation studies suggested that JNK activation was negatively regulated by PKC. Conversely, ERK activation by both m2 and m3 AChRs required PKC, suggesting a novel mechanism for PKC activation by PTX-sensitive m2 AChRs. In summary, mAChRs activate JNK and ERK via divergent mechanisms involving either Ca^{2+} or PKC respectively.

Key words: calcium, G-protein-coupled receptor, mitogen-activated protein kinase, protein kinase C, stress-activated protein kinase.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are activated by a diverse array of extracellular stimuli and regulate a variety of cellular responses (for reviews see [1–4]). MAPK family members include the extracellular signal-regulated kinases (ERKs) [1,2], the c-Jun N-terminal kinases (JNKs) and p38 kinases [3–6]. MAPKs are activated by parallel sequential protein kinase cascades, comprising the MAPK (e.g. ERK1), a MAPK kinase (e.g. MEK1), and a MAPK kinase kinase (e.g. Raf-1) [2–4]. The ERK pathway is activated by receptor tyrosine kinases and G-protein-coupled receptors (GPCRs) that promote mitogenesis, differentiation or hypertrophy. ERKs mediate these effects by phosphorylating various substrates, including cytoplasmic enzymes (e.g. phospholipase A_2, p90 ribosomal protein S6 kinase) and nuclear transcription factors (e.g. ELK-1) [1,2]. JNKs and p38 kinases are activated by cellular stress (e.g. UV- and γ-radiation, osmotic and heat shock, protein synthesis inhibitors) and inflammatory cytokines (e.g. tumour necrosis factor-α, interleukin-1), but also weakly by growth factors (e.g. epidermal growth factor) [3–6]. JNK activation has been implicated in the immune response, oncogenic transformation and apoptosis. Current evidence suggests that JNKs mediate these effects by increasing gene expression; transcription factors activated by JNK include c-Jun, activating transcription factor 2 and ELK-1 [3–6].

MAPK regulation by GPCRs appears to be a widespread phenomenon and is likely to mediate the proliferative and hypertrophic responses of cells to various hormones, neurotransmitters and local mediators that act at this class of receptors [7]. Receptors involved include those linked to phosphoinositide-specific phospholipase C (PLC) activation via pertussis toxin (PTX)-insensitive G_q-proteins and those preferentially coupled to inhibition of adenylyl cyclase via PTX-sensitive G_i-proteins. In most cases, ERK activation by PTX-sensitive G-proteins is independent of protein kinase C (PKC), which is consistent with a lack of involvement of receptor-mediated phosphoinositide hydrolysis [7–9]. Transient expression studies of α_2 adrenergic and m2 muscarinic acetylcholine receptors (mAChRs) in COS-7 cells suggest that $\beta\gamma$ subunits liberated from PTX-sensitive G-proteins couple these receptors to ERK activation via a pathway requiring the monomeric G-protein Ras [7–12]. Indeed, ex-

Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium ion concentration; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated protein kinase; fura-2/AM, fura-2 acetoxymethyl ester; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; KH buffer, Krebs–Henseleit buffer; mAChR, muscarinic acetylcholine receptor; MCh, methacholine; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MAPKKK, MAPK kinase kinase; MEKK, MAPK/ERK kinase kinase; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; PDBu, phorbol dibutyrate; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin.

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pression of free $\beta_1\gamma_2$ subunits in COS-7 cells, but not mutationally activated α_{i2}, is sufficient to activate ERK1 [9,11] and ERK2 [8] in a Ras-dependent manner [8,9]. In contrast, activated α_{i2} can transform Rat-1 fibroblasts [12] and activate ERK2 [11], and may contribute to Ras-dependent ERK activation by the m2 AChR in these cells [13].

Heterogeneity also exists in the mechanisms by which PTX-resistant Ca^{2+}-mobilizing receptors activate ERK, and both Ras-dependent and -independent pathways have been implicated [7]. PKC appears to be important, as its down-regulation or inhibition can partly attenuate or fully inhibit ERK activation by receptors coupled to PLC [7–9]. Consistent with this, acute treatment of cells with tumour-promoting phorbol esters, or overexpression of conventional or novel PKC isoenzymes, causes ERK activation [2,14,15]. Persistent activation of m1, m3 or m5 receptors [7], or expression of mutationally activated α_q [16,17], can induce proliferation and neoplastic transformation of NIH3T3 cells, although expression of activated α_q alone causes modest [11] or no [8] activation of ERK in COS-7 cells. However, manipulations to sequester $\beta\gamma$ subunits have provided evidence for both α_q and $\beta\gamma$ subunit involvement in ERK activation mediated by the G_q-coupled m1 AChR [8], α_{1B}-adrenoceptor [9,18] and bombesin receptor [11] in COS-7 cells. The mechanisms by which $\beta\gamma$ subunits activate the ERK pathway may include Ras guanine nucleotide exchange factors [19] and cytoplasmic tyrosine kinases [18,20–22].

Recently, JNK activation has also been demonstrated for several GPCRs, including m1 and m2 ACh [23–25], angiotensin II [26], α_1 adrenergic [27], thrombin [28] and endothelin-1 [28,29] receptors. For example, the m1 AChR has been shown to activate both JNK and ERK pathways in NIH3T3 cells and cause expression of immediate early genes involved in cell growth (*c-Jun*, *Jun D*, *c-Fos*, *Fos B*) [23]. In COS-7 cells, the m1 and m2 AChRs stimulate JNK by a mechanism involving $\beta\gamma$ subunits acting via Ras and Rac1 [25]. Consistent with this, overexpression of $\beta_1\gamma_2$ is sufficient to activate JNK in COS-7 cells, whereas constitutively active α_{i2} is without effect [25]. α_q has also been reported to activate JNK in COS-1 [30] and PC-12 [31] cells, but does not appear to do so in COS-7 cells [26]. Phorbol esters have little effect on JNK activity [5], indicating that PKC stimulation alone is insufficient to activate JNK. Some studies support a role for Ca^{2+} in receptor-mediated JNK activation [24,26], and the Ca^{2+}-dependent tyrosine kinase Pyk2 has been implicated in JNK activation by cellular stress in PC12 cells but not in COS or HEK293 cells [32]. Finally, mutationally active α_{12} and α_{13} are also able to activate JNK, but not ERK, via Ras [30] and Rac1 [33] or Cdc42 [34]. Although the effectors for α_{12} and α_{13} have not been defined, this pathway may account for thrombin-induced AP1-dependent gene expression and/or mitogenesis [35,36].

Five distinct mAChRs show preferential coupling to at least two classes of G-protein [37]. Thus, the m1, m3 and m5 AChRs are primarily linked to PLC activation via PTX-resistant G_q-proteins. Subsequent hydrolysis of PtdIns(4,5)P_2 provides the second messengers Ins(1,4,5)P_3 and 1,2-diacylglycerol, which mobilize intracellular calcium and activate certain PKC isozymes respectively [38,39]. Conversely, m2 and m4 AChRs are coupled to the activation of potassium channels and to the inhibition of adenylyl cyclase via PTX-sensitive G_i-proteins, thereby lowering cAMP levels [37]. Clearly, MAPK regulation by GPCRs may be downstream or independent of second messenger generation mediated by individual G-protein subunits. We have examined the role of Ca^{2+} and PKC in the regulation of JNK and ERK by human m2 and m3 AChRs expressed in Chinese hamster ovary (CHO) cells. We find that the m3 AChR is coupled to JNK and ERK activation in these cells, whereas the m2 receptor only activates ERK. We demonstrate that Ca^{2+}, mainly derived from extracellular influx, contributes to m3-mediated JNK activation, whereas ERK activation by both receptors is Ca^{2+}-independent. We also demonstrate that PKC is necessary for ERK activation by both m2 and m3 receptors, suggesting a novel mechanism for PKC activation by PTX-sensitive m2 AChRs.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO-K1) cells stably expressing either the recombinant human m2 or m3 AChR [40] were maintained in minimal essential medium-α (Gibco-BRL) at 37 °C in a 5% CO_2/95% 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). Before agonist addition, cells were incubated in a Krebs-Henseleit (KH) buffer (pre-equilibrated with 5% CO_2/95% O_2 and containing 5 mM Hepes, pH 7.4/10 mM glucose/25 mM $NaHCO_3$/1.2 mM K_2HPO_4/118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/1.3 mM $CaCl_2$) for 30 min at 37 °C under 5% CO_2/95% air.

ERK immunoprecipitation and assay

ERK proteins were isolated by immunoprecipitation from CHO cell lysates and assayed for activity according to the following procedure. Cells were solubilized with lysis buffer containing 20 mM Tris/HCl (pH 8.0)/0.5% Nonidet P40/250 mM NaCl/3 mM EDTA/3 mM EGTA/2 mM Na_3VO_4/1 mM dithiothreitol/1 mM PMSF/20 μg/ml aprotinin/5 μg/ml leupeptin. Insoluble material was removed by centrifugation at 14000 ***g*** for 10 min at 4 °C. Lysates were incubated for 90 min at 4 °C with a 1:100 dilution of a rabbit polyclonal antiserum (200 μg/ml) directed against ERK1 or ERK2 [C16: sc-93 or C14: sc-94 respectively (Santa Cruz)]. Immune complexes were incubated for a further 90 min at 4 °C with 70 μl of a 15% (v/v) slurry of Protein A–Sepharose (Pharmacia-LKB) 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 $MgCl_2$/1 mM dithiothreitol/50 μM Na_3VO_4. Immune complex ERK assays were initiated by addition of 40 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol) and 200 μM of a synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor receptor [41]. Reactions were incubated for 20 min at 30 °C and terminated by addition of 10 μl of 25% (w/v) trichloroacetic acid. Mixtures were centrifuged at 14000 ***g*** for 2 min and spotted onto P81 cation-exchange paper (Whatman). Papers were washed as described [41] and counted by liquid-scintillation counting.

JNK assay

JNK activity was assessed using a recombinant protein fragment of c-Jun as an affinity ligand and substrate {glutathione S-transferase (GST)–c-Jun [1–79]} [6]. Cleared cell lysates were prepared exactly as described above and incubated for 60 min at 4 °C with 20 μl of a 25% (v/v) slurry of glutathione–Sepharose (Pharmacia-LKB), pre-coupled to GST–c-Jun (5 μg of protein). Beads were collected by centrifugation and washed twice in 200 μl of lysis buffer and twice in 200 μl of kinase buffer. Reactions were initiated by addition of 40 μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol). After incubation for 20 min at 30 °C, reactions were terminated by addition of

40 μl of Laemmli sample buffer and boiling. Phosphorylated proteins were resolved by PAGE through 12 % acrylamide in the presence of 0.1 % SDS, stained with Coomassie Blue R250 and visualized by autoradiography. Radioactivity incorporated into GST–c-Jun was quantified by liquid-scintillation counting of the excised bands.

Measurement of intracellular calcium

The concentration of intracellular calcium, $[Ca^{2+}]_i$, was estimated as described previously [42], with some modifications. Confluent cells from a 175 cm^2 flask were collected in 5 ml of buffer containing 10 mM Hepes, pH 7.4, 154 mM NaCl and 0.54 mM EDTA, and then washed and resuspended in 3.5 ml of KH buffer. A 0.5 ml aliquot of this suspension was diluted to 2 ml in KH buffer to measure cellular autofluorescence. To the remainder, fura-2 acetoxymethyl ester (fura-2/AM) was added to a final concentration of 2 μM. After incubation for 45 min at room temperature, 0.5 ml aliquots of the cell suspension were centrifuged at 4000 ***g*** for 1 min and the cell pellet was resuspended for analysis in 1.5 ml of KH buffer at room temperature. To make the intracellular and extracellular Ca^{2+} concentrations approximately equivalent, some cells were resuspended in KH buffer which lacked added $CaCl_2$ but contained 100 μM EGTA. Routinely, 100 μM methacholine (MCh) was added and the 340/380 nm excitation ratio was recorded at 509 nm every second using a luminescence spectrometer (Perkin-Elmer). After data collection, maximal and minimal 340/380 nm ratios were recorded in the presence 2 mM $CaCl_2$/0.1 % Triton X-100 and 80 mM EGTA respectively. $[Ca^{2+}]_i$ was determined using the equation described by Grynkiewicz et al. [43].

Ins(1,4,5)P_3 mass assay

Ins(1,4,5)P_3 mass was determined using a radioligand-receptor competition assay [44], with slight modifications. Briefly, incubations of cells were terminated in 300 μl of 0.5 M trichloroacetic acid and subsequently processed by addition of 75 μl of 10 mM EDTA and extraction with 600 μl of tri-n-octylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v). To 200 μl of the upper aqueous phase was added 50 μl of 10 mM $NaHCO_3$ and the mixture was stored overnight at 4 °C for subsequent mass measurement of Ins(1,4,5)P_3 using 30 μl of this mixture as described [44].

Measurement of *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) binding

Total mAChR number was determined by [^{3}H]NMS saturation-binding to cell membrane preparations [45]. Confluent cells from a 175 cm^2 flask were collected in buffer containing 10 mM Hepes, pH 7.4, 154 mM NaCl and 0.54 mM EDTA, resuspended in buffer A (10 mM Hepes, pH 7.4/10 mM EDTA) at 4 °C and homogenized using a Polytron tissue disrupter. The particulate fraction was obtained by centrifugation at 30000 ***g*** for 15 min at 4 °C and resuspended in buffer B (10 mM Hepes, pH 7.4/0.1 mM EDTA) at 4 °C using the Polytron homogenizer. The cell membrane preparation was resuspended in buffer B following centrifugation to a final concentration of 1 mg of protein/ml and stored at −80 °C. Radioligand binding was performed for 1 h at 37 °C in a final volume of 200 μl of buffer C (10 mM Hepes, pH 7.4/100 mM NaCl/10 mM $MgCl_2$) containing 20 μg of membrane protein and various concentrations of [^{3}H]NMS (0.03–3 nM). For each concentration, non-specific binding was determined in the presence of 1 μM atropine and was less than 10 % of total [^{3}H]NMS binding. Bound and free ligand were separated by rapid filtration through GF/B paper (Whatman). Filters were washed in buffer C and then dried and counted by liquid-scintillation counting. Maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) values were determined by saturation analysis using the Prism program (GraphPad Software, San Diego, CA, U.S.A.).

Western blot analysis

Analysis was performed either on cleared cell lysates or on proteins isolated from CHO-m3 cells that specifically bound to GST–c-Jun using the incubation and washing procedure described in the JNK assay section. Proteins were separated by SDS/12 %-PAGE and transferred onto a nitrocellulose membrane for 30 min at 12 V in transfer buffer (48 mM Tris/HCl/39 mM glycine/1.3 mM SDS/20 % methanol) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5 % non-fat milk powder in TTBS [50 mM Tris/HCl (pH 8.0)/0.1 % Tween-20/150mM NaCl] and incubated overnight at 4 °C with primary antibody [anti-ERK1 (C16: sc-93), 1:1000; anti-ERK2 (C14: sc-94), 1:1000; anti-JNK1 (C17: sc-474), 1:500; (Santa Cruz)] in TTBS. After washing in TTBS, blots were incubated for 1 h at room temperature with a 1:1000 dilution in TTBS of anti-rabbit IgG coupled to peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham International).

Fusion protein expression

The construct for bacterial expression of c-Jun (amino acids 1–79) fused to GST was a gift from Dr. Roger J. Davis [6]. The GST–c-Jun protein was isolated on a glutathione–Sepharose affinity matrix (Pharmacia-LKB). Purified protein was resolved by SDS/PAGE through 12 % polyacrylamide and quantified by comparative Coomassie Blue R250 staining using BSA as a standard. In all other cases, protein concentrations were determined by a modified Bradford procedure [46].

Statistical analysis

The statistical differences between data sets was assessed by one-way analysis of variance for multiple comparisons, followed by Duncan's multiple-range test at $P < 0.05$ using SPSS version 6.1 software (Chicago, IL, U.S.A.).

RESULTS

Time- and concentration-dependence of agonist-stimulated ERK and JNK activities in CHO-m2 and CHO-m3 cells

CHO cells separately expressing recombinant human m2 and m3 AChRs at comparable levels of 1.5 ± 0.1 pmol/mg and 2.2 ± 0.2 pmol/mg (mean $\pm$ S.E.M., $n = 6$) were stimulated with the muscarinic agonist MCh. ERK was isolated using an antiserum to ERK1 and assayed using a synthetic peptide substrate [41]. Stimulation of either m2 or m3 AChRs with 100 μM MCh caused dramatic ERK activation (Figure 1A). ERK activation by both m2 and m3 AChRs was observed 2 min after agonist addition and was maximal by 5 min. In CHO-m2 cells, ERK activity returned to basal by 20 min of MCh stimulation, whereas ERK activation in CHO-m3 cells was more sustained. The MCh concentration dependence for ERK activation was similar in both cell types (Figure 1B). Mean EC_{50} values for the m2- and m3-mediated responses were 4 μM MCh [log EC_{50} (M) -5.4 ± 0.1] and 5 μM MCh [log EC_{50} (M) -5.3 ± 0.1] respectively (mean $\pm$ S.E.M., $n = 3$–9), where the M in parentheses refers to molarity.

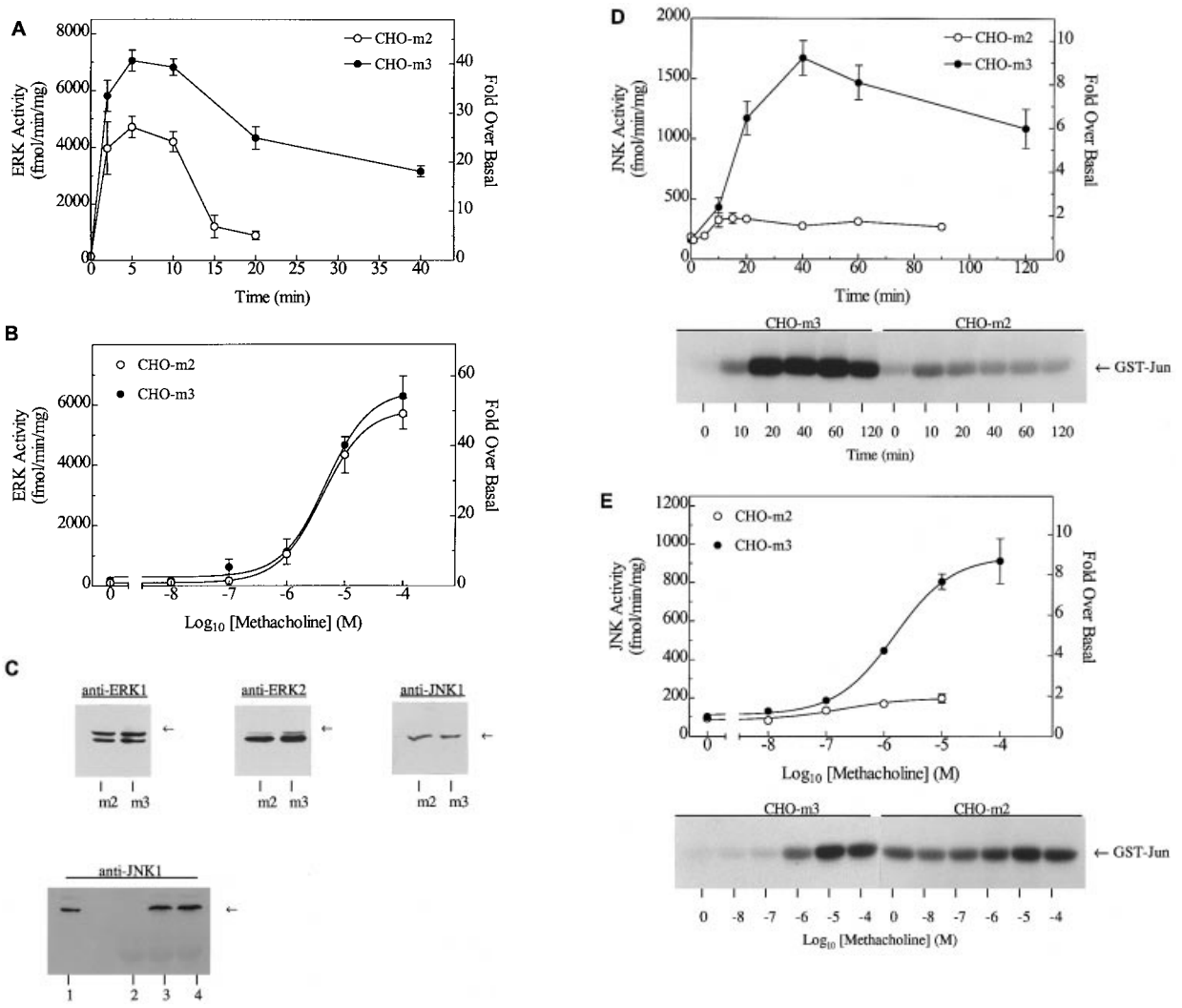

Figure 1 Time- and concentration-dependence of agonist-stimulated ERK and JNK activities in cells expressing human m2 and m3 AChRs

(**A**) CHO cells expressing either the m2 or m3 AChR were stimulated with 100 μM MCh for the times indicated. ERK activity was isolated using anti-ERK1 and assayed as described in the Materials and methods section. Basal ERK activity in CHO-m2 and CHO-m3 cells was 149 ± 38 and 186 ± 30 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. (**B**) Cells were incubated with various concentrations of MCh for 5 min and assayed for ERK activity. Basal ERK activity in CHO-m2 and CHO-m3 cells was 102 ± 35 and 187 ± 88 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. (**C**) p44-ERK1, p42-ERK2 and JNK1 were detected in CHO-m2 (m2) and CHO-m3 (m3) lysates (10 μg of protein) by Western blot analysis using selective antisera. The lower portion of panel (**C**) shows immunoblot analysis using anti-JNK1 antiserum of GST–c-Jun affinity captured proteins isolated from lysis buffer as a control (lane 2), from unstimulated CHO-m3 cells (50 μg of protein, lane 3) and from CHO-m3 cells stimulated for 30 min with MCh (50 μg of protein, lane 4.). Immunoblot analysis was also performed on CHO-m3 cell lysate (10 μg of protein) using the same antiserum (lane 1). The relative position of the 45 kDa ovalbumin marker is indicated by an arrow. (**D**) Cells were stimulated with 100 μM MCh for the times indicated and JNK activity was assessed by GST–Jun phosphorylation. Basal JNK activity in CHO-m2 and CHO-m3 cells was 187 ± 36 and 161 ± 43 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. (**E**) CHO-m2 and CHO-m3 cells were stimulated for either 10 min or 30 min respectively with the indicated concentrations of MCh and assayed for JNK activity. Basal JNK activity in CHO-m3 cells was 101 ± 10 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. The lower portions of panels (**D**) and (**E**) show representative autoradiograms of GST–Jun phosphorylation. Activities represent the means $\pm$ S.E.M. for 3–7 separate determinations.

Immunoblot analysis indicated that the level of ERK proteins in the two cell types was similar (Figure 1C). The ERK1 antiserum recognized both p44 ERK1 and p42 ERK2, whereas an antiserum directed to the C-terminal sequence of ERK2 was specific for this isoenzyme (Figure 1C). Immune complex assays of kinases isolated by each antibody, either alone or in combination, suggested that the ERK1 antiserum measured both ERK1 and ERK2 activities, whereas the ERK2 antiserum was more selective (results not shown). Thus, results obtained using the ERK1 antiserum are likely to represent the combined activity of both ERK1 and ERK2.

JNK1, JNK2 and JNK3 are indistinguishable based on their ability to bind and phosphorylate the N-terminal domain of c-Jun [6]. Therefore, JNK activity was measured using a recombinant fragment of c-Jun (amino acids 1–79) fused to GST as an affinity ligand and substrate. Immunoblot analysis using an antiserum to JNK1 showed the presence of similar amounts of the 46 kDa enzyme in CHO-m2 and CHO-m3 cells (Figure 1C). This analysis also showed that the affinity ligand, GST–c-Jun, bound equal quantities of the 46 kDa JNK1 protein from unstimulated and MCh-stimulated CHO-m3 cells (Figure 1C). As shown in Figure 1(D), MCh induced a marked and sustained JNK activation in CHO-m3 cells that was delayed in onset relative to ERK activation (Figure 1A). In contrast, the m2 receptor failed to activate JNK greatly, giving a maximal stimulation of 2-fold. JNK was activated in CHO-m3 cells with

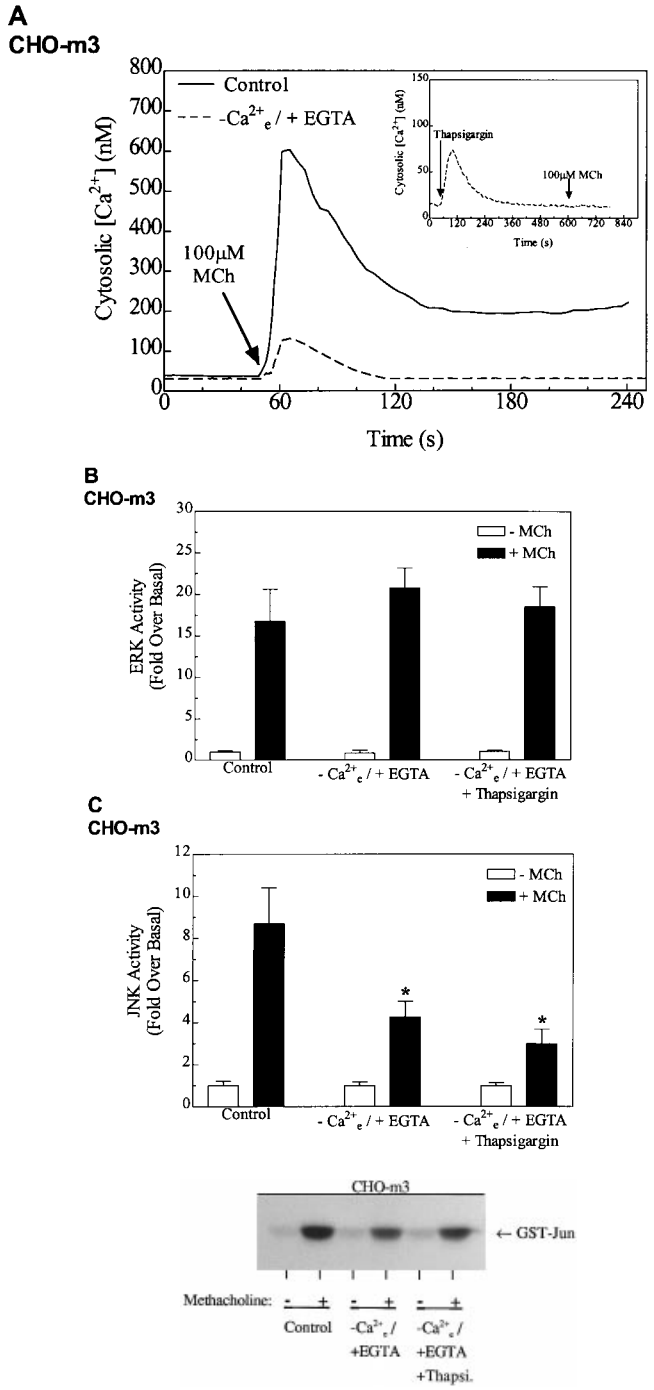

Figure 2 Calcium-dependence of agonist-stimulated ERK and JNK activities in CHO-m3 cells

(**A**) Cytosolic Ca^{2+} concentration was measured in fura-2/AM-loaded CHO-m3 cells stimulated with 100 μM MCh in the presence of 1.3 mM $CaCl_2$ (control, solid line) or under calcium-depleted conditions [i.e. $-Ca^{2+}_e/+$EGTA (100 μM) broken lines]. The inset shows the effect of 2 μM thapsigargin under calcium-depleted conditions. (**B**) and (**C**) CHO-m3 cells were prepared in the presence of 1.3 mM $CaCl_2$ (control) or under conditions to prevent Ca^{2+} entry [$-Ca^{2+}_e/+$EGTA (100 μM)] and intracellular Ca^{2+} release [i.e. $-Ca^{2+}_e/+$EGTA (100 μM) + thapsigargin (2 μM)]. ERK and JNK activities were assayed at 5 min and 30 min respectively after addition of 100 μM MCh (solid bars) or carrier (open bars). Non-stimulated activities for each condition were assigned a fold activity of 1. A representative autoradiogram of JNK activity is shown below (**C**). Results are means $\pm$ S.E.M. of 3–6 separate determinations. $^*P < 0.05$ by Duncan's multiple-range test.

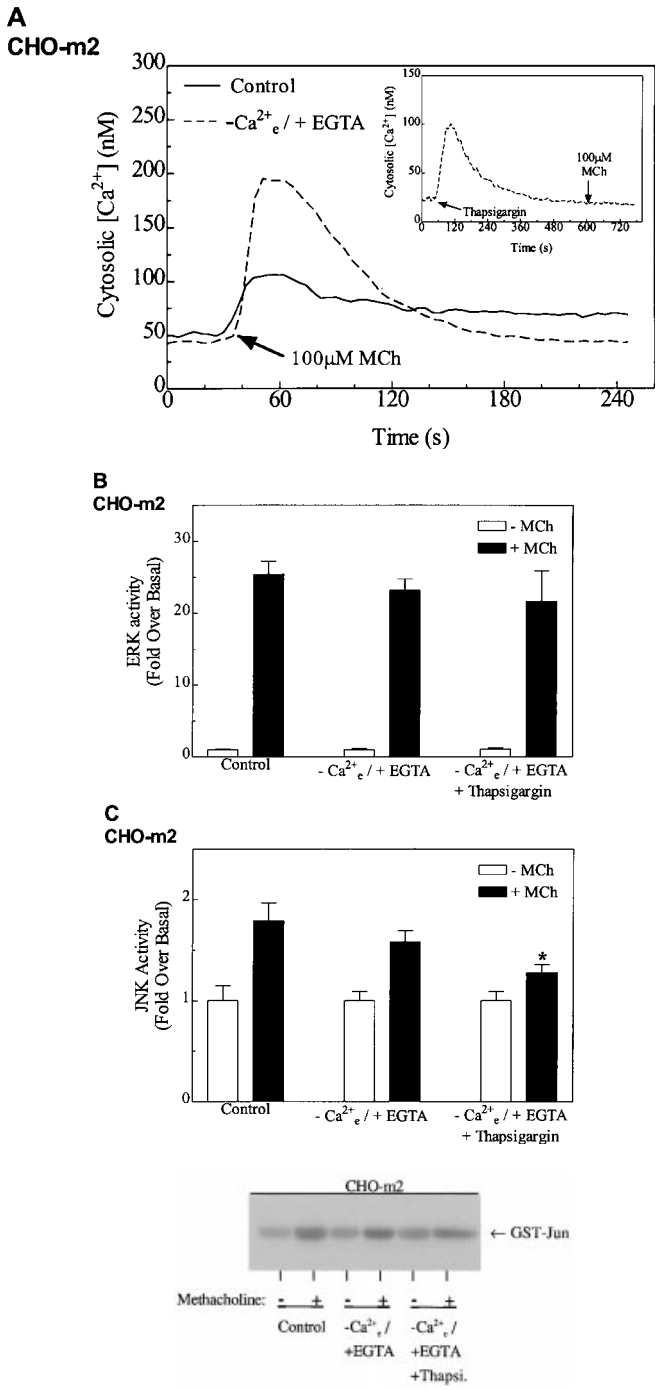

Figure 3 Calcium-dependence of agonist-stimulated ERK and JNK activities in CHO-m2 cells

(**A**) Fura-2/AM-loaded CHO-m2 cells were analysed for agonist-induced intracellular Ca^{2+} elevation as described in the legend to Figure 2. (**B**, **C**) MCh-induced ERK and JNK activities in CHO-m2 cells were measured under the conditions described in the legend to Figure 2, except that JNK activity was measured at 10 min after addition of 100 μM MCh (solid bars) or carrier (open bars). A representative autoradiogram of JNK activity is shown below (**C**). Results are means $\pm$ S.E.M. of 4–8 separate determinations. $^*P < 0.05$ by Duncan's multiple-range test.

an EC_{50} value of 2 μM MCh [log EC_{50} (M) -5.8 ± 0.2; mean $\pm$ S.E.M., $n = 3$–9] (Figure 1E). An equivalent EC_{50} value could not be determined in CHO-m2 cells. MCh-stimulated ERK and JNK activities in both cell types were abolished by the antagonist

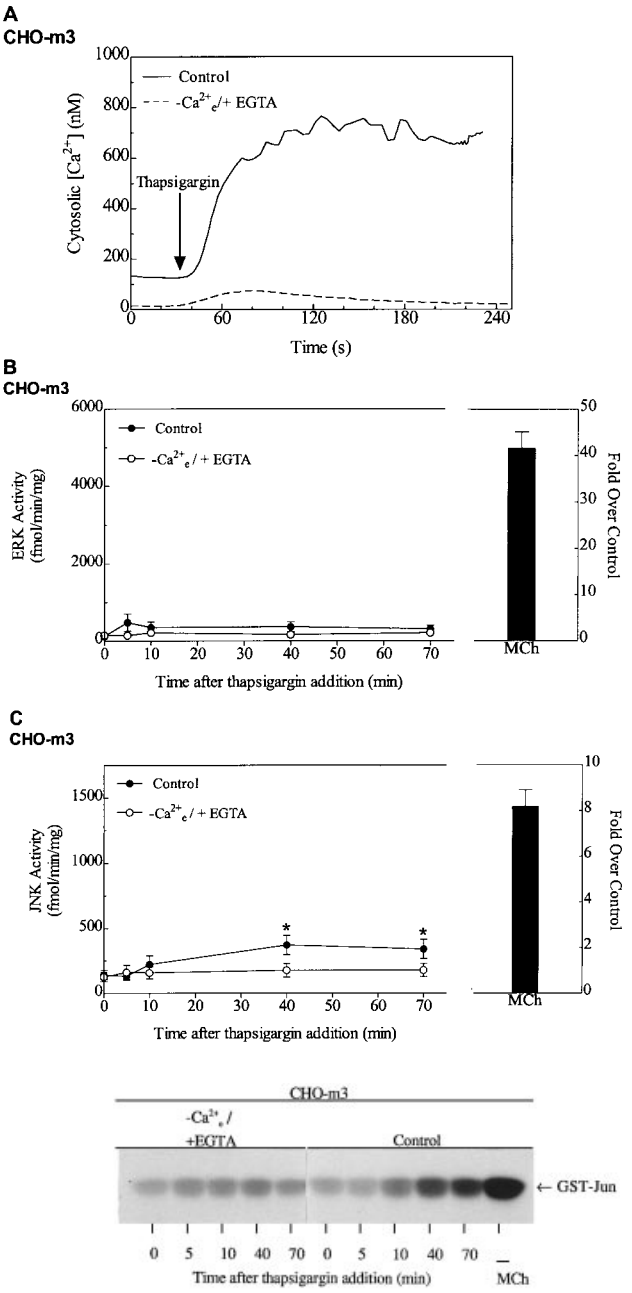

Figure 4 Effect of thapsigargin-mediated intracellular calcium elevation on ERK and JNK activities

(**A**) The effect of 2 μM thapsigargin on $[Ca^{2+}]_i$ was measured in fura-2/AM-loaded CHO-m3 cells in the presence of 1.3 mM $CaCl_2$ (control, solid line) or under Ca^{2+}-depleted conditions [i.e. $-Ca^{2+}_e/+$EGTA (100 μM), broken line]. (**B**) and (**C**) ERK and JNK activities were measured in the absence (○) and presence (●) of 1.3 mM $CaCl_2$, after addition of 2 μM thapsigargin. ERK and JNK activities were also determined after 5 and 30 min of 100 μM MCh stimulation in the presence of 1.3 mM $CaCl_2$ (solid bars). An autoradiogram of JNK activity is shown below (**C**). Results are means of four separate determinations. *Indicates JNK activation (●) at $P < 0.05$ by Duncan's multiple-range test.

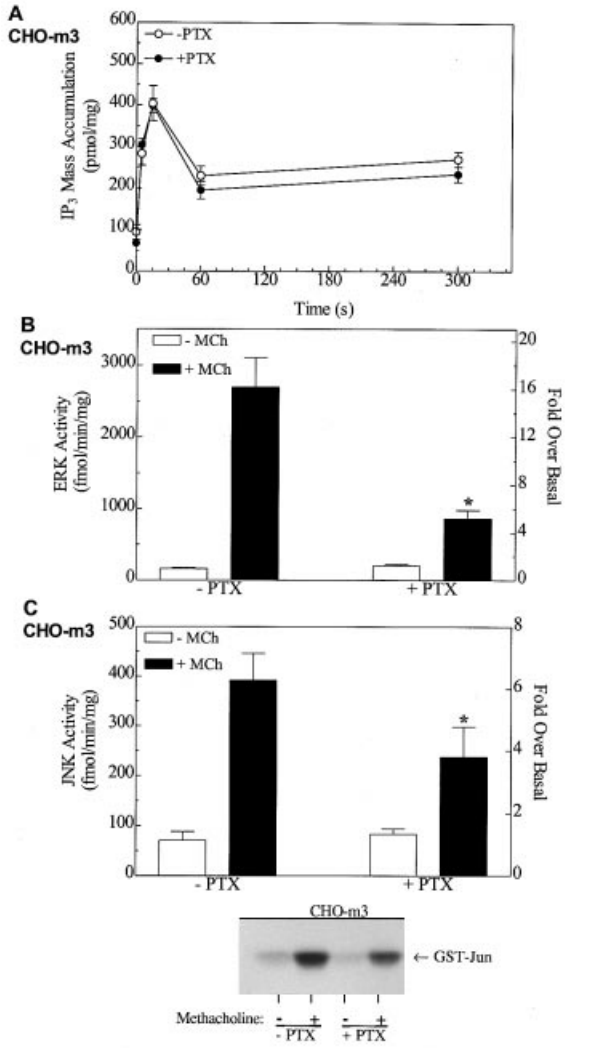

Figure 5 PTX sensitivity of agonist-stimulated ERK and JNK activities in CHO-m3 cells

(**A**) Ins(1,4,5)P_3 mass was determined in either untreated (○) or PTX-treated (75 ng/ml, ●) CHO-m3 cells after stimulation with 100 μM MCh for the times indicated. Values represent the means ± S.E.M. of four separate assay points and were essentially identical with those obtained in a similar experiment. (**B**) and (**C**) ERK and JNK activities were measured in untreated and PTX-treated cells at 5 min and 30 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). *$P < 0.05$ by Duncan's multiple-range test. Basal ERK and JNK activities in non-PTX-treated cells were 159 ± 34 and 81 ± 39 fmol·min^{-1}·mg^{-1} respectively. A representative autoradiogram of JNK activity is shown below (**C**). Values represent the means ± S.E.M. of 4–9 separate determinations. *$P < 0.05$ by Duncan's multiple-range test.

atropine (results not shown), confirming that the responses were muscarinic receptor-mediated. Immunoblot analysis also showed that the amounts of ERK and JNK were unchanged by MCh (results not shown).

Calcium dependence of agonist-stimulated ERK and JNK activities in CHO-m3 and CHO-m2 cells

Since m1, m3 and m5 AChRs are Ca^{2+}-mobilizing receptors coupled to PLC [37], we determined the contribution of Ca^{2+} to ERK and JNK activation in CHO-m3 cells. $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive dye fura-2 and, for comparative purposes, CHO-m2 cells were analysed similarly. MCh (100 μM) induced a rapid elevation of $[Ca^{2+}]_i$ in CHO-m3 cells that was maximal within 10 s of agonist addition (Figure 2A). Removal of the Ca^{2+} gradient across the plasma membrane using 100 μM EGTA in the extracellular buffer indicated that Ca^{2+} influx contributed the major component to the agonist-induced rise in

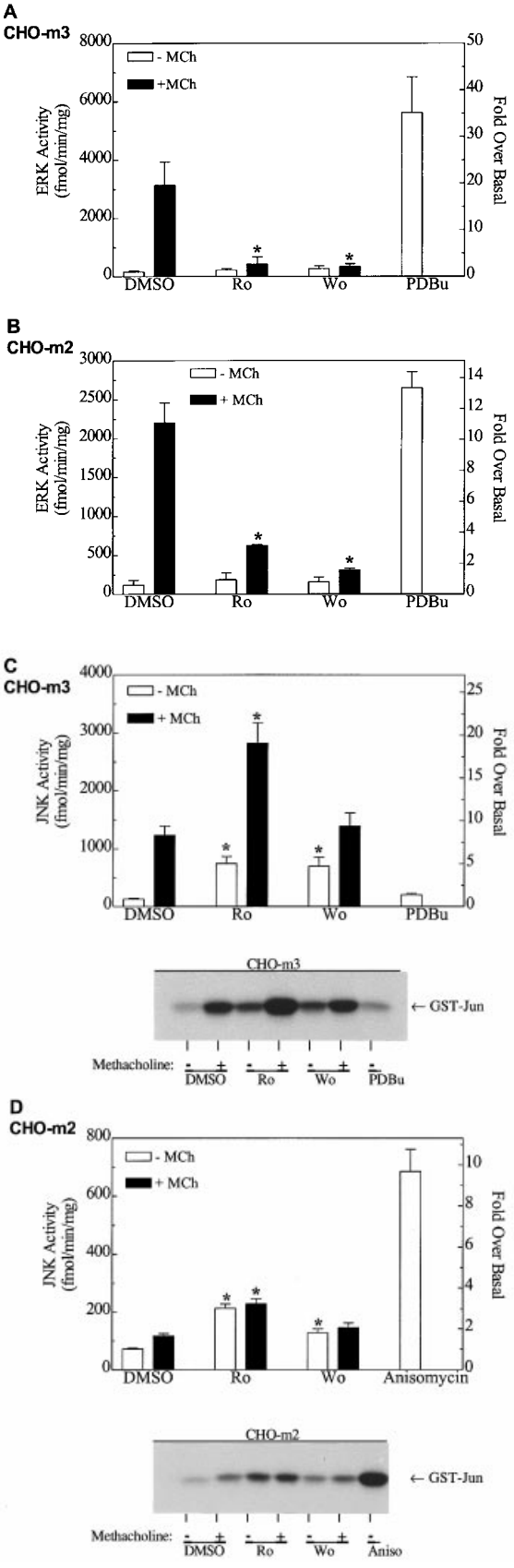

Figure 6 Role of PKC in agonist-stimulated ERK and JNK activities in CHO-m2 and CHO-m3 cells

Cells were pre-treated for 10 min with 10 μM Ro-31-8220 (Ro), 10 μM wortmannin (Wo) or the carrier DMSO before agonist addition. (**A**) and (**B**) ERK activity was measured in CHO-m3 and CHO-m2 at 5 min after addition of 100 μM MCh (white bars) or carrier (solid bars). Cells were separately treated for 10 min with 1 μM PDBu. Basal ERK activities in CHO-m3 and CHO-m2 cells were 157 ± 80 and 118 ± 106 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. (**C**) and (**D**) JNK activity was measured in CHO-m3 and CHO-m2 at 30 min and 10 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). Cells were separately treated for 10 min with 1 μM PDBu (**C**) or for 15 min with 50 ng/ml anisomycin (**D**). Basal JNK activities in CHO-m3 and CHO-m2 cells were 148 ± 30 and 71 ± 6 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. Representative autoradiograms of JNK activity are shown (**C** and **D**). Values represent the means $\pm$ S.E.M. of 3–9 separate experiments. *Indicates significant effects of Ro-31-8220 or wortmannin on basal and agonist-stimulated activities at $P < 0.05$ by Duncan's multiple-range test.

$[Ca^{2+}]_i$. Furthermore, treatment of cells under Ca^{2+}-depleted conditions with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, caused a small transient increase in $[Ca^{2+}]_i$ but, importantly, abolished that induced by MCh (Figure 2A). These manipulations show that ERK activation by the m3 receptor is Ca^{2+}-independent (Figure 2B). In contrast, agonist-induced JNK activity in CHO-m3 cells was reduced by greater than 50 % in the absence of intracellular Ca^{2+} elevation (Figure 2C).

The G_i -coupled m2 AChR also caused a small increase in $[Ca^{2+}]_i$ in CHO cells that was abolished by thapsigargin (Figure 3A). Surprisingly, the Ca^{2+} elevation induced by MCh was reproducibly enhanced when extracellular Ca^{2+} was chelated (Figure 3A). ERK activation was not affected by these conditions or when Ca^{2+} mobilization was abolished by thapsigargin (Figure 3B), whereas JNK activation was attenuated (Figure 3C). Thus, ERK activation by m2 and m3 AChRs appears to be independent of Ca^{2+} elevation in CHO cells, whereas a significant component of the JNK response to these receptors is Ca^{2+}-dependent.

Effect of agonist-independent calcium elevation on ERK and JNK activities

Under Ca^{2+}-depleted conditions, thapsigargin causes a small transient rise in $[Ca^{2+}]_i$ that can be dramatically enhanced by the presence of external Ca^{2+} (Figure 4A). These manipulations were used to assess the effect of $[Ca^{2+}]_i$ elevation, independently of agonist, and showed that ERK was not significantly activated by Ca^{2+} (Figure 4B). However, activation of JNK by thapsigargin could be observed, although the effect was less than that induced by agonist (Figure 4C). Furthermore, this activation was abolished by depletion of external Ca^{2+} (Figure 4C), confirming specificity of the Ca^{2+} effect. Essentially identical results were obtained in CHO-m2 cells (results not shown). Thus elevation of $[Ca^{2+}]_i$ alone is not sufficient to activate JNK maximally, supporting a role for both Ca^{2+}-dependent and Ca^{2+}-independent components in JNK activation by the m3 AChR.

PTX sensitivity of mAChR-mediated responses

PTX catalyses the ADP-ribosylation of α subunits of G_i and G_o, uncoupling them from activated receptors [47]. Although the m1 AChR can be considered a prototypic GPCR linked to PTX-insensitive G_q-proteins, ERK activation by this receptor may be mediated by G_o in CHO cells [48]. In CHO-m3 cells, MCh stimulated a rapid Ins(1,4,5)P_3 accumulation which was maximal at 15 s and which was unaffected by PTX (Figure 5A). Similarly, the EC_{50} for MCh-induced Ins(1,4,5)P_3 accumulation was unaffected by PTX [log EC_{50} (M) -5.6 ± 0.2 and -6.0 ± 0.1 in the absence and presence of PTX; results not shown]. Thus coupling of the m3-AChR to PLC probably involves G_q. In contrast, a large component of ERK activation by the m3 AChR was PTX-

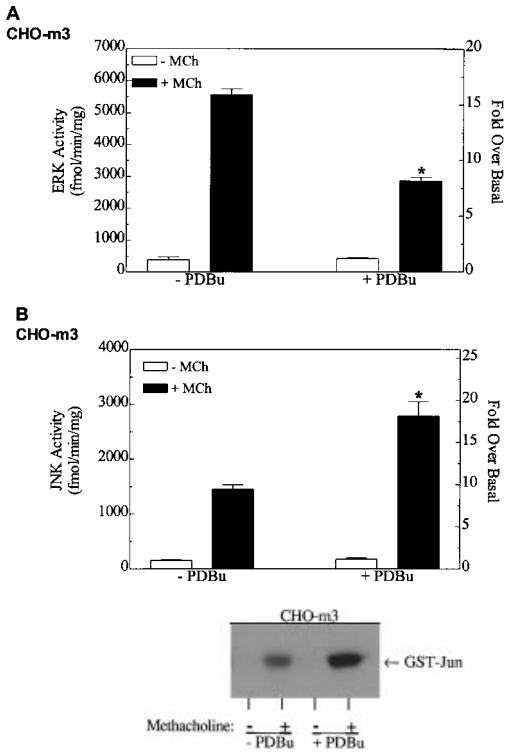

Figure 7 Effect of PKC down-regulation on m3 receptor-mediated activation of ERK and JNK

CHO-m3 cells were pre-treated for 18 h with 1 μM PDBu (+PDBu) or the carrier DMSO (—PDBu) before agonist addition. (**A**) and (**B**) ERK and JNK activities were measured at 5 min and 30 min respectively after addition of 100 μM MCh (white bars) or carrier (solid bars). Basal ERK and JNK activities were 385 ± 108 and 171 ± 55 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively. A representative autoradiogram of JNK activity is shown in below (**B**). Values represent the means $\pm$ S.E.M. of 3–5 separate experiments. *$P < 0.05$ by Duncan's multiple-range test.

sensitive (Figure 5B), indicating that distinct G-proteins couple the m3 receptor to Ins(1,4,5)P_3 formation and ERK activation. JNK activation by the m3 receptor was also partly PTX-sensitive (Figure 5C), indicating that the JNK pathway also involves a G_i/G_o component. PTX treatment had no effect on ERK or JNK expression by Western blotting (results not shown). As expected, PTX abolished MCh-induced ERK and JNK activation in CHO-m2 cells (results not shown).

Inhibition of PKC and phosphoinositide 3-kinase (PI-3K) demonstrates divergent mechanisms for ERK and JNK activation by mAChRs

In agreement with previous reports [2,14,15], PKC stimulation with phorbol ester activates ERK (Figure 6A and 6B). Inhibition of either PKC by 10 μM Ro-31-8220 or PI-3K by 10 μM wortmannin essentially abolished ERK activation induced by MCh in CHO-m3 cells (Figure 6A). Interestingly, Ro-31-8220 also dramatically reduced m2-mediated ERK activation, implicating PKC in the pathway by which PTX-sensitive G-proteins activate ERK (Figure 6B). Whereas JNK can be activated by cellular stress, such as that induced by the protein synthesis inhibitor anisomycin (Figure 6D), phorbol ester had little effect on JNK (Figure 6C), which is consistent with previous work [5]. Furthermore, elevation of $[Ca^{2+}]_i$ by thapsigargin concurrent with activation of PKC by phorbol dibutyrate (PDBu) had less than an additive effect on JNK activity (results not shown). Experiments to determine the involvement of PKC and PI-3K in activation of JNK by mAChRs were confused by the marked stimulatory effects Ro-31-8220 and wortmannin had on basal JNK activity (Figures 6C and 6D). However, PI-3K inhibition had no effect on the level of JNK activity in the presence of MCh in either CHO-m3 (Figure 6C) or CHO-m2 (Figure 6D) cells, whereas a marked enhancement of JNK activity by Ro-31-8220 was apparent in CHO-m3 cells stimulated with MCh (Figure 6C). As the stimulatory effect on JNK of Ro-31-8220 may not be mediated by PKC inhibition, CHO-m3 cells were alternatively treated for 18 h with 1 μM PDBu to down-regulate PKC. Under these conditions, ERK activation induced by MCh was significantly attenuated, confirming a stimulatory role for PKC in this pathway (Figure 7A). In contrast, JNK activation was enhanced by MCh after chronic PDBu treatment (Figure 7B), further suggesting that PKC is a negative regulator of the JNK pathway.

DISCUSSION

Evidence to date points to considerable heterogeneity in the mechanisms by which MAPK signalling pathways are regulated by GPCRs [7]. This heterogeneity can be attributed in part to the nature of the heterotrimeric G-proteins to which individual receptors couple, and also to cell-type-specific differences in the complement of intracellular molecules that participate in the signalling cascades. The current study has used CHO cells as a model to examine the regulation of two distinct subfamilies of MAPK by recombinant human m2 and m3 AChRs that preferentially couple to inhibition of adenylyl cyclase and activation of PLC respectively [37]. We found that the Ca^{2+}-mobilizing m3 AChR is efficiently coupled to both ERK and JNK activation in CHO cells, whereas the m2 AChR activates ERK to a comparable level yet fails to activate JNK significantly. The EC_{50} values for MCh-induced ERK activation in CHO-m2 and CHO-m3 cells were essentially identical (4 μM and 5 μM respectively), and similar to that for JNK activation in CHO-m3 cells (2 μM). Agonist displacement studies of NMS binding in membranes derived from CHO-m2 and CHO-m3 cells have provided agonist occupation curves with K_D values of 1 μM and 40 μM carbachol respectively [40]. In addition, the EC_{50} values for carbachol-stimulated early- and late-phase Ins(1,4,5)P_3 production in intact CHO-m3 cells are approx. 5 μM carbachol [42], whereas this agonist is more potent at elevating intracellular Ca^{2+} by at least one order of magnitude [42]. These observations suggest that the potential for second-messenger-mediated amplification of ERK or JNK activities may be limited by additional factors, such as the stoichiometry of intracellular proteins that are required for activation of these pathways.

We also find that JNK activation by the m3 AChR is delayed in onset and more sustained relative to ERK activation induced by either the m3 or m2 AChR subtype. In Rat1 cells, the m1 AChR activates JNK with a similar time-course as described here, although in these cells ERK is not activated [13,24]. However, in NIH3T3 cells where m1 AChR activates both JNK and ERK, a similar temporal pattern of rapid ERK activation with delayed JNK activation has been reported [23]. Although the G_i-coupled m2 AChR can cause a modest (3-fold) activation of JNK in Rat1 [24] and COS-7 [25] cells, receptors that cause significant JNK activation are primarily coupled to PtdIns(4,5)P_2 hydrolysis, including angiotensin II [26], α_1 adrenergic [27], thrombin [28] and endothelin-1 [28,29] receptors, and induce JNK activity with a similar time-course to that observed here [23–27]. Interestingly, maximal activation of JNK mediated by the m3 receptor requires constant receptor occupation by agonist (P. G. Wylie, R. A. J. Challiss and J. L. Blank, unpublished

work), suggesting that JNK activation is not solely dependent on an early signalling event such as rapid second messenger generation. Indeed maximal JNK activation occurred at times when a significant proportion of m3 receptors would be predicted to be desensitized, raising the possibility that JNK activation may be partly dependent on receptor internalization. In this regard, Daaka et al. [49] have recently provided evidence that receptor endocytosis is required for ERK activation mediated by the β_2-adrenoceptor and lysophosphatidic acid receptor in HEK 293 cells. Although ERK and JNK activation pathways involve distinct intracellular components, it will be of interest to examine the role of m3 receptor internalization in JNK activation in the light of these observations.

Ca^{2+} plays a key role in ERK activation in neuronal cells via pathways requiring the monomeric G-protein Ras, and mechanisms involving tyrosine kinase activation and activation of guanine nucleotide exchange factors for Ras have been proposed [50,51]. In addition, T-lymphocytes have an apparently unique mechanism to allow Ca^{2+}-dependent activation of JNK when PKC is co-activated [52], and JNK activation in B-lymphocytes appears to be dependent on the size and duration of the Ca^{2+} transient [53]. Since the m3 AChR activates PLC, we have also examined the role of Ca^{2+} and PKC in the regulation or ERK and JNK by this receptor. In CHO-m3 cells, MCh can release Ca^{2+} from a thapsigargin-sensitive internal store and activate a large extracellular Ca^{2+} entry pathway. Depletion of internal Ca^{2+} and/or removal of extracellular Ca^{2+} demonstrated that m3 AChR-mediated JNK activation involved both a Ca^{2+}-dependent and a Ca^{2+}-independent component, whereas muscarinic receptor activation of ERK did not require Ca^{2+}. Similar manipulations that should abolish Ca^{2+} mobilization by angiotensin II in GN4 rat liver epithelial cells have also provided evidence for a Ca^{2+}-dependent and a Ca^{2+}-independent mechanism of JNK activation [26], whereas JNK activation by the m1 and m2 AChR has been reported to be entirely Ca^{2+}-dependent in Rat 1 cells [24]. Our observation that increasing $[Ca^{2+}]_i$ independently of agonist can cause significant JNK activation, yet negligible ERK activation, relative to that induced by agonist, supports a differential role for Ca^{2+} in activation of these two MAPK pathways by the m3 AChR. In COS-7 cells, the m1 and m2 AChRs stimulate JNK through G-protein $\beta\gamma$ subunits acting via Ras and Rac1 [25], and such a mechanism may account for the Ca^{2+}-independent component of the m2- and m3-mediated signal in CHO cells. Activating mutants of α_q have also been reported to activate JNK in COS-1 [30] and PC-12 [31] cells, presumably by stimulating β isoenzymes of PLC with consequent elevation of $[Ca^{2+}]_i$. The Ca^{2+}-activated protein tyrosine kinase Pyk2 can be activated by GPCRs for bradykinin, lysophosphatidic acid and carbachol in PC12 cells [22], and by angiotensin II in GN4 cells [54], and can mediate ERK [22] and JNK [32] activation. We have yet to establish whether activation of JNK in CHO-m3 cells requires a Ca^{2+}-dependent tyrosine kinase or whether a Ca^{2+}/calmodulin-dependent protein kinase may be involved [55].

As demonstrated here, agonist-stimulated Ins(1,4,5)P_3 formation in CHO-m3 cells is resistant to PTX, consistent with G_q/PLC-β involvement, whereas a significant component of ERK and JNK response was toxin sensitive. Measurements of agonist-stimulated guanosine 5′-[γ-thio]triphosphate (GTP[S]) binding in CHO-m1 and CHO-m3 cells membranes following PTX treatment has indicated that these G_q-coupled receptors are also linked to G_i and/or G_o [40]. Interestingly, G_o is not widely expressed outside the nervous system [56] but is present in CHO cells where it has been implicated in coupling the m1 AChR to ERK activation [48]. These observations indicate that both the m1 and m3 receptor can couple to at least two distinct classes of G-protein to mediate activation of separate but overlapping signalling pathways.

As evidence exists for the involvement of PI-3K in ERK activation by GPCRs [57], we examined the effect of the PI-3K inhibitor wortmannin on muscarinic regulation of ERK and JNK. Our results suggest that ERK activation by m2 and m3 AChRs is dependent on the activity of PI-3K, which is consistent with the notion that specific isoforms of PI-3K link G-protein $\beta\gamma$ subunits to activation of the ERK pathway [57]. Very recently, PI-3K has also been proposed to mediate JNK activation by the m2 AChR in COS-7 cells [58] and can apparently form a complex with JNK in γ-irradiated U937 myeloid leukaemia cells [59]. Our studies examining PI-3K involvement in JNK activation by muscarinic receptors were inconclusive due to the marked stimulatory effects of wortmannin on JNK as reported previously [59].

Finally, we have also examined the involvement of PKC in muscarinic activation of the ERK and JNK pathways by using the PKC inhibitor Ro-31-8220 or by prolonged phorbol ester treatment to deplete the protein. These experiments indicate that ERK activation by the m3 AChR is mainly mediated via a PKC-dependent pathway and are consistent with work demonstrating that PKC activation by phorbol ester or overexpression of the novel or conventional isoforms of PKC is sufficient alone to activate ERK [2,14,15]. Surprisingly, we also found that ERK activation by the m2 AChR required PKC, despite operating exclusively through a PTX-sensitive pathway. Our observation that MCh could increase intracellular Ca^{2+} in CHO-m2 cells is consistent with PLC activation being mediated by $\beta\gamma$ subunits derived from PTX-sensitive G-proteins, thereby providing a mechanism to account for this PKC-dependence. In support of our observations, the PTX-sensitive G_o protein has been shown to couple the m1 AChR and platelet-activating factor receptor to ERK activation via PKC in CHO cells [48].

In NIH3T3 cells, JNK activation by the m1 AChR does not require PKC [23]. However, we find that PKC inhibition by Ro-31-8220 or by chronic phorbol ester treatment results in enhanced JNK activity in CHO-m3 cells stimulated with MCh, suggesting that PKC contributes an inhibitory component to this pathway. In CHO-m3 cells that were pretreated with PDBu for 5 min to activate PKC, stimulation of JNK by MCh was attenuated by approx. 50% (P. G. Wylie, R. A. J. Challiss and J. L. Blank, unpublished work), whick is consistent with an inhibitory effect of PKC on JNK. In accord with our observations, other studies have shown that PKC inhibition can potentiate JNK activation by endothelin-1 in Rat 1 fibroblasts [60] and angiotensin II in GN4 epithelial cells [26]. Although Ro-31-8220 may also have an effect on JNK activity that is independent of PKC inhibition [61], these results suggest that PKC plays opposing roles in the regulation of JNK and ERK by m3 AChRs in CHO cells.

Biochemical and molecular biological studies have identified several intracellular protein kinase cascades that provide the potential for parallel regulation of the ERK, JNK and p38 subgroups of MAPKs in response to extracellular stimuli and various forms of cellular stress [1–4,7]. Whereas the role of PKC and Ca^{2+} in ERK regulation via pathways involving Ras and the Raf family of MAPKKKs have been examined extensively, the upstream elements involved in JNK and p38 regulation by GPCRs remain to be determined. Recent evidence has established that Rac1 and Cdc42, two members of the Rho family of monomeric G-proteins, are key intracellular regulators of JNK and p38 [3,4,7]. In addition, a family of MAPK/ERK-activating kinase kinases (MEKKs) have been identified as MAPKKKs that preferentially regulate JNK and p38 [2–4,7]. This distinguishes MEKKs from Raf, which activates ERK but not JNK

or p38 [4]. Importantly, Rho family G-proteins have also been implicated in muscarinic receptor signalling [7,25,62] and in the regulation of MEKKs [63,64] and functionally related MAPKKKs [3,4]. Future studies will examine the involvement of MEKKs and of Rho family G-proteins in JNK regulation by muscarinic receptors in CHO cells and address the mechanisms by which Ca^{2+} and PKC may influence this pathway.

We are grateful to Mr. Rick Davis for assisting with the calcium measurements and to Mr. Raj Mistry for performing the Ins(1,4,5)P_3 mass assays. We also thank Dr. Roger Davis for providing the GST–c-Jun expression plasmid. We are grateful to Dr. Karl Deacon and Professor Stefan Nahorski for valuable discussions. This work was supported by the Medical Research Council and the National Asthma Campaign. P. G. W. holds a Medical Research Council postgraduate training studentship.

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Received 1 October 1998/2 December 1998; accepted 5 January 1999