RESEARCH COMMUNICATION

Differential calcium dependence in the activation of c-Jun kinase and mitogen-activated protein kinase by muscarinic acetylcholine receptors in rat 1a cells

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Carbachol stimulation of the muscarinic acetylcholine m1 receptor (m1R), stably expressed in Rat 1a fibroblasts, resulted in a calcium-dependent activation of c-Jun kinase (JNK). Stimulation of the muscarinic acetylcholine m2 receptor (m2R), stably expressed in Rat 1a fibroblasts, resulted in a G_i-mediated activation of JNK that was weak relative to that observed with the m1R. Chelation of calcium inhibited the m2R-mediated activation of JNK but not the robust m2R stimulation of mitogen-activated protein kinase (MAPK) activity. These findings demonstrate a role for the second messenger, calcium, in the differential regulation of the activity of JNK and MAPK in Rat 1a cells.

INTRODUCTION

Physiological responses to a diversity of extracellular stimuli and stresses involve sequential protein kinase reactions involving members of the mitogen-activated protein kinase (MAPK) family. Characterization of members of these sequential protein kinase pathways in yeast and metazoans indicates that these pathways can be selectively activated to control cell function and phenotype [1–3].

The recent cloning and sequence analysis of the c-Jun kinases (JNKs)/stress-activated protein kinases (SAPKs) has revealed a novel subfamily of proteins related to the MAPKs [3,4]. The JNK/SAPKs are proline-directed serine/threonine kinases that are activated by such stimuli as UV irradiation, heat shock, protein-synthesis inhibitors, cytotoxic cytokines and growth factors [3–6]. Mitogens and phorbol esters, which strongly activate p42 and p44 MAPKs, are generally poor activators of the JNK/SAPKs. It is now evident that the JNK/SAPKs are activated by distinct tyrosine/threonine protein kinases referred to as SAPK/extracellular signal-related protein kinase (ERK)-1 (SEK-1) or MAPK kinase (MKK) [7–9]. Activation of SEK/MKK kinases appears to be independent of Raf-1, the upstream regulator of MAPK/ERK kinase-1 (MEK-1) and MAPK. MEK kinase-1 (MEKK-1) is capable of activating SEK-1 kinase and preferentially activates the JNK/SAPK pathway relative to the p42 and p44 MAPKs pathway [5,9].

In this report we demonstrate differential regulation of the MAPK and JNK activation pathways by the m1 and m2 muscarinic acetylcholine receptor subtypes (m1R and m2R). We show that the m1R robustly activates JNK, even though it does not activate MAPK in Rat 1a cells [10]. The m2R, stably expressed in Rat 1a cells, which strongly activates the MAPK pathway, activates JNK weakly relative to the m1R. Calcium chelation totally abolishes both the m1R and m2R stimulation of JNK/SAPK. In contrast, m2R stimulation of p42 and p44 MAPK activities is calcium independent, defining the differential regulation of these protein kinase pathways by a specific second messenger.

MATERIALS AND METHODS

Cell lines and culture

Transfected Rat 1a clones stably expressing the muscarinic m1R or m2R have been described previously [11]. Cells were made quiescent by incubation for 16 h in Dulbecco's modified Eagle's medium containing 0.1% BSA.

JNK assay

JNK activity was measured by using a previously described assay, with slight modifications [12]. GST (glutathione S-transferase)–c-$Jun_{(1-79)}$ was coupled to glutathione–Sepharose-4B beads by standard procedures and used as both a ligand and a substrate for JNK. Stimulated or control cells were lysed in 0.5% Nonidet P40/20 mM Tris/HCl (pH 7.6)/0.25 M NaCl/3 mM EDTA/3 mM EGTA/1 mM dithiothreitol (DTT)/1 mM phenylmethanesulphonyl fluoride/2 mM Na_3VO_4/20 μg/ml aprotinin/5 μg/ml leupeptin. Nuclei were removed by centrifugation (approx. 15000 *g*, 10 min), the cytoplasmic extracts rotated (4 °C, 1 h) with 10 μl of a slurry of GST–c-$Jun_{(1-79)}$–Sepharose beads (3–5 μg of GST–c-$Jun_{(1-79)}$). The beads were recovered by centrifugation in a microfuge (10 s pulse) and then washed twice in cell-lysis buffer and once in kinase buffer [20 mM Hepes (pH 7.5)/10 mM $MgCl_2$/20 mM β-glycerophosphate/10 mM *p*-nitrophenyl phosphate/1 mM DTT/50 μM Na_3VO_4]. After the final wash the beads were resuspended in 40 μl of kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP and incubated for 20 min at 30 °C. The kinase reactions were quenched by the addition of Laemmli sample buffer, boiled for 3 min, and phosphorylated proteins were resolved on an SDS/10% polyacrylamide gel.

Abbreviations used: BAPTA, bis-(o-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; EGFR, epidermal growth factor receptor; JNK, c-Jun kinase; MAPK, mitogen-activated protein kinase; m1R, muscarinic acetylcholine m1 receptor; m2R, muscarinic acetylcholine m2 receptor; PLCβ, phospholipase Cβ; SAPK, stress-activated protein kinase SEK, SAPK/extracellular signal-related protein kinase-1; GST, glutathione S-transferase; DTT, dithiothreitol; ERK, extracellular signal-related protein kinase.

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MAPK activity

MAPK activity was measured exactly as described previously [13] with the exception that Mono Q FPLC fractionation was replaced by batch elution from a DEAE–Sephacel column using a high salt concentration (0.5 M NaCl). The eluate was assayed in triplicate using the epidermal-growth-factor receptor EGFR-662–681-peptide as a selective substrate for MAPK activity [14].

Quantification and presentation of data

Phosphorimager analysis of phosphorylated GST–c-Jun$_{(1-79)}$ provided a quantitative measure of JNK activity in arbitrary phosphorimaging units. This was performed routinely and was the basis for the fold activation values quoted in the text. All values are representative of at least three independent experiments.

RESULTS AND DISCUSSION

We have previously demonstrated that stimulation of the m2R, expressed stably in Rat 1a cells, strongly activated the Ras/Raf/MAPK pathway [11]. In striking contrast, stimulation of the m1R, expressed stably in Rat 1a cells, did not activate the Ras/Raf/MAPK pathway. Rather, the stimulation of m1R inhibited growth-factor-mediated activation of the MAPK pathway via a calcium-dependent increase in cyclic AMP synthesis and subsequent activation of protein kinase A [10]. Thus we considered it pertinent to examine whether these receptors could exert differential regulation of the JNK/SAPK pathway.

Stimulation of the m1R activates JNK in a time-, dose- and receptor-mediated manner

Carbachol (100 μM) treatment of serum-starved m1R Rat 1a fibroblasts strongly stimulated JNK activity to levels comparable to those observed with UV irradiation (UV-B, 2600 μW/cm^2). Stimulation and recovery conditions were optimized for this dose of UV-B irradiation to give maximal JNK activation. Using these UV-irradiation conditions, no alteration in cell viability was apparent during the time-course of JNK activation measured. m1R-mediated activation of JNK was transient, reaching maximal levels at 15 min, and returned to near-basal levels by 30 min (Figure 1a). Maximal activation of JNK, in response to carbachol stimulation of the m1R or to UV irradiation, ranged from 3–10-fold over basal levels. JNK activation was even greater in cells that had not been serum starved before carbachol stimulation, due to a 2–3-fold lower level of basal JNK activity (results not shown); serum-depletion-induced stress of the cells resulted in increased basal JNK activation. Carbachol-stimulated activation of JNK in m1R Rat 1a cells was receptor mediated, occurring in a dose-dependent manner that was inhibited by pretreatment of the cells with the muscarinic receptor antagonist atropine (Figures 1b and 1c).

The m2R activates JNK in a pertussis-toxin-sensitive manner

The m2R selectively couples to G_i-regulated response pathways [11,15], while the m1R predominantly couples to G_q-regulated phospholipase Cβ (PLCβ) [10,16]. Carbachol (100 μM) treatment of Rat 1a fibroblasts, stably expressing the m2R, resulted in weak activation of JNK that was completely inhibited by pretreatment of the cells with pertussis toxin (Figure 2). The m2R stimulation of JNK activity is G_i-mediated since G_o is not expressed in Rat 1a cells (results not shown). This is the first demonstration that a G_i-coupled receptor can activate JNK. The m2R-mediated JNK activation ranged from 2–3-fold over basal levels compared with 3–10-fold stimulations observed with the m1R and UV irradiation. The m1R-mediated response remained largely unaffected by pertussis-toxin pretreatment, as anticipated for a G_q-coupled receptor (Figure 2).

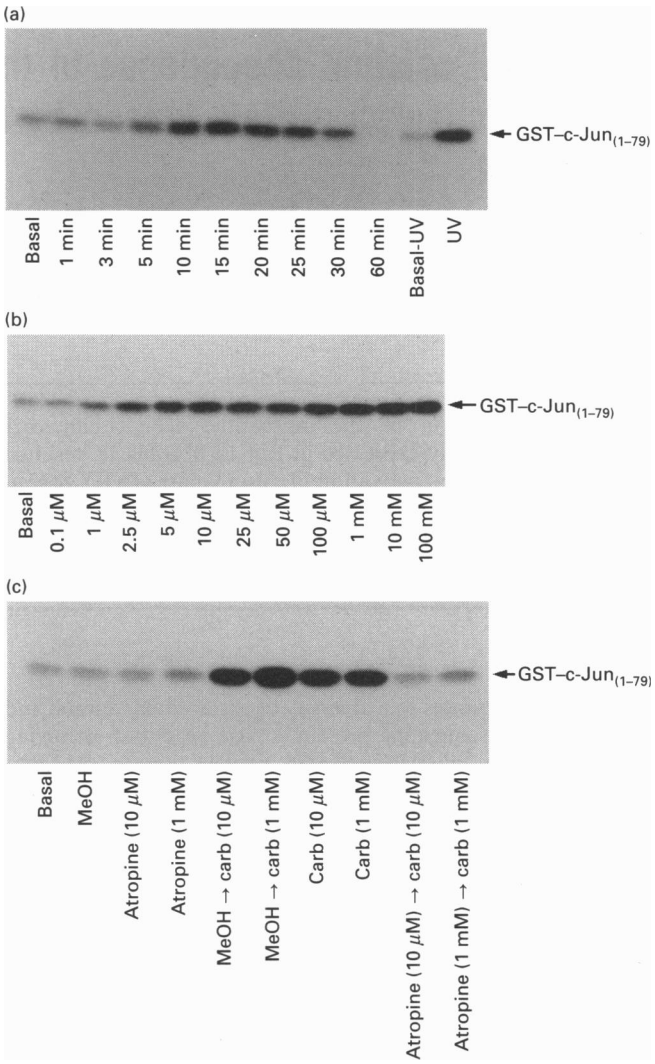

Figure 1 Muscarinic m1 receptor stimulation of JNK activity in Rat 1a cells

(**a**) Serum-starved cells from a Rat 1a clone, stably expressing 4×10^5 m1R per cell (m1R Rat 1a cells), were challenged with 100 μM carbachol for the various times indicated and then assayed for activation of JNK as described in the Materials and methods section. For UV irradiation of the cells, the starvation medium was removed, the cells rinsed twice with PBS at 37 °C, and then dry cells were irradiated with UV-B at 2600 μW/cm^2 for 2 min. The starvation medium was then replaced and the cells returned to the culture incubator for 30 min. The basal-UV lane represents cells that were treated in an identical manner but not subjected to UV irradiation. (**b**) Serum-starved m1R Rat 1a cells were challenged with increasing concentrations of carbachol for 15 min, as indicated, and assayed for activation of JNK as in (**a**). (**c**) Serum-starved m1R Rat 1a cells were treated with the vehicle for atropine, methanol (100%), or atropine (10 μM or 1 mM, as indicated) for 30 min. MeOH → carb indicates a 30 min methanol pretreatment followed by carbachol stimulation for 15 min with the concentrations indicated. Atropine → carb indicates a 30 min atropine pretreatment followed by carbachol stimulation for 15 min with the concentrations indicated. Cells were stimulated with carbachol for 15 min with the concentrations indicated. Following these treatments cells were assayed for activation of JNK as in (**a**).

Calcium chelation abolishes m1R-mediated but not UV-mediated activation of JNK

Activation of the m1 muscarinic receptor characteristically stimulates a large extracellular calcium entry into cells [16]. We

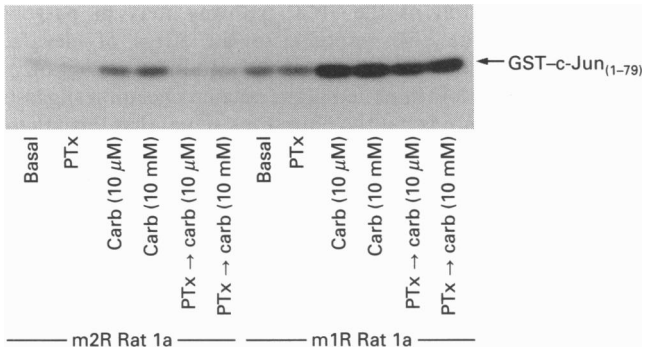

Figure 2 Effect of pertussis toxin on m1R and m2R activation of JNK

A Rat 1a clone, stably expressing 1.4×10^5 m2R per cell, was serum-starved overnight in the presence or absence of 100 ng/ml pertussis toxin (PTx indicates cells treated with toxin). The cells were then challenged with carbachol (10 μM and 10 mM, as indicated) for 15 min. PTx → carb indicates a 15 min stimulation with carbachol of pertussis-toxin-pretreated cells. Cells from the m1R Rat 1a clone, described in the Figure 1 legend, were treated in an identical manner. The stimulated cells were then assayed for activation of JNK as described in the Materials and methods section. The lanes representative of the m1R or m2R Rat 1a cells are indicated.

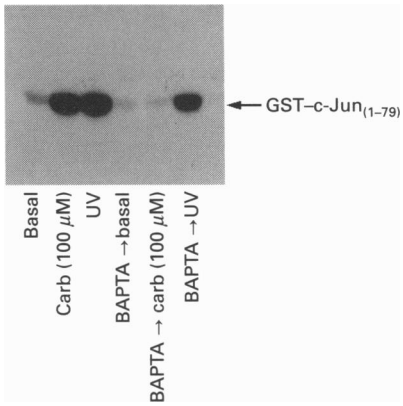

Figure 3 Chelating intracellular calcium with BAPTA inhibits m1R stimulation of JNK

Serum-starved m1R Rat 1a cells were incubated in 1 × Hanks solution (100 μM $CaCl_2$/5.0 mM KCl/0.3 mM KH_2PO_4/0.5 mM $MgCl_2$/0.4 mM $MgSO_4$/37.9 mM NaCl/0.3 mM Na_2HPO_4/5.6 mM D-glucose, pH 7.4) for 60 min and then stimulated with carbachol (100 μM) for 15 min or UV for 2 min, and assayed for JNK as described in the Figure 1 legend. BAPTA → basal indicates a 20 μM BAPTA pretreatment in 1 × Hanks solution for 60 min, followed by 2 mM EGTA for 2 min (to chelate extracellular calcium). BAPTA → carb indicates a 20 μM BAPTA/EGTA pretreatment as described above followed by a 15 min, 100 μM carbachol stimulation. BAPTA → UV indicates a BAPTA/EGTA pretreatment as described above followed by UV stimulation for 2 min.

have demonstrated previously that the m1R activated PLCβ in Rat 1a cells, resulting in mobilization of calcium from intracellular stores. This carbachol-stimulated rise in intracellular calcium was dampened using bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid (BAPTA), an agent that chelates intracellular calcium, in combination with EGTA to chelate extracellular calcium [10]. These calcium chelation conditions completely inhibited the m1R-mediated activation of JNK. Clearly, an elevation of intracellular calcium is crucial to the m1R-mediated activation of JNK. In contrast, UV-stimulated activation of JNK in the same cells was only blunted by approx. 30% with calcium chelation in several independent experiments (Figure 3). The characterization of signal-transduction pathways activated by UV irradiation is rather poorly defined, but recent studies have demonstrated that UV irradiation of cultured cells increases PLC activity [17]. UV- and m1R-mediated activation of JNK was non-additive, suggesting that a common JNK was being activated (results not shown). However, the differential sensitivity to calcium chelation indicated that multiple signalling pathways are involved in regulating JNK activation in Rat 1a cells.

A co-stimulatory role for calcium in the activation of JNK has been suggested from studies by Su et al. [6], where treatment of Jurkat cells with the phorbol ester phorbol 12-myristate 13-acetate and calcium ionophore resulted in synergistic activation of JNK, but not MAPK. Such synergy appears to be unique to T-lymphocytes, as that study also demonstrated that similar treatments in a variety of cell lines did not result in JNK activation [6]. Indeed, treatment of Rat 1a cells with calcium ionophore and phorbol 12-myristate 13-acetate, alone or in combination, did not result in significant activation of JNK (results not shown), indicating that elevation of intracellular calcium and PKC stimulation under these conditions was insufficient for JNK activation.

Calcium chelation differentially affects m2R-mediated activation of JNK and MAPK

The weak m2R-mediated activation of JNK was also inhibited by calcium chelation (Figure 4). Interestingly, although increased PLCβ activity cannot be measured in response to m2R stimulation in Rat 1a cells, we have detected a weak, pulsatile rise in intracellular calcium (S. Winitz and G. L. Johnson, unpublished work). Such waves of low calcium mobilization have been described previously [18] and appear to be important in the m2R-mediated activation of JNK in Rat 1a cells. Importantly, the effect of calcium chelation is selective for the m2R-mediated activation of JNK and caused no inhibition of the m2R-mediated activation of MAPK (Table 1). Thus calcium chelation in Rat 1a cells selectively disrupts JNK, but not p42 and p44 MAPKs activation, demonstrating distinct regulation of the two pathways by the G_i-coupled m2R. Additionally, similar to the G-protein-coupled m2R, the activation of JNK by the endogenously expressed

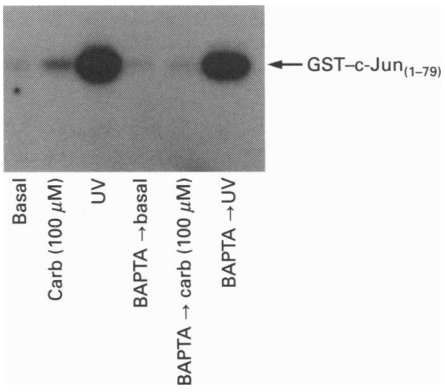

Figure 4 Chelating intracellular calcium with BAPTA inhibits m2R stimulation of JNK

Serum-starved m2R Rat 1a cells were incubated in 1 × Hanks solution for 60 min and then stimulated with carbachol (100 μM) for 15 min or UV for 2 min and assayed for JNK as described in the Figure 1 legend. BAPTA → basal, BAPTA → carb and BAPTA → UV indicate identical treatments to those described in the Figure 3 legend.

Table 1 Chelating intracellular calcium with BAPTA does not inhibit m2R stimulation of MAPK

Serum-starved m2R Rat 1a cells were incubated in 1 × Hanks solution for 60 min and then stimulated with carbachol (100 μM) for 3 min and assayed for MAPK as described in the Materials and methods section (values represent means ± S.E.M). BAPTA → basal indicates a 20 μM BAPTA/EGTA pretreatment as described in the Figure 3 legend. BAPTA → carb indicates a 20 μM BAPTA/EGTA pretreatment as described above followed by a 3 min, 100 mM carbachol stimulation.

Sample	MAPK activity (c.p.m./15 min per 20 μl)
Basal	33929 ± 1628
Carb	152235 ± 15128
BAPTA → basal	55811 ± 3672
BAPTA → carb	193460 ± 9144

tyrosine kinase EGFR is also inhibited upon calcium chelation whereas the robust EGFR-mediated MAPK activation remains unaffected by calcium chelation (results not shown). Thus calcium is critical for the activation of JNK, but not MAPK, for both G-protein-coupled and tyrosine-kinase receptors in Rat 1a cells. Interestingly, Chao et al. demonstrated that both calcium-dependent and -independent pathways are involved in the activation of MAP kinases in human fibroblast and epidermal carcinoma cells lines [19].

Differential regulation of JNK and MAPK pathways is demonstrated in these studies. The strong m1R-mediated activation of JNK, in the absence of Ras and Raf activation in Rat 1a cells [10], clearly separates the signalling requirements for JNK and MAPK activation by G_q-coupled receptors. Interestingly, after completion of our studies, Coso et al. demonstrated that the m1R, when stably expressed in NIH 3T3 cells, stimulated JNK activity [20]. In this cell background the m1R also activates the Ras/RAF/MAPK cascade although in a temporally distinct manner from that of the JNK pathway. Persistent activation of the m1R in NIH 3T3 cells results in malignant transformation of these cells [21]. Clearly, different cell types offer distinct modes of regulation of JNK and MAPK pathways even via the same receptor subtype. Study of the activation of JNK in m1R Rat 1a cells, in the absence of the MAPK activation that is observed in NIH 3T3 cells, should prove insightful with respect to the consequences of JNK activation independent of MAPK activation.

Our previous studies have demonstrated that under conditions of carbachol stimulation of the m1R, identical with those that resulted in activation of JNK, there is a concomitant calcium-dependent cyclic AMP synthesis in these cells [10]. Treatment of m1R Rat 1a cells with forskolin elevates cyclic AMP to levels similar to those induced by carbachol [10]. However, forskolin treatment does not activate JNK in Rat 1a cells (results not shown). Thus, the calcium-dependent activation of JNK in Rat 1a cells is neither positively nor negatively regulated by cyclic AMP. This contrasts with p42 and p44 MAPKs activation in response to growth factors that is clearly inhibited by cyclic AMP in many cell types [10,22–26]. Thus, in addition to calcium, stimuli which activate protein kinase A can potentially exert a selective regulation of JNK and MAPK pathways.

Sustained, high intracellular-calcium levels are generally toxic to cells, and efficient regulatory mechanisms have evolved to maintain low cytoplasmic calcium levels [27]. The calcium-dependent activation of the JNK pathway may in part be involved in a protective response to the stress of elevated intracellular calcium. The fact that the UV-induced activation of JNK is not completely inhibited upon calcium chelation suggests that elevated calcium is not the only stress signal that this kinase pathway responds to. While the MAPK pathway has been well characterized in terms of the control of growth and differentiation responses, the phenotypic consequences of activation of the JNK pathway are currently less well characterized [28]. Nonetheless, the JNK pathway appears to be selectively activated by stresses to the cell and may function in part as a protective response for cell survival. The ability of the second messenger, calcium, to differentially regulate activation of JNK and MAPK provides a mechanism for the cell to differentially modulate these two kinase pathways. In this way the magnitude of the activation of each pathway can be independently regulated for integration of multiple signals and the control of cell phenotype.

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