c-fos and c-jun Are Induced by Muscarinic Receptor Activation of Protein Kinase C but Are Differentially Regulated by Intracellular Calcium*

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It has become increasingly clear that agents classically thought to act as neurotransmitters can also alter gene expression. To understand the early events by which neurotransmitters could effect genetic responses, we have studied the induction of two immediate early genes, c-fos and c-jun. These genes encode proteins that form a dimeric complex (AP-1) active as transcriptional factor. Using the stable acetylcholine analog carbachol to activate muscarinic receptors (mAChR) in a glial cell line (1321N1), we show that c-fos and c-jun mRNA levels are transiently increased, reaching a maximum at 30 min after agonist addition. Experiments in which the actions of carbachol are blocked by adding atropine at various times demonstrate that only 1.5 min of agonist stimulation is needed to give maximal increases in c-fos or c-jun mRNA at 30 min. These results suggest that events previously shown to occur in the first minute of mAChR occupation (the mobilization of intracellular Ca2+, activation of protein kinase C) are sufficient for induction of these immediate early genes. In cells in which protein kinase C has been down-regulated, carbachol no longer stimulates c-fos or c-jun expression, indicating a critical role for protein kinase C in these responses. In cells loaded with bis(0-aminophenoxy)ethane-N,N,N',N"-tetraacetic acid (BAPTA) to buffer increases in cytosolic [Ca2+], mAChR-mediated induction of c-fos is markedly reduced; in contrast there is enhanced c-jun expression. The strong enhancement of c-jun induction by carbachol in BAPTA-treated cells is due at least in part to mRNA stabilization. Experiments using phorbol ester (phorbol 12-myristate 13-acetate) in combination with the Ca2+ ionophore ionomycin confirm that activation of protein kinase C induces c-fos and c-jun expression, and that concomitant increase in cytosolic [Ca2+] potentiates the induction of c-fos while repressing that of c-jun. The data suggest that the ability of neurotransmitters or growth factors to mobilize Ca2+ would modulate the effect of concomitant protein kinase C activation on AP-1 generation and consequent target gene expression.

Several neurotransmitters that transduce signals through the phosphoinositide pathway have been shown to induce mitogenic responses in neuronal and non-neuronal cells. The neurotransmitter serotonin, acting through multiple 5-HT receptor subtypes expressed in fibroblasts, induces DNA synthesis and causes cell transformation (1–3). Activation of the mas oncogene product (an angiotensin receptor) expressed in neuronal cells leads to phosphoinositide turnover and results in mitogenesis (4, 5). In addition the cholinergic agonist carbachol has been demonstrated to stimulate DNA synthesis in astrocytes, neuroblastoma cells and fibroblasts expressing muscarinic acetylcholine receptors (mAChR) that couple to inositol phospholipid metabolism (6). The sequence of events through which these neurotransmitters effect changes in DNA synthesis is not known.

One of the earliest genetic changes elicited by many mitogens is the induction of immediate early gene expression (7–12). Two prominent members of the immediate early gene family are the proto-oncogenes c-fos and c-jun which encode nuclear proteins (13, 14). These proteins function together as a dimeric complex that binds to a specific DNA consensus sequence (AP-1) of target genes to stimulate their transcription (15–20). Stimulation of the muscarinic cholinergic receptor in PC12 and glial cells has been reported to induce c-fos expression (21–23). Most recently Seuwen et al. (58) reported that c-fos and c-jun expression are increased following activation of M1 muscarinic receptors stably expressed in fibroblasts.

The promoter region of the c-fos gene is known to contain specific regulatory elements that confer responsiveness to phorbol esters and calcium ionophores (24–27). Thus activation of protein kinase C and increases in cytosolic [Ca2+] are both capable of inducing c-fos expression. The expression of c-jun is likewise stimulated by phorbol esters and calcium ionophores (28–30). A role for protein kinase C in the induction of c-fos by a number of agents including PDGF has been demonstrated (31–33). In addition increases in cytosolic [Ca2+] have been shown to be important in angiotensin II induced c-fos expression (33). However little is known concerning the relative contribution of Ca2+ and protein kinase C to the induction of c-jun and c-fos by mitogens that activate both of these second messenger pathways.

We have previously shown that activation of muscarinic receptor in 1321N1 cells leads to formation of inositol 1,4,5-triphosphate, mobilization of intracellular Ca2+, and activation of protein kinase C. These second messenger responses all occur within 1 min of receptor stimulation (34–37). In this study, we first examine the kinetics of c-fos and c-jun induc-

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1 The abbreviations used are: mAChR, muscarinic acetylcholine receptor; PMA, phorbol 12-myristate 13-acetate; BAPTA, bis-(o-aminophenoxy)ethane-N,N,N',N"-tetraacetic acid; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me2SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.
tion. The results of our experiments suggest that biochemical changes occurring in the first minute of mAChR occupation are sufficient to induce maximal c-fos and c-jun expression. We then use Ca\textsuperscript{2+} chelators and protein kinase C down-regulation to examine the respective contribution of increases in cytosolic [Ca\textsuperscript{2+}] and activation of protein kinase C to the regulation to examine the respective contribution of increases in cytosolic [Ca\textsuperscript{2+}] and activation of protein kinase C to the regulation to examine the respective contribution of increases in cytosolic [Ca\textsuperscript{2+}] and activation of protein kinase C to the regulation to examine the respective contribution of increases in cytosolic [Ca\textsuperscript{2+}] and activation of protein kinase C to the activation of protein kinase C. However, whereas the muscarinic receptor-mediated increase in cytosolic [Ca\textsuperscript{2+}] enhances c-fos expression, it represses the expression of c-jun at least in part by affecting c-jun mRNA stability.

**EXPERIMENTAL PROCEDURES**

**Materials**—We purchased [\(\alpha\text{-}{^32}\text{P}\)]dCTP (3000 Ci/mmol) from Du Pont-New England Nuclear. Carbamylcholine (carbachol), atropine sulfate, and PMA were purchased from Sigma. Ionomycin, BAPTA, A23187, and culture grade EGF (murine) were obtained from Calbi- ochem. The v-fos 1.0-kilobase PstI/PvuII fragment was purchased from Oncor Probes Inc. (38). The 1.4-kilobase HindIII/EcoRI fragment of human c-jun cDNA, the 1.0-kilobase BamHI/XhoI fragment of jun-B cDNA, and the 1.4-kilobase PstI fragment of mouse a-tubulin cDNA were used as probes for Northern analysis. All cDNA plasmids were generous gifts from Michael Karin (University of California San Diego, LaJolla, CA).

**Cell Culture**—1321N1 Astrocytoma cells were grown on 100-mm plates in Dulbecco’s modified Eagles medium (DMEM) supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). All studies were done on subconfluent cell monolayers approximately five days after culture and following 18 h of serum deprivation in the presence of 0.1% bovine serum albumin (BSA). In some experiments protein kinase C was down-regulated by exposing subconfluent monolayers of cells to 1 \(\mu\)M PMA or to 0.1 M MeSO\(4\) (vehicle) for 18 h.

**Cell Incubations**—All experiments were performed at 37\degree C and were begun by washing cell monolayers twice with fresh DMEM containing 20 mM HEPES buffer. Drugs were added by replacing the wash medium with 4 ml of DMEM/HEPES containing the drug at the final assay concentration. When cell monolayers were exposed to EGF the buffer contained 0.1% BSA. MeSO\(4\) (0.1%), the vehicle for PMA, BAPTA, ionomycin, and A23187, was found to have no effect on the measured responses. Experiments were terminated by aspirating the assay buffer, rinsing once with ice-cold phosphate-buffered saline, and lysing the cells with 1 ml of 4 M guanidine isothiocyanate.

**RNA Isolation and Northern Analysis**—Total RNA was isolated by centrifugation through cesium chloride according to the method of Chirgwin et al. (39). Extracted RNA was quantitated by absorbance measurements at A\textsubscript{260 nm}. The RNA (20 \(\mu\)g) was fractionated by electrophoresis through 1% agarose gels containing 2.2 M formaldehyde, transferred to Hybond-N Nylon membranes (Amersham Corp.) by capillary blotting and immobilized by heating at 80 \degree C for 2 h. Membrane filters were prehybridized for 2–4 h at 42 \degree C using 50% formamide, 5 \times Denhardt’s reagent (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), 5 \times SSPE (1 \times SSPE = 0.15 M NaCl, 0.01 M NaH\textsubscript{2}PO\textsubscript{4}, 0.001 M EDTA, pH 7.4), 1% SDS and 500 \(\mu\)g/ml salmon sperm DNA. The cDNA probes were labeled with [\(\alpha\text{-}{^32}\text{P}\)]dCTP by the random primer method (Pharmacia LKB Biotechnology Inc.). Denatured P-labeled cDNA probes (1–3 \times 10\(^6\) cpm/ml) were added directly to the prehybridization solution. Membrane hybridizations were carried out at 42 \degree C for 24–48 h to detect mRNA transcripts. Following hybridizations membranes were washed twice for 15 min in 0.2 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.2% SDS at 42 \degree C to 50 \degree C and if required were followed by 2 \times 15 min washes in 0.1 \times SSC, 0.1% SDS at 50 \degree C to 65 \degree C. Filters were exposed to Kodak X-Omat AR film overnight at \(-70\) \degree C with one intensifying screen. Relative abundances of specific mRNA transcripts were quantitated by radioanalytic scanning (AMBIS). Total RNA loaded into each lane was normalized by probing the membrane filter with a \(32\text{P}\)-labeled a-tubulin cDNA fragment.

**RESULTS**

**Muscarinic Receptor-mediated Induction of c-fos and c-jun Expression in 1321N1 Cells**—First we determined whether activation of the mACHR leads to changes in the expression of the proto-oncogenes c-fos and c-jun. Activation of the mACHR by the stable acetylcholine analog carbachol results in a dose-dependent increase in the level of 92.2 kilobase c-fos mRNA transcript; a maximal increase (\(\approx\)20-fold) is observed with 1 mM carbachol. The expression of c-fos is barely detectable under control conditions (Fig. 1, 0 min). The carbachol-stimulated increase in c-fos mRNA is detectable as early as 15 min, peaks at 30 min, and is no longer detectable at 120 min (Fig. 1). The response to carbachol (1 mM) is completely blocked by the mACHR-antagonist atropine (1 \(\mu\)M).

We then determined whether c-jun is co-induced with c-fos. In control cells there is a detectable basal level of c-jun mRNA transcripts. Carbachol stimulates an \(\approx\)2-fold increase in c-jun mRNA levels with a time course of induction that is similar to that of c-fos (Fig. 1). We detect an increase in two c-jun mRNA transcripts, with estimated sizes of 2.6 and 3.2 kilobases; these transcripts appear to be generated through alternative splicing of the c-jun gene (40, 41). The mACHR-mediated induction of c-jun expression is also inhibited by the receptor antagonist atropine (Fig. 1).

In 1321N1 cells, mACHR stimulation leads to increases in cytosolic [Ca\textsuperscript{2+}] and to the activation of protein kinase C within 1 min (35–37). To understand whether these early biochemical changes induced by mACHR activation are sufficient to initiate increases in c-fos and c-jun mRNA, we exposed cells to carbachol for very brief periods, removed the agonist-containing medium and replaced it with a medium containing the mACHR antagonist atropine. The level of c-fos and c-jun mRNA transcripts was measured 30 min after the initial addition of agonist. The results of these experiments indicate that 1.5 min of exposure to carbachol is sufficient to induce c-fos and c-jun expression to the same extent.

![Fig. 1. Northern analysis of c-fos and c-jun expression induced by carbachol over a 2-h time course. Subconfluent monolayers of 1321N1 astrocytoma cells were serum deprived for 18 h. Cell cultures were stimulated with 1 mM carbachol or were pretreated with 1 \(\mu\)M atropine for 5 min and then stimulated with carbachol. After the indicated periods of time, total RNA was isolated, and the levels of c-fos and c-jun mRNA transcripts were measured as described under "Experimental Procedures." The RNA filters were then later hybridized with an \(\alpha\)-tubulin cDNA fragment to control the quantity of RNA loaded onto each lane. The levels of c-fos and c-jun mRNA were quantitated by radioanalytic scanning (AMBIS). Increases in c-fos and c-jun mRNA are expressed as "fold" induction relative to control after normalization for total amount of RNA.](image-url)
maximal levels achieved by 30 min of continuous carbachol exposure (Fig. 2). Shorter times (0.15-1.0 min) of carbachol exposure resulted in submaximal induction (data not shown).

Role of mAChR-mediated Protein Kinase C Activation in c-fos and c-jun Expression—The phorbol ester PMA, a direct activator of protein kinase C (42), induces an ≈8-fold increase in the level of c-fos mRNA transcripts in 1321N1 cells (Fig. 3). To determine whether protein kinase C activation is necessary for the induction of c-fos in response to mAChR stimulation, we down-regulated protein kinase C by exposing cells to 1 μM PMA for 18 h. We have found this treatment protocol to be optimal for down-regulating protein kinase C in 1321N1 cells as determined by Western analysis (43). In PMA-pretreated cells, the response to PMA is lost and the ability of carbachol to stimulate c-fos expression is virtually abolished (Fig. 3). In contrast the effect of EGF on c-fos expression is only partially inhibited (reduced by less than 30%) in PMA-pretreated cells (data not shown).

We also examined the role of protein kinase C in the regulation of c-jun expression in 1321N1 cells. PMA induces an ≈3-fold increase in c-jun mRNA levels indicating that protein kinase C can mediate c-jun expression in these cells (see Fig. 6B). The ability of carbachol to increase c-jun mRNA levels is lost in cells in which protein kinase C is down-regulated (Fig. 4). In contrast, EGF still induces an ≈4-fold increase in c-jun mRNA relative to control in PMA-pretreated cells (Fig. 4). These effects of protein kinase C down-regulation implicate protein kinase C in the sequence of events leading to increases in c-fos and c-jun expression following mAChR stimulation.

Role of mAChR-mediated Increases in Intracellular [Ca²⁺] in c-fos and c-jun Expression—The increase in c-fos expression induced by a maximal concentration of PMA is always less than that induced by carbachol (see Fig. 3). This suggests that intracellular signals other than activation of protein kinase C are involved in mAChR-stimulated c-fos expression. To assess the importance of Ca²⁺ mobilization resulting from mAChR activation, we used the Ca²⁺ chelator BAPTA to buffer the rise in cytosolic [Ca²⁺]. When cells are loaded with 20 μM BAPTA for 30 min, carbachol no longer induces an increase in cytosolic calcium (37). In BAPTA-loaded cells, the mAChR-mediated increase in c-fos mRNA levels is reduced by at least 75% (compare lanes 2 and 4, Fig. 5). These data suggest that the increase in cytosolic [Ca²⁺] resulting from stimulation of the mAChR is required to maximally induce c-fos expression.

In experiments using PMA and ionomycin we confirm that both activation of protein kinase C and increases in intracellular [Ca²⁺] are needed to maximally increase c-fos expression. Ionomycin at a concentration of 100 nM causes a rapid and transient increase in cytosolic [Ca²⁺] comparable with that induced by carbachol (44). This treatment causes a significant

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**Fig. 2.** Increases in c-fos and c-jun expression following brief exposures to carbachol. Serum-deprived 1321N1 cells were exposed to 1 mM carbachol for 1.5 or 3 min, after which time the carbachol containing medium was then removed and replaced with a medium containing 1 μM atropine. Other cells were exposed to carbachol continuously for 30 min. The levels of c-fos and c-jun mRNA were measured 30 min after the initial addition of agonist. In control cells, neither changing the medium (ctrl) nor changing medium and adding atropine (ctrl atr) at 5 min affected the levels of c-fos and c-jun mRNA measured 30 min later. Increases in c-fos and c-jun mRNA levels were quantitated and normalized for total amount of RNA as described in Fig. 1.

**Fig. 3.** Effect of protein kinase C down-regulation on mAChR-mediated induction of c-fos. Cells were treated with 1 μM PMA or 0.1% Me₂SO vehicle as control for 18 h in DMEM without serum but containing 0.1% BSA. After pretreatments cells were exposed to 1 mM carbachol, 1 μM PMA, or vehicle. After 30 min, total RNA was isolated and the levels of c-fos and c-jun mRNA were measured, quantitated, and normalized for the amount of RNA in each lane (see Fig. 1). Similar findings were made in four other experiments.

**Fig. 4.** Effect of protein kinase C down-regulation on mAChR-stimulated increase in c-jun expression. Protein kinase C was down-regulated by long-term PMA-pretreatment (see Fig. 3). Control and PMA-pretreated cell cultures were exposed to 1 mM carbachol, 1 μg/ml EGF, or vehicle, and the levels of c-jun mRNA were measured 30 min later. In this experiment basal c-jun mRNA levels are reduced in PMA pretreated cells; this was not routinely observed. Increases in c-jun mRNA levels induced by carbachol or EGF in control or PMA-pretreated cells are expressed as fold induction relative to the respective control.
Muscarinic Receptor-mediated Induction of c-fos and c-jun

FIG. 5. Role of cytosolic [Ca2+] in carbachol-stimulated c-fos induction. Cell cultures were exposed to 20 μM BAPTA or 0.1% Me2SO vehicle for 30 min. After BAPTA loading, cells were stimulated with 1 mM carbachol or exposed to vehicle and the levels of c-fos mRNA were measured at 30 min (see “Experimental Procedures”). Treatment of 1321N1 cells with BAPTA does not change resting [Ca2+] levels and has no effect on basal c-fos mRNA levels as shown. These results are representative of two other experiments. A similar observation was made in experiments using the Ca2+ chelator Quin 2-AM (data not shown).

 increased gene transcription or increased mRNA stabilization. To assess the latter possibility, actinomycin D was used to block further transcription and we determined the half-life (τ1/2) of the c-jun mRNA formed in response to carbachol in control and BAPTA treated cells. Our results indicate that in BAPTA-loaded cells the estimated c-jun mRNA half-life is increased to 2.5 times that of control (see Fig. 8). Furthermore, in cells treated with the protein synthesis inhibitor cycloheximide, c-jun is superinduced, as reported by others (41, 45, 46), but not further enhanced by BAPTA (data not shown). This suggests that the effect of increases in cytoplasmic Ca2+ is not on c-jun transcription. Thus the mACHR-mediated increase in cytosolic [Ca2+] apparently represses c-jun expression through a pathway affecting c-jun mRNA degradation.

FIG. 6. Induction of c-fos and c-jun mRNA by ionomycin and PMA. A, monolayers of 1321N1 cells were exposed to 100 nM ionomycin, 1 μM PMA, ionomycin (100 nM) and PMA (1 μM) together, or 0.1% Me2SO as control for 30 min. Following cell incubations total RNA was isolated and the levels of c-fos and α-tubulin mRNA were measured as described under “Experimental Procedures.” Changes in specific mRNA transcripts were quantitated by radiologic scanning. B, cell incubations were done essentially as described above. Total RNA was isolated and specific changes in c-jun mRNA were measured and quantitated as described previously. Increases in c-fos and c-jun mRNA are expressed as fold induction relative to control after normalization for total amount of RNA. Similar findings were made in two other experiments.

DISCUSSION

The idea that acetylcholine can act as a mitogen is suggested by the finding of Ashkenazi et al. (6) that activation of the mACHR in various cell systems leads to an increase in DNA synthesis. In contrast Seuwen et al. (58) concluded that mACHR stimulation is not sufficient to cause cell proliferation in fibroblasts expressing M1 mACHR. The nature and interactions of various signals that control DNA synthesis are not clearly understood. The induction of immediate early gene expression is a primary event that has been correlated with the initiation of DNA synthesis by growth factors (8, 9, 12). In this study we demonstrate that activation of the mACHR leads to an increased expression of the proto-oncogenes c-fos and c-jun, two members of the immediate early gene family. The c-Fos and c-Jun proteins have been identified as major

(≈10-fold) but smaller increase in c-fos expression than does carbachol (Fig. 6A). A maximal dose of PMA (1 μM) alone induces an (≈8-fold) increase in c-fos expression. When ionomycin and PMA are added simultaneously the increase in c-fos mRNA (Fig. 6A) is comparable with that generally observed with carbachol. These observations indicate that increases in cytosolic [Ca2+] act in a coordinate fashion with activation of protein kinase C to stimulate c-fos expression.

To determine whether the induction of c-jun expression is similarly dependent on changes in the intracellular [Ca2+], we compared the effect of carbachol on c-jun expression in control and BAPTA-loaded cells. Surprisingly, carbachol stimulates a significantly greater increase in c-jun mRNA levels in cells loaded with BAPTA than in control cells (compare lanes 2 and 4, Fig. 7). Because buffering the rise in cytosolic [Ca2+] potentiates the response to carbachol, the mACHR-mediated mobilization of intracellular Ca2+ would appear to be involved not in activation but, rather, in repression of the c-jun gene. To further test whether an increase in intracellular [Ca2+] is inhibitory to the induction of c-jun expression, we examined the effect of ionomycin on the PMA-mediated increase in c-jun mRNA levels. In contrast to what is observed for c-fos expression, ionomycin reduces PMA-stimulated c-jun expression by 30% (see Fig. 6B). This finding provides further evidence that cytoplasmic Ca2+ is a negative modulator of c-jun expression. The induction of another member of the c-jun family, jun B, by mACHR stimulation is not altered in BAPTA treated cells (see Fig. 7).

The marked enhancement of c-jun expression seen in response to carbachol in Ca2+-buffered cells could result from
to 0.1% Me2SO affected basal c-jun or jun-B mRNA levels. Increases in c-jun and jun-B mRNA were quantitated by radioanalytic scanning in control and BAPTA treated cells. Serum-deprived 1321N1 cells were pretreated with 20 μM BAPTA or 0.1% Me2SO for 30 min. Following pretreatment cell cultures were incubated with 1 mM carbachol. The levels of c-jun and jun-B mRNA transcripts were measured 30 min after initial exposure to agonist (see "Experimental Procedures"). Under control conditions, neither exposure to BAPTA nor exposure to 0.1% Me2SO affected basal c-jun or jun-B mRNA levels. Increases in c-jun and jun-B mRNA were quantitated by radioanalytic scanning and expressed as fold induction relative to control. These results are representative of two other experiments.

**FIG. 7.** Effects of buffering mAChR-stimulated increases in cytosolic [Ca2+] on c-jun and jun-B expression. Cells were loaded with 20 μM BAPTA as described in Fig. 5. Following 30 min of BAPTA pretreatment cells were stimulated with 1 mM carbachol. The amount of c-jun mRNA remaining relative to the amount measured following 30 min of carbachol treatment. The amount of c-jun mRNA at the indicated times was normalized to α-tubulin mRNA to control for total RNA. The data are expressed as percent of c-jun mRNA remaining relative to the amount measured following 30 min of carbachol treatment. The t1/2 of c-jun mRNA was determined by linear regression.

Constituents of the transcription factor AP-1 that binds to the promoter element of various target genes to stimulate transcription (47, 48). Other studies from our laboratory have demonstrated that the c-Fos protein is synthesized in cells treated with carbachol and co-immunoprecipitates with a prominent 39 kDa protein likely to be the product of the c-jun proto-oncogene (20, 49, 50). Thus, it appears likely that activation of the muscarinic receptor leads to synthesis of known transcription factors which could in turn effect changes in target gene expression.

The time courses of mAChR-mediated increases in c-fos and c-jun mRNA are typical of those described for other mitogens (7–10, 12). However, the minimal period of receptor activation required for maximal c-fos and c-jun expression has not to our knowledge been previously delineated. Our findings show that only 1.5 min of exposure to carbachol is needed to induce the expression of c-fos and c-jun to a level equivalent to that observed if agonist had been present for the entire 30 min (see Fig. 2). Immediate early gene expression is generally thought to require longer periods of receptor stimulation, because increases in mRNA levels are usually not observed until at least 15 min after agonist addition. However, Greenberg and colleagues (21, 51) have shown that increases in transcription of c-fos and c-jun can be measured within 5 min of receptor stimulation. The rapid activation of transcription is consistent with our data, supporting the conclusion that early receptor-associated events are sufficient to initiate both c-fos and c-jun expression.

The data presented here indicate that the induction of both c-fos and c-jun expression occur through a protein kinase C-dependent mechanism. The involvement of protein kinase C is suggested by the ability of PMA to stimulate significant increases in both c-fos and c-jun mRNA levels. The strongest evidence in support of the importance of protein kinase C in the mAChR-mediated responses is that the induction of c-fos expression is virtually abolished and c-jun expression is completely inhibited in protein kinase C down-regulated cells (see Figs. 3 and 4). Other studies from our laboratory have demonstrated that the protocol used for down-regulation leads to complete loss of soluble and particulate protein kinase C immunoreactivity but does not affect mAChR number, agonist binding, or phosphoinositide hydrolysis (43). Thus, the inability of carbachol to induce c-fos and c-jun expression in PMA-pretreated cells is not due to changes in mAChR function. Furthermore, because induction of c-fos and c-jun expression by EGF, which does not activate protein kinase C in these cells (22), is relatively unimpaired, more distal processes involved in regulating c-fos and c-jun expression do not appear to be compromised.

Contrary to our findings, Blackshear et al. (22) previously reported that activation of the mAChR in 1321N1 cells leads to c-fos induction that is onl partially inhibited by protein kinase C down-regulation (16 μM PMA for 16 h). Furthermore, in their studies the Ca2+ ionophore A23187 did not increase c-fos expression, whereas we find that it is as effective as ionomycin at inducing this response. Although our experiments demonstrate that down-regulation of protein kinase C (1 μM PMA for 18 h) leads to a nearly complete loss in the mAChR-mediated induction of c-fos, there is a slight residual effect of carbachol (10% of control level by radioanalytic scanning) on c-fos expression in PMA-pretreated cells. For reasons that are not yet clear, this protein kinase C-independent pathway may predominate in the 1321N1 cell cultures used by Blackshear and co-workers (22). The failure of A23187 to induce c-fos in their cell cultures could also be explained by the relatively minor role of the protein kinase C-dependent pathway because increases in intracellular

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[Ca\textsuperscript{2+}]\textsuperscript{2} appear to act through a pathway involving protein kinase C as discussed below.

Cytoplasmic [Ca\textsuperscript{2+}] is increased following mAChR stimulation (35) and our data clearly demonstrate that this signal is important in the induction of c-fos expression. The primary evidence implicating cytoplasmic Ca\textsuperscript{2+} is that the carbachol-stimulated increase in c-fos mRNA is substantially reduced when the rise in cytosolic [Ca\textsuperscript{2+}] is buffered by BAPTA (see Fig. 5). In addition, we find that direct activation of protein kinase C and EGF or NGF (51).

The pathway by which increases in intracellular [Ca\textsuperscript{2+}] regulates c-fos expression has been most extensively studied in A12 cells. A specific DNA sequence element of the c-fos promoter was initially shown to be important for induction by the Ca\textsuperscript{2+} ionophore A23187 (25). More recently, Sheng et al. (27) established that increases in intracellular Ca\textsuperscript{2+} and cAMP mediate c-fos induction through this same DNA sequence element by a mechanism involving phosphorylation of the cAMP-responsive element binding protein (CREB). It is unlikely that increases in intracellular [Ca\textsuperscript{2+}] stimulate c-fos through this pathway in 1321N1 cells, because we find that interventions that increase CAMP do not induce c-fos expression. On the other hand, carbachol no longer increases c-fos expression when protein kinase C is down-regulated although it still increases inositol 1,4,5-trisphosphate and mobilizes Ca\textsuperscript{2+} (43). Thus the mAChR-mediated increase in cytosolic [Ca\textsuperscript{2+}] apparently enhances the induction of c-fos by acting through a mechanism involving protein kinase C. Recent studies in NIH3T3 cells also suggest that increases in intracellular [Ca\textsuperscript{2+}] mediate c-fos induction through the same region of the c-fos promoter (serum-response element) that is responsive to phorbol esters.

An unexpected observation was that the transient increase in intracellular [Ca\textsuperscript{2+}] which contributes to the induction of c-fos appears to lead to the repression of c-jun. The initial evidence for this is that there is enhanced mAChR-mediated c-jun expression in cells where the increase in cytosolic [Ca\textsuperscript{2+}] is buffered by BAPTA (see Fig. 7). Buffering the rise in cytosolic [Ca\textsuperscript{2+}] also enhances c-jun expression induced by bradykinin, another agent that acts through the phosphoinositide pathway to increase intracellular [Ca\textsuperscript{2+}] and activate protein kinase C in 1321N1 cells (37, 52). However, the response to EGF, which does not stimulate phosphoinositide hydrolysis or mobilize intracellular Ca\textsuperscript{2+} (22), is not enhanced in BAPTA treated cells. Thus the effect of intracellular Ca\textsuperscript{2+} chelation is specific for agents that mobilize intracellular Ca\textsuperscript{2+} in 1321N1 cells. Further evidence supporting an inhibitory effect of cytoplasmic Ca\textsuperscript{2+} on c-jun expression is the finding that c-jun induction by PMA is reduced by ionomycin (Fig. 6B). These findings are consistent with our observation that carbachol is less effective at eliciting c-jun expression than PMA and EGF, agents that do not elevate intracellular [Ca\textsuperscript{2+}] in 1321N1 cells. Membrane depolarization, which increases cytosolic [Ca\textsuperscript{2+}], is less effective at inducing c-jun than EGF or NGF (51).

A member of the c-jun family, Jun-B, has recently been demonstrated to compete with c-Jun for the AP-1 site within the c-jun promoter, leading to the repression of the c-jun gene (53, 54). Thus, changes in the expression of jun-B could provide a mechanism for the modulatory effect of cytoplasmic Ca\textsuperscript{2+} on c-jun expression. However, since the induction of jun-B mRNA by mAChR stimulation is not altered in BAPTA-loaded cells, increases in cytoplasmic Ca\textsuperscript{2+} do not appear to regulate jun-B mRNA expression (Fig. 7). It remains possible nonetheless that changes in cytoplasmic Ca\textsuperscript{2+} could affect c-jun expression through posttranslational modification of the Jun B protein.

The stability of c-jun mRNA has been suggested to be an important mechanism controlling c-jun gene expression because AU rich sequence elements, which target for rapid mRNA degradation, are present in the 3' untranslated region of the c-jun transcript (40, 41). To test the hypothesis that cytoplasmic Ca\textsuperscript{2+} inhibits c-jun expression by affecting mRNA stability, we used actinomycin D to block transcription and estimated the half-life of the c-jun mRNA in control and BAPTA-treated cells. Our studies demonstrate that when Ca\textsuperscript{2+} increases are buffered by BAPTA treatment, activation of mAChR leads to an ≈2.5-fold increase in the half-life of c-jun mRNA (Fig. 8). This is comparable with the increase in the levels of c-jun mRNA (Fig. 7). The effect of BAPTA on c-jun mRNA stability appears to be specific, since the half-life of c-fos mRNA is not significantly affected. Further evidence indicating that Ca\textsuperscript{2+} affects c-jun mRNA degradation is that BAPTA does not further increase carbachol-stimulated c-jun expression in cells treated with cycloheximide, which has been shown to stabilize c-jun messenger RNA (41, 45, 46). A possible mechanism for the effect of cytoplasmic Ca\textsuperscript{2+} on c-jun mRNA stability may be a change in the synthesis of labile repressors or mRNA degrading proteins suggested to be important factors controlling mRNA levels (55–57).

We conclude that the mAChR-mediated activation of protein kinase C leads to the coordinate induction of c-fos and c-jun. However, the concomitant mAChR-stimulated increase in cytosolic [Ca\textsuperscript{2+}] enhances c-fos expression but is involved in the repression of c-jun. These data suggest that the effects of neurotransmitters or growth factors on AP-1 responsive target genes would differ depending on the relative abilities of the agents to activate protein kinase C and to mobilize intracellular [Ca\textsuperscript{2+}]. Further studies are now in progress to examine the effect of mAChR stimulation and the roles of Ca\textsuperscript{2+} and protein kinase C in the induction of AP-1 responsive target genes in gial cells.

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REFERENCES

11. Kujubu, D. A., Lim, R. W., Varnum, B. C., and Herschman, H.

P. M. McDonough and J. H. Brown, unpublished observations.