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The role of protein kinase C α and ε isozymes in DNA synthesis induced by muscarinic receptors in a glial cell line

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Abstract

Acetylcholine has been shown to induce proliferation of human astrocytoma cells by activating muscarinic receptors, particularly the m3 subtype. In the present study the role of protein kinase C in DNA synthesis induced by carbachol has been investigated. Carbachol-induced [methyl-^{3}H]thymidine incorporation was inhibited by the protein kinase C inhibitors GF 109203X and staurosporine. However, carbachol-induced DNA synthesis was only partially reduced by protein kinase C down-regulation by phorbol 12-myristate 13-acetate (PMA), and maximal concentrations of carbachol and PMA had an additive effect on [methyl-^{3}H]thymidine incorporation. Exposure for 24 h to maximally effective concentrations of carbachol did not induce down-regulation of protein kinase C α, and caused a small but significant down-regulation of protein kinase C ε; cells exposed for 24 h to carbachol were still able to respond with protein kinase C translocation to PMA stimulation. Carbachol caused a significant increase of phorbol ester binding, but did not stimulate protein kinase C α translocation, while it caused a short-lasting translocation of protein kinase C ε; however, protein kinase C ε translocation was not correlated with the time-course of carbachol-induced increase in [methyl-^{3}H]thymidine incorporation. On the other hand, the time-course of translocation/down-regulation of protein kinase C α and protein kinase C ε induced by PMA was in good correlation with the time-course of PMA-induced [methyl-^{3}H]thymidine incorporation. These results suggest that protein kinase C α may not be involved in DNA synthesis induced by muscarinic receptors stimulation in 132-1N1 astrocytoma cells, while protein kinase C ε appears to play a role in the initial exit from G0/G1 phase, though it cannot be considered the major determinant for sustained proliferation.

Keywords: Cell proliferation; Protein kinase C; Astrocytoma cell; Muscarinic receptor

1. Introduction

Astrocytes express receptors for several neurotransmitters which regulate a variety of second messenger systems and ion channels. The family of acetylcholine muscarinic receptors includes five receptor subtypes: the muscarinic m1, m3, and m5 receptors are coupled to guanine-nucleotide-binding-proteins$_q$ (G_q proteins) whose activation induces stimulation of phospholipase C, while the muscarinic m2 and m4 receptors are coupled to G_i proteins which cause inhibition of adenylyl cyclase (Felder, 1995).

Rat cortical astrocytes in primary culture and 132-1N1 astrocytoma cells appear to express three subtypes of muscarinic receptors, m2, m3 and m5, as inferred by identification of their mRNAs (Guizzetti et al., 1996). The observation that stimulation of muscarinic receptors leads to proliferation of both these cell types (Ashkenazi et al., 1989; Guizzetti et al., 1996; Zohar and Salomon, 1992), primarily by activation the m3 subtype (Guizzetti et al., 1996), has suggested that they may play relevant roles during brain development and brain tumor growth (Guizzetti and Costa, 1996). The understanding of the mechanisms leading to cell proliferation in response to acetylcholine may thus be relevant to the areas of developmental neurobiology, as well as neurooncology. Furthermore, the study of muscarinic receptor-mediated cell proliferation may increase knowledge in the general area of mitogenesis associated with the activation of G protein-coupled receptors (Gutkind, 1998; Post and Brown, 1996).

The α subunit of heterotrimeric G proteins belonging to the pertussis toxin-insensitive family G_q, G α_q and G α_{11} coupled to seven-transmembrane-domain receptors, such

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as those for peptide hormones and neurotransmitters, including acetylcholine, has been long thought to play an important role in stimulating cell growth by activating phospholipase C, with the ensuing formation of inositol 1,4,5-trisphosphate (IP_3) and the release of intracellularly stored Ca^{2+}, and of diacylglycerol, which activates various protein kinase C isozymes (Berridge, 1987; Nishizuka, 1995). However, the full picture of the mitogenic signaling via G protein-coupled receptors appears to be more complex (VanBiesen et al., 1996). In particular, receptor-mediated phosphoinositide hydrolysis may not serve, by itself, as a major signaling function for driving quiescent cells into DNA synthesis (Moolenaar, 1991), as activation of phospholipase D is also thought to play a role in the proliferative response of cells to G protein-coupled receptors. Indeed, phosphatidic acid, the product of phosphatidylcholine hydrolysis, can be converted to diacylglycerol, and thus provide an additional sustained stimulation of protein kinase C (Exton, 1997).

Protein kinase C is a family of protein kinases, usually subdivided in three groups: the classical protein consistly of the α, βI, βII and γ isoforms, which are stimulated by calcium, diacylglycerol and phosphatidylserine; the new isoforms (δ, ε, η and μ) which are not regulated by calcium; and the atypical enzymes (ζ and λ) which are not regulated by diacylglycerol (Nishizuka, 1995).

Several lines of evidence indicate that protein kinase C plays a most relevant role in the proliferation of glioma cells (Baltuch and Yong, 1996). For example, phorbol esters are potent mitogens in several glioma cell lines, and protein kinase C inhibitors inhibit their proliferation (Benzil et al., 1992; Bhat, 1989). Furthermore, glioma cells display inherent high levels of protein kinase C (especially the α isozyme) which correlate with their growth rate (Couldwell et al., 1992), and an antisense to protein kinase C α inhibits proliferation of glioma cells (Baltuch et al., 1995).

We had previously found that the non-specific protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) could inhibit carbachol-induced proliferation of astrocytoma cells, suggesting an involvement of protein kinase C in the mitogenic effect

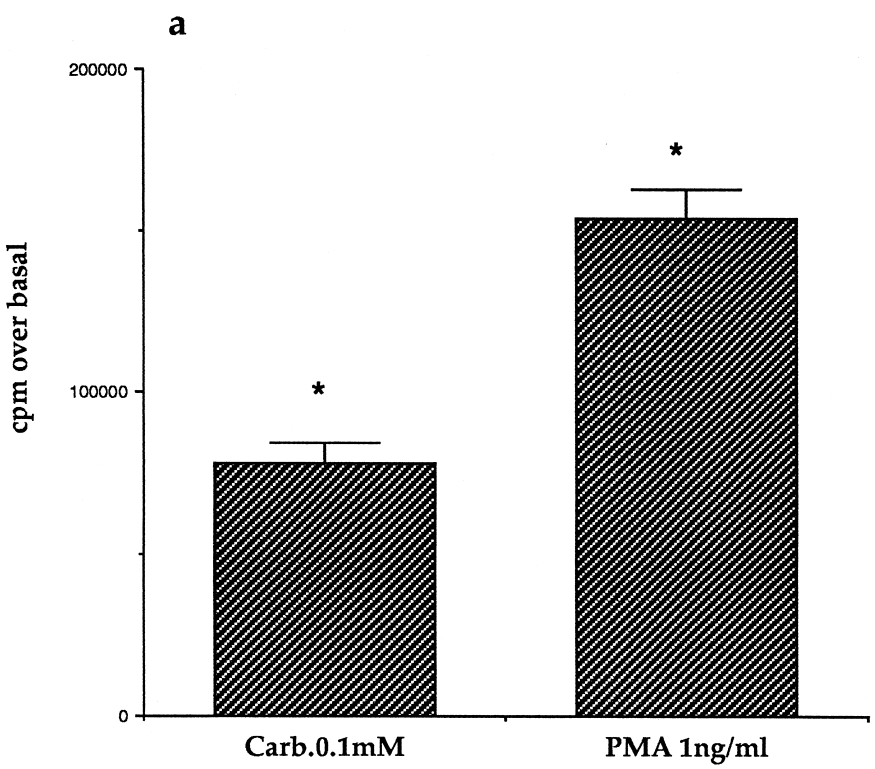

Fig. 1. Mitogenic effect of carbachol in astrocytoma cells. (a) Quiescent cultures of 132-1N1 astrocytoma cells were exposed to carbachol (0.1 mM), or PMA (1 ng/ml) in serum-free medium for 24 h. During the last 6 h of incubation 1 μCi [methyl-^{3}H]thymidine was added as described in Section 2. Data represent the means ± S.E.M. of at least three independent determinations. Basal [methyl-^{3}H]thymidine incorporation was 6539 cpm ± 432. * Significantly different from basal, $P < 0.01$. (b) Representative flow cytometry diagram of cell distribution in the different phases of the cell cycle. Hoechst 33258 (abscissa) and ethidium bromide (ordinate) fluorescence of 132-1N1 astrocytoma cells were analyzed after a 48 h incubation with 5-bromodeoxyuridine in serum-free medium in the absence (upper panel), or in the presence of 1 mM carbachol (bottom panel). Quantification of the number of cells in different phases yielded the following values: Control: 98.6% (±0.30) cells in G0/G1, 0.99% (±0.19) cells in S/G2, 0.39% (±0.14) cells in new G1; Carbachol: 86.14% (±1.44) cells in G0/G1, 12.31% (±1.12) cells in S/G2, 1.56% (±0.35) cells in new G1. These data represent the mean of at least four independent experiments. Data for cells in each phase of the cell cycle are significantly different from control, $P < 0.05$.

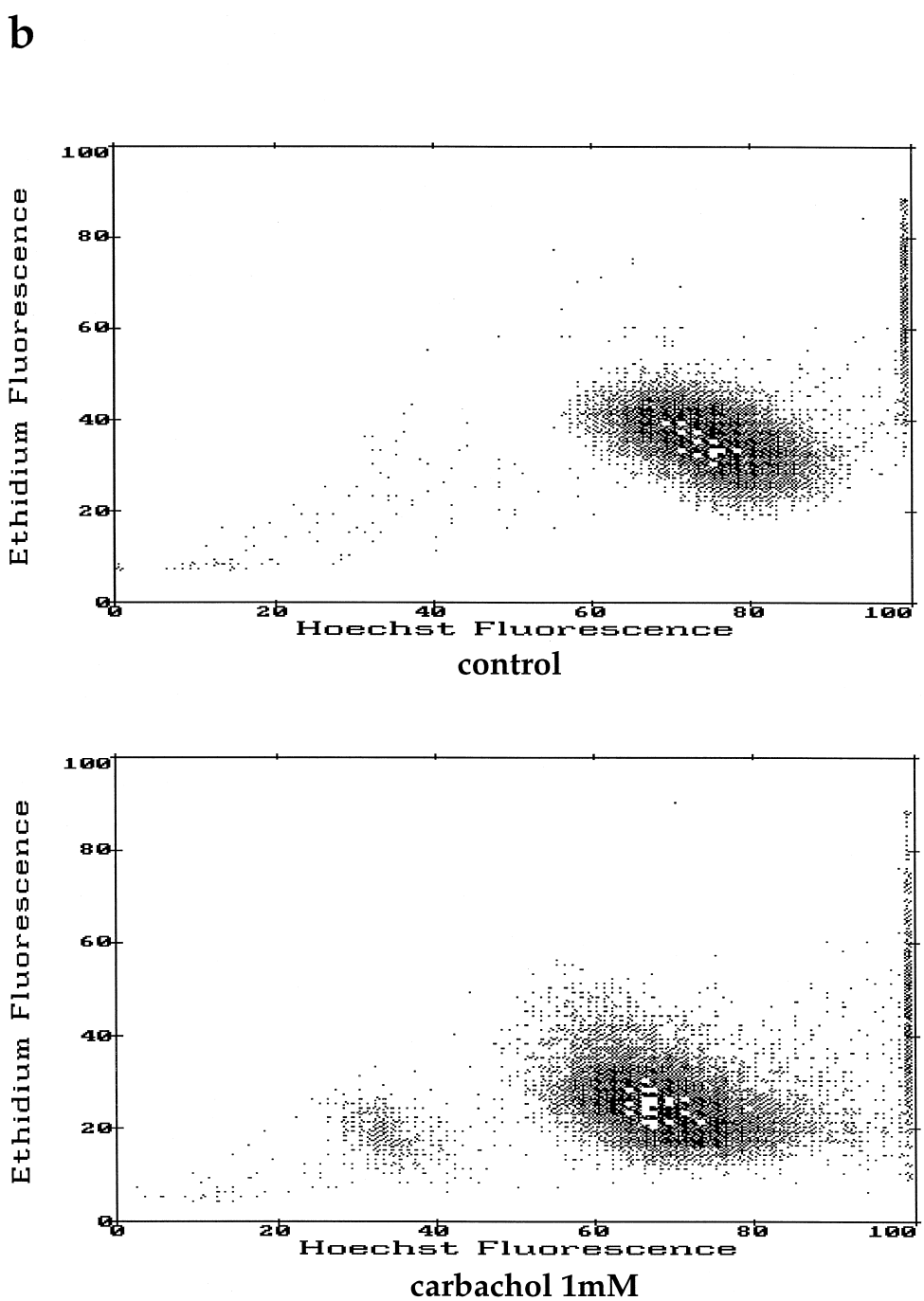

Fig. 1 (continued).

elicited by muscarinic receptor agonists. 132-1N1 astrocytoma cells express the α, ε and ζ protein kinase C isozymes (Post et al., 1996); in these cells, muscarinic receptors have been shown to activate phospholipase C and phospholipase D, to cause Ca^{2+} mobilization from IP_3-sensitive stores, and to activate protein kinase C (Gafni et al., 1997; Martinson et al., 1990; Post et al., 1996).

The goal of the present study was to investigate the role of the two diacylglycerol-sensitive protein kinase C isozymes present in these cells (α and ε) in the mitogenic effect of muscarinic receptor agonists.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum and trypsin were purchased from GIBCO

(Grand Island, NY). [Methyl-^{3}H]thymidine (6.7 Ci/mmol) was from New England Nuclear (Boston, MA, USA). Antibodies to protein kinase C α and protein kinase C ε, and the protease inhibitor cocktail were from Boehringer Mannheim (Indianapolis, IN, USA). [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDB, 10–20 Ci/mmol) and the enhanced chemiluminescence (ECL) detection kit were from Amersham (Arlington, IL, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

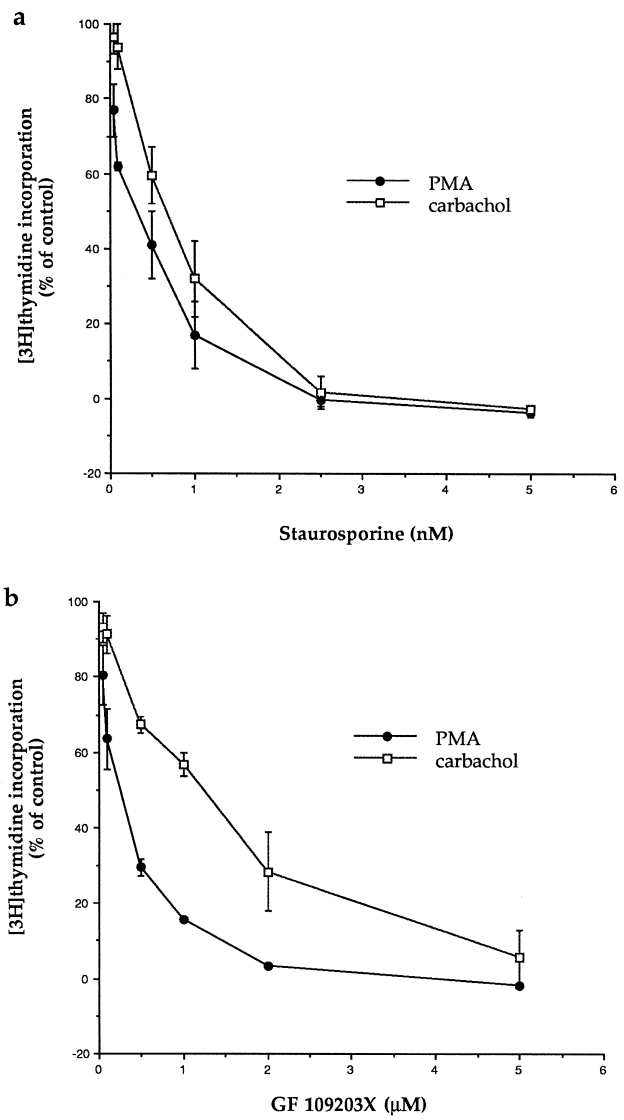

Fig. 2. Inhibition of carbachol- and PMA-induced [methyl-^{3}H]-thymidine incorporation in astrocytoma cells by protein kinase C inhibitors. Dose-dependent inhibition of [methyl-^{3}H]-thymidine incorporation induced by carbachol (0.1 mM) and PMA (1 ng/ml) in 132-1N1 astrocytoma cells by the protein kinase C inhibitors staurosporine (a) and GF 109203X (b). During the last 6 h of incubation 1 μCi [methyl-^{3}H]-thymidine was added as described in Section 2. Data represent the means ± S.E.M. of at least three independent determinations.

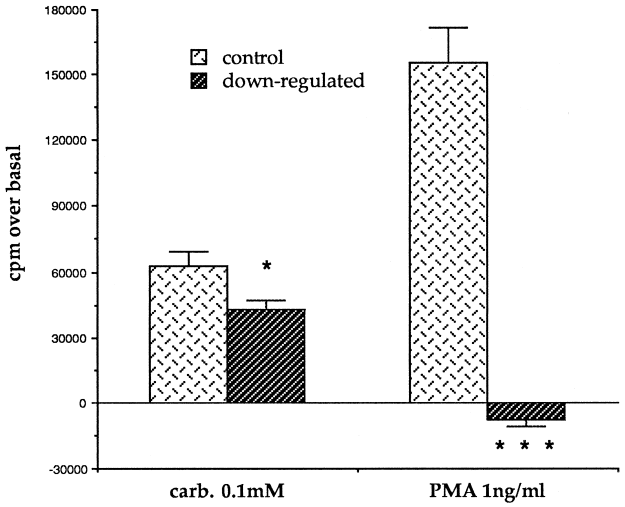

Fig. 3. Effect of protein kinase C down-regulation by PMA on DNA synthesis induced by carbachol, and PMA. Down Regulation of protein kinase C α and ε (see Fig. 6a) was obtained by pre-incubating cells with 185 ng/ml PMA for 24 h. After washing, carbachol (1 mM) or PMA (1 ng/ml) were then added for 24 h. During the last 6 h of incubation 1 μCi of [methyl-^{3}H]thymidine was added as described in Section 2. Data represent the means ± S.E.M. of at least three independent determinations. Basal [methyl-^{3}H]-thymidine incorporation was 6237 cpm ± 650; basal [methyl-^{3}H]-thymidine incorporation after 24 h pretreatment with PMA was 58,751 ± 5,568. * Significantly different from control, $P < 0.05$; * * * significantly different from control, $P < 0.001$.

2.2. Cell culture

The human astrocytoma cell line 132-1N1 (kindly donated by Dr. Joan H. Brown, San Diego, CA, USA) was maintained in DMEM, low glucose, supplemented with 5% fetal bovine serum, 1 U/ml penicillin G and 1 mg/ml streptomycin in 75 cm^2 flasks under a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were subcultured every seven days and the growth medium was changed every 3 or 4 days. For the proliferation experiments, cells were seeded in 24 well plates; for [^{3}H]PDB binding or Western blot experiments cells were seeded in 35 mm or 100 mm dishes, respectively. 48 h before each experiment cells were shifted in the same medium without serum, supplemented with 0.1% bovine serum albumin.

2.3. Proliferation assays

Incorporation of [methyl-^{3}H]thymidine into cell DNA was measured as described previously (Guizzetti et al., 1996). Briefly, 1 μCi/well [methyl-^{3}H]thymidine was included for the last 6 h of the incubation at 37°C under an atmosphere of 5% CO_2/95% air. The monolayer was fixed in methanol and the DNA precipitated with 10% trichloroacetic acid, dissolved in 500 μl of 1 N NaOH and counted in a Beckmann LS 5000 CE scintillation counter.

Flow cytometry analysis was carried out using the 5-bromodeoxyuridine-Hoechst 33258 method as described by Rabinovitch et al., 1988, with slight modifications

(Guizzetti and Costa, 1996). After treatment with the agonist and addition of 150 μM 5-bromodeoxyuridine, cells were incubated for 48 h at 37°C under an atmosphere of 5% CO_2/95% air. At the end of the incubation, cells were harvested by adding 200 μl of 0.05% trypsin. The trypsin was neutralized, and cells were collected and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 500 μl of a solution containing 0.154 M NaCl, 0.1 M Tris (pH 7.4), 0.5 mM $MgCl_2$, 0.2% bovine serum albumin, 0.1% Nonidet-P40 and 5.9 μg/ml 33258 Hoechst; 1.5 μg/ml ethidium bromide was added 15 min before the doubly stained cells were analyzed on a Becton Dickinson FACS Analyzer. Both Hoechst fluorescence and ethidium bromide fluorescence were excited at 375 nm. Hoechst emission was collected at 420–490 nm and ethidium bromide emission at >610 nm. Data were collected and analyzed using the MPLUS AV program (distributed by Phoenix Flow Systems, San Diego, CA, USA).

Cell counting was performed using a Coulter Counter ZM (Coulter Scientific Instruments, Hialeah, FL, USA). Briefly, cells were harvested in 0.05% trypsin, and 250 μl of each sample were diluted in 15 ml ISOTON II (Coulter Diagnostic, Hialeah, FL, USA) and counted.

2.4. [^{3}H]PDB binding to intact cells

Experiments were carried out in 35 mm dishes following the method described by Trilivas and Brown (1989). Briefly, the medium was replaced with a fresh one and carbachol was added at different time-points, while [^{3}H]PDB was added for the last 30 s of incubation at the final concentration of 20 nM. All the binding assays were conducted at 37°C. The assays were terminated by aspirating the binding mixture, rinsing the plates with ice-cold PBS, and solubilizing the cells with 0.5 ml of 1 M NaOH. Samples were counted in a Beckmann LS 5000 CE scintillation counter.

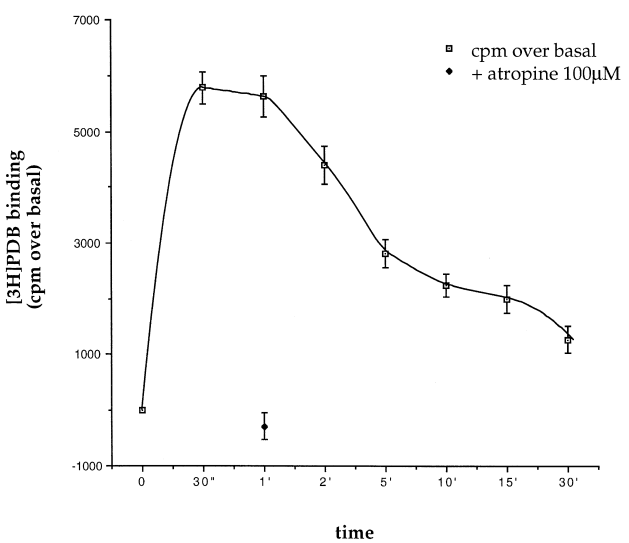

Fig. 4. Time-course of carbachol-induced increase of [^{3}H]PDB binding to intact astrocytoma cells. 132-1N1 astrocytoma cells were incubated with carbachol (1 mM) for different times and the amount of [^{3}H]PDB bound to cells was measured as an index of protein kinase C translocation (Trilivas and Brown, 1989). [^{3}H]PDB (20 nM final concentration) was added for the last 30 s of incubation as described in Section 2. Atropine (10 μM) was added 10 min before the agonist. Data represent the means of at least three independent experiments.

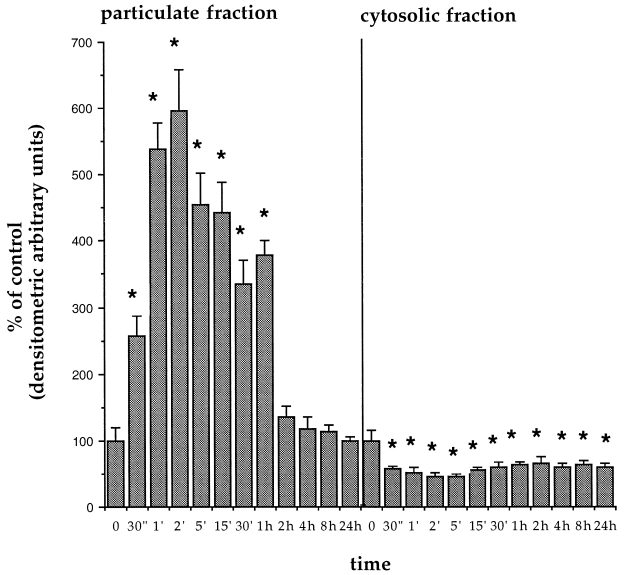

Fig. 5. Time-course of protein kinase C ε translocation induced by carbachol. 132-1N1 astrocytoma cells in serum-free medium were exposed to 1 mM carbachol for different times at 37°C. Cells were broken and the membranes were separated from the cytosol by ultracentrifugation; protein kinase C ε was detected in each fraction by immunoblotting using specific antibodies, and visualized by using ECL detection as described in Section 2. Results are expressed as percent of total optical density in the control after quantification of the relative optical density (O.D.) by densitometer. Data represent the means ± S.E.M. of three independent experiments. * Significantly different from control, $P < 0.05$.

2.5. Western blot analysis

After agonist treatment, cells were scraped in buffer containing Tris/HCl 20 mM (pH 7.4), ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N',-tetraacetic acid (EGTA) 0.5 mM, β-mercaptoethanol 0.1%, and a protease inhibitor cocktail (Boehringer Mannheim). Cells were broken by two cycles of freezing and thawing, and cytosolic and membrane fractions were obtained by sample centrifugation at $100\,000 \times g$. The supernatant was collected as cytosol. The pellet was resuspended in a buffer containing Tris/HCl 20 mM (pH 7.4), EGTA 2 mM, (ethylenedinitrilo)tetraacetic acid (EDTA) 10 mM, Triton-X 0.3%, β-mercaptoethanol 0.1%, and protease inhibitors, shaken at 4°C for 30 min, and centrifuged at $15\,000 \times g$ for 10 min. The supernatant was collected as particulate fraction. Proteins were quantified by the Bradford's method, and a 5× sample buffer was added; 50 μg proteins were loaded on a

8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After separation, proteins were transferred to Immobilion membranes (Millipore, Bedford, MA), which were incubated in the presence of polyclonal antibodies to protein kinase C α or protein kinase C ε (dilution 1:1500) (Boehringer Mannheim) and horseradish peroxidase-conjugated secondary antibody (dilution 1:2000). Bands were revealed by the enhanced chemiluminescence (ECL) kit (Amersham) and densitometrically quantified using a Millipore Image System. Protein molecular weight markers (Bio-Rad) were run with each gel.

2.6. Statistical analysis

One way analysis of variance (ANOVA), followed by the Fisher's Least Significance Difference test was used to determine statistically significant ($P < 0.05$) differences from controls.

3. Results

Carbachol caused a 10- to 13-fold increase in [methyl-^{3}H]thymidine incorporation in 132-1N1 astrocytoma cells, and was also able to induce cell cycle progression, measured by flow cytometry (Fig. 1a, b), thus confirming our previous observations (Guizzetti and Costa, 1996; Guizzetti et al., 1996). Additionally, measurement of the absolute cell number by an electronic cell counter also indicated the ability of carbachol to increase cell proliferation; number of cells ($\times 10^4$) was: control, 5.84 ± 0.29; carbachol 0.1 mM, 6.82 ± 0.14; carbachol 1 mM, 7.67 ± 0.19 ($n = 3$; both treatments were significantly different from controls, $P < 0.01$).

We had previously observed that the broad spectrum protein kinase inhibitor H7 diminished carbachol-induced DNA synthesis (Guizzetti et al., 1996). In the present study we utilized staurosporine, a potent, but not totally specific, protein kinase C inhibitor, and GF 109203X, which is considered a very specific protein kinase C inhibitor (Toullec et al., 1991). Both compounds inhibited [methyl-^{3}H]thymidine incorporation induced by carbachol with IC_{50}s of 0.84 nM (± 0.15) and 1.67 μM (± 0.12), respectively; both compounds were slightly more potent in inhibiting [methyl-^{3}H]thymidine incorporation induced by the phorbol ester PMA (phorbol 12-myristate 13-acetate), with IC_{50}s of 0.38 nM (± 0.01) and 398 nM (± 13),

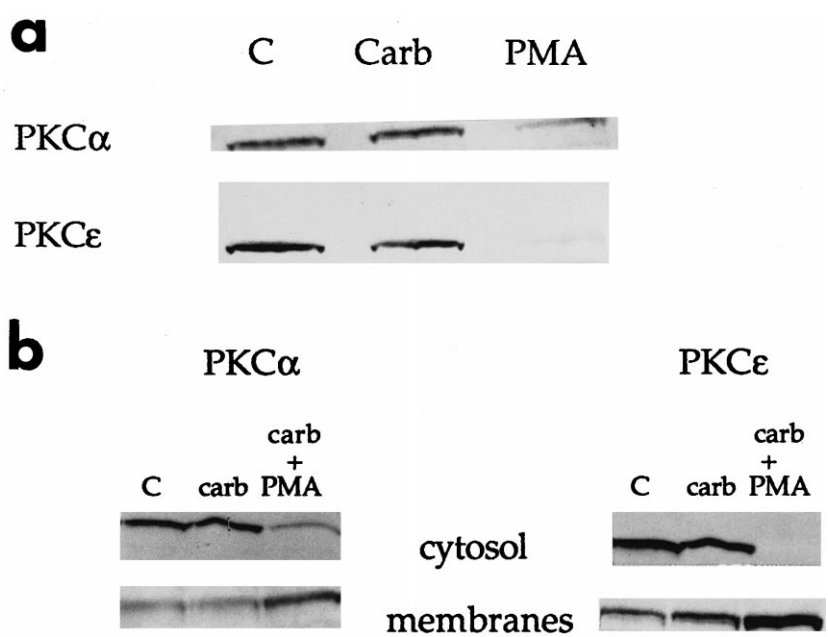

Fig. 6. Effect of carbachol and PMA on protein kinase C α and ε levels and functionality. (a) 132-1N1 astrocytoma cells were exposed to 185 ng/ml PMA or 10 mM carbachol for 24 h, after which cells were broken and the levels of protein kinase C α and protein kinase C ε were measured by Western Blotting. The following results expressed as percent of total optical density in the control were obtained by quantification of the bands' relative optical density (O.D.) by densitometer. Protein kinase C α: Control: 100% ± 12.1; Carb: 100.35% ± 10.45; PMA: 38.3% ± 4.3 (**); protein kinase C ε: Control: 100% ± 9.5; Carb: 70.02% ± 12.2 (*); PMA: 20.98% ± 4.8 (**). Data represent the means ± S.E.M. of three independent experiments. *Significantly different from control, $P < 0.05$; **significantly different from control, $P < 0.01$. The apparent molecular weight of protein kinase C α and protein kinase C ε were 80 and 92 kDa respectively. Molecular mass markers run with each gel were: phosphorilase B (97.4 kDa), bovine albumine (66 kDa) and ovalbumin (45 kDa). (b) 132-1N1 astrocytoma cells were exposed to 10 mM carbachol for 24 h and then stimulated by 100 ng/ml PMA for 20 min. The membranes and the cytosol were separated by centrifugation; experimental conditions are as described above. Densitometric analysis of the protein kinase C bands yielded the following distribution in cytosolic (c) and particulate (m) compartments. protein kinase C α: Control: c 67.3%/m 32.7%; Carbachol: c 63.3%/m 36.7%; Carb + PMA: c 21.7%/m 78.2%; protein kinase C ε: Control: c 89.2%/m 20.8%; Carbachol: c 65.1%/m 34.9%; Carb + PMA: c 1.6%/m 98.3%. This is one representative experiment out of three which gave similar results.

respectively (Fig. 2a, b). It should be noted that staurosporine, at concentrations higher than 0.5 nM, significantly reduced [methyl-^{3}H]thymidine incorporation in unstimulated cells in a dose-dependent manner, suggesting a cytotoxic effect; similarly, GF 109203X inhibited basal incorporation at concentrations of 2 μM and higher (not shown).

A 24 h treatment with PMA (185 ng/ml) caused down-regulation of protein kinase C α and ε (Fig. 6a); this treatment totally abolished the mitogenic response of cells to PMA, while it reduced the effect of carbachol by only 30% (Fig. 3). Furthermore, maximal effective doses of PMA (100 ng/ml) and carbachol (1 mM; Guizzetti et al., 1996) in combination, had an additive, or more than additive, effect on DNA synthesis, as compared to the each compound alone. PMA increased [methyl-^{3}H]thymidine incorporation by 2644 ± 248% of basal, carbachol by 1254 ± 85% of basal and their combination by 4323 ± 305% of basal ($n = 3$; $P < 0.01$ vs. each compound alone).

Altogether, these results suggest that while the conventional protein kinase C α and the novel protein kinase C ε play a central and pivotal role in PMA-induced [methyl-^{3}H]thymidine incorporation, other protein kinases C, neither activated nor down-regulated by PMA, and/or protein kinase C-independent pathways, may be involved in carbachol-induced DNA synthesis in 132-1N1 astrocytoma cells.

To further investigate the carbachol/protein kinase C interaction, we directly measured the ability of carbachol and PMA to activate protein kinase C. Carbachol (1 mM) induced a rapid and transient increase of [^{3}H]PDB binding, which peaked between 0.5 and 1 min and decreased after 2 min, though it was still elevated above control after 30 min (Fig. 4); the effect of carbachol was blocked by preincubation with 10 μM atropine. Carbachol, in our conditions, did not induce protein kinase C α translocation (not shown). On the other hand, membrane-associated immunoreactivity of protein kinase C ε increased after carbachol stimulation, reaching a peak after 2 min (about a 6-fold increase), and returned to basal level after 2 h (Fig. 5). It is important to note that the buffer used for separating cytosol from membranes contains a low concentration (0.5 mM) of EGTA, in order to reduce protein degradation by Ca^{2+}-dependent proteases. Thus, an earlier but weaker and Ca^{2+}-dependent interaction of protein kinase C α with the plasma membrane may been occurred; however, in similar conditions, bombesin was able to induce Ca^{2+}-mediated protein kinase C α translocation (Florin et al., 1996).

A 24 h treatment with 10 mM carbachol did not cause down-regulation of protein kinase C α, while it reduced the total amount of protein kinase C ε by 30% (Fig. 6a); however, this treatment did not affect the functionality of either isozyme, since cells were still able to respond to PMA with protein kinase C α and protein kinase C ε translocation (Fig. 6b). On the other hand, PMA (100 ng/ml) caused a robust increase in membrane-associated immunoreactivity of both protein kinase C isozymes, which peaked between 30 min and 2 h (protein kinase C α) (Fig. 7a), and between 5 min and 1 h (protein kinase C ε) (Fig. 7b), after which both proteins started to be down-regulated.

A time-course of carbachol- and PMA-induced [methyl-^{3}H]thymidine incorporation (Fig. 8) was also carried out, and compared to the time-course of protein kinase C

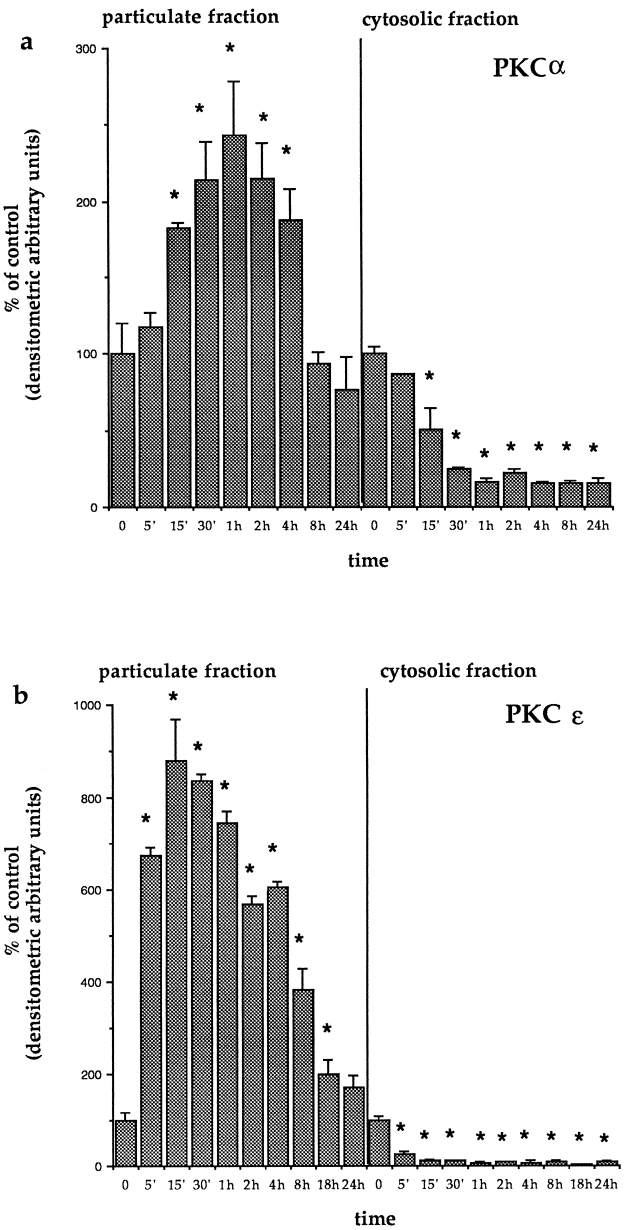

Fig. 7. Time-course of translocation of protein kinase C α and protein kinase C ε induced by PMA. Cultures of 132-1N1 astrocytoma cells in serum-free medium were exposed to 10 ng/ml PMA for different times at 37°C. Cells were broken and the membranes were separated from the cytosol by ultracentrifugation, and protein kinase C α (a) and ε (b) were detected as in Fig. 6. Results are expressed as percent of total optical density in the control after quantification of the relative optical density (O.D.) by densitometer. Data represent the means ± S.E.M. of three independent experiments. * Significantly different from control, $P < 0.01$.

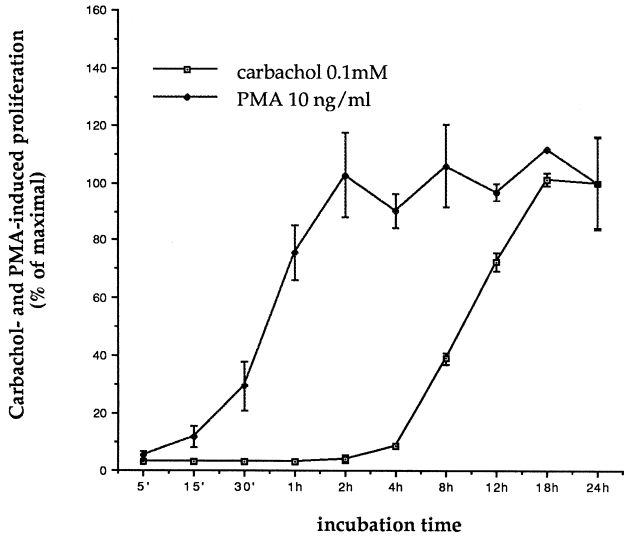

Fig. 8. Time-course of carbachol- and PMA-induced [methyl-^{3}H]thymidine incorporation in astrocytoma cells. [Methyl-^{3}H]-thymidine incorporation was measured following incubation with 10 ng/ml PMA or 0.1 mM carbachol for the indicated length of time. Mitogens were then removed and the incubation was carried out up to 24 h. [Methyl-^{3}H]thymidine was added 6 h before the end of the incubation. Data represent the means ± S.E.M. of at least three independent determinations.

activation. The experiments were performed by incubating cells with agonists for the indicated length of time; the medium was then removed, and cells were washed twice with PBS. A fresh medium was added and the incubation continued up to 24 h. A 5 min incubation with carbachol induced a significant but small (2-fold of basal) increase in [methyl-^{3}H]thymidine incorporation which was constant during the first 4 h incubation. DNA synthesis increased strongly in a time-dependent manner between 4 and 18 h of carbachol incubation, when a maximum plateau was reached. On the other hand, PMA-induced [methyl-^{3}H]thymidine incorporation increased rapidly reaching a plateau after 2 h (Fig. 8). These data suggest that PMA-, but not carbachol-induced DNA synthesis, is temporally correlated with either protein kinase C α or ε activation.

4. Discussion

Activation of acetylcholine muscarinic receptors has been found to elicit a mitogenic response in various cell types. Among others, acetylcholine has been reported to be a mitogen in rat astrocytes and the 132-1N1 human astrocytoma cell line (Ashkenazi et al., 1989; Guizzetti and Costa, 1996; Guizzetti et al., 1996; Zohar and Salomon, 1992), oligodendrocyte progenitors (Cohen et al., 1996), the human prostate cancer cell lines LnCaP, DU145, and PC3 (Luthin et al., 1997; Rayford et al., 1997), the human microcytoma cell line H69 (Fucile et al., 1997), in muscarinic m1 (but not m2) receptor-transfected NIH3T3 cells (Stephens et al., 1993), and in muscarinic m1 and m3, but not m2 and m4 receptor-transfected Chinese hamster ovary (CHO) cell line (Ashkenazi et al., 1989). Furthermore, muscarinic m1, m3 (Gutkind et al., 1991) and m5 receptors (Mattingly et al., 1994), but not m2 and m4 receptors (Gutkind et al., 1991), transfected into NIH3T3 fibroblasts, were able to induce transformation in these cells. On the other hand, other investigators have reported that carbachol is not a mitogen in astrocytoma cells (Brown et al., 1997; Post et al., 1996), and that the Chinese hamster lung fibroblast CCL39 cell line, transfected with the muscarinic m1 receptor, did not proliferate upon carbachol exposure (Seuwen et al., 1988).

In the present study, we confirmed the mitogenic effect of carbachol in human astrocytoma cells, by using three different methods to verify our previous observations. In our experimental conditions, carbachol increased [methyl-^{3}H]thymidine incorporation, caused quiescent cells to reenter the cell cycle, as shown by flow cytometry analysis, and increased total number of cells as evidenced by direct electronic cell counting.

The principal aim of this study was to investigate the role of protein kinase C isoforms activated by Ca^{2+} and/or diacylglycerol, (protein kinase C α and ε in astrocytoma cells), in carbachol-induced DNA synthesis. The protein kinase C inhibitors GF 109203X and staurosporine inhibited both carbachol- and PMA-induced [methyl-^{3}H]thymidine incorporation in a concentration-dependent manner, thus, confirming our previous observation with H7 (Guizzetti et al., 1996). Staurosporine is a potent inhibitor of protein kinases with some selectivity for protein kinase C, while GF 109203X is considered a very selective protein kinase C inhibitor (Toullec et al., 1991), though it was recently reported that this compound inhibits the activity of two other kinases with slightly lower potency (Alessi, 1997). It is important to notice that these compounds have been used indistinctly for inhibiting conventional and novel, but also atypical protein kinase C isozymes (Fagerstrom et al., 1996; Xu and Clark, 1997), since they act on the binding site for ATP (Toullec et al., 1991).

To further evaluate the role of protein kinase C α and ε in carbachol-induced proliferation of astrocytoma cells, we measured the ability of this muscarinic agonist to induce [methyl-^{3}H]thymidine incorporation after a 24 h exposure to 185 ng/ml PMA, which depleted cells of both isozymes. We had previously reported that under this conditions, the mitogenic effect of carbachol was significantly inhibited (Guizzetti et al., 1996). However, a careful reanalysis of those data, as well as additional experiments presented here, indicate that while the mitogenic effect of PMA is completely blocked by protein kinase C down-regulation, carbachol is still capable of inducing a significant, though diminished, proliferative response. We also observed that maximally effective concentrations of carbachol and PMA had an additive effect, suggesting that the two mitogens do not act exclusively through a common pathway.

Carbachol caused a rapid increase in [^{3}H]PDB binding, which was mostly due to an increased translocation of protein kinase C ε rather than protein kinase C α. However, unlike PMA, prolonged stimulation with maximal concentrations of carbachol did not increase the rate of protein kinase C α degradation, and caused a small reduction in the levels of protein kinase C ε (down-regulation). Moreover, both protein kinase C α and C ε were able to respond with translocation to heterologous stimulation (by PMA) following protracted carbachol exposure, suggesting that carbachol did not cause desensitization of protein kinase C. These data differ in part from those of Post et al. (1996) who reported a rapid and short-lasting translocation of protein kinase C α in the same cell line following stimulation with carbachol. The reason for such difference is not clear, but may be related to the presence of EGTA in the buffer used in this study for separating cytosol from membranes or, more likely, to the experimental protocol of cell cultures. Indeed, Post et al. (1996) observed only a very small mitogenic effect of carbachol, a result that we duplicated utilizing their exact same experimental conditions (data not shown).

Differently from carbachol, PMA caused a strong translocation of both protein kinase C α and ε. Furthermore, in case of PMA, the kinetic of protein kinase C α and ε activation/down-regulation correlated with the kinetic of PMA-induced [methyl-^{3}H]thymidine incorporation (which was very rapid, as expected for an agonist acting totally throughout protein kinase C). On the other hand, in addition to the lack of protein kinase C α activation, maximal protein kinase C ε activation by carbachol occurred within the first few min and lasted for about one hour, a period during which the effect of this agonist on [methyl-^{3}H]thymidine incorporation was minimal. Thus, activation of protein kinase C ε may not be sufficient for the full mitogenic action of carbachol to be manifest, though it could be involved in the initial DNA synthesis, and may be important in leading the cells to reenter the cell cycle, as suggested by the small, but consistent and significant, increase in [methyl-^{3}H]thymidine incorporation during the first hours of incubation in the presence of the muscarinic receptor agonist, and by the decrease in carbachol-induced [methyl-^{3}H]thymidine incorporation after protein kinase C α and ε depletion.

Protein kinase C α and ε are the only Ca^{2+}- and diacylglycerol-dependent, and Ca^{2+}-independent but diacylglycerol-dependent, protein kinases C, respectively, expressed in 132-1N1 astrocytoma cells (Post et al., 1996). Muscarinic receptor agonists activate phospholipase C in these cells leading to increases of IP_3, diacylglycerol and Ca^{2+} levels (Martinson et al., 1990; Trilivas and Brown, 1989; Catlin and Costa, unpublished). However, our results indicate that these two protein kinases C may not the major determinants of sustained proliferation of astrocytoma cells induced by activation of muscarinic receptors. The only other protein kinase C expressed in these cells is the Ca^{2+}- and diacylglycerol-independent protein kinase C ζ, which is activated by different mechanisms, and whose role in muscarinic receptor-induced proliferation is currently being investigated in our laboratory.

The present results are in agreement with observations by others; indeed, several investigators have shown that conventional and/or novel protein kinases C do not always play a main or unique role in cell proliferation induced by G-protein-coupled receptors. For example, it has been shown that phospholipase C-activated protein kinase C is not completely responsible for proliferation of rat astrocytes induced by insulin-like Growth Factor I (IGF-I) (Tranque et al., 1992), of Swiss 3T3 fibroblasts by bombesin (Florin et al., 1996), of osteoblast-like MC3T3-E1 cells by thrombin (Suzuki et al., 1996), or of NIH 3T3 cells transfected with muscarinic m1 receptor (Stephens et al., 1993). The observation that the diacylglycerol-dependent and Ca^{2+}-independent protein kinase C ε seems to be activated by carbachol more that the Ca^{2+}- and diacylglycerol-dependent protein kinase C α, suggests that the pathway activated by phospholipase D may be more important than that activated by phospholipase C. Many hormones stimulate the phospholipase D-catalyzed hydrolysis of phosphatidylcholine, which in several systems appears to be the major source of diacylglycerol without increasing Ca^{2+} levels (Exton, 1997), and in 132-1N1 astrocytoma cells, it has been indeed demonstrated that phospholipase D is the major pathway for the generation of diacylglycerol following muscarinic receptor stimulation (Martinson et al., 1990).

In conclusion, our data suggest that the 'classical' Ca^{2+}- and diacylglycerol-dependent protein kinase C α is not activated by the muscarinic agonist carbachol in human astrocytoma cells, while the Ca^{2+}-independent and diacylglycerol-dependent protein kinase C ε is activated only during the first hour of exposure. The results from down-regulation experiments support a small contribution of protein kinase C ε to carbachol-induced DNA synthesis in 132-1N1 astrocytoma cells, while results obtained with isozyme-nonspecific protein kinase C inhibitors suggest that the other protein kinase C isozyme present in this cell line, protein kinase C ζ, may play a relevant role in leading cells to reenter the cell cycle. There are in fact other instances when cellular effects have been antagonized by GF 109203X, but not by down-regulation of diacylglycerol-dependent protein kinases C by PMA (Fagerstrom et al., 1996; Xu and Clark, 1997). Furthermore, as GF 109203X has also been shown to inhibit MAPKAP-K1b (mitogen-activated protein kinase-activated protein kinase-1b) and p70 S6 kinase (Alessi, 1997), other intracellular pathways may also be involved in the mitogenic effect of muscarinic agonists. An alternative hypothesis is that activation of muscarinic m2 receptors, also present in astrocytoma cells (Guizzetti et al., 1996) may be also involved in the mitogenic action of acetylcholine by virtue of a G_i-initiated cascade of second messengers,

including mitogen-activated protein kinases (Johnson et al., 1994). Though our previous data suggest that muscarinic m3, rather than m2, receptors are primarily involved in the mitogenic action of acetylcholine, this possibility also deserves further investigations.

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