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Possible Role of Protein Kinase C ζ in Muscarinic Receptor-Induced Proliferation of Astrocytoma Cells

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ABSTRACT. Recent studies have shown that protein kinase C ζ (PKC ζ) is part of a pathway that plays a key role in a wide range of physiological processes including mitogenesis, cell survival, and transcriptional regulation. Most studies on PKC ζ have been done by stimulating cells with tyrosine kinase receptor agonists, or by transfecting the cells with either constitutively active PKC ζ or negative mutants of PKC ζ. Less is known about the ability of endogenous G-protein-coupled receptors to generate a mitogenic signal through activation of endogenous PKC ζ. In the present paper, we showed that in 123–1N1 human astrocytoma cells, which express the G-protein-coupled M2, M3, and M5 muscarinic receptors, PKC ζ is activated by carbachol in a concentration-dependent manner, resulting in the translocation of PKC ζ from the cytoplasm to granules in the perinuclear region. The effect of carbachol was long-lasting (up to 24 hr) and appeared to be mediated by activation of M3 muscarinic receptors. A selective PKC ζ inhibitor peptide (peptide Z) inhibited PKC ζ translocation as well as carbachol-induced DNA synthesis. Inhibition of both phosphatidylinositol 3-kinase and phospholipase D decreased carbachol-induced [^{3}H]thymidine incorporation and blocked carbachol-induced PKC ζ translocation, suggesting an involvement of both pathways in these effects. BIOCHEM PHARMACOL **60**;10: 1457–1466, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. muscarinic receptor; protein kinase C ζ; cell proliferation; 132–1N1 astrocytoma cells

Several G-protein-coupled receptors, such as those for thrombin, acetylcholine, serotonin, bombesin, bradykinin, endothelin, and adrenaline, have been shown to display mitogenic and/or transforming activity in various cell lines [1, 2], but the intracellular events involved in G-protein-coupled receptor-induced proliferation are not understood fully. The mAChR§ family consists of five genetically defined subtypes, which preferentially couple to $G\alpha_q/G\alpha_{11}$ (M1, M3, and M5 muscarinic receptors) and to $G\alpha_i$ /$G\alpha_o$ (M2 and M4 muscarinic receptors) [3, 4]. A variety of cells expressing mAChRs proliferate in response to stimulation of muscarinic receptors [5–10]. Moreover, studies performed with NIH 3T3 cells transfected with different subtypes of mAChRs have confirmed that M1, M3, and M5 muscarinic receptors induce cell proliferation and transformation, whereas the M2 and M4 receptors do not [11].

We have shown previously that rat astrocytes and 132–1N1 human astrocytoma cells express M2, M3, and, to a lesser extent, M5 receptors [8]. Activation of muscarinic receptors induces DNA synthesis in both of these cell types, and this effect is mediated mostly by the activation of M3 receptors [8]. Studies carried out in 132–1N1 astrocytoma cells have shown that carbachol induces PI hydrolysis, which generates DAG, and increases intracellular Ca^{2+} [12, 13]. However, Ca^{2+}-dependent and/or DAG-dependent PKCs do not appear to be major determinants for the proliferation induced by carbachol in astrocytoma cells [14]. The 132–1N1 astrocytoma cells express the conventional PKC α (activated by Ca^{2+}, DAG, and phospholipids), the novel PKC ε (activated by DAG and phospholipids, but lacking the putative Ca^{2+}-binding domain), and the atypical PKC ζ (activated by phospholipids, but insensitive to Ca^{2+}, DAG, and phorbol esters) [12].

PKC ζ activates several signaling pathways, such as the transcriptional factor NF-κB [15–18], mitogen-activated protein kinase [19, 20], and the ribosomal translational factor p70 S6 kinase [21]. PKC ζ has been shown to mediate several cellular functions, such as glucose transport induced by insulin in L6 myotubes [22], carbachol-stimulated insulin secretion in RINm5F cells [23], adhesion and chemotaxis in polymorphonuclear neutrophils [24], and vascular endothelial growth factor expression [25]. PKC ζ is also involved in antiapoptotic signals in COS, HeLa, and NIH 3T3 cells [16, 25–27], in the maturation of *Xenopus laevis* oocytes [28], in the mitogenic activation of *X. laevis* oocytes

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§ *Abbreviations*: mAChR, muscarinic acetylcholine receptor; DAG, diacylglycerol; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_3, phosphatidylinositol 3,4,5-P_3; PDK-1, phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; PMA, phorbol 12-myristate 13-acetate; and PTX, pertussis toxin.

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and NIH 3T3 fibroblasts [29], and in the proliferation of endothelial cells induced by vascular endothelial growth factor [30]; furthermore, PKC ζ activation is observed in airway smooth muscle cells during proliferation [31]. Nevertheless, the role of this PKC isoform in cell growth control has been questioned [32, 33].

The mechanisms of activation and regulation of PKC ζ are not understood fully. It has been proposed that the product of PLD activity, PA, may activate PKC ζ [34]. Also, PI 3-kinase has been shown to play an important role in PKC ζ activation: the PI 3-kinase lipid product PIP_3 stimulates autophosphorylation of PKC ζ [35], and PDK-1 phosphorylates threonine 410 in the PKC ζ activation loop [36].

The current study was undertaken to examine whether carbachol activates PKC ζ in 132–1N1 astrocytoma cells and whether this activation may be involved in the mitogenic effect of this muscarinic agonist. The hypothesis that activation of PKC ζ may occur through activation of PLD and subsequent generation of PA, and/or via activation of PI 3-kinase, also was addressed.

MATERIALS AND METHODS

Materials

Myristoylated peptides corresponding to the pseudosubstrate regions of PKC ζ (peptide Z: positions 116–124; sequence: myrRRGARRWRK) and PKC α (peptide A: positions 22–30; sequence: myrRKGALRQKN) were custom-synthesized by United Biochemical Research, Inc. Gallamine, 4-DAMP, and PTX were purchased from RBI. DMEM, fetal bovine serum, and trypsin were purchased from GIBCO. [*Methyl*-^{3}H]thymidine (6.7 Ci/mmol) was obtained from New England Nuclear. Antibodies to PKC α, ε, and ζ, and the protease inhibitor mixture, were obtained from Boehringer Mannheim. The enhanced chemiluminescence (ECL) detection kit was purchased from the Amersham Corp. The phospho-Akt (Ser473) polyclonal antibody was obtained from New England Biolabs. All other chemicals were purchased from the Sigma Chemical Co.

Cell culture

The human astrocytoma cell line 132–1N1 (donated by Dr. Joan H. Brown) was maintained in DMEM, low glucose, supplemented with 5% fetal bovine serum, 100 U/mL of penicillin G, and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°. For [^{3}H]thymidine experiments, cells were reseeded in 24-well plates (5×10^4 cells/well); for immunocytochemistry experiments, cells were reseeded in two-well coverslips (10^5 cells/well); and for immunoblot experiments, cells were reseeded in 100 mm dishes (2×10^6 cells/dish). The 132–1N1 astrocytoma cells were maintained for 4 days in the complete medium, and then the cells were shifted to the same medium without serum, supplemented with 0.1% bovine serum albumin, for 48 hr. Cells were 60–80% confluent at the beginning of every experiment.

[^{3}H]Thymidine Incorporation Assay

Incorporation of [*methyl*-^{3}H]thymidine into cellular DNA was measured as an index of DNA synthesis, as previously described [8]. In some experiments, a 45-min preincubation in the presence of peptides A or Z was carried out before the 24-hr treatment in the presence of agonists. The monolayer was fixed in methanol, and the DNA was precipitated with 10% trichloroacetic acid, dissolved in 500 μL of 1 M NaOH, and counted in a Beckman LS 5000 CE scintillation counter.

Immunocytochemistry

After carbachol treatments for 30 min, cells were fixed in 4% paraformaldehyde and permeabilized with methanol. The polyclonal antibody to PKC ζ (1:400 dilution) was added overnight at 4°. Fixed cells were then incubated in the dark with an FITC-conjugated IgG for 1 hr and 0.1% Hoechst for 15 min. Stained cells were analyzed by an attached-cell analysis and sorter (ACAS) Ultima instrument (Meridian Instruments). Using a 60X objective, ten 1.5 μm thick sections were analyzed for each field. The field was excited at 488 nm, and emission was recorded between 500 and 560 nm to detect the fluorescence from FITC bound to PKC ζ. The same field and sections were scanned with the Hoechst excitation wavelength of 351–362 nm and emission wavelength in the range of 420–500 nm, to determine the locations of the nuclei. In the data presented here, the sections corresponding to the middle of the nuclei (maximum of Hoechst fluorescence) were compared for their distribution of PKC ζ.

Subcellular Fractionation

After agonist treatment, cells were scraped in sonication buffer [20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 10 mM EDTA, 0.1% β-mercaptoethanol, and a protease inhibitor mixture]. Cells were sonicated in five 10-sec bursts with a W-220 cell disruptor (Heat Systems Ultrasonics, Inc.) at power setting 3. After 5 min of centrifugation at 1000 *g*, the supernatant was centrifuged at 100,000 *g* for 20 min. The supernatant was collected as cytosol. The membrane pellet was resuspended in the sonication buffer containing 1% SDS and shaken at 4° for 30 min.

Western Blot Analyses

Proteins were quantified by the Bradford method, and a 5× sample buffer was added; 50 μg protein was loaded on an 8% SDS–PAGE. Protein transfer, detection, and quantification were performed as previously described [14].

Assay for AKT/PKB Activation

After agonist treatment, cells were scraped in buffer containing 50 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.8% IGEPAL CA-630, 0.1% SDS, 0.1% Triton-X 100, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, and a protease inhibitor mixture. After protein separation by electrophoresis and protein transfer, Akt activation was evaluated by observing changes in the phosphorylation of serine 473 using a phospho-Akt (Ser473) polyclonal antibody.

Statistical Analysis

One-way ANOVA followed by the Fisher's test was used to determine statistically significant ($P < 0.05$) differences from controls.

RESULTS

PKC ζ immunoreactivity was measured by western blotting. A band corresponding to molecular weight 76,000 was identified as PKC ζ, since it disappeared when the polyclonal antibody was incubated in the presence of the peptide used to generate the antibody. It has been reported previously that the polyclonal antibody to PKC ζ cross-reacts with PKC α [37]. We found that PKC α runs separately from PKC ζ in an 8% SDS–PAGE gel, with an apparent molecular weight of 81,000, as previously reported [38]. Furthermore, the affinity of the polyclonal antibody against PKC ζ that we used was higher for PKC ζ than for PKC α, and, therefore, the band corresponding to PKC α was visible only in overexposed films; also, in our hands, PKC α does not translocate following carbachol exposure [14], and PKC ζ does not translocate following exposure to PMA (not shown). The third kind of PKC expressed in these cells, PKC ε, is not detected by this antibody in our experience, and it is bigger than PKC ζ (molecular weight 90,000). PKC ζ distribution between cytosolic and particulate fractions in resting and carbachol-stimulated 132–1N1 astrocytoma cells was measured by western blotting. An increase in membrane-bound and a decrease in soluble PKC ζ was observed after a 30-min incubation with 1 mM carbachol; this effect was antagonized by the nonselective muscarinic antagonist atropine (10 μM) and by the M3 muscarinic antagonist 4-DAMP (500 nM), but not by PTX (100 nM), which ADP-ribosylates G_i proteins, or by the M2 muscarinic antagonist gallamine (500 nM), suggesting M3 receptor involvement in PKC ζ translocation by carbachol (Fig. 1). As expected, PMA was ineffective in translocating PKC ζ (not shown), as previously reported [23].

Concentration- and time-dependent effects of carbachol on PKC ζ translocation are shown in Fig. 2; after a 30 min exposure, maximal translocation of PKC ζ occurred at a carbachol concentration of 1 mM. Time–course experiments indicated that short-term (<30 min) incubation

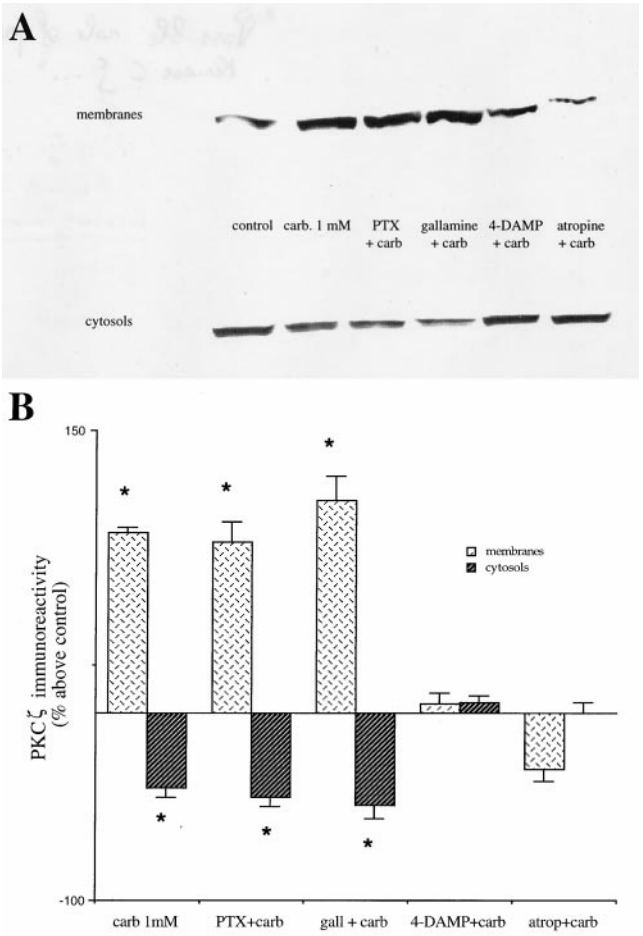

FIG. 1. Translocation of PKC ζ to cell membranes following muscarinic receptor stimulation. 132–1N1 Human astrocytoma cells were stimulated for 30 min with 1 mM carbachol alone or in the presence of atropine (10 μM), 4-DAMP (500 nM), gallamine (500 nM), or PTX (100 nM). (A) Protein immunoblots of cytosol and particulate fractions separated by ultracentrifugation at 100,000 *g* and probed with anti-PKC ζ antibody. (B) Quantification of relative band density. Values are the means ± SEM of three independent experiments. Key: (*) $P <$ 0.05 vs control.

with carbachol had no effect on PKC ζ translocation (not shown). Carbachol caused a long-lasting (30 min–24 hr) increase in membrane-associated PKC ζ (Fig. 2). Interestingly, whereas cytosolic PKC ζ was decreased after 30-min and 1 hr incubations with carbachol, at later time points (2–24 hr) this was not the case, suggesting that the total PKC ζ content increased during incubation. When the level of PKC ζ was measured in whole cells after incubation for 24 hr with 10 mM carbachol, a significant increase in PKC ζ immunoreactivity was observed (not shown).

After a 48-hr serum deprivation, as previously observed by flow cytometry, 98% of astrocytoma cells are in G_0/G_1 phase (quiescent cells) [39]. Quiescent 132–1N1 astrocytoma cells observed with a phase-contrast microscope appeared long and thin; the nuclei were located in the middle of the cells, which were, therefore, thicker in the center; carbachol treatment did not change cell morphol-

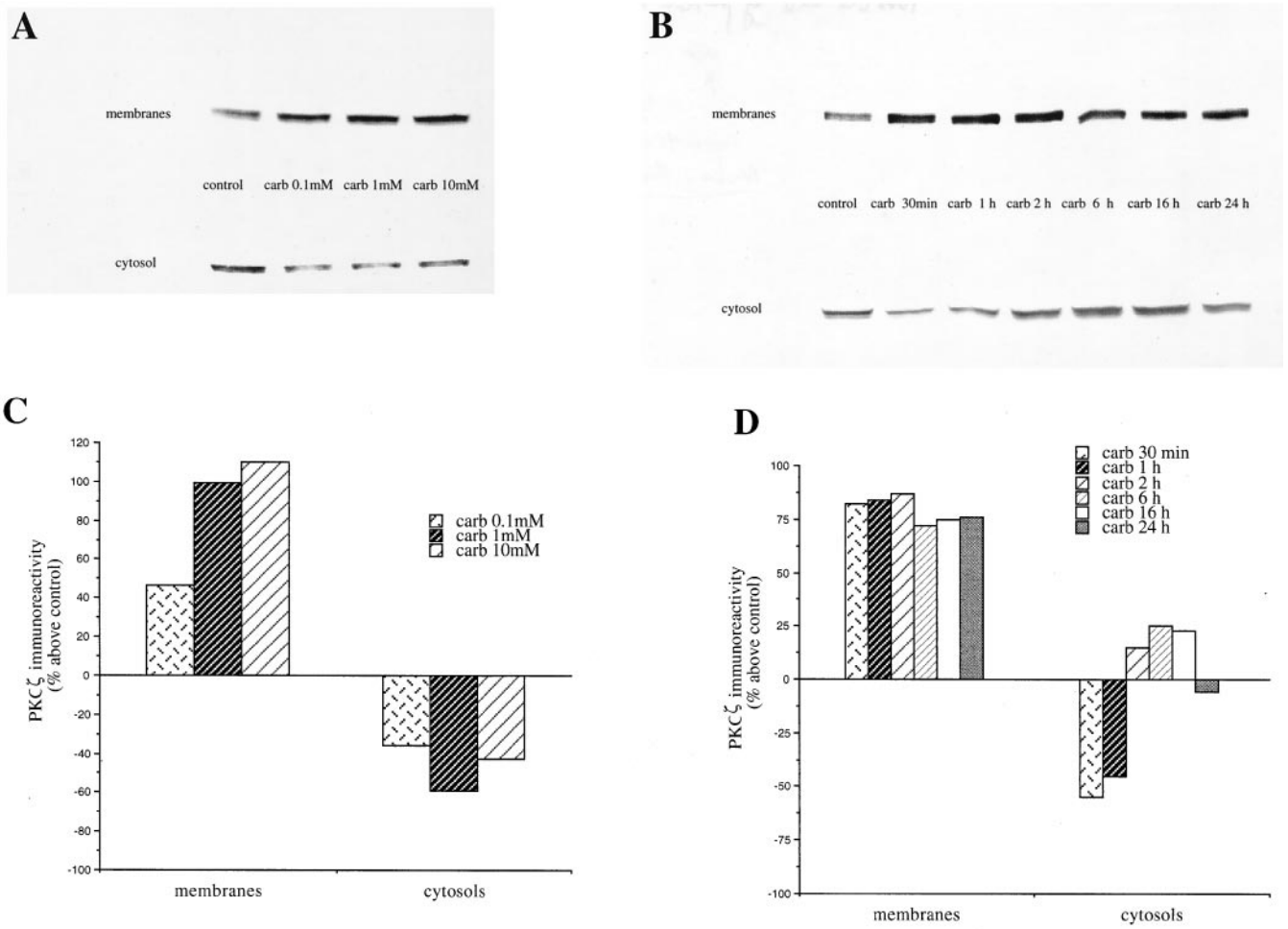

FIG. 2. Concentration- and time-dependent effects of carbachol on PKC ζ translocation and level. (A, C) 132–1N1 Human astrocytoma cells were stimulated for 30 min with 0.1, 1, or 10 mM carbachol. (B, D) 132–1N1 Human astrocytoma cells were stimulated for 30 min, and for 1, 2, 6, 16, and 24 hr with 1 mM carbachol. In panels A and B, proteins of cytosol and particulate fractions were separated by ultracentrifugation at 100,000 *g* and probed on immunoblots with anti-PKC ζ antibody; in panels C and D, quantification of relative band density is shown. Similar results were obtained in three independent experiments.

ogy (not shown), as previously reported [40]. Confocal microscopy of cells immunostained for PKC ζ revealed that in resting 132–1N1 astrocytoma cells PKC ζ was diffused across the whole cytoplasm, including the extremities (Fig. 3A). After exposure to 1 mM carbachol for 30 min, greater fluorescence was observed in the perinuclear area, while the extremities of the cells did not contain PKC ζ, and were, therefore, not visible; furthermore, round formations displaying intense fluorescence were observed in the perinuclear area (Fig. 3B). The effect of carbachol on PKC ζ localization clearly was antagonized by atropine (Fig. 3C). Incubation with anti-PKC ζ antibody in the presence of a competing peptide resulted in a drastic decrease of FITC fluorescence in both control and carbachol-stimulated cells (data not shown). Since it has been reported that antibodies against the C-terminal sequence of PKC ζ cross-react with the PKC α isozyme [37], the same experiments were carried out by labeling the cells with a polyclonal antibody to PKC α. No difference was observed in PKC α localization between control and carbachol-treated cells (data not shown).

To investigate the involvement of PKC ζ in the proliferative effect of carbachol, a myristoylated 9-mer peptide corresponding to the autoinhibitory domain of PKC ζ (peptide Z) was utilized (Fig. 4) [24, 28]. Basal [^{3}H]thymidine uptake (4860 ± 300 dpm) was not affected significantly by peptide Z (25 μM). Carbachol induced an 8-fold increase in [^{3}H]thymidine incorporation, and peptide Z inhibited carbachol-induced DNA synthesis (Fig. 4). On the other hand, peptide Z had no effect on serum-induced [^{3}H]thymidine incorporation in 132–1N1 astrocytoma cells (Fig. 4 inset). To verify the specificity of peptide Z, western blot analyses of PKC ζ, PKC ε, and PKC α were carried out. Peptide Z was able to block the carbachol-induced translocation of PKC ζ from the cytosol to the membranes (Fig. 5), without affecting the carbachol-induced translocation of PKC ε or the PMA-induced translocation of PKC α (Fig. 5 insets). Furthermore, a myristoylated 9-mer peptide corre-

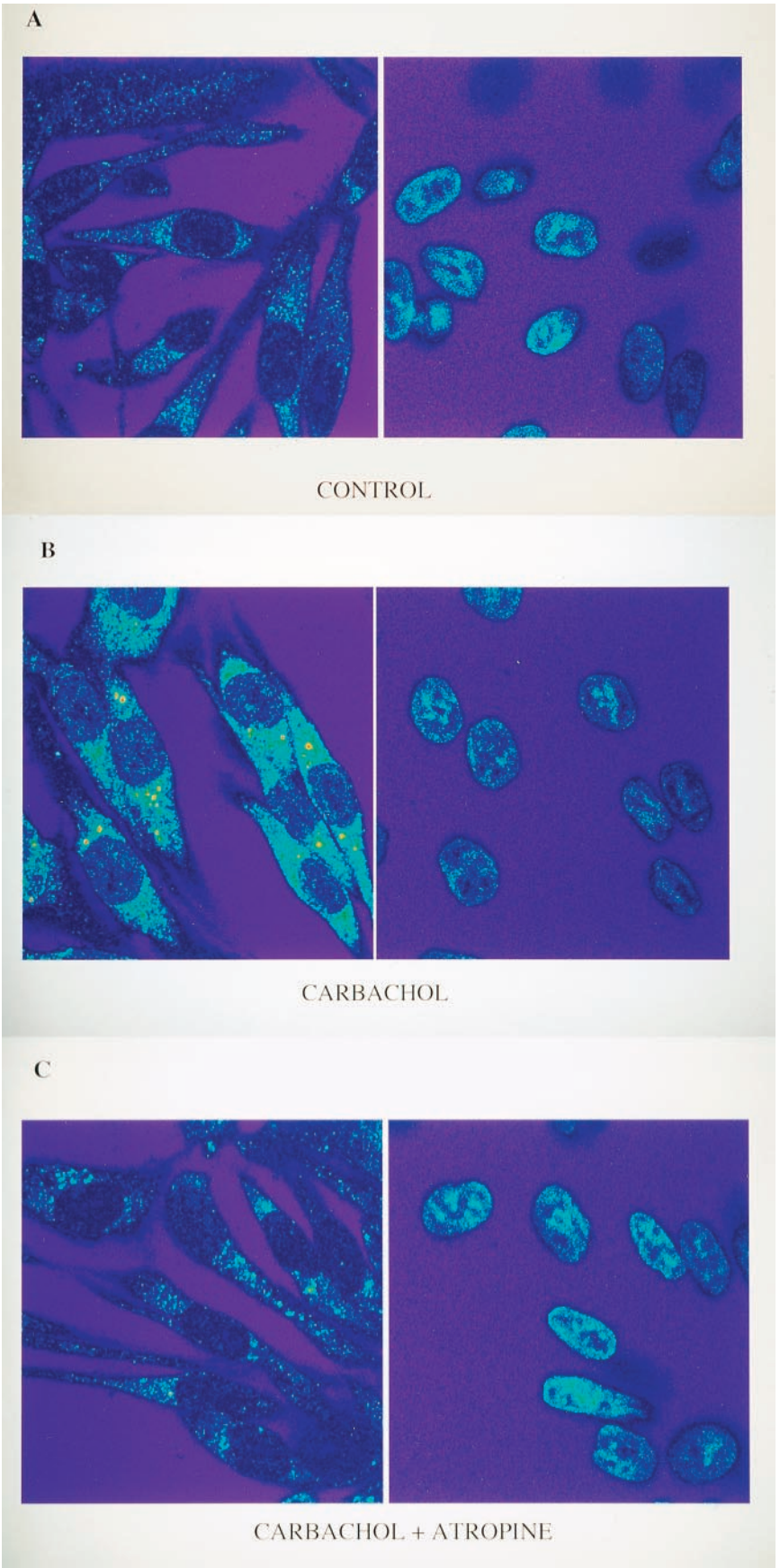

FIG. 3. Immunocytochemical determination of carbachol-induced changes in PKC ζ distribution. 132–1N1 Human astrocytoma cells were incubated in serum-free medium (A) or stimulated for 30 min with 1 mM carbachol (B) or carbachol + 10 μM atropine (C). After treatments, cells were fixed and stained with anti-PKC ζ antibody, with a secondary FITC-conjugated antibody (left images), and with the nuclear dye Hoechst (right images). Shown are representative fields out of five fields analyzed for each treatment. This experiment was repeated four times with similar results.

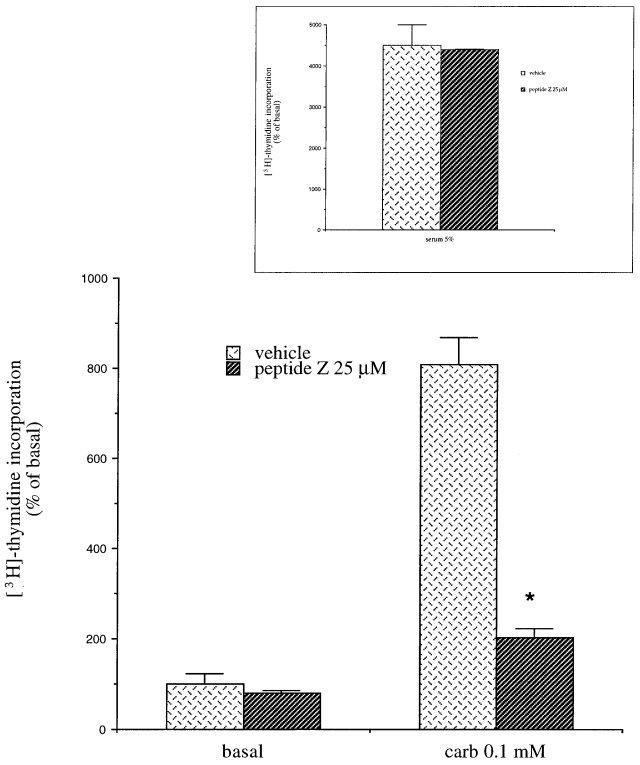

FIG. 4. Effect of peptide Z on [^{3}H]thymidine incorporation induced by carbachol and serum. 132–1N1 Human astrocytoma cells were pretreated for 45 min with 25 μM peptide Z and then incubated for 24 hr in the presence of peptide Z and DMEM/0.1% BSA or 0.1 mM carbachol. Control cells were incubated with DMEM/0.1% BSA and/or 0.1 mM carbachol. Six hours before the end of the incubation, 1 μCi [^{3}H]thymidine was added to the medium. The trichloroacetic acid-precipitated material was assayed for [^{3}H]thymidine content. Values are the means ± SEM of five independent experiments. Key: (*) $P < 0.05$ vs carbachol-stimulated cells. Inset: 132–1N1 Human astrocytoma cells were pretreated for 45 min with 25 μM peptide Z and then incubated for 24 hr in the presence of peptide Z and 5% serum. Control cells were incubated with DMEM/5% FBS. Basal [^{3}H]thymidine uptake was 4860 ± 300 dpm.

sponding to the autoinhibitory domain of PKC α (peptide A), used as a control, did not inhibit carbachol-induced DNA synthesis in astrocytoma cells (not shown).

To investigate the role of PA (generated from PC hydrolysis by PLD) and of PI 3-kinase in carbachol-induced translocation of PKC ζ, additional experiments were carried out. Carbachol-induced PLD activation in 132–1N1 astrocytoma cells has been demonstrated previously [41, 42]. Figure 6 shows that in these cells carbachol also induced phosphorylation of Akt/PKB, a major substrate for PI 3-kinase, and this effect was blocked by the PI 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). A high concentration of ethanol (100 mM), which inhibits PLD by competing with water in a transphosphatidylation reaction [43], inhibited both carbachol-induced [^{3}H]thymidine incorporation and PKC ζ translocation (Fig. 7). Similarly, LY294002 antagonized both DNA synthesis and PKC ζ translocation induced by carbachol.

DISCUSSION

The aim of the present study was to investigate the potential role of PKC ζ in carbachol-induced 132–1N1 astrocytoma cell proliferation. Our previous studies had shown that activation of muscarinic M3 receptors in these cells leads to a sustained mitogenic response [8] and that activation of classical and/or novel PKCs cannot fully explain this effect [14].

We first measured PKC ζ translocation from the cytosol to the membrane fraction as a marker for PKC ζ activation. One recent study has demonstrated that PKC ζ binding to membranes is necessary and sufficient for PKC ζ activation [36], and other studies have shown that PKC ζ translocation to membranes is always associated with PKC ζ activation [24, 44, 45]. However, it has also been reported that the atypical PKC ζ, in contrast to conventional and novel PKCs, does not translocate to the membrane when it is activated [46].

After carbachol stimulation of 132–1N1 astrocytoma cells, an increase in membrane-bound PKC ζ and a decrease in the soluble, cytosolic PKC ζ was observed (Fig. 1). This effect was antagonized by an M3 receptor antagonist but not by an M2 antagonist or by PTX, suggesting an involvement of the M3 muscarinic receptor in PKC ζ translocation by carbachol. Translocation of PKC ζ was long-lasting, with an increase in membrane-associated enzyme still present at 24 hr. While activation of PKC ε by carbachol in these cells is a short-term phenomenon, with an initial increase at 15 min and a decrease by 2 hr [14], the slow and prolonged activation of PKC ζ correlates well with the time course of carbachol-induced [^{3}H]thymidine incorporation [8, 14]. An interesting finding is that upon 24-hr exposure to carbachol there was an increase of total PKC ζ levels. Although the mechanisms involved in such an effect are unclear, this finding suggests that PKC ζ is important during the carbachol-induced transition from the G_0/G_1 phase to the S phase of the cell cycle. Similarly, Carlin *et al.* [31] reported an increase in PKC ζ (but not PKC α) levels in human airway smooth muscle cells stimulated by serum or platelet-derived growth factor. The studies of endogenous PKC ζ translocation kinetics and endogenous PKC ζ up-regulation by stimulation of endogenous muscarinic receptors described in this paper provide information on PKC ζ activation and regulation that would not be possible to obtain using cells transfected with wild-type or mutated PKC ζ.

To verify the role of PKC ζ in carbachol-induced proliferation, we used a myristoylated peptide corresponding to part of the pseudosubstrate region of PKC ζ (peptide Z). Similar pseudosubstrates have been used successfully before [24, 28]. Peptide Z inhibited carbachol-induced [^{3}H]thymidine incorporation and PKC ζ translocation without affecting the activation of other PKC isozymes or

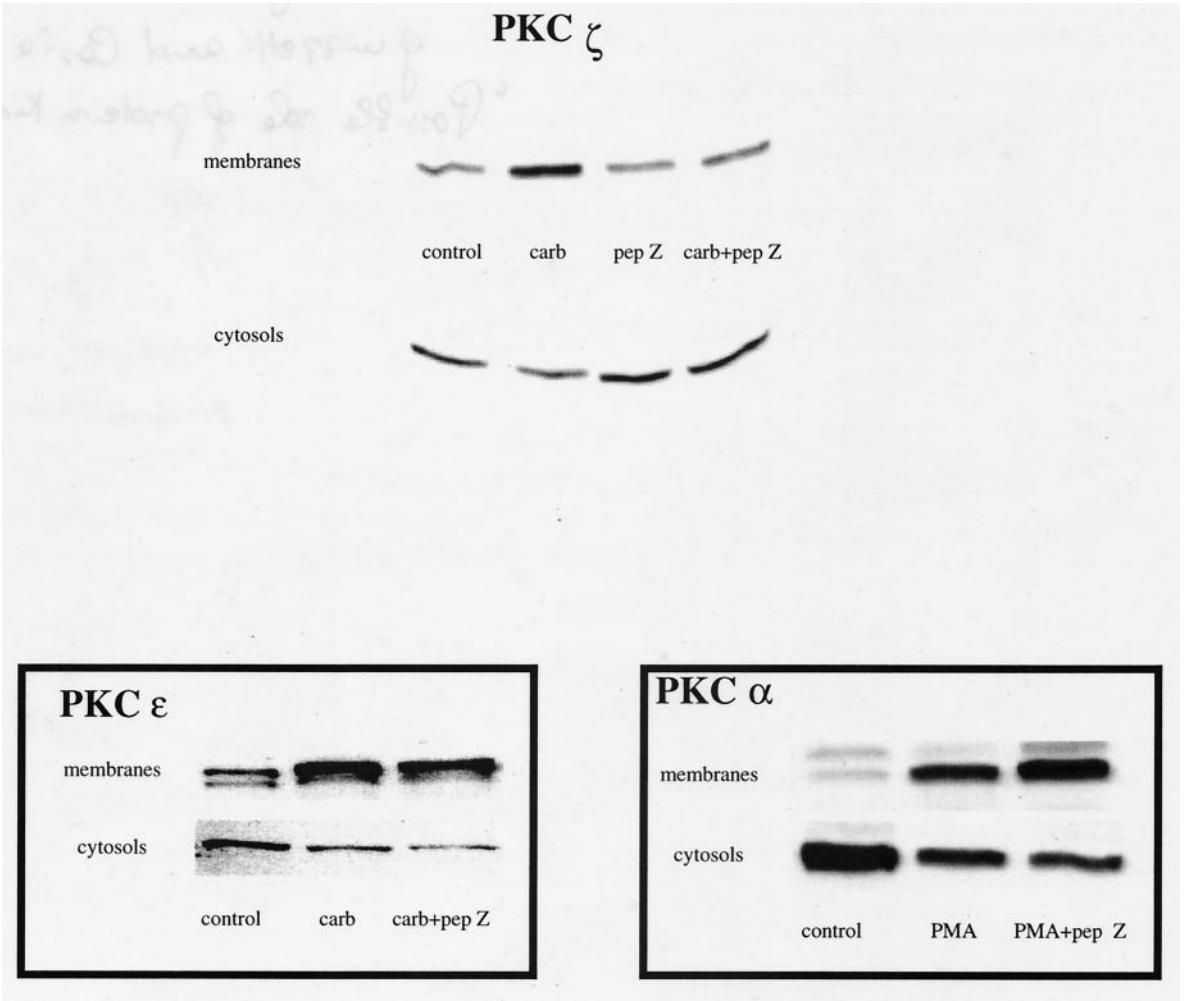

FIG. 5. Effect of peptide Z on translocation of PKC α, ε, and ζ. 132–1N1 Human astrocytoma cells were pretreated for 45 min with vehicle or 50 μM peptide Z and then incubated in the presence of 1 mM carbachol. Shown are protein immunoblots of cytosol and particulate fractions separated by ultracentrifugation at 100,000 *g* and probed with anti-PKC ζ antibody. Similar results were obtained in at least two independent experiments. Left inset: 132-1N1 human astrocytoma cells were pretreated for 45 min with vehicle or 50 μM peptide Z and then incubated in the presence of 1 mM carbachol. Shown are protein immunoblots of cytosol and particulate fractions separated by ultracentrifugation at 100,000 *g* and probed with anti-PKC ε antibody. Right inset: 132–1N1 human astrocytoma cells were pretreated for 45 min with vehicle or 50 μM peptide Z and then incubated in the presence of 10 ng/mL of PMA. Shown are protein immunoblots of cytosol and particulate fractions separated by ultracentrifugation at 100,000 *g* and probed with anti-PKC α antibody.

proliferation induced by serum. These results suggest that PKC ζ activation plays a relevant role in carbachol-induced proliferation of astrocytoma cells.

It has been shown that the PI 3-kinase lipid product PIP_3 activates PKC ζ [35], and that the PI 3-kinase substrate PDK 1 phosphorylates PKC ζ in the activation loop [36]. Additionally, the PLD product PA binds to and activates PKC ζ [34]. PLD-induced formation of PA by carbachol in 132–1N1 astrocytoma cells and its inhibition by ethanol (which competes with water as a substrate of PLD, leading to the formation of phosphatidylethanol via a transphosphatidylation reaction) have been established previously [41, 42]. To date there has been no direct evidence of PI 3-kinase activation by carbachol in 132–1N1 astrocytoma cells. However, we observed that carbachol induced phosphorylation of Akt/PKB, which is a well recognized substrate for PI 3-kinase [47], and this effect was inhibited by the PI 3-kinase-specific inhibitor LY294002. Both ethanol (100 mM) and LY294002 inhibited carbachol-induced DNA synthesis as well as PKC ζ translocation, suggesting

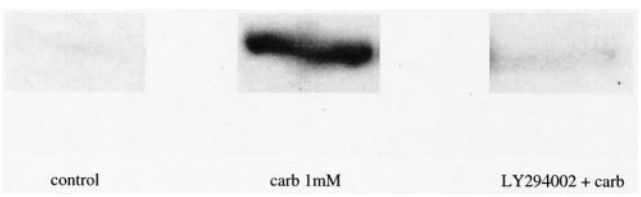

FIG. 6. Effect of carbachol on Akt/PKB phosphorylation. 132–1N1 Human astrocytoma cells were pretreated for 45 min with vehicle or 2 μM LY294002 and then incubated for 30 min with 1 mM carbachol. Shown is a protein immunoblot probed with anti-Akt antibody, representative of three experiments that gave similar results.

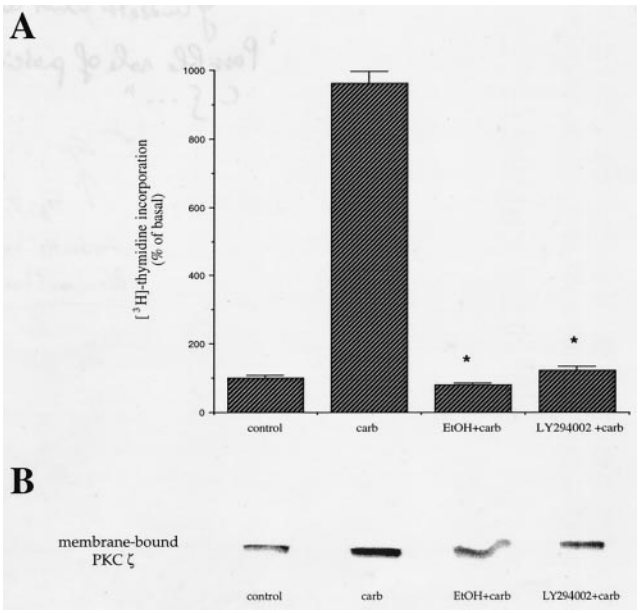

FIG. 7. Effect of ethanol and LY294002 on carbachol-induced [^{3}H]thymidine incorporation and PKC ζ translocation. (A) 132–1N1 Human astrocytoma cells were pretreated for 45 min with vehicle, 100 mM ethanol, or 2 μM LY294002 and then incubated for 24 hr in the presence of 0.1 mM carbachol. Six hours before the end of the incubation, 1 μCi of [^{3}H]thymidine was added to the medium. The trichloroacetic acid-precipitated material was assayed for [^{3}H]thymidine content. Values are the means ± SEM of four independent experiments. Key: (*) $P < 0.05$ vs carbachol-stimulated cells. Basal [^{3}H]thymidine uptake was 4540 ± 230 dpm. (B) 132–1N1 Human astrocytoma cells were pretreated for 45 min with vehicle, 100 mM ethanol, or 2 μM LY294002 and then incubated for 30 min with 1 mM carbachol. Shown is a protein immunoblot of the particulate fraction separated by ultracentrifugation at 100,000 *g* and probed with anti-PKC ζ antibody. The result shown is representative of two experiments, which gave similar results.

that both pathways contribute to activation of this PKC isozyme and to the proliferative response. It has been reported recently that in astrocytes treated with different concentrations of ethanol, both proliferation and PA formation are inhibited in a concentration-dependent manner, supporting the conclusion that PLD activation is important for proliferation in glial cells [48].

Muscarinic receptors have been shown to cause PKC ζ activation in other systems: M3 muscarinic receptors induce activation of PKC ζ in RINm5F cells [23] and possibly mediate the carbachol-induced increase in PKC ζ immunoreactivity in SK-N-SH human neuroblastoma cells [49]. Furthermore, the βγ subunit of G_i protein coupled to the M2 muscarinic receptor activates PKC ζ via PI 3-kinase [50].

Altogether, our results indicate that PKC ζ is activated in 132–1N1 cells after muscarinic receptor stimulation, that this activation is important for muscarinic receptor-mediated DNA synthesis, and that both PLD and PI 3-kinase contribute to PKC ζ activation and to cell proliferation.

The atypical PKC ζ has been shown to be involved in oocyte maturation [28], in fibroblast proliferation [29], and in endothelial cell proliferation induced by vascular endothelial growth factor [30], and to be activated in human airway smooth muscle cells during proliferation [31]. Furthermore, PKC ζ contributes to the activation of p70 S6 kinase [21], which plays an important role during progression through the G_1 phase of the cell cycle [51]. On the other hand, PKC ζ overexpression does not induce cell transformation or tumorigenicity [32] and does not affect the growth characteristics of NIH 3T3 fibroblasts [33], suggesting that PKC ζ may be necessary but not sufficient for proliferation of some cell types.

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