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# Regulation by Phorbol Esters of Amyloid Precursor Protein Release from Swiss 3T3 Fibroblasts Overexpressing Protein Kinase Cα*

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**Release of large soluble $NH_2$-terminal fragments of the amyloid precursor protein (APP) of Alzheimer's disease was measured in two Swiss 3T3 fibroblast cell lines (designated SF1.4 and SF3.2), overexpressing the α subtype of protein kinase C, and in two control cell lines (SC1 and SC2) (Eldar, H., Zisman, Y., Ullrich, A., and Livneh, E. (1990) *J. Biol. Chem.* 265, 13290–13296). Basal release of APP was significantly increased in SF1.4 cells, but not in SF3.2 cells, relative to controls. Phorbol 12-myristate 13-acetate, an activator of protein kinase C, elicited a concentration-dependent increase in APP release in all four cell lines. However, the estimated $EC_{50}$ for this effect was lower in the two cell lines overexpressing protein kinase C-α (7 and 6 nM, in SF1.4 and SF3.2 cells, respectively) than in control SC1 and SC2 cells (56 and 22 nM, respectively). The absolute amount of APP released by maximal concentrations of phorbol ester was not altered by overexpression of protein kinase Cα. The protein kinase C inhibitor H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) significantly reduced the response to phorbol esters in control (SC1) cells but not in cells (SF1.4) that overexpress protein kinase Cα. Levels of cell-associated APP were slightly elevated, and rates of APP turnover were unchanged, in SF1.4 cells relative to controls. However, cell-associated APP levels were lower in SF3.2 cells than in controls. The results demonstrate that protein kinase Cα regulates APP release in Swiss 3T3 fibroblasts, and perhaps in other tissues, including brain, and may be the isozyme that mediates receptor-evoked release of APP.**

The amyloid precursor protein (APP)[1] of Alzheimer's disease (AD) is an integral membrane glycoprotein (Kang *et al.*, 1987; Dyrks *et al.*, 1988; Weidemann *et al.*, 1989) found in a wide variety of cell types including brain neurons and glia, cultured neuronal cells, platelets, fibroblasts, and embryonic kidney cells. Proteolytic processing of APP generates a 39–42/43-amino acid peptide, the amyloid β peptide (Aβ), that is the principal component of the amyloid which accumulates in brains of AD and Down's syndrome patients (Glenner and Wong, 1984). Most cell types release APP into the medium via the action of a protease that cleaves the parent molecule at an extracellular site, releasing the $NH_2$-terminal portion, and leaving a smaller COOH-terminal fragment associated with the cell (Weidemann *et al.*, 1989; Esch *et al.*, 1990; Oltersdorf *et al.*, 1990; Sisodia *et al.*, 1990; Anderson *et al.*, 1991; Lowery *et al.*, 1991). Since this secretory processing pathway involves cleavage of the molecule outside the membrane but within the Aβ sequence (Esch *et al.*, 1990; Sisodia *et al.*, 1990; Anderson *et al.*, 1991; Lowery *et al.*, 1991), amyloid production must result from an alternative process. The recent discovery that the Aβ peptide is released from normal cells (Citron *et al.*, 1992; Haass *et al.*, 1992; Shoji *et al.*, 1992) raises the possibility that overactivity of this latter pathway might lead to amyloid deposition in AD. However, the factors regulating the generation of various APP fragments are still incompletely understood.

Treatment of cultured cells with phorbol esters increased APP mRNA expression after 24 h in human endothelial cells (Goldgaber *et al.*, 1989). Phorbol esters are potent activators of protein kinase C (PKC) that, in addition to increasing the expression of the APP gene, also stimulate APP proteolysis. Thus, treatment of PC12 cells with phorbol esters increased (within 1 h) levels of cell-associated COOH-terminal fragments of APP (Buxbaum *et al.*, 1990) and release of large $NH_2$-terminal fragments (Caporaso *et al.*, 1992; Gillespie *et al.*, 1992). These results suggest that normal secretion of APP may be regulated via PKC-mediated phosphorylation.

The PKC enzyme family contains at least 10 distinct proteins with varying sensitivities to activators including phospholipids, diacylglycerol, calcium, and fatty acids (Nishizuka, 1992). The α subtype (PKCα) is the most widely distributed isozyme, and is found in most tissues, including brain (Ohno *et al.*, 1987). It is activated by diacylglycerol in the presence of phosphatidylserine and by arachidonic acid in the presence of elevated calcium concentrations (Nishizuka, 1988). In order to investigate the role of PKCα in the regulation of APP processing, fibroblasts overexpressing this subtype as a result of the introduction of the cDNA coding for PKCα, and control cells, were treated with the PKC activator phorbol 12-myristate 13-acetate (PMA), and APP levels in the medium were measured. Our findings confirm and extend previous reports that PKC regulates cleavage and release of APP (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992). Moreover, they suggest that the PKCα subtype may participate in this process.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Swiss 3T3 fibroblasts were infected with virus particles containing the full-length PKCα gene and a neomycin resist-

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[1] The abbreviations used are: APP, amyloid precursor protein; AD, Alzheimer's disease; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

ance gene or the neomycin resistance gene alone (Eldar *et al.*, 1990). Four of the resulting neomycin-resistant clones were used in this study; two overexpressing PKCα and designated SF 1.4 and SF 3.2, and two control lines, designated SC1 and SC2 (Eldar *et al.*, 1990). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum (Life Technologies, Inc.), in an atmosphere of 5% $CO_2$. Prior to experiments, cells were subcultured onto 35- or 60-mm plastic dishes and grown to confluence. The medium was replaced with serum-free Dulbecco's modified Eagle's medium for measurement of APP release. Test substances were added to the medium at the beginning of the incubation period. PMA (Sigma) was dissolved in dimethyl sulfoxide. Medium containing dimethyl sulfoxide alone (0.1%) served as a vehicle control. H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; Seikagaku America; Rockville, MD) and cycloheximide (Sigma) were dissolved in water.

*Measurement of APP Release*—Media were collected and centrifuged at the end of the incubation period to remove cells and debris, then kept on ice. Phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, and the media were applied to Sephadex G-25 columns (14 × 1 cm) and eluted in a volume of 4 ml of $H_2O$. The column eluates were frozen and dried by vacuum centrifugation and then resuspended in an extraction buffer (50 mM Tris buffer, 150 mM NaCl, 5 mM EDTA, pH 7.6), containing 2% Triton X-100 and 2% Nonidet P-40, and diluted 1:1 with loading buffer (125 mM Tris buffer, 4% sodium dodecyl sulfate, 20% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue, pH 6.8). After boiling, the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on mini-gels (Bio-Rad). The volume of sample loaded was corrected for total cell protein per dish. Following electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in 5% powdered milk (Carnation Co., Los Angeles, CA) and incubated overnight in anti-PreA4 monoclonal antibody (mAb clone 22C11; Weidemann *et al.*, 1989) supplied by Boehringer Mannheim and diluted with Tris-buffered saline (10 mM Tris buffer, 150 mM NaCl) containing 0.05% Tween 20. In some experiments, membranes were incubated with a polyclonal antibody directed against the COOH terminus of APP (Selkoe *et al.*, 1988). After washing for 5 × 5-min periods, the membranes were incubated in peroxidase-linked secondary antibody (anti-mouse from sheep, Amersham Corp. or anti-rabbit from goat, Sigma) for 1–2 h, washed again, and exposed to phosphate-buffered saline containing 0.5 mg/ml diaminobenzidine and 0.001% hydrogen peroxide to visualize the APP. Alternatively, the blots were developed using a chemiluminescence (ECL) method (Amersham). The bands were quantitated by laser scanning densitometry (LKB, Bromma, Sweden). Measurements fell within the linear range of detection as determined using dilution curves of cell protein extracts. All quantitative comparisons between groups were derived by comparing samples processed simultaneously within one blot.

Following aspiration of the medium, cells were rinsed and collected in phosphate-buffered saline with 2 mM phenylmethylsulfonyl fluoride. Aliquots of the suspension were taken for total cell protein determination. The cells were pelleted in a microcentrifuge and resuspended in a small volume of extraction buffer. The extracts were centrifuged again, and aliquots of the supernatant were taken for protein determination. The remainder was diluted 1:1 in loading buffer for SDS-PAGE as described above.

*Measurement of Phosphatidylcholine Labeling*—Cell phosphatidylcholine synthesis from labeled choline was measured as described previously (Slack *et al.*, 1991).

*Statistical Analysis*—The statistical significance of differences was estimated by *t* test. Differences were taken to be significant at $p < 0.05$. Data given in text are expressed as means ± S.E. unless otherwise stated.

## RESULTS

Both control and PKCα-overexpressing cell lines constitutively released APP into the medium. The released protein appeared as two bands on Western blots: a major band with an approximate molecular mass of 111 kDa and a minor species of about 100 kDa (Figs. 1*A* and 4*A*). These are similar in size to the APP released from HeLa cells, fibroblasts, PC12 cells, and human embryonic kidney cells (Saitoh *et al.*, 1989; Weidemann *et al.*, 1989; Caporaso *et al.*, 1992; Nitsch *et al.*, 1992). The medium obtained from both control and PMA-treated fibroblast cultures contained only large $NH_2$-terminal fragments of APP (Fig. 1); cell-associated APP, but not APP in the medium, could be detected on Western blots probed with an antibody directed against the COOH terminus (Selkoe *et al.*, 1988) (Fig. 1*B*). Basal APP release from the PKCα-overexpressing cell line SF 1.4 was significantly higher (by 84 ± 23%) than that from control (SC1) cells (this result is based on a 1-h collection period and represents the mean ± S.E. of 12 separate experiments). However, the basal release of APP from a second line overexpressing PKCα (SF3.2) was unchanged relative to control SC1 cells (Fig. 2*A*). Basal release from a second control line (SC2) was somewhat lower than that from the other lines (Fig. 2*A*). PMA caused a dose-dependent increase in APP released into the medium in both PKCα-overexpressing and control fibroblasts. When levels of APP released by PMA from PKCα-overexpressing cell lines and from controls were directly compared by analyzing the samples within one blot, it could be seen that the maximum amount of APP released was the same for all cell lines. Dose-response curves were therefore normalized by expressing APP release at each concentration of PMA as a percentage of the maximum amount released by PMA from each cell line within

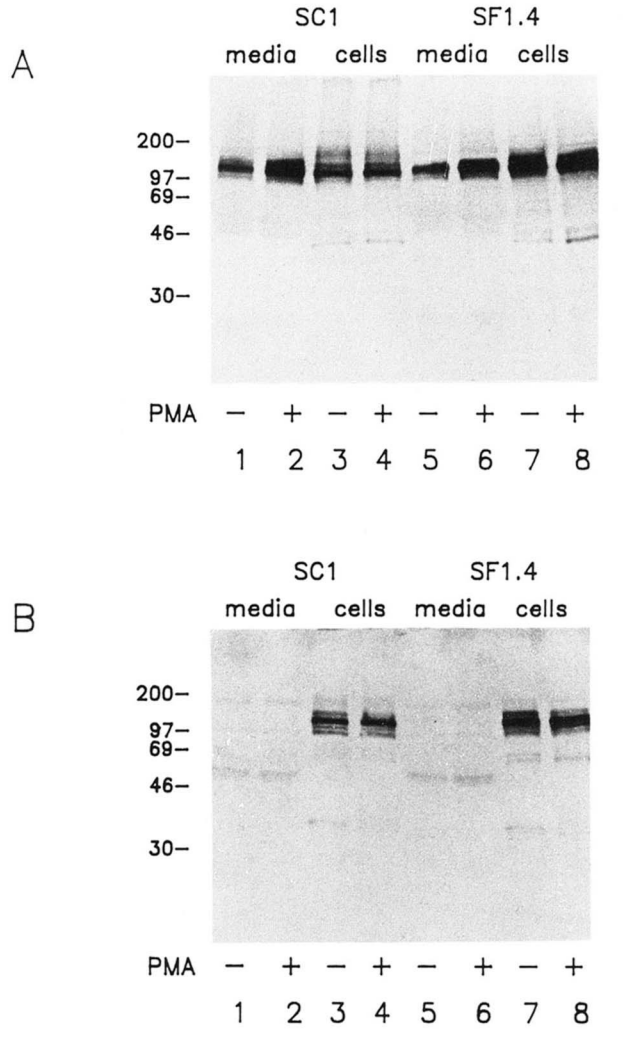


FIG. 1. **APP released into the medium is a cleavage product of full-length APP.** Control (SC1) and PKCα-overexpressing (SF1.4) cells were treated for 1 h with PMA (1 μM) or dimethyl sulfoxide (0.1%). Media and cells were extracted and separated by SDS-PAGE. Immunoblots were prepared with antibodies directed against the $NH_2$-terminal of APP (*A*) or the COOH-terminal of full-length APP (*B*) (see "Experimental Procedures").

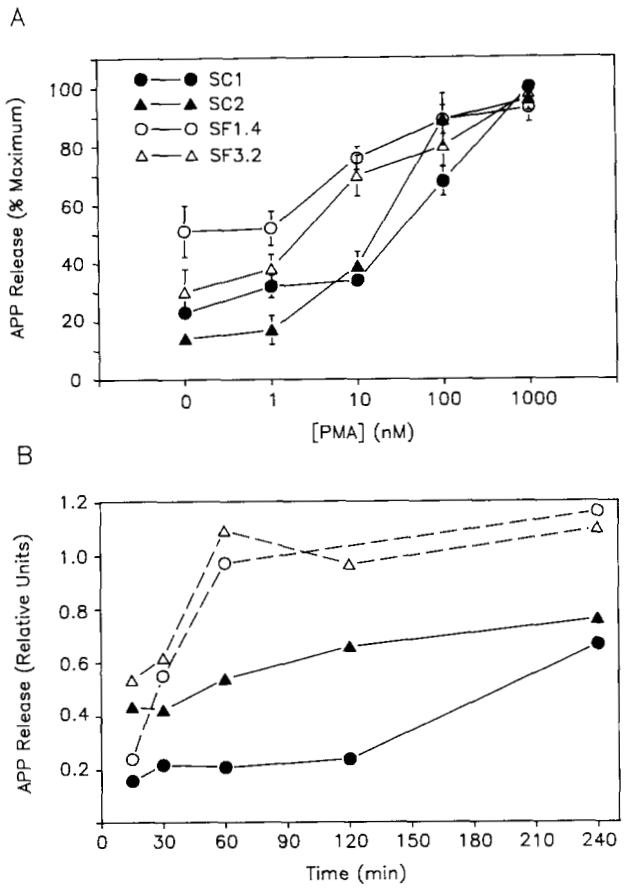


FIG. 2. **Effect of PMA on APP release into the medium.** *A*, control cell lines (*closed circles*, SC1; *closed triangles*, SC2) and cell lines overexpressing PKCα (*open circles*, SF1.4; *open triangles*, SF3.2) were incubated for 1 h in serum-free medium containing 0–1 μM PMA. Data are expressed as a percent of maximum APP released and represent means ± S.E. from three to six separate experiments performed in duplicate. *B*, time course of APP release in control (*circles*, SC1) and PKCα-overexpressing (*triangles*, SF1.4) cells treated for varying periods of time with 100 nM PMA (*open symbols*) or dimethyl sulfoxide vehicle (*closed symbols*). Data were obtained from a representative experiment performed three times.

a given experiment, and the results from three to six experiments were averaged (Fig. 2*A*). In the lines that overexpressed PKCα, the mean estimated $EC_{50}$ for PMA was considerably lower (7 and 6 nM in SF1.4 and SF3.2 cells, respectively) than in control cells (56 and 22 nM in SC1 and SC2 cells, respectively) (Fig. 2*A*). The greatest difference in the responses of control and PKCα-overexpressing cells to PMA was observed at a concentration of 10 nM. At this concentration the amount of APP released within 1 h was equivalent to 38 ± 3% and 39 ± 5% of maximal levels in SC1 and SC2 control lines, respectively, whereas this value was increased to 76 ± 4% and 70 ± 7% of maximum levels in SF1.4 and SF3.2 cells, respectively (Fig. 2*A*).

The response to PMA was time-dependent; an increase in APP secretion was apparent within 30 min of treatment (Fig. 2*B*). It was shown previously in PC12 cells that exposure to phorbol ester increased APP release severalfold within 1 h (Caporaso *et al.*, 1992). It is likely that these events are preceded by activation of PKC, which is associated with translocation of this enzyme to the membrane, and occurs within minutes in many cell types (Kraft and Anderson, 1983; Chida *et al.*, 1986).

The cell-associated APP protein was resolved into three to four bands with molecular masses ranging from approximately 106 to 130 kDa on 12% or 4–20% gradient polyacrylamide gels (Figs. 1 and 4). The level of cell-associated APP was slightly but significantly higher in SF1.4 cells than in control SC1 cells (133 ± 15%, *cf.* control cells, $n = 12$). SF3.2 cells, in contrast, contained reduced levels of cell-associated APP relative to both SC1 and SC2 cells. (APP levels in SF3.2 cells were 48% lower, on average, than in SC1 cells ($n = 2$) and 56 ± 4% lower than in SC2 cells ($n = 3$)). Acute treatment with PMA (100 nM) reduced cell-associated levels of the higher molecular mass band to 73 ± 4% and 63 ± 8% of control values, in control and SF1.4 cells, respectively, within 1 h (means ± S.E., $n = 4$).

PMA has been shown previously to stimulate synthesis of APP mRNA in cultured cells (Goldgaber *et al.*, 1989). In order to confirm that the increase in APP release elicited by PMA was due to alterations in processing of pre-existing protein, and not secondary to increased synthesis, the response to PMA both in the presence and absence of the protein synthesis inhibitor cycloheximide was assessed. The results indicated that although cycloheximide caused a decrease in basal levels of APP released within 1 h, the magnitude of the increase elicited by PMA was not significantly altered (Fig. 3*A*).

The specificity of PMA-stimulated APP release was tested in cells pretreated with the PKC inhibitor H-7. In the pres-

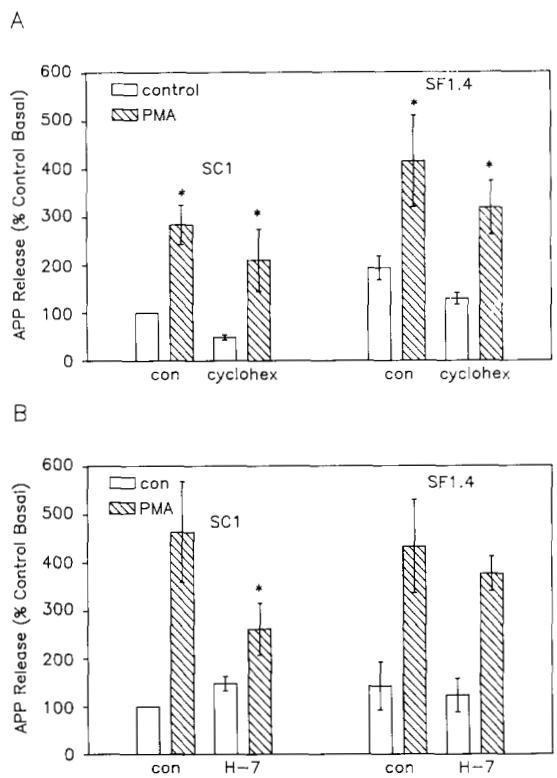


FIG. 3. *A*, effect of cycloheximide on APP release. Cells were incubated with or without cycloheximide (50 μg/ml) for 1 h in the presence or absence of PMA (100 nM), and APP release was measured. Results are expressed as a percent of basal release from control cells and represent means ± S.E. from three to four separate experiments. *, $p < 0.05$; values significantly different from corresponding controls by paired *t* test. *B*, effect of H-7 on APP release. Cells were pretreated for 30 min in the presence or absence of the PKC inhibitor H-7, then incubated for 1 h in the presence or absence of PMA (100 nM) with or without H-7 (100 μM). APP released into the medium during the 1-h incubation period was measured. Data are expressed as a percent of basal release from control cells and represent means ± S.E. of three experiments. *, $p < 0.05$; values significantly different from corresponding cultures treated with PMA alone.

ence of H-7 (100 μM), the response of SC1 cells to 100 nM PMA was significantly reduced (by 43 ± 8%; mean ± S.E., in three experiments). However, the response to PMA of SF1.4 cells was reduced by only 23 ± 6% in cells pretreated with H-7; this latter effect was not significant (Fig. 3*B*).

The turnover rates of APP protein were measured in control and PKCα-overexpressing cell lines treated with cycloheximide. Under these conditions, levels of cell-associated APP decreased by approximately 50% within 75 min (Fig. 4, *B* and *D*). The rate of decline was almost identical in both SF1.4 and SC1 cells, indicating that APP degradation was not altered by PKCα overexpression. Rates of APP release into the medium were also similar in the two cell lines (Fig. 4, *A* and *C*). The turnover rate of cell-associated APP in these fibroblast lines was similar to that reported for radioactive APP in PC12 cells pulse-labeled with [$^{35}$S]methionine (Weidemann *et al.*, 1989).

## DISCUSSION

These data show that release of large soluble $NH_2$-terminal fragments of APP from fibroblasts is increased in a concentration-dependent manner by activators of PKC and that the sensitivity of this response is consistently higher in fibroblasts that overexpress PKCα. Hence, the $EC_{50}$ for the phorbol ester PMA in control cells was approximately 3–9-fold greater than in PKCα-overexpressing cell lines. (It should be noted that PKCε is expressed in Swiss 3T3 fibroblasts in addition to PKCα but that levels of PKCε are not changed by the introduction of the gene for PKCα (Eldar *et al.*, 1993).) Basal APP release was increased in only one (SF1.4) of the two PKCα-overexpressing lines examined, although the maximum amounts of APP released in response to PMA were the same in all four lines. This pattern of response suggests that, although PKCα is clearly involved in the regulation of APP release, the availability of one of the downstream targets of PKCα (perhaps APP itself or a protease that cleaves APP following activation) is limited. (In contrast, PKCα overexpression increased the maximum degree of stimulation of membrane phosphatidylcholine synthesis by PMA in these fibroblast lines (data not shown), suggesting that the magnitude of a given response is limited by the concentration of a target molecule (or effector) rather than by the availability of PKCα.)

PKC phosphorylates APP both *in vitro* (Gandy *et al.*, 1988) and in semi-intact cells (Suzuki *et al.*, 1992), and treatment of cultured cells with phorbol esters, which directly activate PKC (Nishizuka, 1984), is associated with increased release of a large soluble $NH_2$-terminal fragment of APP from cultured cells, and the appearance of a short cell-associated COOH-terminal fragment (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992). PKC activity can also be regulated by stimulation of membrane receptors linked to phospholipase C, a process

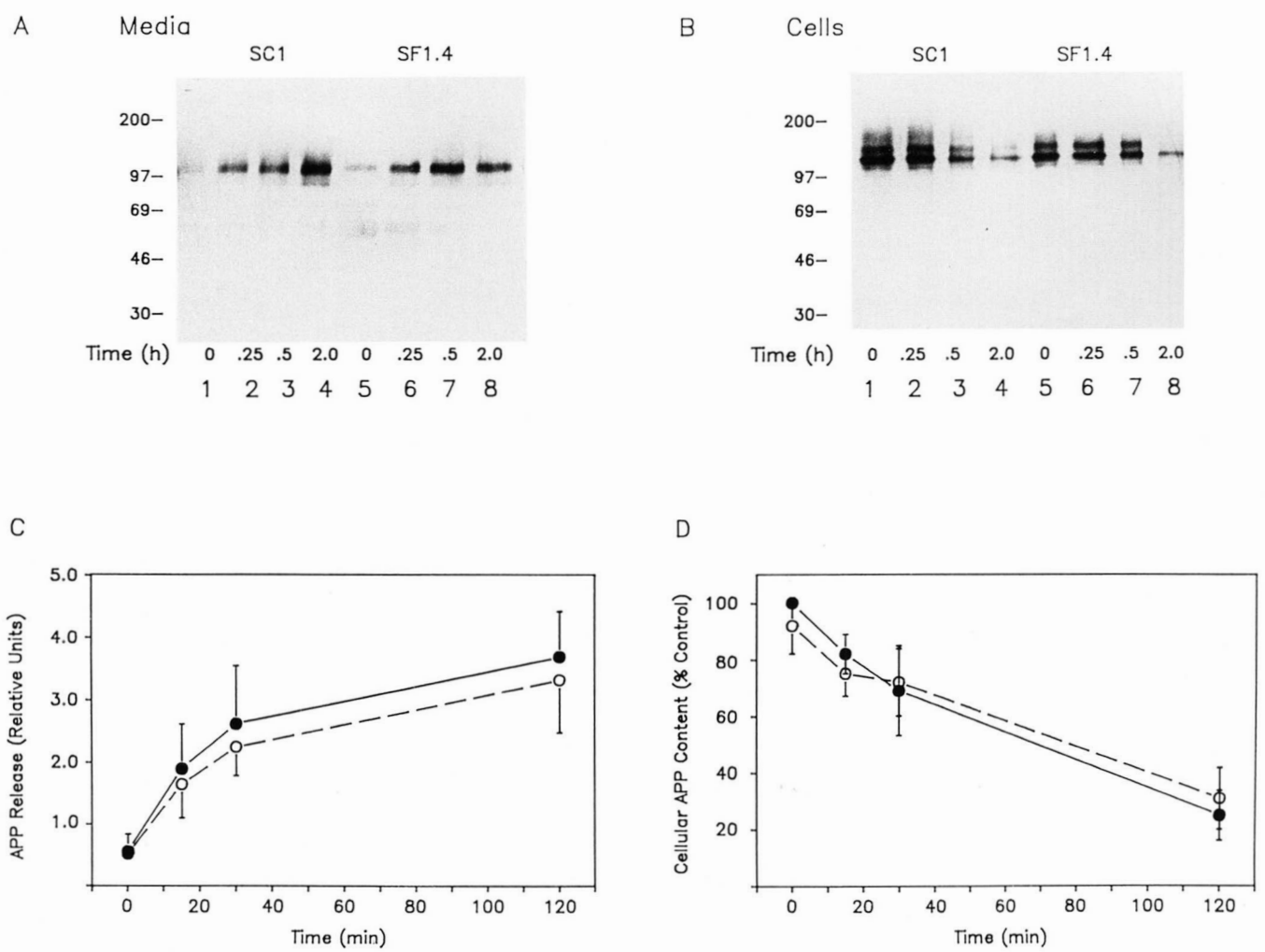


FIG. 4. **Levels of released APP (*A, C*) and cell-associated APP (*B, D*) as a function of time of exposure to cycloheximide (50 μg/ml).** APP in the medium is expressed in relative units, and cell-associated APP is expressed as a percent of APP levels in SC1 cells at 0 min. Values represent means ± S.E. from four separate experiments. *Closed circles*, SC1 cells; *open circles*, SF1.4 cells.

known to generate diacylglycerols, the physiological activators of PKC (Nishizuka, 1984, 1988, 1992), and recently it was demonstrated that activation of such receptors stimulates APP release (Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992). Taken together, the results indicate that APP release from the cell is regulated by well described signal transduction mechanisms and may be differentially affected by various factors, including receptor type and protein kinase C subtype.

Although treatment of human endothelial cells with phorbol esters increased levels of APP mRNA after 24 h (Goldgaber *et al.*, 1989), the enhancement in APP release elicited by PMA in the present study was maximal within 1 h and was not blocked by the protein synthesis inhibitor cycloheximide, indicating that the acute effect of PMA is attributable to altered processing of pre-existing APP rather than to changes in synthesis. APP levels in the SF1.4 PKCα-overexpressing cell line were slightly increased relative to controls, whereas the rates of decline of cell-associated APP levels in cycloheximide-treated SF1.4 and SC1 cells were almost identical (Fig. 4*D*), suggesting that APP turnover was not affected by overexpression of PKCα. The SF3.2 cell line actually contained lower levels of APP than either control clone. Overall, the results suggest that PKCα is not the subtype responsible for the PMA-stimulated increase in APP synthesis observed by others (Goldgaber *et al.*, 1989).

The PKC antagonist H-7 reduced the amount of APP released in response to PMA by approximately 50% in control cells (Fig. 3*B*), but it did not alter basal levels of APP release, or the amount of APP released by PMA, in SF1.4 cells. It is likely that the reduced effectiveness of H-7 in the latter may be due to the high levels of PKCα present in these cells.

The results presented here clearly implicate the α subtype of PKC in the regulation of APP proteolysis and release. This conclusion receives further support from the observation that PKCα stimulates phosphorylation of APP in an insect cell-baculovirus transfection system (Shapiro *et al.*, 1992). This subtype is widely distributed in many mammalian tissues, including brain (Ohno *et al.*, 1987), and regional variations in the levels of PKC subtype might be associated with different patterns of constitutive and regulated APP release. The relevance of these findings to the etiology of AD cannot yet be assessed. However, it was reported that the proportions of PKCα and PKCβ associated with membranes (measured by Western blot) were depressed in hippocampal tissue obtained from brains of patients with AD (Masliah *et al.*, 1990); and PKC activity was decreased in fibroblasts from AD patients (Bruel *et al.*, 1991), raising the possibility that alterations in the activity of one or more subtypes of PKC might contribute to the abnormalities in APP processing that are believed to underlie the pathophysiology of AD.

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