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Effect of Continuous Phorbol Ester Treatment on Muscarinic Receptor-Mediated Calmodulin Redistribution in SK-N-SH Neuroblastoma Cells

Z. Shariat-Madar, A. M. Goldsmith, and M. E. Gnegy

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

Abstract: Stimulation of muscarinic receptors by carbachol and activation of protein kinase C elicits the translocation of calmodulin (CaM) from membranes to cytosol in the human neuroblastoma cell line SK-N-SH. Our previous studies have suggested a role for protein kinase C in the regulation of CaM redistribution. To explore further the role of protein kinase C in carbachol-induced calmodulin translocation, we treated cells for 17 h with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to down-regulate protein kinase C isozymes or 72 h to differentiate the cells. Treatment of SK-N-SH cells for 17 h with 70 n*M* TPA nearly abolished the effect of carbachol on CaM redistribution. After 72 h of TPA, however, the cells appeared differentiated, and the ability of carbachol to increase cytosolic CaM levels was restored. In untreated control cells, the carbachol-mediated increase in cytosolic CaM content was mimicked by TPA and blocked by pretreatment with the selective protein kinase C inhibitor Ro 31-8220 at 10 μM. In the 72-h TPA-treated cells, however, the ability of TPA to increase cytosolic CaM levels was significantly reduced, and the action of carbachol was no longer blocked by Ro 31-8220. The effect of prolonged TPA treatment on select protein kinase C isozymes was examined by immunoblotting. Treatment of cells for either 17 or 72 h abolished the α-isozyme in the cytosol and reduced (17 h) or abolished (72 h) the content in the membranes. In both 17- and 72-h TPA-treated cells, the ϵ-isozyme was nearly abolished in the cytosol and slightly reduced in the membranes. Some protein kinase C activity may have been maintained during TPA treatment because the basal level of phosphorylation of the protein kinase C substrate myristoylated alanine-rich C kinase substrate was enhanced in cells treated for either 17 or 72 h with TPA. The potential dissociation of carbachol and protein kinase C in eliciting increases in cytosolic CaM content was a function of prolonged TPA treatment and not differentiation per se because carbachol-mediated increases in cytosolic CaM levels were inhibited by Ro 31-8220 in retinoic acid-differentiated SK-N-SH cells. This study demonstrates that continuous TPA treatment, although initially down-regulating the protein kinase C-mediated effect of carbachol on CaM redistribution, uncouples carbachol and protein kinase C at longer times. **Key Words:** Translocation—Protein kinase C—Myristoylated alanine-rich C kinase substrate—Carbachol—Differentiation—43-kDa growth-associated protein.
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Calmodulin (CaM) was initially characterized as a Ca^{2+}-dependent activator of cyclic nucleotide phosphodiesterase and has since been shown to mediate many effects of Ca^{2+} in various cell reactions and processes. CaM is an intracellular Ca^{2+} binding protein that is involved in regulation of cyclic nucleotide metabolism, phosphorylation–dephosphorylation cascades, cytoskeletal functions, ion channel activity, and cell proliferation (Manalan and Klee, 1984). Knowledge of the intracellular locations of CaM and changes in response to stimuli, such as receptor activation, is important to understanding functions and regulation of CaM (Gnegy, 1993). The fact that binding of CaM to various proteins can be altered by modifications such as phosphorylation suggests the possibility that CaM localization is regulated by intracellular signal-processing mechanisms (Gnegy, 1993). In human neuroblastoma SK-N-SH cells, activation of muscarinic receptors mediates a translocation of CaM from membrane into cytosol (Mangels and Gnegy, 1990). The carbachol-mediated redistribution of CaM from membrane into cytosol was attributed to the involvement of both protein kinase C (PKC) activation and the rise in intracellular Ca^{2+} concentration (Mangels and

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Address correspondence and reprint requests to Dr. M. E. Gnegy at Department of Pharmacology, 2220E MSRB III, University of Michigan Medical School, Ann Arbor, MI 48109-0632, U.S.A.

Abbreviations used: CaM, calmodulin; DMEM, Dulbecco's modified Eagle's medium; GAP-43, 43-kDa growth-associated protein; MARCKS, myristoylated alanine-rich C kinase substrate; PCA, perchloric acid; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Gnegy, 1990; McGinnis and Gnegy, 1996). The carbachol effect was predominantly mimicked by a phorbol ester and blocked by a PKC inhibitor.

Continuous treatment of cells with a phorbol ester such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) initially down-regulates PKC activity but can also lead to cellular differentiation (Blackshear, 1988; Påhlman et al., 1990). Although SK-N-SH cells have a neuronal morphology, they can be induced to differentiate further along a neuronal lineage (Påhlman et al., 1990). Differentiation of SK-N-SH cells and their clonal cell line SH-SY5Y by TPA leads to neurite outgrowth and increased expression of the neuronal 43-kDa growth-associated protein, GAP-43 (Jalava et al., 1988; Påhlman et al., 1990). Phorbol ester-induced differentiation of SK-N-SH cells and the clonal cell line, SH-SY5Y, decreases levels of components important in muscarinic receptor-mediated translocation of CaM, such as muscarinic receptor density, G protein-stimulated phosphoinositide turnover, and PKC activity and differentially regulates expression of PKC isoforms (Cioffi and Fisher, 1990; Leli et al., 1993).

The objective of this study was to investigate the effect of continuous TPA treatment leading to down-regulation of PKC on muscarinic receptor-induced changes in CaM localization. We found that treatment with TPA for 17 h down-regulated specific PKC isozymes and completely abolished carbachol-stimulated increases in cytosolic CaM levels. Despite continuous down-regulation of some specific PKC isozymes, the carbachol effect was restored after treatment with TPA for 72 h. Experiments with the specific PKC inhibitor Ro 31-8220 suggested that the carbachol effect was uncoupled from PKC after 72 h of TPA treatment. This uncoupling was probably due to continuous TPA treatment and was not a general property of differentiated SK-N-SH cells.

MATERIALS AND METHODS

Materials

Tissue culture supplies were from Corning Glass Works (Corning, NY, U.S.A.). Cell culture reagents, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, EGTA, bovine serum albumin, carbachol, Lubrol-PX, and TPA were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monoclonal antibody to GAP-43 was from Boehringer Mannheim. Affinity-purified isozyme-specific antisera against the α, β, ϵ, and ζ isozymes of PKC were purchased from GibcoBRL (Grand Island, NY, U.S.A.). ^{32}P-Inorganic phosphate ($^{32}P_i$) was purchased from Du Pont NEN (Boston, MA, U.S.A.). Ro 31-8220 and genistein were obtained from Calbiochem (San Diego, CA, U.S.A.).

Cell growth studies

The human neuroblastoma SK-N-SH cells were seeded, grown, and harvested according to the technique of Mangels and Gnegy (1990). Cells were treated with 70 n*M* TPA in the culture medium for 17 or 72 h (Spinelli et al., 1982). Differentiation of the cells has been reported after treatment with 70 n*M* TPA for 72 h (Spinelli et al., 1982; Åkerman et al., 1984). TPA was serially diluted in media from a 1 m*M* stock dissolved in dimethyl sulfoxide such that the final concentration of dimethyl sulfoxide in the growth media was <0.01%. Cells were differentiated with retinoic acid by addition of 1 μM retinoic acid 48 h after seeding; growth was continued for 6 days. Retinoic acid was serially diluted in media from a 10 m*M* stock dissolved in ethanol such that the concentration of ethanol in the growth media was 0.1%. Control media contained 0.1% ethanol. All cultures were fed every 48 h. Cell viability was determined using trypan blue exclusion. Differentiation was monitored by visually noting increased neurite extension. Cell number was determined by hemocytometer. Prominent neurite extension was found in cells treated with either TPA for 72 h or retinoic acid. Although the SK-N-SH cell line has been reported to exhibit both neuroblast and epithelial phenotypes (Ross et al., 1983), under our culture conditions, i.e., relatively prolonged culture at low initial subculture densities, only the neuroblast phenotype is found (Sadee et al., 1987). All cells were used within 8–10 days of subculture. Cells were seeded at a density of ~400,000 cells per flask.

Cell treatment and subcellular fractionation

Cells were dislodged from the flasks in Puck's buffer, pelleted, and resuspended in incubation buffer (142 m*M* NaCl, 30 m*M* sodium HEPES, 5.6 m*M* KCl, 3.6 m*M* $NaHCO_3$, 2.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, and 5.6 m*M* D-glucose, pH 7.4). Cells were treated with 100 μM carbachol for variable intervals at 37°C. After incubation, cells were homogenized in buffer containing 320 m*M* sucrose, 40 m*M* Tris, 3 m*M* $MgCl_2$, 1 μM leupeptin, 1 μM pepstatin, and 1 m*M* phenylmethylsulfonyl fluoride (pH 7.4), and 100,000-*g* particulate (membrane) and supernatant (cytosol) fractions were prepared as described (Mangels and Gnegy, 1990). In inhibitor studies, cells were preincubated with either 10 μM Ro 31-8220 for 5 min or 100 μM genistein for 40 min before addition of 100 μM carbachol.

Radioimmunoassay

CaM was quantified by using affinity-purified sheep anti-CaM (Sweet et al., 1991) as described (Roberts-Lewis et al., 1986). Cytosol samples were heated for 6 min and particulate fractions for 12 min before assay. Treatment and control groups were always assayed in the same experiment. This was important because the basal levels of CaM would vary from experiment to experiment.

Immunoblotting

Cell membrane and cytosol fractions were prepared, diluted in sample buffer, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the percentages of polyacrylamide indicated. Proteins were electrophoretically transferred to nitrocellulose membranes at 8 mA overnight. Blots were incubated in blocking buffer [3% (wt/vol) dry milk, 0.2% Tween 20, 0.15 *M* NaCl, and 20 m*M* Tris-HCl, pH 7.5] for 1 h. Antibodies were diluted in blocking buffer as indicated in figure legends and incubated with blots for 2 h. Alkaline phosphatase-conjugated anti-rabbit IgG (1:5,000) was incubated with blots for 2 h and washed extensively. The antigen–antibody complex was detected with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All steps were performed at room temperature.

Myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation

On the day of the experiment, medium was removed, monolayers were washed twice with 2 ml of phosphate-free (and serum-free) DMEM per well, and cells were incubated for 1 h in phosphate-free DMEM at 37°C. Medium was removed and replaced with 0.5 mCi of $^{32}P_i$ in 1 ml of phosphate-free DMEM per well, and incubation was continued as above for 2 h. Drugs (or vehicle) were added directly to the medium for indicated times. Drug (TPA or carbachol) preincubations were included as part of the 2-h labeling period. The reaction was stopped by removing the radioactive media by aspiration and washing the monolayers twice with cold (4°C) Tris-buffered saline. The Tris-buffered saline was removed, cells were extracted with 150–300 μl of 2.5% perchloric acid (PCA), 150 m*M* NaCl, and 1% Triton X-100 (Deloulme et al., 1990), removed with a cell scraper, and transferred into microfuge tubes on ice. Extracts were sonicated for 15 min in a water bath sonicator, centrifuged for 10 min at 15,000 *g* at 4°C, and then neutralized with 2 *M* NaOH. Protein concentration of supernatants (PCA-soluble fraction) was measured using the Pierce BCA protein assay with bovine serum albumin as a standard. Data were quantified using either a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) or a Hoefer model GS365W scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Peak areas were quantified by Gaussian integration using the Hoefer model GS365W electrophoresis data system.

MARCKS immunoprecipitation

Immunoprecipitation of MARCKS from $^{32}P_i$-labeled cells was performed as described (Lobaugh and Blackshear, 1990) using 5 μg of neutralized PCA-soluble extract per condition. Samples were precleared by incubating while rotating for 1 h at 4°C with a 1:100 dilution of normal rabbit serum and 50 μl of a 20% slurry of protein A-Sepharose in TEG buffer [10 m*M* TES (pH 7.6), 50 m*M* NaCl, 4 m*M* EDTA, and 10% glycerol]. Samples were spun at 15,000 *g* for 5 min, and the pellet was discarded. Supernatants were then incubated overnight with rotation at 4°C with a 1:100 dilution of MARCKS antiserum (generously provided by P. Blackshear, Duke University), after which 50 μl of a 20% slurry of protein A-Sepharose was added to each tube. Incubation was continued for 2 h, and samples were centrifuged as above. The supernatants were removed, and the immune pellets were washed three times with 500 μl of TEG buffer. The final pellets were subjected to electrophoresis on a 7% sodium dodecyl sulfate-polyacrylamide gel, dried, and autoradiographed.

RESULTS

Effect of carbachol on CaM localization in TPA-pretreated SK-N-SH cells

In control cells, carbachol elicited a robust increase in cytosolic CaM and a decrease in particulate CaM that was maximal at 15 min (Fig. 1). After 17 h of TPA, carbachol was unable to alter the localization of CaM (Fig. 1). TPA was similarly unable to redistribute CaM in cells treated for 17 h with TPA (data not shown). The ability of carbachol to redistribute CaM was restored on continuing TPA treatment for 72 h

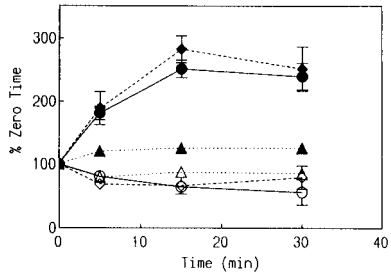

FIG. 1. Time course of carbachol-mediated CaM translocation in untreated or 17- or 72-h TPA-treated SK-N-SH cells. Cells were untreated (circles) or treated with 70 n*M* TPA for 17 (triangles) or 72 h (diamonds). Cells were treated with 100 μ*M* carbachol for variable intervals at 37°C, and CaM was quantified in 100,000-*g* supernatants (solid symbols) and pellets (open symbols) as stated in Materials and Methods. Results for control and 72-h TPA-treated cells are expressed as percentages of values at zero-time and are mean ± SEM (bars) values of triplicate determinations from three different experiments. Results for 17-h TPA-treated cells are the average of triplicate determinations from two different experiments that did not differ by >5%. Zero-time values for CaM (in ng per 10^6 cells) in cytosol and membrane fractions are as follows: from nondifferentiated cells, 12 ± 1.3 and 47 ± 7; from 17-h TPA-treated cells, 16 and 53; and from 72-h TPA-treated cells, 12 ± 1 and 32 ± 5, respectively.

(Fig. 1). In 72-h TPA-treated cells, carbachol elicited an elevation of cytosolic CaM content that was not significantly different from that in untreated cells.

Localization and immunoblotting of PKC isozymes

We wished to examine whether select PKC isozymes were down-regulated by the TPA treatment and whether changes in isozyme content of PKC would explain the difference in the carbachol response between 17- and 72-h TPA-treated cells. The contents of the Ca^{2+}-dependent PKC isozymes, α and β, the Ca^{2+}-independent PKC isozyme, ε, and the TPA- and diacylglycerol-independent PKC isozyme, ζ, were determined by immunoblots in the membrane and cytosol fractions of untreated cells and cells treated for 17 or 72 h with TPA. These are the predominant isozymes in SK-N-SH cells (Baumgold and Dyer, 1994; Sproull et al., 1995). In untreated control cells, α-PKC was mainly localized in the cytosol, although a measurable amount was detected in the particulate fraction (Fig. 2). Treatment of cells for either 17 or 72 h with TPA abolished α-PKC in cytosol fractions and reduced (17 h) or abolished (72 h) α-PKC in membrane fractions. In untreated cells, β-PKC was predominantly localized in the cytosol, although a low but detectable amount was detected in the particulate fraction. In cells treated for either 17 or 72 h with TPA, the β-PKC in the cytosol remained virtually unchanged from that in nondifferentiated controls, and some β-PKC was faintly detected in the membranes. In nondifferentiated cells, ε-PKC was more evenly distributed between cytosol and membranes. In both 17- and 72-h TPA-treated

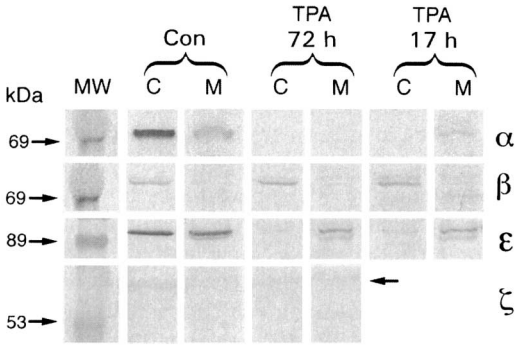

FIG. 2. Immunoblot analysis of subcellular distribution of α-, β-, ε-, and ζ-PKC isozymes in untreated SK-N-SH cells (Con) and cells treated for 17 or 72 h with TPA. Immunoblot analysis of the PKC isozymes was performed in the 100,000-*g* cytosol (C) and membrane (M) fractions prepared as described in Materials and Methods. Cytosol (25 μg) and membrane (40 μg) fractions from SK-N-SH cells were separated by SDS-PAGE using 8.75% polyacrylamide. The molecular weight (MW) markers are given in the far left lane.

cells, ε-PKC was nearly abolished in the cytosol and reduced in the membranes. In contrast, ζ-PKC, which was present at low levels in the cells, was not altered by 72-h TPA treatment.

Effect of TPA treatment on activity of PKC as assessed by phosphorylation of MARCKS

To assess the effect of the TPA treatments on PKC activity, the phosphorylation of a PKC substrate, the CaM-binding protein MARCKS, was determined. The MARCKS protein was visible as a phosphoprotein band at 86 kDa in a PCA extract prepared from $^{32}P_i$-preincubated SK-N-SH cells. Identity of MARCKS as the 86-kDa band was confirmed using immunoprecipitation with MARCKS-specific antisera generously donated by Dr. Perry Blackshear (Duke University) (Fig. 3C). As shown in Fig. 3A, in nontreated cells, phosphorylation of MARCKS was increased by 100 μ*M* carbachol and was robustly stimulated by TPA. In cells treated for either 17 or 72 h with TPA (Fig. 3B and C), phosphorylation of MARCKS was significantly enhanced under basal conditions, and the protein could not be further phosphorylated by either carbachol or TPA.

Effect of PKC inhibition on carbachol-mediated increases in cytosolic CaM content after TPA treatment

To explore further the role of PKC in carbachol-mediated CaM redistribution in 72-h TPA-treated SK-N-SH cells, the effect of the specific PKC inhibitor Ro 31-8220 was determined. As shown in Table 1, both 100 μ*M* carbachol and 25 n*M* TPA increased cytosolic CaM content in untreated cells. Preincubation of the cells with 10 μ*M* Ro 31-8220 significantly blocked the effect of both carbachol and TPA in increasing cytosolic CaM. In cells treated for 72 h with TPA, the ability of carbachol to elicit increases in cytosolic CaM was similar to that of the control, but the effect of 25 n*M* TPA was significantly reduced. The weak stimulating effect of TPA was further reduced by preincubation with 10 μ*M* Ro 31-8220. The effect of carbachol, however, was not blocked by preincubation with Ro 31-8220, suggesting that the carbachol action was no longer mediated by PKC. On the other hand, carbachol-mediated increases in cytosolic CaM content in both untreated and 72-h TPA-treated SK-N-SH cells were blocked by treatment with 100 μ*M* genistein.

Effect of retinoic acid differentiation on carbachol-mediated increases in cytosolic CaM content

Treatment of SK-N-SH cells and its clonal cell line, SH-SY5Y, for 72 h with 70 n*M* TPA differentiates the

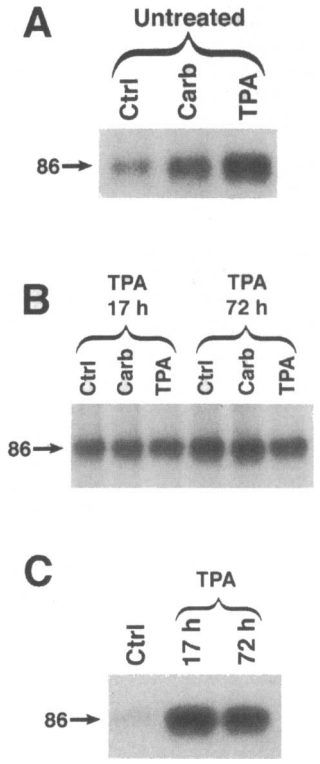

FIG. 3. Phosphorylation of MARCKS in (**A**) untreated control (Ctrl) or (**B**) 17- and 72-h TPA-treated SK-N-SH cells. Cells were treated with TPA as described in Materials and Methods. For the experiment, cells were prelabeled with $^{32}P_i$ for 2 h as described in Materials and Methods including incubation with 100 μ*M* carbachol for 30 s or 100 n*M* TPA for 15 min. The reaction was stopped with 2.5% PCA and neutralized as described in Materials and Methods, and 5 μg of PCA-soluble protein per lane was applied to SDS-PAGE using 7.5% polyacrylamide. **C:** MARCKS was immunoprecipitated from neutralized $^{32}P_i$-labeled PCA-soluble fractions from either control SK-N-SH cells or cells treated for 17 or 72 h with 70 n*M* TPA. MARCKS was immunoprecipitated from 5 μg of precleared PCA-soluble protein as described in Materials and Methods. Immunoprecipitates were electrophoresed on 7% polyacrylamide gels, which were then dried and autoradiographed.

TABLE 1. *Inhibition of carbachol- and TPA-mediated increases in cytosolic CaM content*

Cell pretreatment	% Stimulation with activator	
	100 μM Carbachol	25 nM TPA
Untreated cells		
None	274 ± 26[a]	315 ± 34[a]
10 μM Ro 31-8220	91 ± 33	140 ± 27
100 μM genistein	99 ± 18	ND
72-h TPA-treated cells		
None	250 ± 21[a]	151 ± 10[a]
10 μM Ro 31-8220	200 ± 19[a]	111 ± 13
100 μM genistein	90 ± 1	ND
Retinoic acid-treated cells		
None	252 ± 52[a]	287 ± 52[a]
10 μM Ro 31-8220	115 ± 23	139 ± 49

Cells were untreated or treated with 70 nM TPA for 72 h or retinoic acid for 6 days as described in Materials and Methods. Cells were incubated with 10 μM Ro 31-8220 for 5 min or 100 μM genistein for 40 min before addition of buffer, 100 μM carbachol, or 25 nM TPA for an additional 15 min. Results are expressed as percent stimulation of cytosolic CaM content over buffer controls and are mean ± SEM values (n = 4). Neither Ro 31-8220 nor genistein significantly affected basal cytosolic CaM content. ND, not determined.

[a] $p < 0.005$, significantly different from 100%.

cells (Spinelli et al., 1982; Åkerman et al., 1984). We wished to determine whether the alteration in coupling of carbachol-mediated increases in cytosolic CaM to PKC was a general function of differentiation or a result of continuous TPA treatment. Therefore, we differentiated SK-N-SH cells with retinoic acid and examined the effect of Ro 31-8220 on carbachol-mediated increases in cytosolic CaM. In both TPA- and retinoic acid-differentiated cells, we found responses denoting differentiation: neurite extension (data not shown) and an increase in the growth-associated protein, GAP-43 (Fig. 4) (Kuramoto et al., 1981; Påhlman et al., 1990). In retinoic acid-differentiated cells, increases in cytosolic CaM elicited by both carbachol and TPA were blocked by preincubation with 10 μM Ro 31-8220 (Table 1). Therefore, carbachol-mediated CaM redistribution in retinoic acid-differentiated cells appeared coupled to PKC as in untreated cells.

DISCUSSION

In this study we examined the effect of long-term TPA treatments on carbachol-mediated CaM redistribution in human neuroblastoma SK-N-SH cells. Short-term incubation (15 min) with TPA mimicks carbachol in causing an increase in CaM in the cell cytosol (Mangels and Gnegy, 1990; present study). In addition, the carbachol-mediated redistribution of CaM is inhibited by the PKC inhibitor H-7 (Mangels and Gnegy, 1990) and the more specific inhibitor Ro 31-8220 (present study). Increasing the time of incubation with TPA to 17 h resulted in a total blockade of the carbachol effect, probably through down-regulation of protein kinase isozymes. After 72 h of TPA treatment, the efficacy of carbachol in increasing cytosolic CaM was the same as in untreated cells but was no longer blocked by the PKC inhibitor Ro 31-8220. At 72 h of TPA treatment, the PKC isozymes were still down-regulated. This suggests that the mechanism by which carbachol elicits increases in cytosolic CaM in 72-h TPA-treated cells has become uncoupled from PKC.

Decreases in PKC content and activity have been reported in cells treated for prolonged times with TPA (Cioffi and Fisher, 1990; Trejo and Brown, 1991; Leli et al., 1993). We found reductions in content of both α and ϵ isozymes of PKC in cytosol and membranes from cells treated for 17 and 72 h. There was no decrease, and perhaps even a slight enhancement, in content of the β-PKC isozyme after TPA treatment. The same results were found by Leli et al. (1993) in SH-SY5Y cells differentiated with 10 nM TPA. Parrow et al. (1995), however, found that the α- and ϵ-PKC forms were enriched in growth cones of TPA-differentiated SH-SY5Y cells. On the other hand, some PKC activity is maintained throughout the TPA treatment. We found an increased level of phosphorylation of MARCKS in 17- and 72-h TPA-treated cells. Because it occurred in cells treated for only 17 h with TPA, this increase began before cells were significantly differentiated. Elevated MARCKS phosphorylation has been reported in TPA-differentiated SH-SY5Y cells (Parrow et al., 1992). Blackshear (1988) found that a TPA treatment that down-regulated 95% of the PKC activity in human astrocytoma cells resulted in an

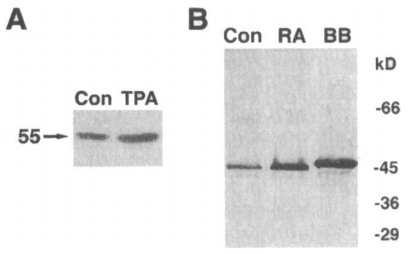

FIG. 4. Western blot analysis of GAP-43. GAP-43/neuromodulin was quantified by immunoblot in homogenate fractions from (**A**) untreated control (Con) and 72-h TPA-treated SK-N-SH cells (10 μg each) and (**B**) untreated and retinoic acid (RA)-treated SK-N-SH cells using a monoclonal antibody to GAP-43 as described in Materials and Methods. In A, the protein was obtained from the PCA-soluble fraction as described in Materials and Methods and separated by SDS-PAGE using 7.5% polyacrylamide. In B, the following concentrations were used: untreated Con SK-N-SH cells, 40 μg; RA-differentiated SK-N-SH cells, 40 μg; and bovine brain homogenate (BB), 40 μg. Proteins (non-PCA-precipitated) were separated by SDS-PAGE using 13% polyacrylamide. Molecular sizes are shown. The difference in apparent molecular size of GAP-43 between A and B is due to the difference in percentage of polyacrylamide in the SDS-PAGE (Benowitz et al., 1987).

enhanced phosphorylation of MARCKS at the PKC-phosphorylated sites. Although MARCKS is a substrate for other protein kinases, such as a proline-directed kinase (Taniguchi et al., 1994) and a PKC-related kinase (Palmer et al., 1996), there was no further phosphorylation in the presence of TPA, suggesting that the PKC substrate sites were already occupied. The PKC phosphorylation site in $pp60^{src}$ is also phosphorylated early in TPA differentiation in SH-SY5Y cells (Bjelfman et al., 1990; Påhlman et al., 1990).

Despite the apparent maintenance of a constitutive PKC activity, the decrease in carbachol- and TPA-mediated redistribution of CaM in 17-h TPA-treated cells is likely to be due to the observed decrease in content of α- and ϵ-PKC. The decrease in TPA-mediated CaM redistribution is maintained in 72-h TPA-treated cells, reflecting the continued down-regulation of α- and ϵ-PKC. The observed effectiveness of carbachol in 72-h TPA-treated cells may not be due to PKC activation as the PKC inhibitor Ro 31-8220 did not inhibit the carbachol effect. Carbachol stimulates several signal transduction activities in SK-N-SH and SH-SY5Y cells. Both carbachol and TPA increase tyrosine kinase activity (Baumgold and Fishman, 1988), and carbachol can increase cyclic AMP formation (Offermanns et al., 1993). We have shown that cyclic AMP can alter CaM localization in SK-N-SH cells, although it is not as active as carbachol (Mangels and Gnegy, 1992). The ability of genistein to inhibit the carbachol effect in untreated cells further suggests that several signal transduction events can be important in carbachol-mediated CaM redistribution. Genistein, at concentrations that inhibit tyrosine kinase, has been shown to inhibit Ca^{2+} fluxes in several cell types (Lee et al., 1993; Sargeant et al., 1993*a,b*). Studies suggest that genistein selectively blocks agonist-induced entry of extracellular Ca^{2+} into cells (Lee et al., 1993; Sargeant et al., 1993*a,b*). Although Ca^{2+} is important for carbachol-mediated CaM redistribution, our studies suggest that external Ca^{2+} is not able to mediate CaM redistribution and that Ca^{2+} released from internal stores is required (McGinnis and Gnegy, 1996). Interactions between tyrosine kinases and PKC activities have been reported; for instance, activation of PKC leads to activation of tyrosine phosphorylation in hippocampal slices (Stratton et al., 1989; Siciliano et al., 1994). Therefore, it is possible that a signal transduction event in addition to PKC activation is involved in CaM redistribution and that this event, perhaps tyrosine kinase activation, is operative in untreated and 72-h TPA-treated cells. It is also likely that this signal transduction event is not dependent on intracellular Ca^{2+} release because a decrease in carbachol-mediated release of intracellular Ca^{2+} in TPA-differentiated SH-SY5Y cells has been reported (Cioffi and Fisher, 1990). We have replicated this finding (K. M. McGinnis, personal communication). This uncoupling from PKC is a result of TPA treatment and not a function of differentiation per se, as Ro 31-8220 was active in blocking the carbachol-mediated increases in cytosolic CaM level in retinoic acid-differentiated cells.

Potential substrates for PKC phosphorylation resulting in CaM redistribution in cells are MARCKS and GAP-43 (neuromodulin, B-50, P57, F1) (Coggins and Zwiers, 1991; Aderem, 1995). Both proteins are CaM-binding proteins that have been proposed to act as CaM depot proteins. Phosphorylation of the proteins by PKC results in a dissociation of CaM (Liu and Storm, 1990; Coggins and Zwiers, 1991; Aderem, 1995). MARCKS is an actin-binding protein that binds and cross-links actin filaments. The cross-linking is disrupted by both phosphorylation and Ca^{2+}/CaM. The high level of basal phosphorylation and the lack of further response to TPA or carbachol suggest that MARCKS plays no role in carbachol-stimulated CaM redistribution in 72-h TPA-treated cells. The constitutive phosphorylation of MARCKS could suggest altered actin cross-linking in TPA-treated cells. GAP-43 is a neuron-specific protein bound to the cytoskeleton and membrane skeleton (Liu and Storm, 1990; Coggins and Zwiers, 1991). Despite elevation of GAP-43 content in TPA-differentiated cells, there is no enhancement in carbachol-mediated CaM redistribution as compared with untreated cells. In the TPA-differentiated cells, however, carbachol appears uncoupled from PKC. It is likely, as suggested by the experiments with genistein, that other substrates and enzymes are involved in CaM redistribution in 72-h TPA-treated cells.

In conclusion, PKC activation appears to be an important intermediate for muscarinic receptor-mediated CaM redistribution in untreated SK-N-SH cells. Treatment of cells for 17 h, a time at which PKC is down-regulated, also down-regulates the muscarinic receptor-mediated CaM redistribution. In cells treated with TPA for 72 h, which are differentiated, the carbachol effect is restored but appears to be uncoupled from TPA-stimulated PKC activity. It is possible that another signal transduction event becomes more prominent in mediating the carbachol action in cells treated for 72 h with TPA.

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