*Journal of Neurochemistry*
Lippincott–Raven Publishers, Philadelphia
© 1998 International Society for Neurochemistry

# Cytosolic Calmodulin Is Increased in SK-N-SH Human Neuroblastoma Cells Due to Release of Calcium from Intracellular Stores

K. M. McGinnis, Z. Shariat-Madar, and M. E. Gnegy

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

**Abstract:** Muscarinic receptor stimulation elicits a redistribution of calmodulin (CaM) from the membrane fraction to cytosol in the human neuroblastoma cell line SK-N-SH. Increasing the intracellular $Ca^{2+}$ concentration with ionomycin also elevates cytosolic CaM. The aim of this study was to investigate the roles of extracellular and intracellular $Ca^{2+}$ pools in the muscarinic receptor-mediated increases in cytosolic CaM in SK-N-SH cells. Stimulus-mediated changes in intracellular $Ca^{2+}$ were monitored in fura-2-loaded cells, and CaM was measured by radioimmunoassay in the 100,000-*g* cytosol and membrane fractions. The influx of extracellular $Ca^{2+}$ normally seen with carbachol treatment in SK-N-SH cells was eliminated by pretreatment with the nonspecific $Ca^{2+}$ channel blocker $Ni^{2+}$. Blocking the influx of extracellular $Ca^{2+}$ had no effect on carbachol-mediated increases in cytosolic CaM (168 ± 18% of control values for carbachol treatment alone vs. 163 ± 28% for $Ni^{2+}$ and carbachol) or decreases in membrane CaM. Similarly, removal of extracellular $Ca^{2+}$ from the medium did not affect carbachol-mediated increases in cytosolic CaM (168 ± 26% of control). On the other hand, prevention of the carbachol-mediated increase of intracellular free $Ca^{2+}$ by pretreatment with the cell-permeant $Ca^{2+}$ chelator BAPTA/AM did attenuate the carbachol-mediated increase in cytosolic CaM (221 ± 37% of control without BAPTA/AM vs. 136 ± 13% with BAPTA/AM). The effect of direct entry of extracellular $Ca^{2+}$ into the cell by $K^+$ depolarization was assessed. Incubation of SK-N-SH cells with 60 m*M* $K^+$ elicited an immediate and persistent increase in intracellular free $Ca^{2+}$ concentration, but there was no corresponding alteration in CaM localization. On the contrary, in cells where intracellular $Ca^{2+}$ was directly elevated by thapsigargin treatment, cytosolic CaM was elevated for at least 30 min while particulate CaM was decreased. In addition, treatment with ionomycin in the absence of extracellular $Ca^{2+}$, which releases $Ca^{2+}$ from intracellular stores, induced an increase in cytosolic CaM (203 ± 30% of control). The mechanism for the CaM release may involve activation of the $\alpha$ isozyme of protein kinase C, which was translocated from cytosol to membranes much more profoundly by thapsigargin than by $K^+$ depolarization. These data demonstrate that release of $Ca^{2+}$ from the intracellular store is important for the carbachol-mediated redistribution of CaM in human neuroblastoma SK-N-SH cells. **Key Words:** Calmodulin—Calcium—Protein kinase C.
*J. Neurochem.* **70,** 139–146 (1998).

Calmodulin (CaM) is a ubiquitous, multifunctional mediator of $Ca^{2+}$ signaling in cells. It was originally discovered as a $Ca^{2+}$ binding activator of cyclic nucleotide phosphodiesterase but has since been found to act as a $Ca^{2+}$-dependent regulator of protein phosphorylation–dephosphorylation cascades, ion transport, cytoskeletal function, and cell growth and proliferation (Manalan and Klee, 1984). Knowledge of the intracellular localization of CaM and mechanisms for changes in its localization in response to stimuli is important for understanding the functions and regulation of CaM. Several laboratories have shown that neurotransmitters and hormones can evoke changes in the subcellular distribution of CaM (Gnegy, 1993). The binding of CaM to various proteins can be altered by changes in target protein phosphorylation state or changes in intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (Estep et al., 1990; Liu and Storm, 1990; Hartwig et al., 1992; Sheu et al., 1995). This suggests that CaM localization may be regulated by intracellular signal-processing mechanisms (Gnegy, 1993). Stimulus-mediated changes in CaM localization would allow CaM to regulate the $Ca^{2+}$/CaM-dependent enzyme systems at various loci.

We have shown that activation of $M_3$ muscarinic

Received June 19, 1997; revised manuscript received September 8, 1997; accepted September 9, 1997.

Address correspondence and reprint requests to Dr. M. E. Gnegy at Department of Pharmacology, 2240 MSRB III, University of Michigan School of Medicine, Ann Arbor, MI 48109-0632, U.S.A.

The current address of Dr. Z. Shariat-Madar is Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI 48109, U.S.A.

*Abbreviations used:* BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl) ester; $[Ca^{2+}]_i$, intracellular free $Ca^{2+}$ concentration; CaM, calmodulin; $IP_3$, inositol 1,4,5-trisphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C.

receptors in SK-N-SH human neuroblastoma cells by the muscarinic agonist carbachol mediates an increase in cytosolic CaM (Mangels and Gnegy, 1990). Activation of $M_3$ receptors in these cells leads to the formation of inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol via phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate. $IP_3$ formation evokes a pronounced increase in $[Ca^{2+}]_i$ after binding to its receptor on the endoplasmic reticulum (Fisher et al., 1989; Lambert and Nahorski, 1990; Berridge, 1993). The $Ca^{2+}$ signal is characterized by a rapid peak rise due to the release of $Ca^{2+}$ from $IP_3$-sensitive intracellular stores followed by a sustained plateau phase that is dependent on the influx of extracellular $Ca^{2+}$ ($[Ca^{2+}]_e$) (Baird et al., 1989; Lambert et al., 1991). Diacylglycerol formation leads to the activation of several protein kinase C (PKC) isozymes. A number of CaM binding proteins are PKC substrates. Of particular interest are neuromodulin (also called F-1, GAP-43, and B-50) and myristoylated alanine-rich C kinase substrate (MARCKS). Both of these proteins are found in relative abundance in the brain and have been postulated to act as CaM ''sinks'' in unstimulated cells (Estep et al., 1990; Liu and Storm, 1990; Hartwig et al., 1992; Slemmon and Martzen, 1994).

The carbachol-mediated increase in cytosolic CaM involves both PKC activation and the increase in $[Ca^{2+}]_i$. The carbachol effect was mimicked by treatment with a phorbol ester and the $Ca^{2+}$ ionophore ionomycin and was blocked by a PKC inhibitor (Mangels and Gnegy, 1990). The aim of this study was to further elucidate the role of $Ca^{2+}$ fluxes in muscarinic receptor-mediated redistribution of CaM. We find that the rise in cytosolic CaM and decrease in membrane-bound CaM are dependent on increases in intracellular $Ca^{2+}$ due to release from an intracellular store. This is the first report demonstrating the crucial role of the release of $Ca^{2+}$ from the intracellular pool in the muscarinic receptor-mediated redistribution of CaM.

## EXPERIMENTAL PROCEDURES

### Materials

Tissue culture supplies were from Corning Glass Works (Corning, NY, U.S.A.). Cell culture reagents, Dulbecco's modified Eagle's medium, fetal bovine serum, EGTA, $NiCl_2$, KCl, thapsigargin, and carbachol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fura-2-acetoxymethyl ester (fura-2/AM) and 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) were from Molecular Probes (Eugene, OR, U.S.A.). All compounds used were reagent grade or better. $^{125}$I-CaM (~2,000 Ci/mmol; prepared by Bolton–Hunter radioiodination) was purchased from NEN Du Pont (Boston, MA, U.S.A.).

### Cell culture and sample preparation

Human SK-N-SH neuroblastoma cells were grown to confluency in Corning Ti-75 polystyrene tissue culture flasks in 20 ml Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 5% $CO_2$ atmosphere at 37°C (Fisher et al., 1989). Although the SK-N-SH cell line has been reported to exhibit both neuroblast and epithelial phenotypes (Ross et al., 1980), under our culture conditions, i.e., relatively prolonged culture at low initial subculture densities, only the neuroblastoma phenotype is found (Sadée et al., 1987). Cultures were fed every 48 h and were used within 8–10 days of subculture. Cells were detached with Puck's D1 solution (Honegger and Richelson, 1976) and collected by centrifugation. They were resuspended in 30 m*M* Na-HEPES buffer (pH 7.4), 142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, and 5.6 m*M* D-glucose. The indicated concentration of drug was added to cell suspension and incubated in a shaking water bath at 37°C. All carbachol treatments were for 15 min. In experiments with $Ni^+$ or BAPTA, cells were pretreated with 50 μ*M* BAPTA/AM for 15 min or 3 m*M* $Ni^{2+}$ for 3 min prior to the 15-min 100 μ*M* carbachol treatment. Incubations were stopped on ice with the addition of cold saline followed by rapid centrifugation to collect cells. Some experiments were performed by treating cells on the dish rather than in suspension. We found absolutely no difference in the results whether cells were treated in suspension or on the dish. The cells were homogenized in a buffer containing 40 m*M* Tris-HCl (pH 7.4), 0.32 *M* sucrose, 1 μ*M* leupeptin, 1 μ*M* pepstatin, and 1 m*M* phenylmethylsulfonyl fluoride buffer using a Teflon/glass homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min to remove cell debris. The resulting supernatant was centrifuged at 100,000 *g* for 1 h to obtain cytosol and membrane fractions.

### CaM quantification

The CaM content of the fractions was determined by radioimmunoassay. Cytosol (100,000-*g* supernatant) and membrane fractions, prepared as described above, were solubilized in 0.5% Lubrol PX. The fractions then were diluted in the homogenization buffer and heated for 10 min at 95°C prior to CaM quantification. The radioimmunoassay was performed as fully described by Roberts-Lewis et al. (1986) using affinity-purified sheep anti-CaM (Sweet et al., 1991) and Bolton–Hunter radioiodinated CaM (NEN Du Pont). Bound and free CaM were separated using donkey anti-sheep IgG and polyethylene glycol immunoprecipitation. Following centrifugation, the supernatant was discarded, and the $^{125}$I-CaM in the pellets was quantitated using gamma counting.

Results obtained from the preheated CaM standards were used to construct a standard curve, from which values of the unknown samples were obtained by interpolation. Assays contained 5 μg of membrane or cytosol protein. Treatment and control groups were always measured in the same experiment to control for interassay variability. Data were initially calculated as nanograms of CaM per microgram of protein and then expressed as percent control due to interassay variability. CaM was purified from bovine testis as described (Dedman et al., 1978).

### Calcium measurement

Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was measured in fura-2-loaded cells using dual-wavelength spectrofluorometry. Cells were incubated with 2 μ*M* fura-2/AM for 30 min at 37°C. The fura-2-loaded cells were washed twice

and resuspended in incubation buffer for a final protein concentration of ~3 mg/ml. Fluorescence measurements were made on 1-ml aliquots of cells maintained at 37°C and constantly stirred. Changes in $[Ca^{2+}]_i$ were monitored as changes in the fluorescence ratio of the 340/380-nm excitation wavelength in a Shimadzu RF-5000 spectrofluorometer. Calcium concentrations, given in the figure legends, were calculated by the method of Grynkiewicz et al. (1985).

### PKC isozyme immunoblotting

The 100,000-*g* supernatant (15 $\mu g$) and pellet (30 $\mu g$) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a 4–20% acrylamide gel using Tris/glycine running buffer and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, U.S.A.). Membranes were blocked for 30 min with 5% nonfat dry milk in 20 m*M* Tris-HCl and 0.05% Tween and incubated in blocking buffer with a 1:1,000 dilution of the following antibodies: monoclonal mouse anti-PKC-$\alpha$ (1:1,000 dilution; a generous gift from Karen L. Leach, Upjohn Laboratories, Kalamazoo, MI, U.S.A.), affinity-purified rabbit anti-PKC-$\beta$ (Life Technologies, Grand Island, NY, U.S.A.), or affinity-purified rabbit anti-PKC-$\epsilon$ (Life Technologies) or -$\delta$ (Trans Lab, Lexington, KY, U.S.A.). Protein bands were visualized by incubation with biotinylated secondary antibody and strepavidin/alkaline phosphatase conjugated with tertiary antibody (Amersham, Arlington Heights, IL, U.S.A.) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma).

## RESULTS

### Effect of $Ni^{2+}$ pretreatment or extracellular EGTA on carbachol-mediated changes in CaM redistribution

The role of extracellular $Ca^{2+}$ on carbachol-mediated changes in CaM distribution was assessed by blocking the entrance of extracellular $Ca^{2+}$ using $Ni^{2+}$, a nonspecific $Ca^{2+}$ channel blocker. Cells were loaded with the cell-permeant ratiometric $Ca^{2+}$ fluorophore fura-2. Treatment with 100 $\mu M$ carbachol induced an instantaneous rise in $[Ca^{2+}]_i$ in SK-N-SH cells followed by a sustained plateau (Fig. 1A). The initial peak corresponds to the release of $Ca^{2+}$ from $IP_3$-sensitive stores, while the plateau is due to the influx of extracellular $Ca^{2+}$. After pretreatment with 3 m*M* $Ni^{2+}$ for 3 min (Fig. 1B), the sustained plateau due to influx of extracellular $Ca^{2+}$ (Lambert and Nahorski, 1990) was no longer apparent, but the instantaneous carbachol- and $IP_3$-mediated rise in intracellular $Ca^{2+}$ was unaffected (cf. Fig. 1A and B).

CaM in the cytosolic and membrane fractions was measured in SK-N-SH cells pretreated with buffer or 3 m*M* $Ni^{2+}$ for 3 min followed by a 15-min treatment with 100 $\mu M$ carbachol. Results were compared with changes in CaM induced by carbachol in cells with no $Ni^{2+}$ pretreatment. As shown in Fig. 1C, carbachol induced a significant increase in cytosolic CaM and decrease in particulate CaM that were not altered by pretreatment with $Ni^{2+}$. Pretreatment with $Ni^{2+}$ alone did not alter basal levels of CaM.

In a separate experiment, the effect of extracellular $Ca^{2+}$ on carbachol-mediated increases in cytosolic CaM was examined further by deleting $Ca^{2+}$ from the extracellular buffer and addition of 20 $\mu M$ EGTA. As shown in Fig. 1D, the increase in cytosolic CaM elicited by 100 $\mu M$ carbachol was the same in the presence or absence of extracellular $Ca^{2+}$.

### Effect of pretreatment with cell-permeant $Ca^{2+}$ chelator BAPTA/AM on carbachol-mediated redistribution of CaM

The role of intracellular $Ca^{2+}$ in the carbachol-mediated increase in cytosolic CaM was assessed by treating cells with BAPTA/AM, a cell-permeant $Ca^{2+}$-selective chelator (Tsien, 1980). BAPTA pretreatment ablates the $IP_3$-mediated $Ca^{2+}$ increase in neuronal cells (Markram and Segal, 1992). To confirm this, SK-N-SH cells were loaded with fura-2/AM and treated for 15 min with 50 $\mu M$ BAPTA/AM prior to incubation with 100 $\mu M$ carbachol. Pretreatment with BAPTA/AM completely blocked the carbachol-induced increase in $[Ca^{2+}]_i$ (Fig. 2A). An increase in $[Ca^{2+}]_i$ was apparent, however, following addition of the $Ca^{2+}$ ionophore ionomycin.

As shown in Fig. 2B, pretreatment with 50 $\mu M$ BAPTA for 15 min attenuated the carbachol-mediated increases in cytosolic CaM. Treatment with 50 $\mu M$ BAPTA alone for 30 min did not significantly alter the levels of cytosolic CaM.

### Effect of extracellular $Ca^{2+}$ on redistribution of CaM

The experiments with carbachol and $Ni^{2+}$ suggested that carbachol-stimulated entry of $Ca^{2+}$ through voltage-sensitive $Ca^{2+}$ channels did not elicit a redistribution of CaM from membrane to cytoplasm. To investigate this further, we used $K^+$ depolarization as a method to directly elicit the entry of extracellular $Ca^{2+}$ through activation of voltage-sensitive $Ca^{2+}$ channels at the plasma membrane (Smart et al., 1995). SK-N-SH cells were treated with 60 m*M* $K^+$. To ensure isotonicity, the $Na^+$ concentration in the buffer was lowered commensurately. Upon exposure to high $K^+$, $[Ca^{2+}]_i$ increased immediately (Fig. 3A). Subsequent addition of carbachol evoked a sharp $Ca^{2+}$ spike that corresponds to the release of $Ca^{2+}$ from the intracellular store (Fisher et al., 1989).

CaM in the 100,000-*g* membrane and cytosol fractions was quantified in cells exposed to 60 m*M* $K^+$ or vehicle for 1, 5, 15, or 30 min. As shown in Fig. 3B, there was no significant change in CaM in cells exposed to 60 m*M* $K^+$ at any time point.

### Effect of direct release of intracellular $Ca^{2+}$ stores on redistribution of CaM

The role of $Ca^{2+}$ released directly from the intracellular store was investigated using thapsigargin, an inhibitor of endoplasmic reticulum $Ca^{2+}$ transport ATPases (Thastrup et al., 1987). Treatment of SK-N-SH cells with 1 $\mu M$ thapsigargin results in an increase of

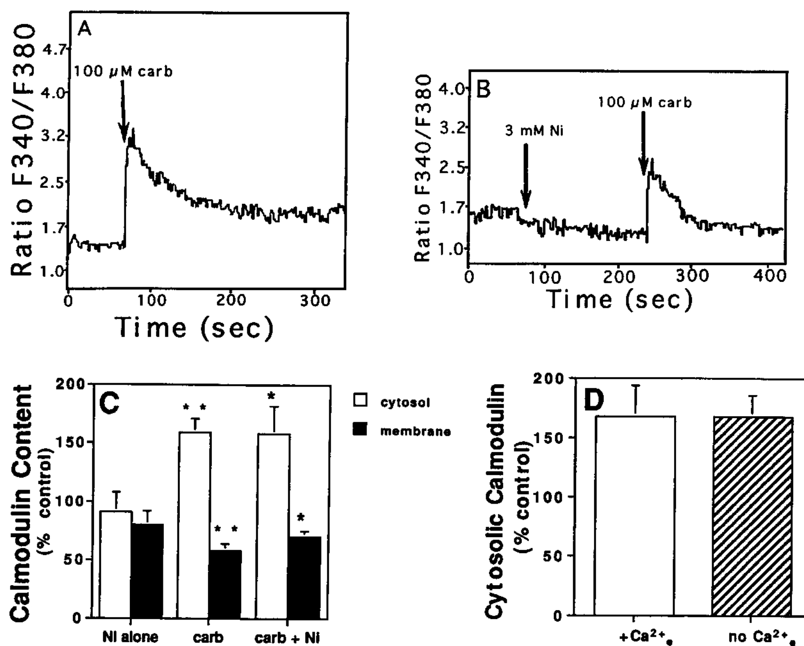



**FIG. 1.** Effect of $Ni^{2+}$ on carbachol (carb)-mediated increases in intracellular $Ca^{2+}$ (A and B) and redistribution of CaM (C). **A** and **B:** Fura-2-loaded SK-N-SH cells in suspension were pretreated with buffer alone (A) or 3 m*M* $Ni^{2+}$ (B) for 3 min prior to the addition of 100 μ*M* carb. In A, basal, carb peak, and plateau $Ca^{2+}$ values were 59, 369, and 115 n*M*, respectively. In B, basal, $Ni^{+}$ alone, peak carb, and plateau $Ca^{2+}$ values were 91, 40, 254, and 51 n*M*, respectively. **C:** SK-N-SH cells were treated with 3 m*M* $Ni^{2+}$ for 3 min, after which 100 μ*M* carbachol was added for 15 min. CaM was quantified in the 100,000-*g* cytosol (white columns) and membrane (black columns) fractions as described in Experimental Procedures and reported as percent control. **D:** SK-N-SH cells were treated with 100 μ*M* carbachol for 15 min in the incubation buffer as described ($+Ca^{2+}{}_e$) or the buffer containing 20 μ*M* EGTA with no added calcium. CaM was quantified in the 100,000-*g* cytosol and results are given as percent control. Results are given as means ± SEM for n = 3–4. Control values of CaM in supernatant and pellet were 0.38 ± 0.05 and 0.23 ± 0.02 μg/mg of protein, respectively. $^*p \le 0.05$ as compared with 100%; $^{**}p \le 0.01$ as compared with 100%.

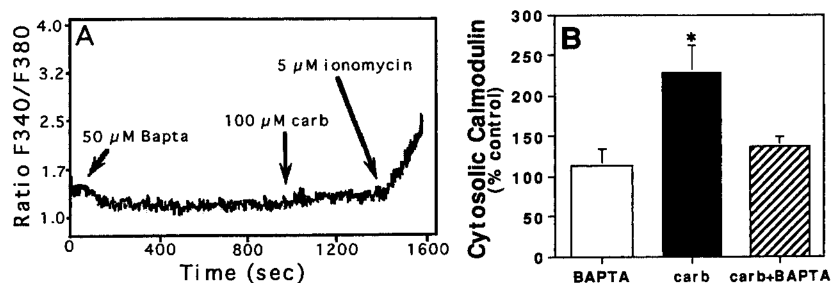
intracellular $Ca^{2+}$ via depletion of intracellular stores through uncompensated leakage (Thastrup et al., 1990; Inesi and Sagara, 1992). As shown in Fig. 4A, treating fura-2-loaded SK-N-SH cells with 1 μ*M* thapsigargin induced an increase in $[Ca^{2+}]_i$ that slowly declines. Treatment of SK-N-SH cells with 1 μ*M* thapsigargin for various times produced a significant increase in cytosolic CaM and a decrease in particulate CaM (Fig. 4B). The increase in cytosolic CaM was maximal at 15 min (152 ± 13% of control) and declined to control levels by 120 min.

To assess further the role of intracellular $Ca^{2+}$ stores, cells were treated with the $Ca^{2+}$ ionophore ionomycin in the absence of extracellular $Ca^{2+}$. In the absence of extracellular $Ca^{2+}$, ionomycin will release $Ca^{2+}$ from the intracellular stores alone (Augustine and Neher, 1992). SK-N-SH cells were incubated with 20 μ*M* EGTA with no added $Ca^{2+}$ for 5 min. Cells were subsequently treated for 15 min with 1 μ*M* ionomycin, and cytosolic CaM levels were quantified. In the absence of extracellular $Ca^{2+}$, treatment with 1 μ*M* ionomycin increased cytosolic CaM by 203 ± 30% of control ($p < 0.05$).

## Translocation of PKC isozymes in response to thapsigargin or $K^+$ depolarization

Our previous results demonstrated that activation of PKC was involved in CaM redistribution in SK-N-SH cells (Mangels and Gnegy, 1990). To begin to elucidate the mechanism by which different sources of $Ca^{2+}$ differentially release CaM, we investigated the activation of PKC isozymes in SK-N-SH cells by the release of intracellular $Ca^{2+}$ or the influx of extracellular $Ca^{2+}$. SK-N-SH cells were treated for various times with 1

**FIG. 2.** Effect of BAPTA/AM on carbachol (carb)-mediated increases in intracellular $Ca^{2+}$ (A) and cytosolic CaM (B). **A:** Fura-2-loaded SK-N-SH cells were pretreated with 50 μ*M* BAPTA/AM for 15 min followed by 100 μ*M* carb and 5 μ*M* ionomycin as indicated. Basal $Ca^{2+}$ values and those after BAPTA/AM treatment were 60 and 35 nM, respectively. **B:** CaM was quantified in the 100,000-*g* cytosol in SK-N-SH cells pretreated with 50 μ*M* BAPTA for 15 min followed by 15 min of 100 μ*M* carbachol as described in Experimental Procedures. Results are given as means ± SEM for n = 5–6 experiments. $^*p < 0.01$ as compared with control.



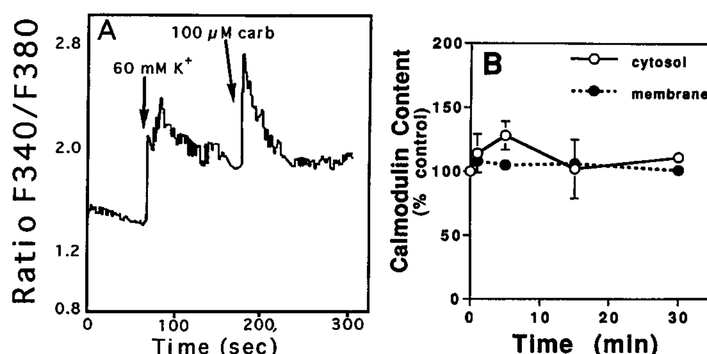



**FIG. 3.** Effect of depolarization with 60 m*M* KCl on the redistribution of CaM in SK-N-SH cells. **A:** Fura-2-loaded SK-N-SH cells were treated with 60 m*M* $K^+$ and 100 μ*M* carbachol (carb) as indicated. Basal, $K^+$ peak, carb peak, and plateau $Ca^{2+}$ values were 50, 218, 273, and 140 n*M*, respectively. **B:** SK-N-SH cells were treated with 60 m*M* $K^+$ for the indicated times. Following the incubations, the 100,000-*g* cytosol and membrane fractions were prepared as described in Experimental Procedures. Results are given as means ± SEM of the percent control. No values differed significantly from control.

μ*M* thapsigargin or 60 m*M* $K^+$. As shown in Fig. 5, thapsigargin treatment elicited the translocation of the $\alpha$ isozyme of PKC within 30 s, and the translocation was still apparent at 15 min. The effect of thapsigargin on the translocation of PKC-$\alpha$ was much more profound than that elicited in response to treatment with 60 m*M* KCl at times up to 15 min. Neither thapsigargin nor 60 m*M* KCl elicited translocation of the $\beta$, $\delta$, or $\epsilon$ isozymes of PKC in SK-N-SH cells (data not shown).

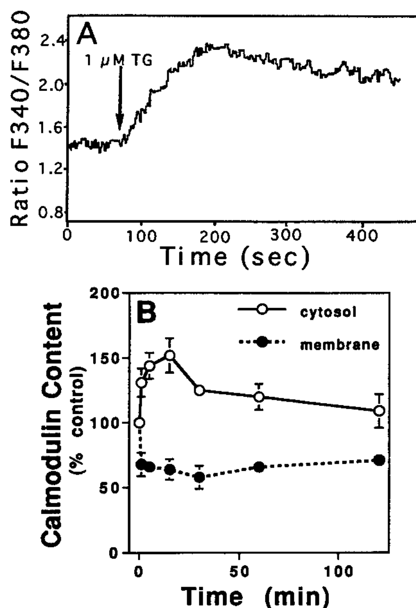



**FIG. 4.** Effect of thapsigargin on increases in intracellular $Ca^{2+}$ (A) and redistribution of CaM in SK-N-SH cells (B). **A:** Fura-2-loaded SK-N-SH cells were treated with 1 μ*M* thapsigargin. Basal and thapsigargin peak $Ca^{2+}$ values were 50 and 209 n*M*, respectively. **B:** CaM levels in the 100,000-*g* cytosol and membrane fractions were measured in cells treated for the indicated times with 1 μ*M* thapsigargin. Results are given as means ± SEM of the percent control for six separate experiments. For supernatant values, $p < 0.05$ (1 min) and $p < 0.005$ (5, 15, and 30 min) as compared with control. For membrane values at 5, 15, and 30 min, $p < 0.01$ as compared with control.

We used SK-N-SH human neuroblastoma cells to investigate the role of intracellular and extracellular $Ca^{2+}$ fluxes on stimulus-induced increases in cytosolic CaM. This study demonstrates that the release of $Ca^{2+}$ from intracellular stores is highly important for muscarinic $M_3$ receptor-mediated redistribution of CaM in SK-N-SH cells. This finding serves to further elucidate the mechanism for muscarinic receptor-mediated elevations in cytosolic CaM previously reported by this laboratory (Mangels and Gnegy, 1990).

Our results indicate that $Ca^{2+}$ is required for carbachol-mediated redistribution of CaM, but entry of extracellular $Ca^{2+}$ did not appear to have a significant role in this process. Neither activation of voltage-sensitive $Ca^{2+}$ channels through depolarization nor inhibition of these channels by $Ni^{2+}$ altered basal or carbachol-mediated redistribution of CaM. The inability of extracellular $Ca^{2+}$ to influence carbachol-mediated increases in cytosolic CaM was further demonstrated by the ability of carbachol to increase cytosolic CaM after extracellular $Ca^{2+}$ had been removed from the medium. Carbachol can increase both $[Ca^{2+}]_i$ and $IP_3$ formation in the absence of extracellular $Ca^{2+}$ (Fisher et al., 1989). An elevation in $[Ca^{2+}]_i$, regardless of the source, appeared to mediate the carbachol-stimulated redistribution of CaM. The cell-permeant $Ca^{2+}$ chelator BAPTA/AM blocked the ability of carbachol to increase cytosolic CaM. In addition, a redistribution

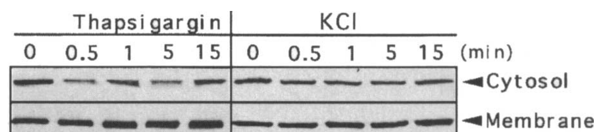



**FIG. 5.** Translocation of the $\alpha$ isozyme of PKC in response to thapsigargin and KCl. SK-N-SH cells were treated with 1 μ*M* thapsigargin or 60 m*M* KCl for the given times. Cytosol and membrane fractions were prepared as described in Experimental Procedures, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to Immobilon-P. Blots were incubated with monoclonal anti-PKC-$\alpha$ as described.

of CaM was elicited by direct elevation of $[Ca^{2+}]_i$ either by blocking refilling of the intracellular store with thapsigargin or by releasing $Ca^{2+}$ from intracellular stores using low concentrations of ionomycin in the absence of $Ca^{2+}$ in the medium. These results suggest that a general elevation of $[Ca^{2+}]_i$, independent of the source, is not mediating the carbachol-dependent redistribution of CaM. In addition, the duration of the elevated $[Ca^{2+}]_i$ is not critical because both $K^+$ depolarization and thapsigargin elevated $[Ca^{2+}]_i$ for many minutes, but only thapsigargin was effective in redistributing CaM. It would appear that $IP_3$ formation and release of $Ca^{2+}$ from intracellular stores are important factors for carbachol-induced increases in cytosolic CaM. It has been reported, however, that $K^+$ depolarization of the SK-N-SH subclone SH-SY5Y, in addition to opening voltage-sensitive calcium channels, also leads to activation of phospholipase C and formation of $IP_3$ (Smart et al., 1995). There are at least two possibilities to explain the inability of the $IP_3$ generated by $K^+$ depolarization to influence cytosolic CaM concentrations. First, the $K^+$-induced increase in $IP_3$ is both small and brief (Smart et al., 1995) as compared with the increase in response to carbachol (Fisher et al., 1989). Second, $IP_3$ pools may be compartmentalized. In tracheal smooth muscle, it was found that not all $IP_3$ formed had access to $IP_3$ receptors on the endoplasmic reticulum (Baron et al., 1992). Therefore, not all events leading to an increase in $[Ca^{2+}]_i$ or $IP_3$ will influence CaM localization. Thapsigargin elicited a much more pronounced translocation of the $\alpha$ isozyme of PKC from cytosol to membranes than did $K^+$ depolarization. This demonstrates that PKC isozymes may be selectively activated in response to increases in $[Ca^{2+}]_i$. Since PKC has been strongly implicated in the mechanism of CaM translocation in SK-N-SH cells (Mangels and Gnegy, 1990), these data suggest that selective activation of PKC-$\alpha$ may be important for CaM redistribution in these cells.

Even though the release of intracellular $Ca^{2+}$ appears to be crucial for the carbachol-mediated redistribution of CaM, it may not totally mimic the effect of carbachol. As shown previously (Mangels and Gnegy, 1990), the carbachol-mediated increase of CaM in the supernatant lasted 4 h. The thapsigargin-mediated increase in cytosolic CaM peaked at 30 min and then declined to control level. Therefore, another factor besides release of intracellular $Ca^{2+}$ activated by carbachol may be important in helping to maintain an increase in soluble CaM.

It has been postulated that increases in $[Ca^{2+}]_i$ would lead to an increase in the amount of CaM available for activation, or "free" CaM (Means, 1988). The carbachol-mediated redistribution of CaM likely represents a dissociation of CaM from a predominantly particulate set of binding proteins and rebinding to a more soluble set of target proteins. Several proteins, such as neuromodulin (Estep et al., 1990) and MARCKS (Ouimet et al., 1990), are considered to be CaM "sinks," proteins that bind CaM in the unstimulated cell and release it upon stimulation (Liu and Storm, 1990). Neuromodulin, a neuron-specific protein bound to the cytoskeleton and membrane skeleton, exists in brain in concentrations similar to that of CaM (Liu and Storm, 1990; Zwiers and Coggins, 1990). Neuromodulin binds CaM more tightly in the absence of $Ca^{2+}$. Both an elevation of $Ca^{2+}$ levels (Alexander et al., 1988) and phosphorylation of neuromodulin by PKC result in dissociation of CaM (Alexander et al., 1988; Liu and Storm, 1990). Thus, neuromodulin could regulate CaM binding upon stimulus of the cell. A neuromodulin/CaM complex was detected in mouse anterior pituitary AtT-20 cells (Gamby et al., 1996). MARCKS, on the other hand, requires $Ca^{2+}$ to bind CaM (Graff et al., 1989). MARCKS is an actin binding protein that binds CaM while loosely bound to the cytoskeleton. PKC-mediated phosphorylation of MARCKS dissociates CaM from MARCKS and releases MARCKS and possibly CaM into the soluble fraction (Aderem, 1992). Our previous studies suggested that both $Ca^{2+}$ and PKC activation are important in SK-N-SH cells for the carbachol-mediated release of CaM from membranes to cytosol (Mangels and Gnegy, 1990).

Compartmentalization of the proteins binding CaM in the unstimulated cell could also account for the inability of depolarization to evoke increases in cytosolic CaM. These proteins may be compartmentalized in the cell and thereby shielded from influxes of extracellular $Ca^{2+}$. In nondifferentiated PC-12 cells, for example, the plasma membrane is devoid of neuromodulin. With use of immunohistochemistry, labeled neuromodulin was found in lysosomal organelles and Golgi stacks (Van Hooff et al., 1989). In brain, MARCKS is prominently localized to microtubules but is also found in the cytoplasm and plasma membrane (Ouimet et al., 1990). In addition, release of $Ca^{2+}$ from intracellular stores could activate localized PKC isozymes. PKC isozymes are localized on intracellular membranes such as nuclear membrane, endoplasmic reticulum, and Golgi membranes in addition to cytoplasm and plasma membranes (Thomas et al., 1988; James and Olson, 1992; Chida et al., 1994; Lehel et al., 1995). Both thapsigargin (Kiley et al., 1992) and ionomycin (Kiley and Jaken, 1990) activate selective PKC isozymes. Microdomains consisting of CaM depots and PKC isozymes could be localized near intracellular $Ca^{2+}$ pools. The importance of the $Ca^{2+}$ source on signaling outcome is increasingly recognized. For example, gene expression in hippocampal neurons is differentially regulated by distinct $Ca^{2+}$ signaling pathways (Bading et al., 1993; Lerea and McNamara, 1993).

We have demonstrated that an increase in $[Ca^{2+}]_i$ due to release of $Ca^{2+}$ from intracellular stores is important in mediating $M_3$ muscarinic receptor-stimu-

lated redistribution of CaM. A rise in $[Ca^{2+}]_i$ due to influx of extracellular $Ca^{2+}$ after depolarization was ineffective in changing CaM distribution. It is possible that specific microdomains of CaM bound to depot proteins, perhaps in conjunction with PKC isozymes, could exist near intracellular $Ca^{2+}$ pools.

**Acknowledgment:** The authors thank Dr. Steve Fisher for his generous help with the fura-2 measurements and his valuable discussions. We also thank Dr. Karen Leach (Pharmacia and Upjohn Pharmaceutical Inc.) for her generous donation of monoclonal anti-PKC-$\alpha$. This work was supported by NIH grant MH36044, NIH training grant 5T32GM07767, and a National Science Foundation graduate research fellowship to K.M.M.

## REFERENCES

Aderem A. (1992) The MARCKS brothers: a family of protein kinase C substrates. *Cell* **71,** 713–716.

Alexander K. A., Wakim B., Doyle G. S., Walsh K. A., and Storm D. R. (1988) Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. *J. Biol. Chem.* **263,** 7544–7549.

Augustine G. J. and Neher E. (1992) Calcium requirements for secretion in bovine chromaffin cells. *J. Physiol. (Lond.)* **450,** 247–271.

Bading H., Ginty D. D., and Greenberg M. E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* **260,** 181–186.

Baird J. G., Lambert D. G., McBain J., and Nahorski S. R. (1989) Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH. *Br. J. Pharmacol.* **98,** 1328–1334.

Baron C. B., Pompeo J. N., and Azim S. (1992) Inositol 1,4,5-trisphosphate compartmentalization in tracheal smooth muscle. *Arch. Biochem. Biophys.* **292,** 382–387.

Berridge M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature* **361,** 315–325.

Chida K., Sagara H., Suzuki Y., Murakami A., Osada S., Ohno S., Hirosawa K., and Kuroki T. (1994) The eta isoform of protein kinase C is localized on rough endoplasmic reticulum. *Mol. Cell Biol.* **14,** 3782–3790.

Dedman J. R., Welsh M. J., and Means A. R. (1978) $Ca^{2+}$-dependent regulator. Production and characterization of a monospecific antibody. *J. Biol. Chem.* **253,** 7515–7521.

Estep R. P., Alexander K. A., and Storm D. R. (1990) Regulation of free calmodulin levels in neurons by neuromodulin: relationship to neuronal growth and regeneration. *Curr. Top. Cell Regul.* **31,** 161–180.

Fisher S. K., Domask L. M., and Roland R. M. (1989) Muscarinic receptor regulation of cytoplasmic $Ca^{2+}$ concentrations in human SK-N-SH neuroblastoma cells: $Ca^{2+}$ requirements for phospholipase C activation. *Mol. Pharmacol.* **35,** 195–204.

Gamby C., Waage M. C., Allen R. G., and Baizer L. (1996) Analysis of the role of calmodulin binding and sequestration in neuromodulin (GAP-43) function. *J. Biol. Chem.* **271,** 26698–26705.

Gnegy M. E. (1993) Calmodulin in neurotransmitter and hormone action. *Annu. Rev. Pharmacol. Toxicol.* **32,** 45–70.

Graff J. M., Young T. N., Johnson J. D., and Blackshear P. J. (1989) Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* **264,** 21818–21823.

Grynkiewicz G., Poenie M., and Tsien R. Y. (1985) A new generation of calcium indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260,** 3440–3450.

Hartwig J. H., Thelen M., Rosen A., Janmey P. A., Nairn A. C., and Aderem A. (1992) MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* **356,** 618–622.

Honegger P. and Richelson E. (1976) Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell culture. *Brain Res.* **109,** 335–354.

Inesi G. and Sagara Y. (1992) Thapsigargin, a high affinity and global inhibitor of intracellular $Ca^{2+}$ transport ATPases. *Arch. Biochem. Biophys.* **298,** 313–317.

James G. and Olson E. (1992) Deletion of the regulatory domain of protein kinase C alpha exposes regions in the hinge and catalytic domains that mediate nuclear targeting. *J. Cell Biol.* **116,** 863–874.

Kiley S. C. and Jaken S. (1990) Activation of alpha-protein kinase C leads to association with detergent-insoluble components of GH4C1 cells. *Mol. Endocrinol.* **4,** 59–68.

Kiley S. C., Parker P. J., Fabbro D., and Jaken S. (1992) Selective redistribution of protein kinase C isozymes by thapsigargin and staurosporine. *Carcinogenesis* **13,** 1997–2001.

Lambert D. G. and Nahorski S. R. (1990) Muscarinic-receptor-mediated changes in intracellular $Ca^{2+}$ and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y. *Biochem. J.* **265,** 555–562.

Lambert D. G., Challiss R. A., and Nahorski S. R. (1991) Accumulation and metabolism of Ins(1,4,5)P3 and Ins(1,3,4,5)P4 in muscarinic-receptor-stimulated SH-SY5Y neuroblastoma cells. *Biochem. J.* **273,** 791–794.

Lehel C., Oláh Z., Jakab G., Szállási Z., Petrovics G., Harta G., Blumberg P. M., and Anderson W. B. (1995) Protein kinase C $\epsilon$ subcellular localization domains and proteolytic degradation sites. A model for protein kinase C conformational changes. *J. Biol. Chem.* **270,** 19651–19658.

Lerea L. S. and McNamara J. O. (1993) Ionotropic glutamate receptor subtypes activate c-fos transcription by distinct calcium-requiring intracellular signaling pathways. *Neuron* **10,** 31–41.

Liu Y. C. and Storm D. R. (1990) Regulation of free calmodulin levels by neuromodulin: neuron growth and regeneration. *Trends Pharmacol. Sci.* **11,** 107–111.

Manalan A. S. and Klee C. B. (1984) Calmodulin. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **18,** 227–278.

Mangels L. A. and Gnegy M. E. (1990) Muscarinic receptor-mediated translocation of calmodulin in SK-N-SH human neuroblastoma cells. *Mol. Pharmacol.* **37,** 820–826.

Markram H. and Segal M. (1992) The inositol 1,4,5-trisphosphate pathway mediates cholinergic potentiation of rat hippocampal neuronal responses to NMDA. *J. Physiol. (Lond.)* **447,** 513–533.

Means A. R. (1988) Molecular mechanisms of action of calmodulin. *Recent Prog. Horm. Res.* **44,** 223–259.

Ouimet C. C., Wang J. K. T., Walaas S. I., Albert K. A., and Greengard P. (1990) Localization of the MARCKS (87 kDa) protein, a major specific substrate for protein kinase C, in rat brain. *J. Neurosci.* **10,** 1683–1698.

Roberts-Lewis J. M., Welsh M. J., and Gnegy M. E. (1986) Chronic amphetamine treatment increases striatal calmodulin in rats. *Brain Res.* **384,** 383–386.

Ross R. A., Joh T. H., Reis D. J., Spengler B. A., and Biedler J. L. (1980) Neurotransmitter-synthesizing enzymes in human neuroblastoma cells: relationship to morphological diversity. *Advances in Neuroblastoma Research, Proceedings of the 2nd Symposium* **12,** 151–160.

Sadée W., Yu V. C., Richards M. L., Preis P. N., Schwab M. R., Brodsky F. M., and Biedler J. L. (1987) Expression of neurotransmitter receptors and myc protooncogenes in subclones of a human neuroblastoma cell line. *Cancer Res.* **47,** 5207–5212.

Sheu F.-S., Huang F. L., and Huang K.-P. (1995) Differential responses of protein kinase C substrates (MARCKS, neuromodulin, and neurogranin) phosphorylation to calmodulin and S100. *Arch. Biochem. Biophys.* **316,** 335–342.

Slemmon J. R. and Martzen M. R. (1994) Neuromodulin (GAP-43)

can regulate a calmodulin-dependent target in vitro. *Biochemistry* **33,** 5653–5660.

Smart D., Wandless A., and Lambert D. G. (1995) Activation of phospholipase C in SH-SY5Y neuroblastoma cells by potassium-induced calcium entry. *Br. J. Pharmacol.* **116,** 1797–1800.

Sweet S. C., Gnegy M. E., and Welsh M. J. (1991) A method for the removal of affinity-matrix specific antibody from antigen affinity-purified polyclonal antibodies to calmodulin. *J. Immunol. Methods* **36,** 31–36.

Thastrup O., Foder B., and Scharff O. (1987) The calcium mobilizing tumor promoting agent, thapsigargin elevates the platelet cytoplasmic free calcium concentration to a higher steady state level. A possible mechanism of action for the tumor promotion. *Biochem. Biophys. Res. Commun.* **142,** 654–660.

Thastrup O., Cullen P. J., Drobak B. K., Hanley M. R., and Dawson A. P. (1990) Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2(+)}$-ATPase. *Proc. Natl. Acad. Sci. USA* **87,** 2466–2470.

Thomas T. P., Talwar H. S., and Anderson W. B. (1988) Phorbol ester-mediated association of protein kinase C to the nuclear fraction in NIH 3T3 cells. *Cancer Res.* **48,** 1910–1919.

Tsien R. Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19,** 2396–2404.

Van Hooff C. O., Holthuis J.-C., Oestreicher A. B., Boonstra J., De Graan P. N., and Gispen W. H. (1989) Nerve growth factor-induced changes in the intracellular localization of the protein kinase C substrate B-50 in pheochromocytoma PC12 cells. *J. Cell Biol.* **108,** 1115–1125.

Zwiers H. and Coggins P. J. (1990) Corticotropin (ACTH) inhibits the specific proteolysis of the neuronal phosphoprotein B-50/GAP-43. *Peptides* **11,** 951–954.