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Muscarinic Receptor-Mediated Translocation of Calmodulin in SK-N-SH Human Neuroblastoma Cells

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SUMMARY

The role of muscarinic receptor-mediated polyphosphoinositide hydrolysis and subsequent calcium signals in altering the subcellular localization of calmodulin (CaM) was examined in SK-N-SH human neuroblastoma cells. Upon incubation of the cells with the full agonist carbachol, a 4.5- to 5-fold increase in CaM in the cytosol was observed, from 126 ng of CaM to 629 ng of CaM. There was an accompanying 68% decrease in membrane-bound CaM. The increase in the cytosol was maximal by 15 min, as was a corresponding decrease in membrane-associated CaM. The redistribution of CaM was maintained for at least 2 hr, before returning toward control levels by 4 hr. The EC_{50} values for carbachol in eliciting the translocation were 3.7 μM for the increase in cytosol and 1.3 μM for the decrease in membranes. The maximal changes in CaM concentration in both membranes and cytosol occurred with 10 μM carbachol. Incubation of the SK-N-SH cells with the partial muscarinic agonists bethanechol and arecoline resulted in 27 and 26% decreases in membrane-associated CaM, respectively, and 28 and 35% increases in cytosolic CaM, respectively. Thus, the partial agonists were less efficacious than carbachol in eliciting changes in CaM localization. Atropine completely blocked the carbachol-stimulated translocation, whereas the nicotinic agonist 1,1-dimethyl 4-phenylpiperazinium had no effect on the localization of CaM. Activation of receptors coupled to adenylate cyclase did not affect distribution of CaM. CaM content in membranes and cytosol of cells incubated with prostaglandin E_1 or the α_2-adrenergic agonist UK 14,304 was not different from control values. The ionophore ionomycin (10 μM) and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (50 nM) were both able to elicit changes in CaM distribution. Ionomycin caused a 64% increase in CaM in the cytosol, with no significant change in membrane CaM. TPA elicited a decrease in membrane-associated CaM, with a corresponding increase in CaM in the cytosol. When TPA and ionomycin were incubated together, the translocation was equal in magnitude to that observed with carbachol alone. The protein kinase C inhibitor H-7 blocked the TPA-stimulated response and partially blocked the carbachol-stimulated response. The CaM-binding protein neuromodulin, which demonstrates a decreased affinity for CaM in the presence of Ca^{2+} and when phosphorylated by protein kinase C, was present in both membranes and cytosol of SK-N-SH cells. It is concluded that muscarinic receptor activation resulting in subsequent Ca^{2+} fluxes and protein kinase C-stimulated phosphorylation directs CaM localization in SK-N-SH cells and that the mechanism may involve the neural-specific CaM-binding protein neuromodulin.

CaM and CaM-dependent enzymes are present at high concentrations in brain and may play crucial roles in nerve function (1). CaM itself has been shown to modulate neurotransmitter-stimulated adenylate cyclase activity (2, 3), muscarinic receptor-mediated inhibition of cAMP accumulation in intact 1321N1 human astrocytoma cells (4), and release of neurotransmitters through activation of Ca^{2+}/CaM-dependent protein kinase II activity (5). Several studies have shown that neurotransmitters and hormones can elicit changes in the subcellular distribution of CaM, such as dopamine in the striatum (6) and hippocampus (7), gonadotropin-releasing hormone in the pituitary (8), opiates in NG108-15 cells (9), and isoproterenol in the parotid gland (10). The changes in CaM distribution would allow CaM to regulate cellular processes at various loci. It has been postulated that neurotransmitter-induced calcium fluxes could alter CaM localization in cells (11). Our interest is to investigate the role of neurotransmitter receptor activation and subsequent Ca^{2+} fluxes in altering CaM localization. For these experiments, we chose a cell line with well characterized receptor subtypes and Ca^{2+} fluxes.

SK-N-SH human neuroblastoma cells express M_3 muscarinic, nicotinic, α_2-adrenergic, and PGE_1 receptors (12–14). Fisher and co-workers (15) have demonstrated that activation of the M_3 muscarinic receptors on these cells results in a

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ABBREVIATIONS: CaM, calmodulin; PGE_1, prostaglandin E_1; DMPP, 1,1-dimethyl-4-phenylpiperazinium; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; H-7, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine dihydrochloride; RIA, radioimmunoassay; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; UK 14,304; 6-quinoxalinamine 5-Br-*N*-(4,5-dihydro-1*H*-imidazol-2-yl), PPI, phosphoinositide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate.

pronounced increase in intracellular Ca^{2+} concentration, the extent of which is closely linked to the magnitude of PPI hydrolysis elicited by the agonist. The calcium flux is characterized by a rapid peak rise, followed by a maintained plateau phase that is due to the influx of extracellular Ca^{2+} and lasts for as long as the agonist occupies the receptor. The molecular mechanism of the plateau phase is presently unknown. Partial muscarinic agonists are not fully efficacious in eliciting the PPI response and peak rise in Ca^{2+} but are full agonists in eliciting the plateau phase of the calcium influx.

We have used the SK-N-SH human neuroblastoma cell line to investigate the role of PPI hydrolysis and the resulting Ca^{2+} signals in the alteration of cellular CaM localization. We demonstrate that muscarinic receptor activation results in a 2–3-fold increase in CaM concentration in the cytosol, with a corresponding decrease in the membranes. The mechanism for the release appears to involve increases in both intracellular Ca^{2+} and protein kinase C activity. We additionally demonstrate the presence of the CaM-binding protein neuromodulin (16) in these cells. Neuromodulin shows a decreased affinity for CaM in the presence of increased Ca^{2+} concentrations and upon phosphorylation by protein kinase C. Although it has been postulated that increases in intracellular Ca^{2+} and protein kinase C-mediated phosphorylation can release CaM from membranes into the cytosol (11), this is the first report showing that both limbs of the PPI signalling pathway are involved in altering CaM localization in a physiological system. A preliminary report of this work has appeared elsewhere (17).

Materials and Methods

CaM RIA kits were purchased from New England Nuclear (Boston, MA). SK-N-SH cells were a generous gift from Dr. Stephen K. Fisher, Department of Pharmacology, The University of Michigan. The source of SK-N-SH cells was as previously described by Fisher and Snider (18). Dulbecco's modified Eagle's medium was purchased from Whittaker M.A. Bioproducts (Walkersville, MD). Tissue culture supplies were from Corning Glass Works (Corning, NY). Cell culture reagents, antibodies, fetal bovine serum, EGTA, bovine serum albumin, $MgCl_2$, carbachol, atropine, bethanechol, arecoline, DMPP, ionomycin, $CaCl_2$, Trizma, Lubrol-PX, PGE_1, TPA, and H-7 were all purchased from Sigma Chemical Co. (St. Louis, MO). UK 14,304 was a gift from Dr. Richard Neubig. Department of Pharmacology, The University of Michigan. Immobilon-P transfer membranes were purchased from Millipore (Bedford, MA). The antibody directed against the CaM-binding domain of neuromodulin was a gracious gift from Dr. Daniel Storm, Department of Pharmacology, University of Washington (Seattle, WA).

Cell culture and sample preparation. Human neuroblastoma SK-N-SH cells were grown according to the method of Fisher and Snider (18) in 100-mm tissue culture dishes, in 10 ml of Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 10% fetal bovine serum, in a humidified 5% CO_2 atmosphere at 37°. Cells were grown for 9 days and, following subculture, the medium was changed on days 4, 6, and 8. Cells were used on day 9. Cells were harvested at the indicated times by washing the monolayer three times with 40 mM Tris·HCl, pH 7.4, 1 mM $MgCl_2$ buffer and detaching the cells with Puck's D_1 solution (19). The cells were collected by centrifugation and resuspended in 30 mM NaHEPES buffer, pH 7.4, 142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose. The indicated concentrations of drugs were added to the cell suspension and incubated at 37°. The incubation was stopped by the addition of ice-cold saline, followed by rapid centrifugation to collect the cells. The cells were homogenized in a buffer containing 40 mM Tris·HCl, pH 8, 3 mM $MgCl_2$, and 0.32 M sucrose, using a Teflon-glass homogenizer. The homogenate was centrifuged at 100,000 × *g* for 1 hr. The crude membrane pellet (P) was resuspended in solubilization buffer containing 40 mM Tris·HCl, pH 8, 3 mM $MgCl_2$, and 0.5% Lubrol-PX. For the extraction of CaM, both the resuspended P fraction and the 100,000 × *g* supernatant (S), were diluted in solubilization buffer and heated for 3 min at 95°. The reliability of the separation procedure for membrane-bound and soluble CaM was tested by adding a trace amount of ^{125}I-CaM to the cells during homogenization. We found that 89% of the soluble labeled CaM that was added remained in the soluble fraction and did not artificially associate with membranes during homogenization.

CaM quantitation. The CaM content of the cell fractions was determined using CaM RIA kits (New England Nuclear). The RIA was performed as recommended in the CaM RIA instructions, using the indicated materials. 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 for the preheated CaM standards were used to construct a standard curve, from which values of the unknown samples were obtained by interpolation.

Protein measurement. The amount of protein was quantified according to the method of Peterson (20).

Preparation of tissue extracts for Western blot analysis. SK-N-SH cells were homogenized in cold 20 mM NaHEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol, plus a protease inhibitor cocktail (10 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin). The homogenate was spun at 100,000 × *g* for 60 min. The supernatant from the 100,000 × *g* spin was saved as the "cytosol." The pellet from the 100,000 × *g* spin was washed three times with homogenization buffer, and the final pellet was resuspended in 9 volumes of solubilization buffer (20 mM Tris·HCl, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Lubrol-PX). The preparations were aliquoted and stored at −70° until assayed.

Western blot analysis. SK-N-SH cell membranes (48 μg) and cytosol (41 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins were then electrophoretically transferred by a Hoefer Transphor transfer unit onto an Immobilon membrane for 4 hr at 4°, using 0.4 A. The Immobilon membrane was then incubated for 1 hr at 25° with 2.5% (w/v) bovine serum albumin in TBS containing 0.1% Tween-20, to block nonspecific binding sites. The Immobilon membrane was then incubated overnight at 4° with rabbit polyclonal antiserum (100 μg/ml) directed against the CaM-binding domain of bovine brain neuromodulin, followed by washing with TBS plus Tween-20. The washed membrane was incubated for 8 hr at 4° with goat anti-rabbit horseradish peroxidase-conjugated IgG and washed as described above. Antigen-antibody complexes were visualized by incubating the Immobilon membrane with TBS containing 20% methanol, 3 mg/ml 4-chloro-1-naphthol, and 1% (v/v) 3% hydrogen peroxide. Staining was terminated by washing the membrane with TBS.

Results

Effect of carbachol on CaM distribution. To determine the effects of PPI hydrolysis and Ca^{2+} fluxes on CaM distribution, SK-N-SH cells were incubated with the full muscarinic agonist carbachol and CaM was quantified using RIA. The time course for carbachol-mediated changes in CaM localization (Fig. 1) shows that translocation of CaM from membranes to the cytosol occurred as early as 5 min and was maximal by 15–30 min. The total CaM (Fig. 1A) in the membranes maximally decreased from 310 ng of CaM to 100 ng of CaM by 15 min and returned to control values after 4 hr of incubation with carbachol. The cytosolic CaM increased to a maximum by 30 min, from 126 ng of CaM to 808 ng of CaM, and remained higher than control values after 4 hr of incubation with carbachol.

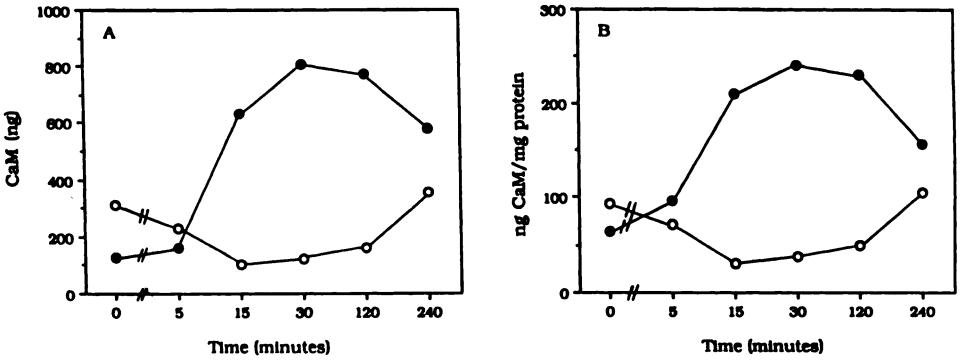

Fig. 1. Time course of CaM translocation in SK-N-SH cells incubated at 37° with 10 μM carbachol. The cells were harvested at the indicated time points, and CaM was quantified using RIA. ○, Membranes; ●, cytosol. A, Data expressed as total CaM (ng); B, data expressed as CaM concentration (ng of CaM/mg of protein). The data shown are representative of four separate experiments that differed by less than 10%.

The magnitude of the decrease in membrane CaM was not equal to the increase in the cytosol, with the increase in the cytosol exceeding the decrease in the membranes. As shown in Fig. 1B, expression of the data on a per protein basis demonstrates that the concentration of CaM increased in the cytosol and decreased in the membranes. The total CaM in the homogenates increased 20–35% with increasing time of exposure to carbachol or increasing concentration of carbachol (Table 1). Recovery of membrane plus cytosol CaM was generally 80–90% in the homogenates but approached 100% in carbachol-treated cell homogenates. The concentration-response curves for carbachol-stimulated CaM translocation are shown in Fig. 2A, expressed as total CaM (ng), and in Fig. 2B, expressed as CaM concentration for the same experiment. The EC_{50}, calculated using a logit plot, for carbachol in decreasing membrane-associated CaM was 1.3 μM, whereas the EC_{50} for the carbachol-stimulated increase in cytosolic CaM was 3.7 μM. For both membranes and cytosol, the maximal response occurred at 10 μM carbachol and appeared to saturate, because increasing the carbachol concentration to 1 mM did not change the effect.

TABLE 1

CaM quantitation in SK-N-SH cell homogenates

The CaM content of homogenates for the time course shown in Fig. 1A and the concentration-response shown in Fig. 2A was quantitated using RIA, as described in Materials and Methods. The data shown are representative of four experiments performed in duplicate.

Time	CaM	Carbachol	CaM
min	*ng*	*μM*	*ng*
0	820	0	820
5	980	0.1	919
15	1100	1.0	959
30	1060	10	980
120	1061	100	1001
240	877	1000	960

Thus, the carbachol-mediated CaM translocation was time and concentration dependent.

Effect of partial muscarinic agonists on CaM redistribution. Partial muscarinic agonists are known to be less efficacious than full agonists in promoting PPI turnover in SK-N-SH cells (18) and elicit a 50% lower peak rise in intracellular Ca^{2+} than that attained with carbachol (15). The plateau phase of the Ca^{2+} signal is not different whether cells are stimulated with partial or full agonists. We measured the effect of partial muscarinic agonists on CaM translocation, using RIA. As shown in Table 2, the partial agonists bethanechol and arecoline were less effective in eliciting a translocation of CaM than was carbachol. Membrane-associated CaM decreased by 48% with carbachol but only by 27 and 26% with bethanechol and arecoline, respectively. Similarly, carbachol elicited a 124% increase in cytosolic CaM, whereas bethanechol and arecoline stimulated a 28 and 35% increase in cytosolic CaM, respectively. With the partial agonists, the decrease in CaM content in the membranes appeared to equal the increase in the cytosol.

Specificity of the carbachol-stimulated translocation. To determine whether the effect of carbachol on CaM distribution was mediated through the muscarinic receptor, cells were incubated with the muscarinic antagonist atropine and the nicotinic agonist DMPP. The response to carbachol was completely blocked by atropine (Fig. 3). Atropine alone did not affect the localization of CaM. The CaM content of membranes and cytosol from cells incubated with the nicotinic agonist DMPP was not different from control.

Effect of agonists for receptors positively and negatively coupled to adenylate cyclase on CaM localization. The M_3 receptors expressed on SK-N-SH cells, to which carbachol binds, are coupled to PPI hydrolysis. To determine whether activation of a different second messenger system

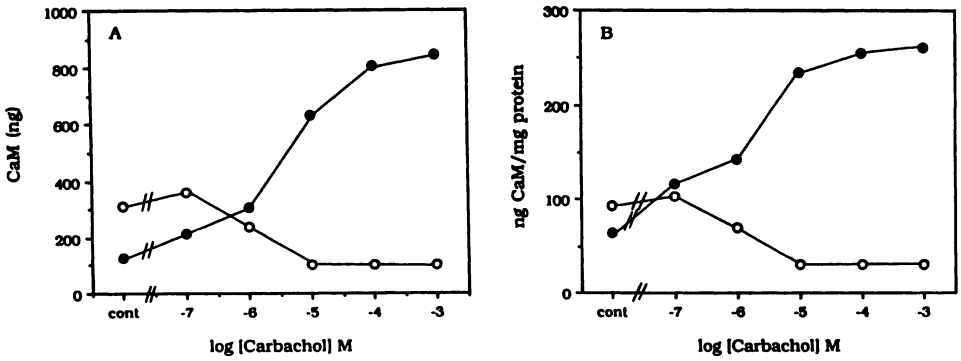

Fig. 2. Concentration-response curves for carbachol-mediated CaM translocation in SK-N-SH cells. Cells were treated with the indicated concentrations of carbachol for 15 min at 37°, CaM was extracted, and CaM content was determined using RIA. ○, Membranes; ●, cytosol. The EC_{50} for the increase in cytosolic CaM concentration was 3.7 μM, and the EC_{50} for the decrease in membrane-associated CaM was 1.3 μM. A, Data expressed as total CaM (ng); B, data expressed as CaM concentration (ng of CaM/mg of protein). The data shown are representative of four separate experiments that differed by less than 10%.

TABLE 2

Differential ability of muscarinic agonists to elicit changes in CaM distribution

Cells were treated with the indicated concentration of drug for 15 min at 37°, and CaM was extracted from membrane and cytosol fractions as described in Materials and Methods. The CaM content of the fractions was determined using RIA. The data are expressed as a percentage of the CaM concentration in control membrane and cytosol fractions. The means ± standard errors of three experiments using RIA are given, and each experiment was performed in duplicate. The control values (100%) in the membranes and cytosol using RIA were 40 ± 5 ng of CaM/10^6 cells and 35 ng of CaM/10^6 cells, respectively.

	CaM content	
	Membranes	Cytosol
	% of control	
Carbachol, 100 μM	52 ± 6	224 ± 12
Bethanechol, 500 μM	73 ± 7	128 ± 4
Arecoline, 100 μM	74 ± 7	135 ± 11

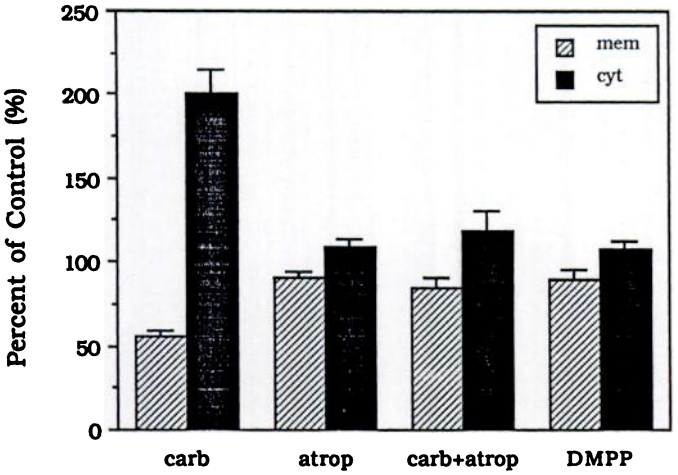

Fig. 3. Effect of atropine and DMPP on carbachol-mediated CaM translocation. Cells were treated with 10 μM carbachol (*carb*), 1 μM atropine (*atrop*), or 100 μM DMPP and harvested after 15 min of incubation at 37°. CaM was quantified using CaM RIA. The data are presented as the mean ± standard error for three experiments performed in duplicate. Control values (100%) were 85 ± 6 ng of CaM/mg of protein for the membranes and 78 ± 8 ng of CaM/mg of protein for the cytosol. *mem*, membrane; *cyt*, cytosol.

TABLE 3

Effect of adenylate cyclase-coupled receptor activation on translocation of CaM in SK-N-SH cells

Cells were treated for 15 min at 37° with 10 μM carbachol, 30 μM PGE_1, or 0.1 μM UK 14,304, as described in Materials and Methods. CaM was extracted from membrane and cytosol fractions and quantified using RIA. The data shown are representative of four separate experiments performed in duplicate.

	CaM content	
	Membranes	Cytosol
	ng/10^6 cells	
Control	42	38
Carbachol, 10 μM	23	79
PGE_1, 30 μM	40	36
UK 14,304, 0.1 μM	51	39

could also affect CaM localization, the SK-N-SH cells were treated with agonists that stimulate or inhibit the formation of cAMP (Table 3). It has been shown that PGE_1 stimulates (21) and α_2-adrenergic receptor agonists inhibit (13) adenylate cyclase activity in these cells. Neither PGE_1 nor the α_2-adrenergic receptor agonist UK 14,304 changed the subcellular localization of CaM, and CaM levels of membranes and cytosol fractions were similar to control levels.

Effect of ionomycin and phorbol ester on CaM localization. PPI hydrolysis results in the formation of inositol 1,4,5-triphosphate and diacylglycerol, which stimulate a rapid rise in intracellular [Ca^{2+}] and the activation of protein kinase C, respectively. To determine whether either or both of these limbs of the PPI cascade play a role in the muscarinic receptor-stimulated translocation of CaM, we measured the CaM content in cells treated with the Ca^{2+} ionophore ionomycin (10 μM) and the phorbol ester TPA (50 nM). Fig. 4 shows that ionomycin elicited a significant increase in CaM in the cytosol, with a much smaller change in membranes that did not achieve statistical significance. TPA was more effective than ionomycin in eliciting the translocation of CaM; a decrease of 33 ng of CaM/10^6 cells was measured in membranes, with an increase of 36 ng of CaM/10^6 cells in the cytosol. Incubation of cells with 100 nM TPA resulted in a similar response to that obtained using 50 nM TPA (data not shown). Thus, protein kinase C-stimulated phosphorylation could elicit a CaM translocation whereby the decrease in membranes and the increase in cytosol were of similar magnitude. When ionomycin and TPA were added together, there was a decrease of 34 ng of CaM/10^6 cells in the membranes and an increase of 66 ng of CaM/10^6 cells in the cytosol. These results were not different from those elicited by 10 μM carbachol, again demonstrating an increase in the cytosol of greater magnitude than the decrease in membranes. To further establish that a component of the carbachol-stimulated response was due to activation of protein kinase C, the ability of the protein kinase C inhibitor H-7 to block the carbachol-stimulated CaM translocation was determined. The data in Fig. 5 demonstrate that addition of H-7 blocked the carbachol-stimulated decrease in membrane-associated CaM, with a final decrease in membranes of 31%. The increase in cytosolic CaM by carbachol was also inhibited by H-7, resulting in a 40% increase in cytosolic CaM over control. Although H-7 inhibited carbachol-stimulated translocation, in no instance was H-7 able to completely block the carbachol response. H-7 completely blocked the redistribution stimulated by TPA, however, and H-7 alone did not affect CaM distribution.

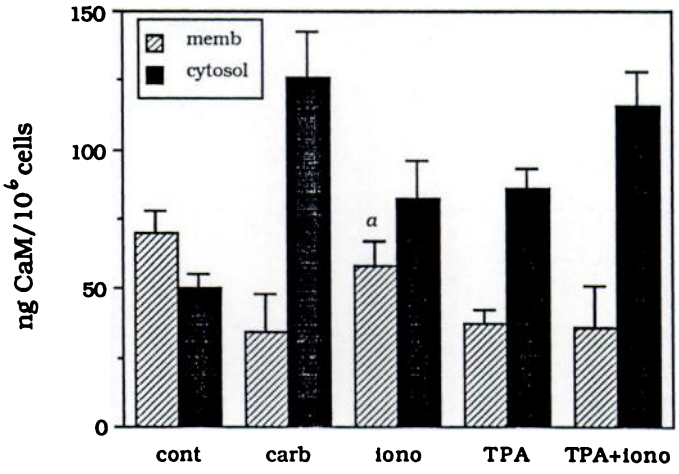

Fig. 4. Effect of TPA and ionomycin on CaM translocation. Cells were incubated with 10 μM carbachol (*carb*), 50 nM TPA, 10 μM ionomycin (*iono*), or combinations for 15 min at 37°. CaM quantitation was by RIA. These results show the mean ± standard error from three experiments performed in duplicate. *a*, Not significantly different from control membranes, $p > 0.05$. *cont*, control; *memb*, membrane.

Presence of the CaM-binding protein neuromodulin in SK-N-SH cells. Membranes and cytosol were assayed for the presence of neuromodulin, using Western blotting and immunodetection with polyclonal antibodies produced against the CaM-binding domain of bovine brain neuromodulin (22). The major immunoreactive protein present in crude membranes (Fig. 6, *lane 3*) shows an apparent molecular weight of 52,000 on the 7.5% polyacrylamide gel. Lower molecular weight immunoreactive proteins are also evident in the membranes and probably represent cleaved forms of the M_r 52,000 protein. The 100,000 × *g* cytosol (Fig. 6, *lane 4*) shows immunoreactive proteins at M_r 52,000 and 61,000. Both bovine (Fig. 6, *lane 1*) and human cortex (Fig. 6, *lane 2*) neuromodulin migrate predominately at M_r 52,000 on the 7.5% polyacrylamide gel.

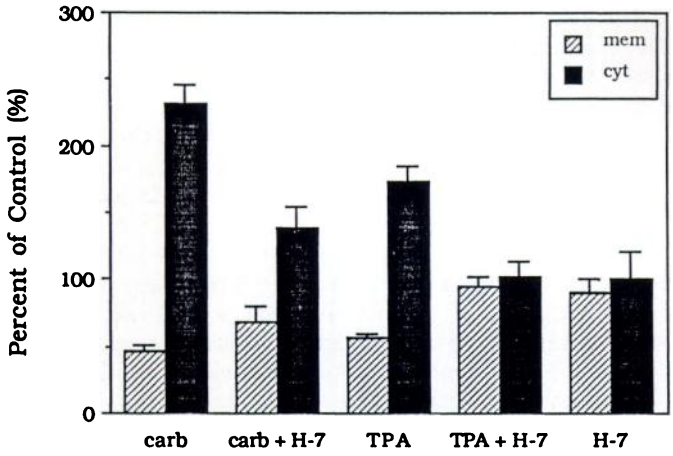

Fig. 5. Effect of H-7 on carbachol- and TPA-elicited changes in CaM translocation. Cells were incubated with 10 μM carbachol (*carb*), 50 nM TPA, 18 μM H-7, or combinations for 15 min at 37°. CaM was quantified using RIA. The data are expressed as a percentage of the CaM concentration of untreated control membranes and cytosol. Control values were 89 ± 7 ng/mg for the membranes and 80 ± 9 ng/mg for the cytosol. The results are the mean ± standard error for three experiments performed in duplicate. *mem*, membrane; *cyt*, cytosol.

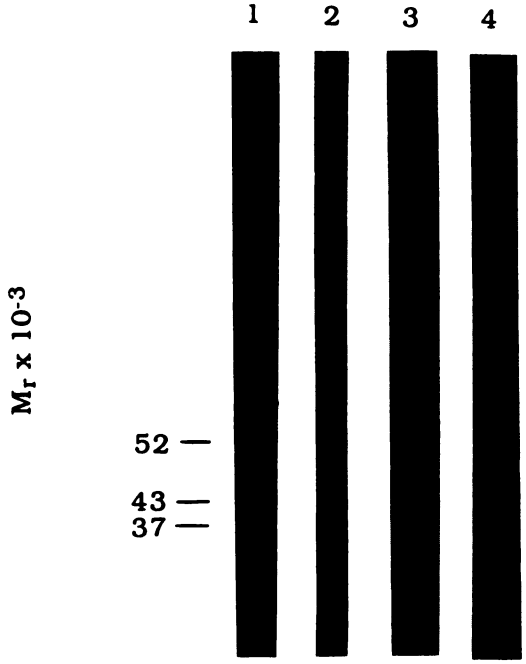

Fig. 6. Western blot analysis of neuromodulin in SK-N-SH cells. *Lane 1*, bovine cortex homogenate (40 μg); *lane 2*, human cortex homogenate (35 μg); *lane 3*, membranes (48 μg); *lane 4*, cytosol (41 μg). Samples were electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to an Immobilon-P (Millipore) filter, and incubated with polyclonal antiserum directed against the CaM binding domain of bovine brain neuromodulin. The immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG.

Discussion

Activation of muscarinic receptors on SK-N-SH cells leads to an increase in PPI hydrolysis and Ca^{2+} fluxes. The Ca^{2+} signal is characterized by a rapid peak rise, followed by a sustained plateau phase during which intracellular Ca^{2+} is elevated above resting levels for as long as agonist occupies the muscarinic receptor. It has been postulated in many systems that increases in intracellular [Ca^{2+}] could cause Ca^{2+}- and CaM-dependent activation of enzymes and changes in CaM localization within cells (23). We have demonstrated that activation of the M_3 muscarinic receptors on SK-N-SH cells elicits a translocation of CaM from the membranes into the cytosol. This translocation was directly dependent upon PPI hydrolysis and probably the initial peak rise in Ca^{2+}, as opposed to the plateau phase of Ca^{2+} influx, because partial agonists do not demonstrate full efficacy in either the PPI response or CaM translocation. The concentration-response curves for carbachol-stimulated translocation of CaM are 10 times more sensitive than those for carbachol-stimulated PPI hydrolysis and Ca^{2+} fluxes (approximate EC_{50} = 10–30 μM) (15). The reasons for the different EC_{50} values measured for carbachol-mediated responses are not clear but may reflect points of signal amplification along the stimulus-response pathway, most likely downstream from PPI hydrolysis and Ca^{2+} release.

The translocation of CaM appears to be due to both an increase in intracellular calcium and production of diacylglycerol for activation of protein kinase C. Although it has been postulated that increases in intracellular Ca^{2+} alone could elicit a translocation of CaM (1), ionomycin could not completely substitute for carbachol in the translocation of CaM. Nor can ionomycin fully substitute for carbachol in eliciting PPI hydrolysis (15). Therefore, the translocation of CaM was not solely due to increases in the concentration of intracellular Ca^{2+}. TPA and ionomycin added together, however, did mimic the effect of carbachol in altering CaM localization. It is interesting that, although ionomycin had a minimal effect, if any, on CaM in the membranes, it increased CaM in the cytosol. Stimulation of protein kinase C-mediated phosphorylation by TPA, however, elicited changes in CaM localization from membranes to the cytosol that were similar in magnitude, suggesting that the protein kinase C-stimulated phosphorylation is important in mediating the simple translocation of CaM from membranes to the cytosol. On the other hand, the increase in intracellular Ca^{2+} may be more important for an additional increase in the cytosol. It is possible that the agonist induces an additional change in cytosolic CaM and this additional change could be mimicked by ionomycin. The discrepancy is also apparent in the time course and concentration-response curves for carbachol, because the magnitude of the increase in the cytosol exceeds the decrease in the membranes (Figs. 1A and 2A). Our studies show a redistribution of CaM occurring maximally as soon as 15 min, suggesting that protein synthesis may not be involved in the large increase in cytosolic CaM observed at this time point. There are several possible expla-

nations for the "extra" CaM appearing in the cytosol. Carbachol may elicit modifications of the CaM molecule that result in altered CaM immunoreactivity, inasmuch as the increase in CaM was measured using RIA. For example, it has been shown that CaM can be phosphorylated (24–29), and it is possible that phosphorylation alters the ability of anti-CaM antibodies to recognize the protein or that phosphorylation itself can elicit changes in CaM localization. Alternatively, CaM bound to membrane or cytosolic proteins could be masked and its detection by RIA hindered until treatment with various agents. New protein synthesis could contribute to the increase in cytosolic CaM concentrations at the later time points.

The fact that TPA plus ionomycin could effectively substitute for carbachol demonstrates that increases in both intracellular Ca^{2+} and protein kinase C-mediated phosphorylation were important for the cellular changes in CaM. It has been demonstrated that some novel CaM-binding proteins dissociate CaM upon elevations in Ca^{2+} and protein kinase C-mediated phosphorylation. Neuromodulin (also known as GAP-43, F1, and B-50), a neurospecific CaM-binding protein, has been shown to bind CaM in the absence of Ca^{2+} as well as serve as a good substrate for protein kinase C (11, 30). It has been proposed that neuromodulin functions to sequester CaM in the vicinity of CaM-activated enzymes under low Ca^{2+}, whereupon elevation of intracellular free Ca^{2+} would promote dissociation of CaM from neuromodulin. Phosphorylation of neuromodulin by protein kinase C also promotes the dissociation of CaM from neuromodulin. We show that neuromodulin is present in both membranes and cytosol of SK-N-SH cells. This unique CaM-binding protein could be involved in the CaM translocation, which we have shown to be dependent on increased intracellular [Ca^{2+}] and protein kinase C-mediated phosphorylation. The detection of neuromodulin in membranes and cytosol is consistent with the suggestions of our data that an increase in intracellular Ca^{2+} may facilitate the release of CaM from binding sites in the membranes and cytosol. The differences in molecular weight between the bovine and human brain neuromodulin and the minor M_r 43,000 SK-N-SH cell membrane neuromodulin probably reflects proteolysis but also may reflect heterogeneity of neuromodulin. Protease inhibitors were present during homogenization. Neuromodulin has been purified as a mixture of molecular isoforms (31). Additionally, the protein is an elongated molecule with a high axial ratio that shows an unusual mobility on SDS-polyacrylamide gels, due to the existence of an atypical conformation in SDS (32).

Recently, Graff *et al.* (33) have described abundant CaM-binding proteins that are cellular substrates for protein kinase C, called myristoylated alanine-rich C kinase substrates (MARCKS). These investigators have proposed that the phosphorylation of CaM-binding proteins by protein kinase C in intact cells may displace bound CaM, thus leading to a significant change in the concentration of "free" CaM. Whether these proteins are present in SK-N-SH cells and play a role in carbachol-stimulated translocation is not known; however, it is interesting that protein kinase C substrates that are also CaM-binding proteins may function to localize CaM in cellular compartments until the cell is stimulated.

The function of the released CaM is not yet known, although it clearly could activate CaM-stimulated enzymes. Activation of muscarinic receptors of 1321N1 human astrocytoma cells (4) and of dog thyroid slices (34) results in attenuation of cyclic AMP accumulation through stimulation of a Ca^{2+}/CaM-stimulated phosphodiesterase. It was proposed that the muscarinic receptor-mediated increase in cytoplasmic Ca^{2+} resulted in the activation of the Ca^{2+}/CaM-stimulated phosphodiesterase. CaM-binding proteins and CaM-dependent enzymes present in the SK-N-SH cells have not been clearly identified, and it is not known with which proteins CaM associates upon carbachol-stimulated release. Carbachol has been shown to be a mitogen in SK-N-SH cells, and the expression of muscarinic subtypes coupled to PPI hydrolysis was shown to be correlated with the mitogenic action of carbachol (35). Similarly, CaM has been demonstrated to have important regulatory functions in the control of cell growth and cell cycle progression (10, 36). It is possible that an increase in CaM plays a role in the initiation of events leading to DNA synthesis in these cells. The importance of neurotransmitter receptors as mitogens in postmitotic cells is unknown.

In summary, our results have demonstrated a muscarinic receptor-mediated redistribution of CaM from the membrane to the cytosol in SK-N-SH cells. This is the first reported physiological system demonstrating that a neurotransmitter-stimulated increase in both Ca^{2+} and protein kinase C activity from neurotransmitter-stimulated PPI hydrolysis leads to translocation of CaM.

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