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# Cyclic AMP accumulation alters calmodulin localization in SK-N-SH human neuroblastoma cells

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In SK-N-SH human neuroblastoma cells, the muscarinic agonist carbachol promotes polyphosphoinositide (PPI) hydrolysis via $M_3$ receptors and increases cyclic AMP levels through an unidentified mechanism. Activation of PPI hydrolysis by carbachol elicits a robust translocation of CaM from membranes into cytosol which was previously shown to be mimicked by the addition of the calcium ionophore ionomycin and the phorbol ester TPA[28]. The effect of agonist-stimulated second messenger production on CaM localization was determined by activating receptors that increase and decrease adenylyl cyclase activity on SK-N-SH cells. VIP (10 $\mu$M), prostaglandin E1 (30 $\mu$M) and forskolin (10 $\mu$M) all increased adenylyl cyclase activity 8- to 10-fold above the activity with 1 $\mu$M GTP. Carbachol (100 $\mu$M) did not stimulate adenylyl cyclase activity. The $\alpha_2$-adrenergic agonist UK 14,304 (0.1 $\mu$M) and the $\delta$ and $\mu$ opioid DPDPE (10 $\mu$M) and DAMGO (10 $\mu$M) inhibited forskolin-stimulated cyclic AMP formation by 27–32%. CaM did not stimulate adenylyl cyclase activity. Incubation of cells with vasoactive intestinal polypeptide (VIP), dibutyryl cyclic AMP and forskolin, resulted in 30% decrease in membrane CaM and an increase in cytosolic CaM of 40–50%. The CaM translocation with the combination of an agent that elevates cyclic AMP levels and a low dose of carbachol was not different from that observed with either agent alone. UK 14,304, DPDPE and DAMGO potentiated carbachol-stimulated increases in cytosolic CaM. Upon the addition of carbachol, a 5-fold increase in intracellular calcium concentration measured with fura-2 fluorescence was observed. VIP and UK 14,304 elevated intracellular calcium concentrations 2 to 3 fold, while forskolin (10 $\mu$M) had no effect. Thus, receptor-mediated cyclic AMP synthesis and $Ca^{2+}$ fluxes alter CaM localization, but with a smaller magnitude than the CaM translocation stimulated by PPI hydrolysis.

## INTRODUCTION

Predominant receptor-mediated signal transduction pathways in neural tissues are the polyphosphoinositide (PPI) hydrolysis pathway and the adenylyl cyclase pathway. Recent studies have suggested that multiple signal transduction systems can be activated upon receptor activation by agonist[12,14,23]. Felder et al.[14] showed that $M_1$ muscarinic receptor-stimulated PPI hydrolysis could lead to the generation of cyclic AMP. In many systems, protein kinase C activators can lead to changes in cyclic AMP accumulation[21]. For example, protein kinase C-stimulated phosphorylation of adenylyl cyclase has been shown to enhance adenylyl cyclase activity[35]. Additionally, agents that elevate intracellular cyclic AMP levels have been shown to affect PPI hydrolysis. Such agents, most likely activating cyclic AMP-dependent protein kinase, have been demonstrated to inhibit muscarinic receptor-stimulated PPI hydrolysis in NCB-20 cells[29] and SK-N-SH cells[1]. SK-N-SH human neuroblastoma cells express predominantly $M_3$ muscarinic receptors, which are coupled to activation of phospholipase C, and a small percentage of $M_2$ muscarinic receptors which inhibit adenylyl cyclase activity. Interestingly, investigators have reported that the muscarinic receptor agonist carbachol and the direct activator of PPI hydrolysis maitotoxin stimulated an increase in intracellular cyclic AMP levels[5,7,8]. This increase in cyclic AMP is not believed to occur through direct coupling of muscarinic receptors to stimulation of adenylyl cyclase. Studies in transfected cells have suggested that the generation of cytosolic $Ca^{2+}$ via muscarinic receptor-stimulated inositol triphosphate ($IP_3$) release could activate CaM and a $Ca^{2+}$/CaM-dependent adenylyl cyclase[12,14].

The ubiquitous $Ca^{2+}$-binding protein CaM mediates many $Ca^{2+}$-dependent cellular functions through activation of a variety of enzyme systems such as those involved in cyclic nucleotide metabolism, protein phosphorylation and dephosphorylation, intracellular calcium homeostasis and smooth muscle contraction[30]. CaM has been shown to modulate neurotransmitter-stimulated adenylyl cyclase activity[20,26] and polyphosphoinositide formation by activation of $IP_3$ kinase[24]. Several studies have described neurotransmitter and hormone-elicited changes in the subcellular distribution of CaM, such as dopamine in the striatum[17] and hippocampus[34], gonado-

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tropin-releasing hormone (GnRH) in the pituitary[11], opiates in NG 108-15 cells[4], and isoproterenol in the parotid gland[9]. The changes in CaM distribution would allow $Ca^{2+}$/CaM to regulate these cellular processes at various loci. It has been postulated that neurotransmitter-induced calcium fluxes and activation of protein kinase C could alter CaM localization in cells[2,18]. We have shown that the localization of CaM is altered upon carbachol-stimulated PPI hydrolysis in SK-N-SH cells[28]. Carbachol mediated a translocation of CaM from membranes increasing the cytosolic CaM 3- to 4-fold. Other studies have suggested that cyclic AMP may play a role in the changes in CaM localization[17,31]. The translocation described by these investigators appears to be mediated through a cyclic AMP-dependent phosphorylation of membrane CaM-binding proteins, thus releasing CaM into the cytosol. In this study, we characterized the effects of second messenger production on the CaM translocation. SK-N-SH cells express receptors coupled through $G_s$ and $G_i$ to the stimulation and inhibition of adenylyl cyclase activity, respectively. Both VIP and prostaglandin $E_1$ ($PGE_1$) receptors stimulate the formation of cyclic AMP, and $\alpha_2$-adrenergic as well as $\mu$ and $\delta$ opioid receptors inhibit the formation of cyclic AMP[5,36]. We found that activation of the adenylyl cyclase as well as the PPI signal transduction systems can change CaM localization and affect the intracellular calcium concentration. In neural tissues and cells, diverse stimuli activate signal transduction systems which must be integrated and regulated in order to produce a physiological effect. Our results suggest that CaM translocation in the SK-N-SH cell line may be coordinately regulated by diverse as well as integrated cellular signals. A preliminary report of this work has appeared elsewhere[27].

## MATERIALS AND METHODS

### *Materials*

CaM radioimmunoassay (RIA) kits were purchased from New England Nuclear (Boston, MA). SK-N-SH human neuroblastoma cells and fura-2 (Molecular Probes, Eugene, OR) were generous gifts 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[16]. 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, $CaCl_2$, Trizma, Lubrol-PX, leupeptin, pepstatin, PMSF, $PGE_1$, VIP and dbcAMP were all purchased from Sigma Chemical Co. (St. Louis, MO). UK 14,304 and yohimbine were gifts from Dr. Richard Neubig, Department of Pharmacology, The University of Michigan. DPDPE and DAMGO were gifts from Dr. Fedor Medzihradsky, Department of Biological Chemistry, The University of Michigan. CaM was prepared from bovine testes by the method of Dedman et al.[13] using phenyl-Sepharose chromatography.

### *Cell culture and sample preparation*

Human neuroblastoma SK-N-SH cells were grown according to Fisher and Snider[16] in 75 $cm^2$ tissue culture flasks in 15 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 °C. Cells were grown and harvested as previously described[28]. Briefly, 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 $MgCl_2$, and 5.6 mM D-glucose (buffer A). The indicated concentrations of drugs or vehicle were added to the cell suspension and incubated at 37 °C. 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 $MgCl_2$, 0.32 M sucrose, 1 $\mu$M leupeptin, 1 $\mu$M pepstatin and 1 mM PMSF using a teflon-glass homogenizer. The homogenate was centrifuged at 100,000 *g* for 1 h. The crude membrane pellet 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 particulate fraction and the 100,000 *g* supernatant were diluted in a 0.125 M borate buffer, pH 8.4, containing 75 mM NaCl, 0.2% BSA, 1 mM EGTA and 0.1% sodium azide and heated for 3 min at 95 °C.

### *CaM quantification*

The CaM content of the homogenate, membrane and cytosol fractions were determined using CaM radioimmunoassay (New England Nuclear, Boston, MA). 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 quantified 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. The membrane-associated plus the cytosolic CaM levels routinely accounted for 75–85% of the homogenate values.

### *Protein measurement*

The amount of protein was quantified according to Peterson[33].

### *Adenylyl cyclase assay*

The cells were washed, collected by centrifugation as above and homogenized in a buffer containing 10 mM Tris-HCL (pH 7.4), 1 $\mu$M leupeptin, 1 mM EDTA, 0.32 M sucrose and 3 mM $MgCl_2$. Membranes were prepared by centrifugation for 20 min, at 27,000 *g*. Membranes were resuspended in sucrose-free homogenization buffer and recentrifuged. The final resuspension was in 50 mM NaHEPES (pH 7.4) and 3 mM $MgCl_2$ buffer to a protein concentration of 5 mg/ml. Adenylyl cyclase activity was measured according to the procedure of Krishna et al.[22]. Each assay tube contained 50 mM NaHEPES buffer pH 7.4, 3 mM $MgCl_2$, 1 mM cyclic AMP, 5 mM phospho*enol*pyruvate, 0.5 mM IBMX, 0.2 mM EDTA, 66 $\mu$g BSA, 40 $\mu$g pyruvate kinase, 0.5 mM [$\alpha$-$^{32}$P]ATP (1 $\mu$Ci) in a final assay volume of 100 $\mu$l. The reaction mixture was incubated at 37 °C for 9 min and stopped by heating for 1 min at 95 °C. All enzyme activities were adjusted for the recovery of [$^3$H]cAMP, which was consistently between 85–95%.

### *Measurement of cytoplasmic calcium concentrations*

Intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) were measured using fura-2 and dual wavelength fluorimetry as described by Fisher et al.[15]. SK-N-SH cells were loaded with 1 $\mu$M fura-2/AM for 15 min at 37 °C. The loaded cells were diluted and washed in buffer A. The wash step was repeated, and the final resuspension was in buffer A to a protein concentration of approximately 3 mg/ml. Fluorescence measurements were made on 1 ml aliquots of the cells constantly stirred and maintained at 37 °C. The fluorimeter was an Aminco-Bowman spectrophotofluorimeter (excitation $\lambda$ = 340 and 380 nm; emission $\lambda$ = 490 nm), interfaced to a strip chart recorder and a LED readout, from which quantitative fluorescence measurements were obtained. Maximal and minimal fluorescence ratios were calculated as described[15]. Under these conditions, $[Ca^{2+}]_i$ =

TABLE I

*Adenylyl cyclase activities in SK-N-SH human neuroblastoma cells*

SK-N-SH cell membranes were prepared and assayed for stimulation (A) or inhibition (B) of adenylyl cyclase activity using the indicated receptor agonists. Basal adenylyl cyclase activity was 4.0 ± 0.9 pmol/min/mg and with 1 μM GTP was 7.3 ± 0.7 pmol/min/mg. Inhibition of adenylyl cyclase activity was measured in the presence of 10 μM forskolin. The data are the mean ± S.E.M. for two experiments performed in triplicate.

| | *Agonist* | *Stimulation above 1 μM GTP (pmol cAMP formed/min/mg protein)* | *% Inhibition* |
|---|---|---|---|
| A. | 10 μM VIP | 76 ± 6 | |
| | 30 μM $PGE_1$ | 62 ± 4 | |
| | 10 μM carbachol | 1.4 ± 1* | |
| | 100 μM carbachol | 1 ± 1* | |
| B. | 10 μM forskolin | 60 ± 5 | |
| | 10 μM carbachol | 53 ± 7 | 12 |
| | 100 μM carbachol | 48 ± 4** | 20 |
| | 0.1 μM UK 14,304 | 42 ± 3** | 30 |
| | 10 μM DPDPE | 44 ± 3** | 27 |
| | 10 μM DAGOL | 41 ± 2** | 32 |

* Not significantly different from 1 μM GTP.
** Significantly different from 10 μM forskolin ($P < 0.01$).

$(R-R_{min}/R_{max}-R)$ $B$ $(K_d)$, where $R$, $R_{min}$, and $R_{max}$ are the ratios of the fluorescence obtained at 340 nm and 380 nm, correlated for autofluorescence. $B$ is the ratio of fluorescence of $Ca^{2+}$-free/$Ca^{2+}$-saturated fluorescence signals at 380 nm.

## RESULTS

### *SK-N-SH cells express receptors coupled to adenylyl cyclase activities*

SK-N-SH cells express receptors coupled to stimulation and inhibition of adenylyl cyclase activity. Both VIP (10 μM) and $PGE_1$ (30 μM) increased adenylyl cyclase activity 9- to 10-fold above the activity with 1 μM GTP (Table IA). Although carbachol has been shown to increase intracellular cyclic AMP levels in these cells[7], carbachol did not stimulate adenylyl cyclase activity at concentrations of 10 μM or 100 μM. The diterpene forskolin, which is thought to directly activate the catalytic activity of adenylyl cyclase, stimulated activity 8.5-fold above GTP. Agonists acting through receptors that are coupled to inhibition of adenylyl cyclase through $G_i$ (Table IB) were assayed for inhibition of forskolin-stimulated adenylyl cyclase activity. Carbachol, acting through $M_2$ muscarinic receptors, modestly inhibited forskolin-stimulated adenylyl cyclase activity by 12% (10 μM) and 20% (100 μM). The $\alpha_2$-adrenergic agonist UK 14,304 (0.1 μM) and the opioid receptor agonists D-pen, D-pen enkephalin (DPDPE) (10 μM) and Tyr-D-Ala-Gly-MePhe-Gly-ol

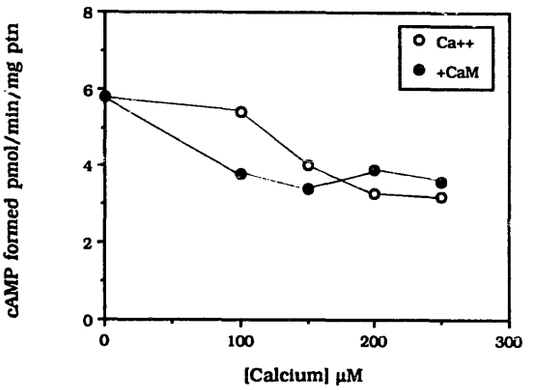


Fig. 1. Effect of $Ca^{2+}$ and CaM on adenylyl cyclase activity in SK-N-SH cell membranes. SK-N-SH cell membranes were prepared and washed twice with a 1 mM EDTA-containing homogenization buffer (see Materials and Methods) to remove endogenous CaM. Adenylyl cyclase activity was measured in the presence of different $CaCl_2$ concentrations without (○) and with (●) 3 μg CaM as described in Materials and Methods. Basal adenylyl cyclase activity was 5.8 pmol/min/mg protein. The final EDTA concentration in the 100 μl assay volume was 200 μM. The data are representative of 5 separate experiments performed in triplicate.

(DAMGO) (10 μM) each inhibited forskolin-stimulated cyclic AMP formation by 27–32%. SK-N-SH cell membranes that were depleted of endogenous CaM were assayed for the presence of CaM-stimulated adenylyl cyclase activity (Fig. 1). Adenylyl cyclase activity was inhibited with increasing concentrations of $CaCl_2$, from a basal activity of 5.8 pmol/min/mg to 3.3 pmol/min/mg at 200 μM $CaCl_2$. The addition of 3 μg of CaM did not significantly change the activity in the presence of $Ca^{2+}$ alone.

### *Increased cyclic AMP levels affect CaM localization*

The ability of cyclic AMP to mediate translocation of CaM in SK-N-SH cells was determined. Both forskolin (10 μM) and the cyclic AMP analogue dibutyryl cyclic AMP (dbcAMP) (1 mM) elicited a translocation of CaM from membranes into cytosol, with a decrease in membranes of 30% and an increase in total CaM in the cytosol of 40–50% (Fig. 2). This result is similar to that observed with the lower dose of 1 μM carbachol which is approximately the $EC_{50}$ for carbachol-stimulated CaM translocation[28]. Receptor-stimulated increases in cyclic AMP levels induced by VIP (10 μM) also changed the distribution of CaM, with a decrease in the membranes of 29% and an increase in the cytosol of 40%. Incubation of cells with $PGE_1$, however, did not affect CaM localization even though it stimulated adenylyl cyclase activity to a similar extent as VIP (data not shown). The effect of the combination of carbachol and agents that elevate intracellular cyclic AMP levels on CaM translocation is shown in Table II. The combinations of carbachol with forskolin, dbcAMP, and VIP did not change

TABLE II

*Effects of combinations of agents that elevate cyclic AMP with carbachol*

Cells were incubated with combinations of agents for 15 min at 37 °C. CaM quantitation was by RIA. The concentrations of the agents employed are the same as those shown in Fig. 2. The data represent the mean ± S.E.M. for three experiments performed in duplicate, except for VIP, the data represent the mean of two experiments performed in duplicate. Control values (100%) were 48 ± 6 ng CaM/$10^6$ cells for the membranes and 47 ± 7 ng CaM/$10^6$ cells for the cytosol. carb, carbachol; Forsk, Forskolin.

| *Agent* | *% Control* | |
|---|---|---|
| | *Membrane* | *Cytosol* |
| 1 μM carb | 69 ± 8 | 142 ± 7 |
| dbcAMP + carb | 66 ± 9 | 148 ± 8 |
| VIP | 75 | 134 |
| PGE1 + | 60 ± 6 | 126 ± 9 |
| 10 μM | 43 ± 3 | 275 ± 18 |
| Forsk ± 10 μM carb | 58 ± 10 | 228 ± 32 |

the degree of translocation of CaM observed with cyclic AMP-elevating agents alone or carbachol alone. The combination of carbachol and $PGE_1$ caused a CaM translocation of similar magnitude to that observed with 1 μM carbachol alone. The combination of forskolin and a nearly maximal concentration of carbachol was also not additive, and did not differ from carbachol alone.

*Agonists that decrease intracellular cyclic AMP levels potentiate carbachol-stimulated increases in cytosolic CaM*

Carbachol did not directly stimulate adenylyl cyclase activity (Table IA), but does increase intracellular cyclic

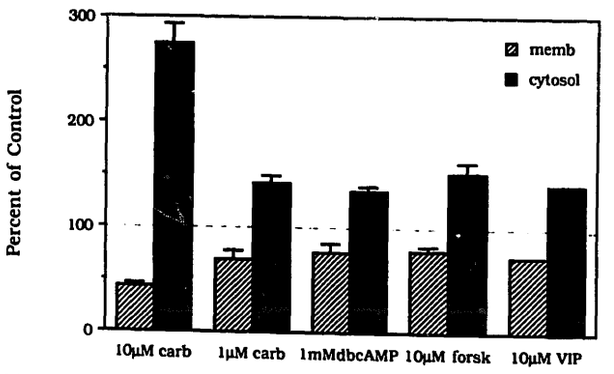


Fig. 2. Increased intracellular cyclic AMP elicits a CaM translocation. Cells were incubated with 10 μM or 1 μM carbachol (carb), 1 mM dibutyryl cyclic AMP (dbcAMP), 10 μM forskolin (forsk), or 10 μM VIP. CaM quantification was by RIA. The data show the mean ± S.E. for three experiments performed in duplicate, except for VIP, for which the data represent the average of two experiments in duplicate. Control values (100%) were 48 ± 6 ng CaM/$10^6$ cells for the membranes and 47 ± 7 ng CaM/$10^6$ cells for the cytosol.

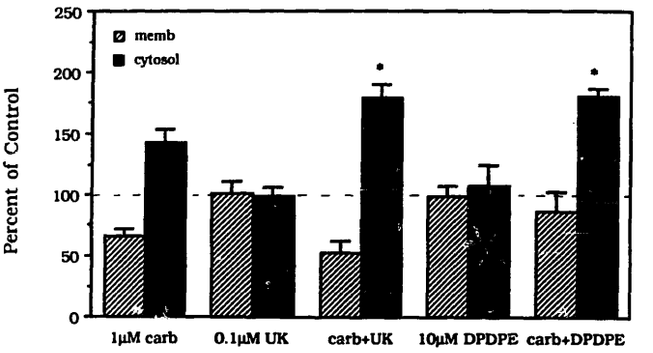


Fig. 3. Effect of agonists negatively coupled to adenylyl cyclase on CaM localization. Cells were incubated with 1 mM carbachol (carb), 0.1 μM UK 14,304 (UK), 10 μM D-pen, D-pen enkephalin (DPDPE) alone or in combination with 1 μM carbachol for 15 min at 37 °C. CaM quantification was by RIA. The data represent three determinations performed in duplicate. Control (100%) values were 57 ± 8 ng CaM/$10^6$ cells for the membranes and 53 ± 15 ng CaM/$10^6$ cells for the cytosol. *Significantly different from 1 μM carbachol $P < 0.05$.

AMP levels in these cells. We investigated the effect of agonists that bind to receptors negatively coupled through $G_i$ to adenylyl cyclase activity on the carbachol-stimulated CaM translocation. Activation of these receptors would directly inhibit cyclic AMP formation. Neither the $\alpha_2$-adrenergic agonist UK 14,304 (0.1 μM) nor the opioid receptor agonists DPDPE (10 μM) and DAMGO (10 μM) affected the cellular distribution of CaM (Fig. 3). However, both UK 14,304 and DPDPE significantly potentiated the carbachol-stimulated increase in cytosolic CaM by 36%. The $\alpha_2$-adrenergic receptor antagonist yohimbine (10 μM) blocked the UK 14,304-mediated potentiation of carbachol-stimulated translocation of CaM (data not shown).

*Measurement of cytoplasmic $Ca^{2+}$ concentrations in agonist-stimulated SK-N-SH cells*

Intracellular calcium $[Ca^{2+}]_i$ is elevated by many hormones and neurotransmitters and is required for CaM activation. Since changes in $[Ca^{2+}]_i$ may be an important stimulus for CaM translocation, basal and agonist-stimulated $[Ca^{2+}]_i$ values in SK-N-SH were monitored using fura-2 and dual wavelength measurements. The basal $[Ca^{2+}]_i$ of quiescent SK-N-SH cells was 40 ± 11 nM ($n$ = 10) which corresponds to values previously reported[15]. Carbachol stimulated a rapid increase in $[Ca^{2+}]_i$ to a peak of 282 ± 10 nM, followed by a gradual decline to a plateau level of 104 ± 7 nM (Fig. 4A). VIP, which stimulated adenylyl cyclase activity (Table IA), also increased $[Ca^{2+}]_i$, however, to a much smaller extent with a peak rise of 186 ± 44 nM ($n$ = 4) (Fig. 4B). VIP-stimulated $Ca^{2+}$ fluxes were not observed if the cells were pre-stimulated with carbachol. Forskolin did not change $[Ca^{2+}]_i$ even using concentrations up to 100 μM.

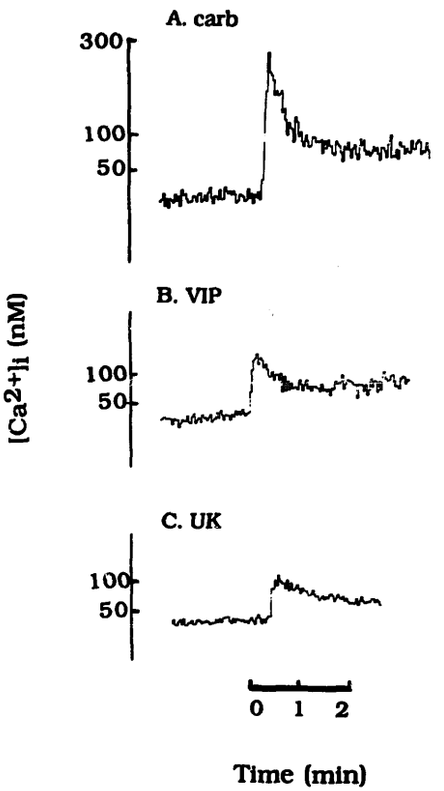


Fig. 4. A–C: effect of agonist stimulation on $[Ca^{2+}]_i$ monitored by fura-2 fluorescence. Cells were loaded with 1 $\mu$M fura-2/AM for 15 min at 37 °C. At the times indicated, the agonists were added. A: 100 $\mu$M carbachol (carb). B: 10 $\mu$M VIP. C: 50 nM UK 14,304 (UK).

The $\alpha_2$ agonist UK 14,304 elicited a rise in $[Ca^{2+}]_i$ to a peak of 130 ± 10 nM ($n$ = 4) (Fig. 4C). Like VIP, UK 14,304 did not affect $[Ca^{2+}]_i$ when carbachol was added to the cells first. The ability of VIP or UK 14,304 to potentiate muscarinic receptor-stimulated $Ca^{2+}$ responses was assessed using 1 $\mu$M carbachol, and little or no potentiation was observed (data not shown).

## DISCUSSION

We have used the SK-N-SH human neuroblastoma cell line to investigate the effect of second messenger production on the distribution of CaM. In these cells, the muscarinic agonist carbachol promotes PPI turnover via $M_3$ receptors[16], modestly inhibits adenylyl cyclase activity via $M_2$ receptors (see Results), and increases intracellular cyclic AMP levels[5,7]. The mechanism for the increased cyclic AMP levels does not appear to be through CaM stimulation of adenylyl cyclase, since we were unable to detect a stimulatory effect of added CaM, and actually observed a slight inhibition of activity. Carbachol can also stimulate a robust translocation of CaM from membranes into the cytosol[28], thus increasing the availability of 'free' CaM to regulate various $Ca^{2+}$/CaM-dependent enzyme systems such as CaM-dependent protein kinases, calcineurin, phosphodiesterase or $Ca^{2+}$ transport systems. The mechanism of the release involves both intracellular $Ca^{2+}$ and protein kinase C activity. Previous work has suggested a role for cyclic AMP-dependent phosphorylation in neurotransmitter-induced changes in CaM localization[17,31]. Many CaM-dependent enzymes and -binding proteins are substrates for cyclic AMP-dependent protein kinase. The current study demonstrates that agents elevating intracellular cyclic AMP levels such as forskolin, dbcAMP and VIP can decrease CaM content in membranes and increase CaM levels in the cytosol of SK-N-SH cells. The CaM translocation mediated by cyclic AMP, however, is significantly lower than that elicited by PPI hydrolysis, which would give the greatest response physiologically.

Since stimulation of both the adenylyl cyclase and PPI[28] systems can affect CaM localization, then the activation of protein kinase C or cyclic AMP-dependent protein kinase may be mechanistically important in translocation. The phosphorylation of a membrane of cytosolic CaM-binding protein which is a substrate for both protein kinase C and cyclic AMP-dependent protein kinase could release CaM. Recent studies have demonstrated that some CaM-binding proteins such as neuromodulin[3], MARCKS protein[18], and neurogranin[6] are substrates for protein kinases, and the phosphorylated forms of the CaM-binding proteins have a lower affinity for CaM. If phoshorylation of a membrane-bound CaM-binding protein were the mechanism of the translocation, then the decrease of CaM in the membrane should approximate the increase in CaM in the cytosol. The PKC-activating phorbol ester TPA decreased membrane CaM and increased cytosolic CaM to the same extent[28]. In this study VIP stimulated a 30% decrease in membrane CaM (15 ng) and 40% increase in cytosolic (20 ng) CaM. Therefore, phosphorylation of CaM-binding proteins by cyclic AMP-dependent protein kinase may initiate these changes in CaM localization. We are currently investigating CaM-binding proteins phosphorylated in response to carbachol and agents that increase cyclic AMP levels.

Elevations in $[Ca^{2+}I_i$ contribute to the mechanism of the translocation of CaM as shown in previous studies using the ionophore ionomycin[28]. Our work and others[32] demonstrate that VIP can elicit a $Ca^{2+}$ signal in these cells. It appears that the mechanism of the VIP-stimulated $Ca^{2+}$ flux is distinct from stimulation of adenylate cyclase activity, however, since forskolin did not change $[Ca^{2+}]_i$. Thus, VIP, forskolin and dbcAMP increased cyclic AMP levels and elicited a CaM translocation probably not via changes in $[Ca^{2+}]_i$, but instead through cyclic AMP-dependent protein kinase activation.

The cyclic AMP and carbachol-stimulated translocations were not additive and there could be several explanations for this finding. Cyclic AMP and PPI hydrolysis may be acting through a common pathway to elicit a CaM translocation, for example, phosphorylation of the same target protein. Alternatively, the cyclic AMP-sensitive pool of CaM could be contained within the larger PPI-sensitive pool; thus the response observed with a combination of agents would not be greater than the larger of the two pools. The data do not indicate if the carbachol or the cyclic AMP response predominates. Interactions between these two signal transduction systems have been described, but appear to differ depending on the tissue or cell type employed in the study. Activation of specific protein kinase C isozymes in PC12 and transfected NIH 3T3 cells induced changes, both facilitatory and inhibitory, in cyclic AMP accumulation[19]. In another study, increased intracellular cyclic AMP levels in SK-N-SH cells inhibited PPI turnover at a site distal to the G protein coupled to phopholipase C[1]. Incubation of the cells with cyclic AMP analogues or forskolin as well as guanine nucleotides in permeabilized cells inhibited PPI hydrolysis by 20–70%. In this same study, $PGE_1$ did not affect PPI turnover, and interestingly, we describe that it does not affect CaM localization. The non-additivity observed with regard to the cyclic AMP and PPI effects on CaM, however, is not likely to result from cyclic AMP-mediated inhibition of the carbachol response since forskolin did not significantly inhibit CaM translocation elicited by 10 $\mu$M carbachol. In addition, carbachol activation of the $M_3$ muscarinic receptors present on these cells was still able to stimulate a robust PPI response[16] and a rise in cyclic AMP levels[7]. Baron and Siegel[5] have shown that stimulation of muscarinic receptors by carbachol actually augments the forskolin and VIP-stimulated cyclic AMP accumulation in SK-N-SH cells. Our data show that VIP and forskolin have effects when added alone and do not require PPI turnover to elicit changes in CaM localization. It has been postulated that the carbachol-induced elevation in cyclic AMP secondary to PPI turnover occurs through a CaM-stimulation of adenylyl cyclase. We found no evidence to support this in SK-N-SH cells, however.

Agonists which inhibit adenylyl cyclase potentiated the carbachol-stimulated CaM translocation, resulting in greater levels of cytosolic CaM. $Alpha_2$-adrenergic receptor activation has been reported to stimulate a variety of second messenger systems in addition to inhibition of adenylyl cyclase activity, such as $Na^+/H^+$ antiport and phospholipase $A_2$ activity[25]. In addition, UK 14,304 has been reported to have a modest ability to stimulate PPI metabolism and increase intracellular $Ca^{2+}$ via a cyclic AMP-independent mechanism as well as inhibiting adenylyl cyclase activity in transfected cells[12]. We and others[5] show that UK 14,304 inhibits forskolin-stimulated adenylyl cyclase activity in SK-N-SH cells. UK 14,304 also generated a $Ca^{2+}$ signal in SK-N-SH cells, but did not appear to potentiate the carbachol-stimulated $Ca^{2+}$ flux, although variable results were obtained. The potentiative effects of UK 14,304 on CaM content may be mediated through multiple interacting signaling systems since UK 14,304 had an effect on CaM only in the presence of added carbachol. Indeed, UK 14,304 was shown to be significantly less effective in inhibiting forskolin-stimulated adenylyl cyclase activity in the presence of carbachol[5]. The mechanism of the opiate receptor-mediated potentiation of carbachol-stimulated CaM translocation is not clear, although DPDPE and DAMGO inhibited adenylyl cyclase activity in these cells. Enkephalins have been reported to stimulate the redistribution of CaM in NG 108-15 cells[4], however, our data show that DPDPE and DAMGO have effects only in the presence of carbachol. Activation of other second messenger pathways has not been linked to these receptors, but it is possible that as yet unidentified interactions between opiate and carbachol-stimulated processes can occur.

The outcome of receptor-G-protein-effector interactions in whole cells is influenced by biochemical events other than those which occur in isolated membranes in response to singular stimuli. In neural tissues and cells, signal transduction systems are activated by diverse stimuli, and these signals are integrated and regulated in order to produce the physiological response. CaM has been described as a 'multifunctional regulator'[10] because of its pivotal ability to regulate activities important in both $Ca^{2+}$ and cyclic AMP signal cascades. We demonstrate that activation of either PPI hydrolysis or adenylyl cyclase activity results in a translocation of CaM. In addition, the two pathways can interact to effect an even greater increase in CaM in the cytosol. These changes in CaM localization could allow altered regulation of CaM-dependent enzyme systems at specified cellular locations.

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## ABBREVIATIONS

| | |
|---|---|
| BSA | bovine serum albumin |
| CaM | calmodulin |
| cAMP | cyclic AMP |
| DAMGO | Tyr-D-ala-Gly-MePhe-Gly-ol |
| dbcAMP | dibutyryl cyclic AMP |
| DPDPE | D-pen, D-pen enkephalin |
| EGTA | ethylene glycol bis-($\beta$-aminoethyl ether)-*N,N,N′,N*-tetraacetic acid |
| fura-2 | 1-[2-carboxyazol-2-yl)-6-amino-benzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N*-tetraacetic acid |
| fura-2/AM | pentaacetoxymethyl ester derivative of fura-2, PKC, protein kinase C |
| GTP | guanosine triphosphate |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| IBMX | isobutylmethylxanthine |
| $IP_3$ | D-myo-inositol trisphosphate |
| $PGE_1$ | prostaglandin $E_1$ |
| PKA | protein kinase A |
| PPI | polyphosphoinositide |
| RIA | radioimmunoassay |
| TPA | 12-*O*-tetradecanoylphorbol 13-acetate |
| UK 14,304 | 6-quinoxalinamine 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl) |
| VIP | vasoactive intestinal polypeptide |

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