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CARBACHOL ENHANCES FORSKOLIN-STIMULATED CYCLIC AMP ACCUMULATION VIA ACTIVATION OF CALMODULIN SYSTEM IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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SUMMARY: We have investigated the modulatory action of carbachol on intracellular cAMP levels in human neuroblastoma SH-SY5Y cells. Carbachol enhanced forskolin-stimulated cAMP levels in a dose-dependent manner (EC_{50}=3 μM). The enhancing effect of carbachol was completely inhibited by pirenzepine and atropine. Pertussis toxin treatment of the cells partially affected the ability of carbachol. Furthermore, carbachol also enhanced the effect of vasoactive intestinal peptide (EC_{50}=3 μM)-, adenosine- and prostaglandin E_1-stimulated cAMP levels. The enhancing response of carbachol was sensitive to trifluoperazine but insensitive to calphostin C. These results suggest that the mechanism for carbachol-induced cAMP levels may act, at least in part, through the activation of calmodulin system in SH-SY5Y cells. Hence we describe for the first time a synergistic interaction between calmodulin- and cAMP-dependent signal transduction pathway mediated by carbachol in neuron-derived cell line.

Activation of muscarinic receptors by the neurotransmitter acetylcholine elicits several physiologically important intracellular events (1,2). Recent evidence indicates that cross-talk between the phosphoinositide (PI) hydrolysis and adenylate cyclase results in increased cyclic AMP (cAMP)

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ABBREVIATIONS: PI, phosphoinositide; cAMP, cyclic AMP; IPs, inositol phosphates; IBMX, isobutylmethylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; PTX, pertussis toxin; CAR, carbachol; FOR, forskolin; VIP, vasoactive intestinal peptide; ADE, adenosine; PGE_1, prostaglandin E_1; PKC, protein kinase C; PMA, 4-ß-phorbol 12-myristate 13-acetate.

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levels in SK-N-SH cells in response to muscarinic stimulation (3). Defining the nature and molecular mechanisms of such potential cross-talk is important for understanding the ultimate functional responses of cells. The human neuroblastoma SH-SY5Y cells are of a neuroblastic subclone from SK-N-SH cells (4). These cells are unique in that they express the neural properties and have muscarinic receptors coupled to PI hydrolysis (5,6,7). Therefore, we used SH-SY5Y cells to determine whether such cross-talk exists. We now report that muscarinic agonist carbachol induces in dose-dependent enhancement of basal, forskolin- and vasoactive intestinal peptide (VIP)-stimulated cAMP levels in SH-SY5Y cells. The enhancement of cAMP levels appears to require calmodulin activation, reflecting cross-talk between the two signal transduction pathways.

MATERIALS AND METHODS

Materials. Forskolin, isobutylmethylxanthine (IBMX), carbachol, atropine, pirenzepine, and adenosine were obtained from Sigma (St.Louis, MO, U.S.A.). Pertussis toxin (PTX) was from Kaken Pharmaceuticals.(Tokyo, Japan). Human vasoactive intestinal peptide (VIP) was from Peptide Institute Inc. (Osaka, Japan). Calphostin C was Kyowa Medex (Tokyo, Japan). Trifluoperazine was from Wako Pure Chemicals.(Osaka, Japan).

Cell Culture. Human neuroblastoma SH-SY5Y cells were obtained from Dr.J.Biedler (Sloan-Kettering Institute, NY,U.S.A.). The cells were grown in platic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, U.S.A.) with 10 % fetal calf serum (Gibco), penicillin (100 u/ml), and streptomycin (100 µg/ml). Cultures were maintained at 37°C in 5 % CO_2/humidified air.

Measurement of Intracellular cAMP. All experiments were carried out in plastic dishes (Falcon 3802, Primaria) with the cells at 80-90 % of confluent growth. Cells were incubated with DMEM medium containing 20 mM Hepes and 100 µM IBMX for 10 min, followed by incubation of 10 µM forskolin. The medium was removed and then the reaction was terminated by the addition of ice-cold 6 % TCA to the cells. Cell extracts in 6 % TCA were centrifuged and cAMP levels in the supernatant were determined by radioimmunoassay using a Yamasa cAMP assay kit (Yamasa Shoyu Co. Chiba, Japan). Protein assays were performed by the BCA method (Pierce Chemical, Rockford, U.S.A.)

RESULTS

To investigate the ability of muscarinic receptors to modulate adenylate cyclase activity, we compared the effects of carbachol on intracellular levels of cAMP following direct stimulation of adenylate cyclase by forskolin. The phosphodiesterase inhibitor IBMX was included to ensure that changes in cAMP levels were not due to changes in phosphodiesterase activity.

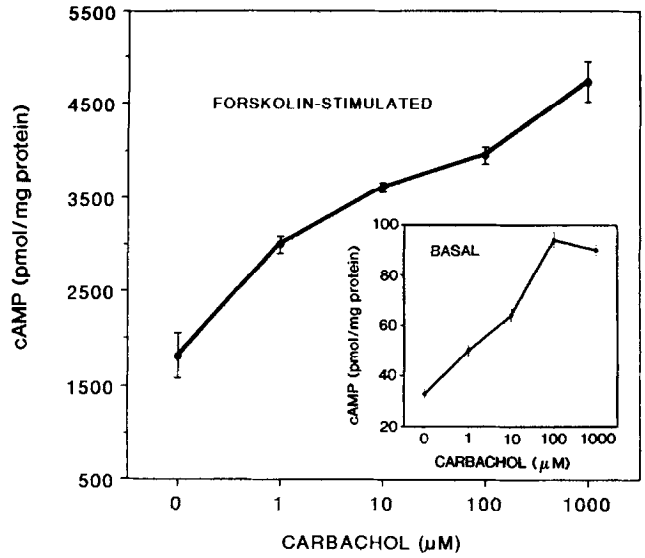

Figure 1. Concentration dependence for carbachol-mediated changes in intracellular levels of cAMP induced by forskolin. SH-SY5Y cells were pretreated with 100 μM IBMX for 10 min. Forskolin (10 μM) and the indicated concentrations of carbachol were added and the cells were incubated for another 10 min. *Inset*, Effect of carbachol on the basal cAMP levels. cAMP was assayed as described in Materials and Methods.

Carbachol enhanced basal and forskolin-stimulated cAMP levels in a dose-dependent manner (Fig. 1). Carbachol produced about 2-3-fold stimulation above the basal cAMP levels with an EC_{50} of 5 μM. Forskolin (10 μM) induced about a 50-fold increase in cAMP levels in SH-SY5Y cells. The maximal increase in forskolin-stimulated cAMP response was about 2-3-fold with an EC_{50} of 3 μM.

The nature of the receptors responsible for cAMP accumulation response was next investigated by using muscarinic agonist/antagonist. As shown in Fig. 2A, oxotremorine (100 μM) and McN-A-343 (100 μM) were not as effective as carbachol in promoting enhanced forskolin-stimulated cAMP levels. The enhancing effect by 100 μM carbachol was completely abolished by two muscarinic antagonists, atropine (10 μM) and pirenzepine (100 μM). The half-maximal inhibitory concentration for pirenzepine was 2 μM (Fig. 2B). This result indicates that the response is due to pirenzepine-sensitive muscarinic receptors.

Since G-proteins can be distinguished by sensitivity to pertussis toxin (PTX), we tested the effect of this toxin on carbachol's ability to enhance forskolin-

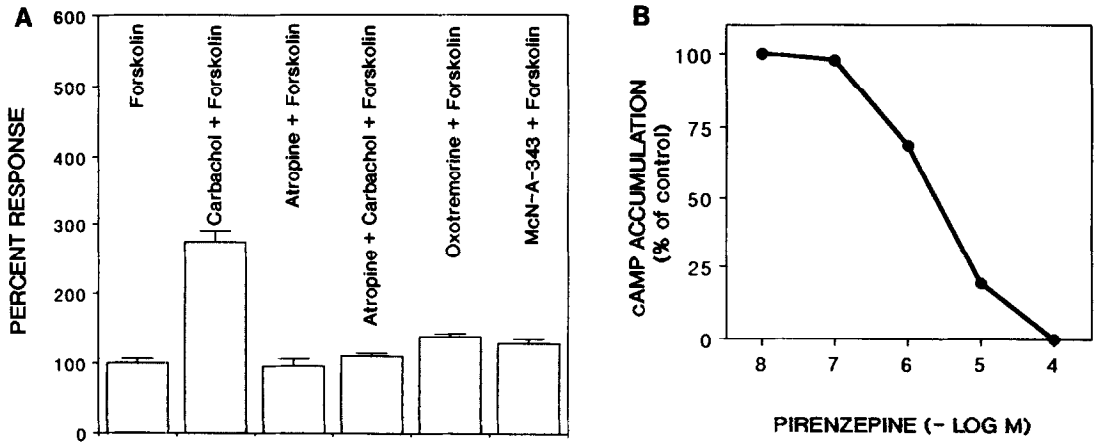

Figure 2. A. Effects of muscarinic antagonist or agonist on intracellular levels of cAMP induced by forskolin in SH-SY5Y cells. Atropine (10 μM) was added 15 sec prior to carbachol (100 μM) and forskolin (10 μM). The concentrations of oxotremorine and McN-A343 were 100 μM. Data were expressed as a percentage of the forskolin alone value in each experiment (mean ± S.E.M., n=5). B. Pirenzepine inhibition of forskolin-stimulated cAMP levels elicited by carbachol. Carbachol (100 μM) and forskolin (10 μM) were added with the indicated concentrations of pirenzepine and the cells were incubated another 10 min.

stimulated cAMP levels in SH-SY5Y cells (Fig.3). PTX-treated cells showed partial inhibition (50 % at maximum), which saturated at 1 ng/ml PTX.

Since SH-SY5Y cells are also sensitive to cAMP stimulation by VIP, adenosine and prostaglandin (PG) E_1, we tested carbachol's ability to enhance VIP (1 μM)-, adenosine (100 μM)- and PGE_1 (10 μM)-stimulated

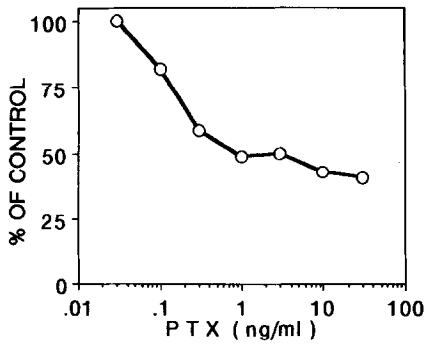

Figure 3. Effect of pertussis toxin on ability of carbachol to enhance forskolin-stimulated cAMP levels. SH-SY5Y cells were incubated for 16 hr with increasing amounts of pertussis toxin. Carbachol and forskolin were present in concentrations of 100 μM and 10 μM, respectively.

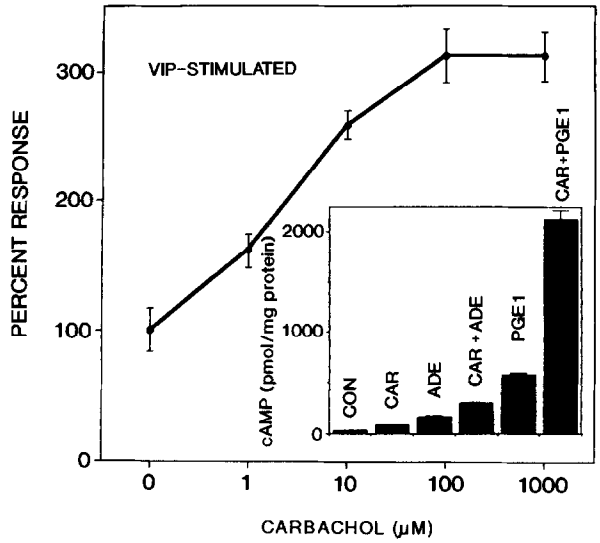

Figure 4. Effect of carbachol on VIP-stimulated cAMP levels in SH-SY5Y cells. VIP (1 μM) and the indicated concentration of carbachol were added and the cells were incubated for 10 min. *Inset*: Effect of carbachol (100 μM) on PGE_1- and adenosine-stimulated cAMP levels. PGE_1 and adenosine were present in concentrations of 10 μM and 100 μM, respectively.

cAMP levels. VIP (1 μM) induced about a 30-fold increase in cAMP levels in SH-SY5Y cells. As shown in Fig.4, carbachol also enhanced the VIP-stimulated cAMP response by 3-fold, with an EC_{50} of 3 μM. The effect was almost completely abolished by atropine (data not shown). Carbachol similarly enhanced adenosine- and PGE_1-stimulated cAMP levels (Fig.4 Inset).

To assess the possible contribution of protein kinase C (PKC) and calmodulin system to the enhancement of forskolin-stimulated cAMP levels elicited by carbachol in SH-SY5Y cells, we tested the effects of PKC inhibitor calphostin C and calmodulin antagonist trifluoperazine. As shown in Fig.5, the enhancement of forskolin-stimulated cAMP levels elicited by carbachol was inhibited with trifluoperazine (100 μM). In contrast, calphostin C (3 μM) had no effect. Thus, enhancing effect of forskolin-stimulated cAMP levels elicited by carbachol appears to be due to activation of calmodulin system.

DISCUSSION

The present report demonstrates that carbachol elicits increases in basal, forskolin-, adenosine-, PGE_1- and VIP-stimulated cAMP levels in SH-SY5Y

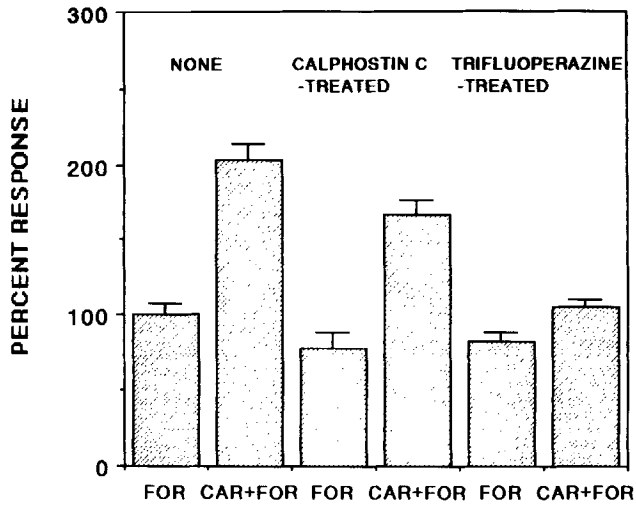

Figure 5. Effects of PKC inhibitor and calmodulin antagonist on intracellular levels of cAMP induced by forskolin in SH-SY5Y cells. Calphostin C (3 μM) and trifluoperazine (100 μM) were added 20 min prior to carbachol (100 μM) and forskolin (10 μM). Data were expressed as percentage of the forskolin alone value in each experiment (mean ± S.E.M., n=5).

cells. Mei et al. reported that carbachol enhances forskolin-stimulated cAMP levels in SH-SY5Y cells (6). However, this enhancing effect by carbachol was not blocked by atropine. The reason for this apparent discrepancy in the effect of antagonist is unknown. In the parent SK-N-SH cells, Baumgold and Fishman have reported that muscarinic antagonists are effective in blocking the enhancement of PGE_1-stimulated cAMP levels elicited by carbachol (3). Interestingly, the enhancing effect of forskolin-stimulated cAMP levels elicited by carbachol was partially inhibited by PTX. Our result closely match those by Mei et al. who found that carbachol-stimulated IPs formation in SH-SY5Y cells was partially sensitive to PTX (8). Moreover, SH-SY5Y cell menbranes were shown to contain Gi , Go that can be specifically ADP-ribosylated by PTX (9).

Conflicting reports exist on the properties of muscarinic receptor subtypes in SH-SY5Y cells. Sera et al. have proposed, on the basis of antagonist affinities, that M1-subtype receptors mediate the PI hydrolysis (10). On the other hand, Lambert et al. have reported that SH-SY5Y cells express an homogenous M3-subtype receptors (11). Our result obtained for pirenzepine sensitivity is in agreement with the competitive curve for N-

[^{3}H]methylscopolamine binding recently presented by Cioffi and Fisher (12). Therefore, it is important in determining the nature of the subtype expressed in SH-SY5Y cells by Northern blot analysis.

In the SH-SY5Y cells, generation of cAMP by carbachol stimulation appeared to be dependent on the activation of calmodulin, since calbachol's effect on forskolin-stimulated cAMP response was blocked by trifluoperazine yet was unaffected by calphostin C. Analogous finding have been observed in several different types of cells such as 132N1 astrocytoma cells, A9L fibroblast cells transfected with m1 gene and Y1 adrenal carcinoma cells transfected with M1 gene (13,14,15). These recent studies suggested that a possible mechanism for carbachol-induced increase in cAMP levels is activation of calmodulin-dependent adenylate cyclase resulting from incresed intracellular calcium levels triggered by increases in inositol phosphate (IPs). Therefore this phenomenon may represent a more general cellular response.

We have preliminary observed that 4-ß-phorbol 12-myristate 13-acetate (PMA) also enhance forskolin-stimulated cAMP levels in SH-SY5Y cells. However, PMA has been reported to be an activator of PKC, but it is not specific because it also can modify phospholipase A_2, phospolipase D and tyrosine protein kinase activity (16,17,18). Thus further studies are in progress.

Muscarinic receptors in this cell line are coupled to the control of cAMP levels and should provide a useful cell model to define the importance of a synergistic interaction between calmodulin- and cAMP-dependent signal transduction pathway. Whether the elevation of cAMP levels observed here is of physiological significance remains to be determined.

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