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Synergistic activation of adenylyl cyclase is dependent upon phospholipase C-mediated processes in human neuroblastoma SK-N-BE(2)C cells

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Abstract

1-[6-[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), an inhibitor of processes involved in the activation of phospholipase C, was used to assess the role of phospholipase C activation in the synergistic elevation of cAMP induced by carbachol and prostaglandin E_2 in human neuroblastoma (SK-N-BE(2)C) cells. Pre-treatment of the cells with U-73122 resulted in inhibition of carbachol-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) rise and inositol 1,4,5-trisphosphate ($InsP_3$) generation, with maximal and half maximal inhibition (IC_{50}) occurring at approximately 15 μM and 3.2 μM, respectively. U-73122 also inhibited the synergistic enhancement of cAMP accumulation induced by carbachol and prostaglandin E_2 in a concentration-dependent manner with maximum and IC_{50} at 12 ± 4 μM and 3.4 ± 0.3 μM, respectively. However, U-73122 did significantly inhibit prostaglandin E_2-induced production. While 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA/AM) treatment decreased the synergistic cAMP accumulation by 28%, addition of U-73122 further decreased it down to complete inhibition. Furthermore, GTPγS- and AlF_4^--induced $InsP_3$ generation in digitonin-mediated permeabilized cells was also inhibited by U-73122 treatment. Pre-treatment of the cells with neomycin, another blocker of the phospholipase C pathway, also resulted in inhibition of the carbachol-induced $[Ca^{2+}]_i$ rise, $InsP_3$ generation, and the enhancing effect on cAMP accumulation, to a comparable extent. But, Ca^{2+} chelation by BAPTA/AM in addition to neomycin treatment further decreased the cAMP accumulation. These results suggest that the increase in cytosolic Ca^{2+} and the coupling process between muscarinic receptor-linked G-protein and phospholipase C are important for the synergistic activation of adenylyl cyclase in SK-N-BE(2)C cells.

Keywords: SK-N-BE(2)C human neuroblastoma; Phospholipase C; U-73122; cAMP production; Ca^{2+} mobilization; Inositol 1,4,5-triphosphate; Neomycin

1. Introduction

A variety of extracellular signals stimulate adenylyl cyclase, one of the principal cell surface signal transduction systems (Hille, 1992). From various studies, it has been learned that there are at least 8 different isoforms of adenylyl cyclase in mammalian tissues (Iyengar, 1993), and that all the subtypes are commonly stimulated via the α-subunit of guanine nucleotide-binding G_s-protein or directly stimulated by forskolin (Tang and Gilman, 1992). However, among the eight subtypes, the type 1, 3 and 8 adenylyl cyclases are specifically activated by Ca^{2+}/calmodulin-dependent kinase (Cali et al., 1994; Xia et al., 1993; Taussig et al., 1993), while type 6 is inhibited by direct binding of Ca^{2+} to the molecule (Yoshimura and Cooper, 1992). In contrast, type 2 and 4 adenylyl cyclases are activated by βγ-subunits (Gao and Gilman, 1991; Feinstein et al., 1991), whereas type 1 is inhibited (Gao and Gilman, 1991; Taussig et al., 1993). Type 2 and 7 adenylyl cyclases are activated by protein kinase C (Hellevuo et al., 1995; Lustig et al., 1993; Choi et al., 1993).

We and other groups have demonstrated that the stimulation of the human neuroblastoma cell lines SK-N-SH, SH-SY5Y, and SK-N-BE(2)C with prostaglandin E_1 or E_2 elevates cAMP levels (Suh and Kim, 1995; Baumgold and Fishman, 1988; Nakagawa-Yagi et al., 1991). Moreover, co-treatment of these cells with prostaglandin E_1 or E_2 and

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carbachol, the muscarinic acetylcholine receptor agonist, results in a markedly higher synergistic elevation of the cAMP level as compared to the treatment with prostaglandin alone, although treatment with carbachol alone does have a small effect on cAMP generation. However, the mechanism underlying this phenomenon of enhancement is still not fully understood. Our previous studies have shown that muscarinic receptor-mediated activation of protein kinase C appears not to be involved in the activation of adenylyl cyclase (Suh and Kim, 1995). Baumgold (1992) proposed that $\beta\gamma$-subunits of G-proteins generated by the activation of muscarinic receptors may play a role in the synergistic responses.

In the present study, we used aminosteroid 1-[6-[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122) and neomycin, inhibitors of processes involved in the activation of phospholipase C in various cells, including human neuroblastoma cells (Thompson et al., 1991), neutrophils and platelets (Bleasdale et al., 1990; Smith et al., 1990), embryonic kidney cells (Sandmann et al., 1991), and rat pituitary cells (Smallridge et al., 1992). Our goal was to investigate the relationship between carbachol-induced phospholipase C activation and the synergistic rise in cAMP production in SK-N-BE(2)C cells. Here we report that U-73122 and neomycin inhibit the carbachol-induced synergistic elevation of cAMP production by blocking the signal transduction pathways to phospholipase C. This strongly suggests that the synergistic activation of adenylyl cyclase is dependent upon the phospholipase C-linked processes.

2. Materials and methods

2.1. Materials

Carbamylcholine (carbachol) chloride, prostaglandin E_2, (±)-sulfinpyrazone, Triton X-100, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), $AlCl_3$, NaF, potassium glutamate, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), ATP, glucose, neomycin, bovine serum albumin and inositol 1,4,5-trisphosphate ($InsP_3$) were obtained from Sigma (St. Louis, MO, USA). U-73122, isobutylmethylxanthine (IBMX) were purchased from Research Biochemicals (Natick, MA, USA). [^{3}H]$InsP_3$ and [^{3}H]adenine were obtained from NEN (Boston, MA, USA) and fura-2 acetoxymethylester (fura-2/AM) and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA/AM) were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

Human neuroblastoma clone SK-N-BE(2)C cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Gaithersburg, MD, USA) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone, Logan, UT, USA) and 1% antibiotics (GIBCO) under a humidified atmosphere of 5% CO_2 at 37°C. Medium was changed every 2 days, and cells were subcultured about once a week.

2.3. Measurement of intracellular Ca^{2+} level

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the fluorescent Ca^{2+} indicator fura-2 as previously reported (Suh and Kim, 1995). Briefly, SK-N-BE(2)C cells were loaded with fura-2 pentaacetoxymethyl ester (fura-2/AM) to a final concentration of 3 μM in complete medium and incubated at 37°C with stirring for 50 min. After the loading, the cells were pelleted and washed twice with Locke's solution (NaCl, 154 mM; KCl, 5.6 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.2 mM; Hepes, 5.0 mM; glucose, 10 mM, pH 7.4) to remove the extracellular dye. Sulfinpyrazone was added to both, the loading medium and the washing solution, to a final concentration of 250 μM to prevent dye leakage (Di Virgilio et al., 1988). Fluorescence ratios were taken by dual excitation at 340 nm and 380 nm and emission at 500 nm with an alternative wavelength time scanning method. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed according to Grynkiewicz et al. (1985).

2.4. Determination of $InsP_3$ in whole cells

$InsP_3$ concentration in the cells was determined by [^{3}H]$InsP_3$ competition assay in binding to $InsP_3$ binding protein (Downes et al., 1986). To determine the $InsP_3$ production induced by carbachol, SK-N-BE(2)C cells were grown in 6-well culture plates to about 95% confluency. The cells were stimulated with agonists for specific periods of time. The reaction was terminated by aspirating the medium off the cells followed by addition of 0.3 ml ice-cold 15% (w/v) trichloroacetic acid containing 10 mM EGTA. The plates were left on ice for 30 min to extract the water-soluble inositol phosphates. The extract was then transferred to an Eppendorf tube and the trichloroacetic acid removed by four extractions with diethyl ether. Finally the extract was neutralized with 200 mM Trizma base and its pH adjusted to about 7.4. 20 μl of the cell extract was added to 20 μl of the assay buffer [0.1 M tris(hydroxymethyl)aminomethane buffer containing 4 mM EDTA and 4 mg/ml bovine serum albumin] and 20 μl of [^{3}H]$InsP_3$ (0.1 μCi/ml). Then, 20 μl of solution containing the binding protein was added and the mixture incubated for 15 min on ice and centrifuged at 2000 × *g* for 5 min. The pellet was resuspended in 100 μl water, and 1 ml of scintillation cocktail was added to measure the radioactivity. $InsP_3$ concentration in the sample was determined based on a standard curve and expressed as pmol/μg protein. The $InsP_3$ binding protein was prepared from bovine adrenal cortex according to the method of Challiss et al. (1990).

2.5. cAMP accumulation in whole cells

Intracellular cAMP was determined by measuring the formation of [^{3}H]cAMP from [^{3}H]adenine nucleotide pools as described previously by Salomon (1991) with some modifications. The cells were grown in 6-well dishes to confluency and loaded with [^{3}H]adenine (2 μCi/ml) in complete medium for 24 h. After the loading, the cells were washed 3 times with Locke's solution and pre-incubated with 1 mM IBMX for 15 min in Locke's solution to inhibit phosphodiesterase. IBMX was also added to the stimulating buffer. The reaction was stopped by aspirating the medium off and adding 1 ml of ice-cold 5% (w/v) trichloroacetic acid containing 1 μM unlabeled cAMP. The plates were left on ice for 30 min to extract the water-soluble cAMP. Then, the extracts were transferred to Eppendorf tubes and centrifuged at 5000 $\times$ g for 5 min to precipitate the cell debris. [^{3}H]cAMP and [^{3}H]ATP were separated by sequential chromatography on dowex AG50W-X4 (200–400 mesh) cation exchanger and a neutral alumina column. The [^{3}H]ATP fraction was obtained by elution with 2 ml distilled water from dowex column, and the sequential elution with 3.5 ml distilled water was loaded onto the alumina column. The alumina column was washed with 4 ml imidazole solution (0.1 M, pH 7.2), and the eluant fractions were collected into scintillation vials containing 15 ml scintillation fluid to count the [^{3}H]cAMP. Increase of intracellular cAMP concentration was calculated as [^{3}H]cAMP/([^{3}H]ATP + [^{3}H]cAMP) $\times$ 10^3.

2.6. Permeabilized cell preparation

SK-N-BE(2)C cells were grown to confluency in 6-well plates. Tissue culture medium was removed, and the cells were washed with Locke's solution. The cells were then permeabilized in KG buffer (139 mM potassium glutamate, 20 mM PIPES, 0.5 mM ATP, 1 mM $MgSO_4$, 5 mM glucose, pH 7) containing 20 μM digitonin for 10 min and then stimulated with GTPγS or $AlCl_3$ and NaF. The amount of $InsP_3$ was determined by competition assay as described above.

2.7. Statistics

The results are expressed as the mean $\pm$ S.E.M. values from the number of determinations indicated. A Student's t test was used for comparing individual treatments with their respective control values. A probability of $P < 0.05$ was accepted as denoting a significant difference.

3. Results

Stimulation of SK-N-BE(2)C cells with 1 mM carbachol resulted in the elevation of $[Ca^{2+}]_i$ through inositol 1,4,5-trisphosphate ($InsP_3$) generation after activation of phospholipase C. U-73122 effectively inhibited the carbachol-induced $[Ca^{2+}]_i$ rise (Fig. 1A). Pre-treatment with U-73122 for 10 min caused, in parallel, the decrease of the carbachol-induced elevation of $[Ca^{2+}]_i$ and the production of $InsP_3$. This occurred in a concentration-dependent manner with the maximum effect and half maximal concentration (IC_{50}) seen at approximately 15 μM and 3.2 μM, respectively (Fig. 1B).

When SK-N-BE(2)C cells were exposed to various concentrations of carbachol, the $[Ca^{2+}]_i$ and $InsP_3$ increased in a concentration-dependent manner. As shown in Fig. 2, the maximum and half maximum response (EC_{50}) were obtained at 1 mM and 1–4 μM, respectively. Pre-treatment with 3 μM U-73122 resulted in a 48% reduction

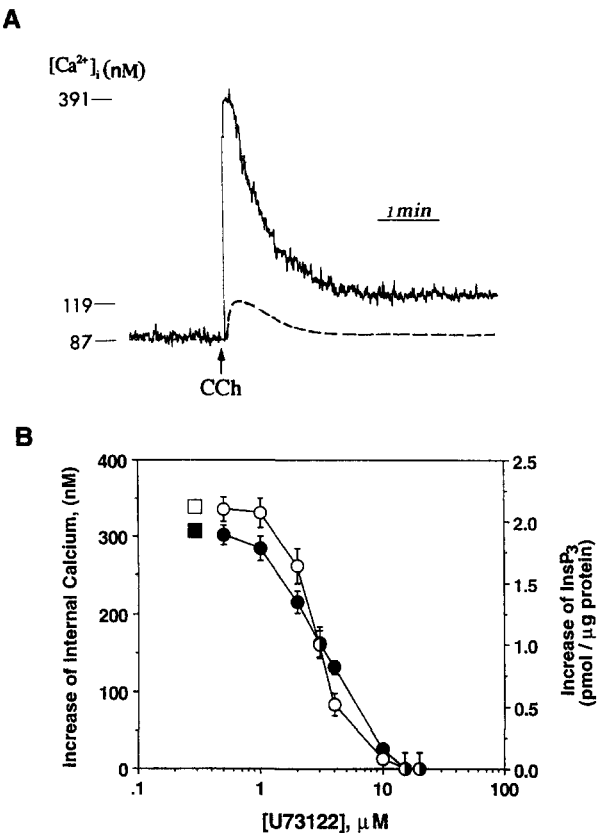

Fig. 1. Inhibition of the carbachol-induced intracellular Ca^{2+} rise and $InsP_3$ production by U-73122 in SK-N-BE(2)C cells. (A) Typical pattern of $[Ca^{2+}]_i$ rise after treatment with carbachol (CCh) (1 mM) in the presence of 2.2 mM extracellular Ca^{2+} (solid trace). U-73122 (5 μM) was added 10 min before the onset of carbachol treatment (dashed trace) ($n = 5$). (B) Concentration-dependent inhibition of the carbachol-induced $[Ca^{2+}]_i$ rise (●) and $InsP_3$ production (○) by U-73122. Fura-2-loaded cells were pre-treated with various concentrations of U-73122 for 10 min and then stimulated with 1 mM carbachol. The peak $[Ca^{2+}]_i$ level was measured. Cells treated with various concentrations of U-73122 for 10 min were also stimulated with 1 mM carbachol for 1 min to measure the $InsP_3$ content in the cells as described in Section 2. Data are presented as increments over the basal levels. The basal Ca^{2+} and $InsP_3$ levels were 75 ± 6 and 0.87 ± 0.07, respectively. The maximal increments of Ca^{2+} (■) and $InsP_3$ (□) levels are presented. Each concentration of carbachol was tested 4 times independently and the mean $\pm$ S.E.M. values are reported.

of the 1 mM carbachol-induced responses, but there were differences in the extent of the inhibition at lower concentrations of carbachol (Fig. 2, insets). As shown in the insets of Fig. 2, 3 μM U-73122 inhibited 65% of the calcium elevation and 95% of the $InsP_3$ generation when the stimulation was done with 10 μM carbachol. The discrepancy might be due to a lower inhibitory effect of U-73122 on Ca^{2+} influx, occurring concomitantly with Ca^{2+} release from intracellular Ca^{2+} pools, than on $InsP_3$ generation.

We tested the effect of U-73122 on the elevation of cAMP by muscarinic stimulation. We have previously demonstrated that in SK-N-BE(2)C cells, 1 mM carbachol slightly increased intracellular cAMP, about 1.5-fold. However, simultaneous treatment of the cells with carbachol and prostaglandin E_2 enhanced cAMP accumulation synergistically, and the enhancing effect of carbachol could be abolished by treatment with 5 μM atropine (Suh and Kim, 1995). Fig. 3A shows that pre-treatment with 10 μM

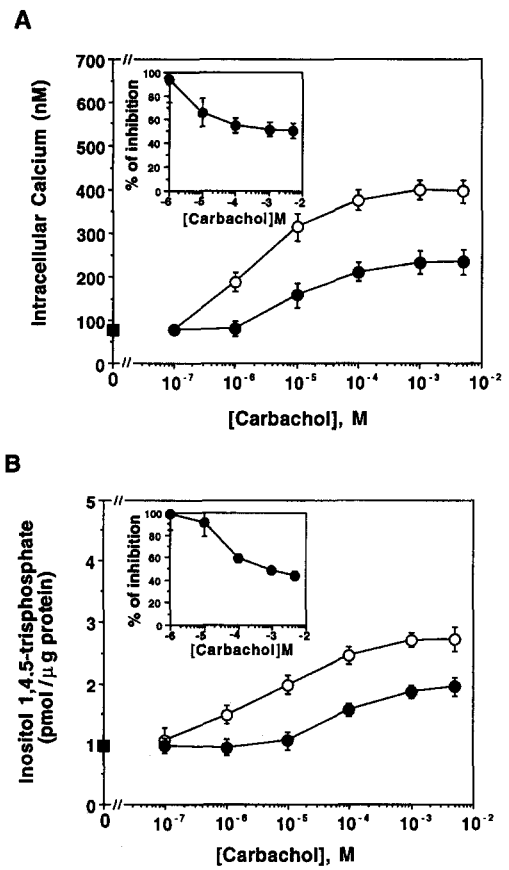

Fig. 2. Inhibitory effects of U-73122 on intracellular Ca^{2+} rise and $InsP_3$ generation evoked by various concentrations of carbachol. Cells pre-incubated with vehicle (○) or 3 μM U-73122 for 10 min (●) were stimulated with various concentrations of carbachol, and the peaks of cytosolic Ca^{2+} (A) and $InsP_3$ generation (B) were measured. The basal Ca^{2+} and $InsP_3$ levels are presented as closed squares (■). U-73122 by itself did not have any effect on both basal levels. Insets: percent inhibitions of carbachol-induced intracellular Ca^{2+} rise and $InsP_3$ generation by 3 μM U-73122. Each data point was obtained from triplicate experiments and the mean ± S.E.M. values are presented.

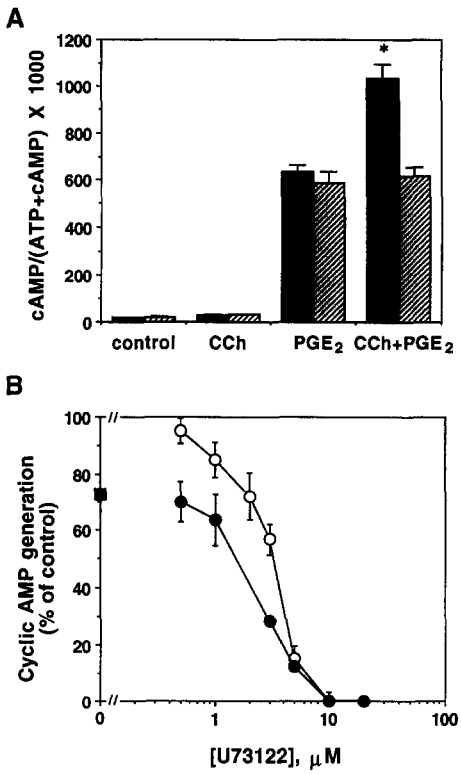

Fig. 3. Effect of U-73122 on the carbachol-induced enhancement of the prostaglandin E_2-stimulated cAMP production in SK-N-BE(2)C cells. (A) The IBMX (1 mM)-pre-treated cells were incubated with carbachol (1 mM), prostaglandin E_2 (10 μM) and carbachol plus prostaglandin E_2 in the absence (solid bar) or presence (hatched bar) of 10 μM U-73122 for 20 min. U-73122 was added to the cells 10 min before the stimulation. * $P < 0.05$, compared with prostaglandin E_2. (B) Concentration-dependent inhibition of carbachol-induced synergistic enhancement of cAMP accumulation by U-73122 in the absence (○) or presence (●) of BAPTA/AM. Control cells or BAPTA/AM-loaded cells (30 min with 50 μM) were treated with various concentrations of U-73122 for 10 min before the cells were stimulated with 1 mM carbachol and 10 μM prostaglandin E_2 for 20 min. The net increases of cAMP obtained by subtracting the prostaglandin E_2-stimulated cAMP level from the agonists-induced cAMP level are presented as percentage of the net increase in the control cells treated with prostaglandin E_2 plus carbachol in the absence of U-73122. The basal, 10 μM prostaglandin E_2-, and 1 mM carbachol and 10 μM prostaglandin E_2-induced cAMP levels were 20 ± 1, 678 ± 49 and 1,165 ± 55, respectively. Synergistic accumulation of cAMP in BAPTA/AM-loaded cells without any incubation with U-73122 (■) was lower than that of control cells. The experiments were done 3 times and the mean ± S.E.M. values are presented.

U-73122 for 10 min completely inhibited the synergistic enhancement of the cAMP accumulation by co-stimulation with 1 mM carbachol and 10 μM prostaglandin E_2. However, U-73122 did not have a significant effect on the cAMP production that resulted from treatment with prostaglandin E_2 alone. U-73122 concentration-dependent inhibition of the carbachol-induced synergistic cAMP production revealed a pattern similar to the inhibition of the $[Ca^{2+}]_i$ rise and $InsP_3$ production (Fig. 3B). The IC_{50} and maximal inhibition were obtained at 3.4 ± 0.3 μM and 12 ± 4 μM, respectively. In addition, pre-treatment with BAPTA/AM, a chelator of Ca^{2+}, also decreased the carbachol-induced

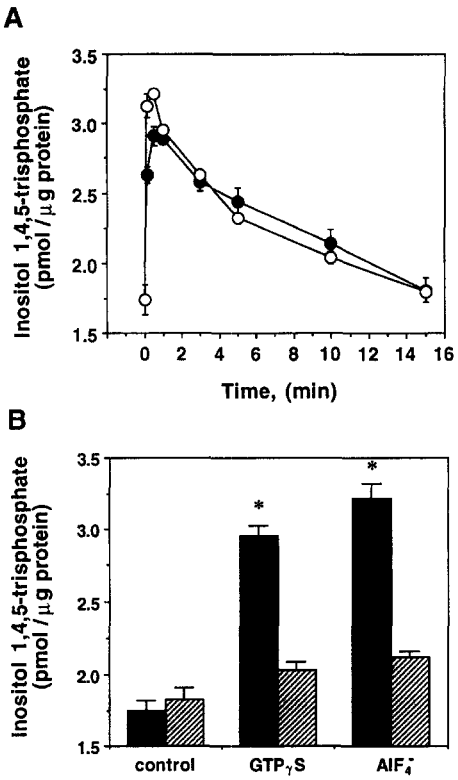

Fig. 4. Effect of U-73122 on G protein-mediated phospholipase C activation. (A). The digitonin-permeabilized SK-N-BE(2)C cells were stimulated with 50 μM GTPγS (●) or 30 μM $AlCl_3$ and 10 mM NaF (○) for indicated periods. The reaction was stopped by the addition of 15% (w/v) trichloroacetic acid containing 10 mM EGTA. (B) Cells pre-incubated with vehicle (solid bar) or 5 μM U-73122 (hatched bar) were permeabilized and stimulated with 50 μM GTPγS or 30 μM $AlCl_3$ and 10 mM NaF for 30 s. The amount of $InsP_3$ was determined by competition assay as described in Section 2. The experiments were done 3 times and the mean ± S.E.M. values are presented. * $P < 0.05$, compared with U-73122-treatment.

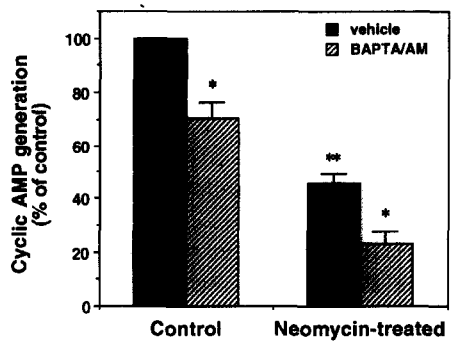

Fig. 5. Inhibition of carbachol-induced synergistic enhancement of cAMP accumulation by neomycin in the presence or absence of BAPTA/AM. Control cells or BAPTA/AM-loaded cells (30 min with 50 μM) were treated with 2 mM neomycin for 5 min before the cells were stimulated with 1 mM carbachol and 10 μM prostaglandin E_2 for 20 min. The net increase of cAMP obtained by subtracting the prostaglandin E_2-stimulated cAMP level from the two agonists-induced cAMP level are presented as percentage of net increase in the control cells treated with prostaglandin E_2 plus carbachol in the absence of neomycin. The basal, prostaglandin E_2 (10 μM)-, and carbachol (1 mM) plus prostaglandin E_2 (10 μM)-induced cAMP levels were 23 ± 2, 501 ± 37 and 987 ± 65, respectively. Synergistic accumulation of cAMP in BAPTA/AM-loaded cells without incubation with neomycin was lower than that of the control cells. The experiments were done 4 times and the mean ± S.E.M. values are presented. * $P < 0.05$, compared with each vehicle. ** $P < 0.01$, compared with control.

effect by 28%, and addition of U-73122 in the presence of BAPTA/AM further reduced the synergistic effect on cAMP production in a concentration-dependent manner (Fig. 3B).

To investigate the site of action for U-73122, we permeabilized the cells with digitonin and directly activated G-protein with agents like GTPγS and AlF_4^-, thus, bypassing receptor activation. Fig. 4A shows the time courses of $InsP_3$ generation by GTPγS and AlF_4^- in permeabilized cells and the maximum effects for both agents observed 30 s after stimulation. However, the GTPγS- or AlF_4^--induced $InsP_3$ generation was also inhibited by U-73122 pre-treatment (Fig. 4B). This result indicates that the site of action for U-73122 might be at a post-receptor site, and that the stimulating effect of carbachol on the synergistic cAMP production occurs at a signal component after muscarinic receptor activation.

Phospholipase C involvement in carbachol-mediated enhancement of cAMP accumulation was further confirmed by using neomycin, another blocker of the phospholipase C pathway. Table 1 shows that pre-incubation of cells with 2 mM neomycin for 5 min resulted in inhibition of the subsequent carbachol-induced $[Ca^{2+}]_i$ rise and $InsP_3$ generation by about 47.3 and about 42.5%, respectively. However, neomycin above a 4 mM concentration has non-

Table 1
Effect of neomycin on carbachol-induced phospholipase C activation

	Control		Neomycin-treated		% Inhibition *
	Basal	Carbachol	Basal	Carbachol	
$[Ca^{2+}]_i$ increase (nM)	78 ± 12	412 ± 34	61 ± 6	237 ± 18	47.3%
$InsP_3$ generation (pmol/μg protein)	0.57 ± 0.04	3.30 ± 0.31	0.45 ± 0.07	2.02 ± 0.15	42.5%

Fura-2-loaded cells were treated with 2 mM neomycin for 5 min and then stimulated with 1 mM carbachol. The peak $[Ca^{2+}]_i$ level was measured. Cells pre-treated with 2 mM neomycin for 5 min were also stimulated with 1 mM carbachol for 1 min and the reaction was stopped by the addition of trichloroacetic acid. The $[Ca^{2+}]_i$ and $InsP_3$ generation were measured as described in Section 2. The values are the mean ± S.E.M. values of three separate experiments done in triplicate. * % Inhibition was calculated by the net increase of values (subtracting basal from carbachol treatment) obtained from the control and neomycin-treated cells.

specific effects on the basal $[Ca^{2+}]_i$ and $InsP_3$ levels, in decreasing both levels (data not shown). Neomycin also inhibited the enhancing effect of carbachol on cAMP accumulation. Fig. 5 shows that neomycin significantly inhibited the carbachol-induced cAMP enhancement, and pre-incubation of the cells with BAPTA/AM in addition to neomycin further decreased the cAMP level. These results suggest that the signal transduction pathways to phospholipase C, including intracellular Ca^{2+} elevation, play an important role in carbachol-mediated activation of adenylyl cyclase in SK-N-BE(2)C cells.

4. Discussion

The results of this study show that inhibition of phospholipase C activation can completely abolish the carbachol-induced synergistic enhancement of cAMP accumulation as well as the $[Ca^{2+}]_i$ rise and $InsP_3$ generation. They also suggest that the $[Ca^{2+}]_i$ rise partially contributes to the synergistic elevation of cAMP induced by carbachol, since the blockage of the $[Ca^{2+}]_i$ rise eliminates 28% of the synergistic effect.

Several previous reports have suggested a role for phospholipase C in muscarinic receptor-mediated activation of adenylyl cyclase (Choi et al., 1992; Felder et al., 1989). There was speculation that the rise in $[Ca^{2+}]_i$ or the generation of diacylglycerol induced by muscarinic receptor-mediated phospholipase C activation may account for the activation of adenylyl cyclase through Ca^{2+}/calmodulin (Cooper and Caldwell, 1988) or protein kinase C (Yoshimasa et al., 1987). This notion is strengthened by the observation that muscarinic stimulation of adenylyl cyclase is mediated by Ca^{2+} and protein kinase C in SH-SY5Y cells (Jansson et al., 1991). However, protein kinase C is not responsible for the synergistic elevation of cAMP induced by either carbachol and prostaglandin E_1 (Baumgold and Fishman, 1988) or by carbachol and forskolin (Nakagawa-Yagi et al., 1991) in SK-N-SH cells. In the above studies, the stimulation of cells with prostaglandin E_1 and phorbol 12-myristate 13-acetate (PMA) does not elicit the synergistic effect. The down-regulation of protein kinase C by long-term pre-treatment with PMA does not affect the synergistic accumulation of cAMP in SK-N-SH cells. Moreover, intracellular Ca^{2+} chelation also does not affect the carbachol-induced synergistic enhancement of prostaglandin E_1-stimulated cAMP production (Baumgold et al., 1992). These results support the possibility that the subunits of G-proteins may be candidates of adenylyl cyclase activators (Baumgold, 1992).

Recently, we demonstrated that carbachol-induced $[Ca^{2+}]_i$ rise, $InsP_3$ generation and synergistic enhancement of cAMP accumulation exhibit similar concentration-dependent patterns in SK-N-BE(2)C cells (Suh and Kim, 1995). This allowed the hypothesis that the synergistically elevated cAMP accumulation may be related to muscarinic receptor-linked phospholipase C activation. Since, in addition, treatment of the cells with ionomycin evoked cAMP production, this indicates that Ca^{2+}/calmodulin-sensitive adenylyl cyclase is expressed. Also, co-treatment with ionomycin and prostaglandin E_2 enhanced the prostaglandin E_2-induced cAMP accumulation, while activation of protein kinase C was not involved in the carbachol-induced synergistic cAMP accumulation.

We used U-73122 and neomycin to selectively inhibit muscarinic receptor-coupled phospholipase C-mediated events. The mechanism by which U-73122 inhibits phospholipase C-mediated processes is not well defined, but recent studies suggest that the action site of U-73122 is to be found at an early stage in the signal transduction pathway, maybe at the level of the GTP-binding protein, G_q (Caulfield, 1993; Taylor et al., 1991; Smrcka et al., 1991), or at the link between G-protein and effector enzyme (Thompson et al., 1991; Yule and Williams, 1992). In the present study, we observed that U-73122 lowered the carbachol-induced synergistic enhancement of cAMP accumulation with an IC_{50} and a maximum effective concentration that was similar to the respective values in its inhibition of the $[Ca^{2+}]_i$ increase and the $InsP_3$ generation. U-73122 also inhibited the GTPγS- and AlF_4^--induced $InsP_3$ generation in permeabilized cells, while prostaglandin E_2-stimulated adenylyl cyclase activation was not affected in the intact cells. These results imply that U-73122 interferes with the interactions between G-protein and phospholipase C that and the carbachol-induced adenylyl cyclase activation is dependent upon the G-protein-mediated phospholipase C activation in SK-N-BE(2)C cells. The experiments with U-73122 were further confirmed by another blocker of phospholipase C, neomycin. Neomycin treatment decreased the carbachol-induced $[Ca^{2+}]_i$ rise, $InsP_3$ production, and cAMP accumulation in comparable patterns. In addition, Ca^{2+} chelation by BAPTA treatment further enhanced the inhibitory effects of neomycin.

We do not know which adenylyl cyclase isozymes are present in SK-N-BE(2)C cells, however, the partial inhibition of the synergistic cAMP accumulation by intracellular Ca^{2+} chelation means that there is a type of adenylyl cyclase involved in the carbachol-induced synergistic cAMP accumulation that is stimulated by Ca^{2+} in the cells. Several studies have demonstrated that type 1 and type 8 adenylyl cyclases are directly activated by elevated Ca^{2+} via Ca^{2+}/calmodulin-dependent kinase, whereas type 3 is synergistically stimulated only when it is simultaneously activated by G_s-protein or forskolin (Tang and Gilman, 1991; Choi et al., 1992; Cali et al., 1994). In our results, internal Ca^{2+} elevation slightly enhanced both the basal and the prostaglandin E_2-induced adenylyl cyclase activity, suggesting that type 1 or type 8 might be expressed and, thus, partly contribute to the synergistic activation of adenylyl cyclase. The major portion of the synergistic cAMP accumulation might be generated by βγ-subunits liberated during phospholipase C-linked G-

protein activation. If muscarinic receptors are co-localized with phospholipase C and type 4 adenylyl cyclase, $\beta\gamma$-subunits generated by muscarinic receptor stimulation would interact effectively in activating a proximal adenylyl cyclase (Inglese et al., 1995). The present results clearly demonstrate that the transduction of signal from G-protein to phospholipase C is necessary and prerequisite to muscarinic receptor-mediated synergistic activation of adenylyl cyclase. They also imply that SK-N-BE(2)C cells express at least two different isozymes of adenylyl cyclase, which are activated in a Ca^{2+}/calmodulin-dependent pathway and by $\beta\gamma$-subunits of G-protein.

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