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Suppressive effect of carbachol on forskolin-stimulated neurite outgrowth in human neuroblastoma NB-OK1 cells

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SUMMARY: We have used human neuroblastoma NB-OK1 cells to investigate the regulation of neurite outgrowth. Carbachol suppressed forskolin-stimulated neurite outgrowth in NB-OK1 cells although forskolin-stimulated cAMP levels were enhanced. The dose-response curve for this suppression was very similar to that for stimulation of inositol monophosphate (IP_1) formation and for stimulation of the initial rise of $[Ca^{2+}]i$ elicited by carbachol. Carbachol-mediated changes in neurite outgrowth, IP_1 formation and $[Ca^{2+}]i$ displayed high sensitivity for pirenzepine but low sensitivity for AF-DX116. Inhibition of intracellular calcium release with TMB-8 prevented the suppressive effect of carbachol on forskolin-stimulated neurite outgrowth. Hence we describe for the first time a relationship between neurite outgrowth and inositol triphosphate-triggered calcium release mediated by carbachol in the human neuron-drived cell line.

The intracellular events that underlie neurotransmitter effects on neurite outgrowth are not clear, but cAMP, calcium and protein kinase C (PKC) are implicated (1,2,3). Considerable evidence on the regulation

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ABBREVIATIONS: PI, phosphoinositide; IP_1, inositol monophosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; cAMP, cyclic AMP; VIP, vasoactive intestinal peptide; AF-DX116, 11-{[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-on; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; $[Ca^{2+}]i$, intracellular free calcium concentration.

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of neurite outgrowth has been obtained from studies of specific identified *Helisoma* neurons (4). However, no clear examples of such effects on neurite outgrowth in human neuronal cells have been described. By chance, we found human neuroblastoma NB-OK1 cell line which extended their neurites quickly in response to forskolin. Since NB-OK1 cell line has binding properties for muscarinic receptors (5), we investigated, in NB-OK1 cells, whether muscarinic agonist carbachol could alter neurite outgrowth. We now report that carbachol-induced suppression of forskolin-stimulated neurite outgrowth in NB-OK1 cells can be regulated by inositol triphosphate-triggered calcium release.

MATERIALS AND METHODS

Materials. Carbachol, atropine, pirenzepine, forskolin, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St.Louis, MO, U.S.A.). AF-DX116 was kindly supplied by Dr.T.Kato (Yokohama City University). Human vasoactive intestinal peptide (VIP) was from Peptide Institute Inc.(Osaka, Japan). 8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) was from Research Biochemicals Inc.(Natick, MA, U.S.A.).

Cell Culture. Human neuroblastoma NB-OK1 cells were kindly supplied by Dr.N.Yanaihara (University of Shizuoka, Japan). NB-OK1 cells were grown in Dulbecoo's modified Eagle's medium (DMEM, Gibco, NY, U.S.A.) enriched with 10 % fetal bovine serum (Gibco) and antibiotics. Cultures were maintained at 37°C in 5 % CO_2/humidified air.

Neurite Outgrowth. The percentage of cells bearing neurites was determined by counting more than 140 cells in four randomly chosen fields under phase-contrast microscopy. Cells with neuritic processes longer than two or more diameters of the cell body were scored. Values are means and S.E. on 3 replicate cultures.

Biochemical Determinations. NB-OK1 cells were incubated with [^{3}H]*myo*-inositol (12.3Ci/mmol, NEN, 4 μCi/4 ml/dish) for 20 hr 37°C in 5 % CO_2/humidified air. Labeled cells were incubated with Krebs-Ringer bicarbonate buffer (114 mM NaCl, 5.5 mM KCl, 0.75 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, and 10 mM glucose) containing 10 mM LiCl for 30 min, followed by incubation of experimental agents. Incubations were terminated after 30 min at 37°C. Inositol monophosphate (IP_1) was quantified by the method of Berridge (6), as described previously (7,8). Measurement of intracellular cAMP was as previously described (9). Protein assays were performed by the BCA method (Pierce Chemical, Rockford, U.S.A.). Intracellular free calcium [Ca^{2+}]i was determined using the fluorescent dye fura-2/AM (

Dojindo, Kumamoto, Japan) as described by Kudo et al.(10). The calcium measurements were performed using a Model FC-200 (Mitsubishi-Ksei, Tokyo, Japan) spectrofluorometer which was connected to a Olympus epifluoroscence microscope. Briefly, the cells were plated on a glass coverslip attached to a Flexiperm-Disc (Heraeus Biotech., Hanau, Germany) and precoated with poly-D-lysine (Sigma). After 3 days culture in growth medium, the cells were labeled with fura-2/AM (20 μM) for 30 min at 37°C in 0.4 ml of HEPES buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose and 20 mM HEPES-NaOH; pH 7.3). Fluorescence of calcium-bound and unbound fura-2 was determined by rapidly alternating (0.3 sec) the exciting radiation between 340 and 360 nm.

RESULTS AND DISCUSSION

After plating NB-OK1 cells, there was a slow spontaneous increase in the proportion of cells with neurites. In the presence of forskolin (10 μM) and in the presence of VIP (1 μM), the proportion of neurite-bearing cells attained 30 and 31 %, respectively. Maximal neurite outgrowth was reached 5 hr after the start of forskolin or VIP treatment (Fig.1). The half-maximum response to forskolin or VIP was obtained at about 2 hr

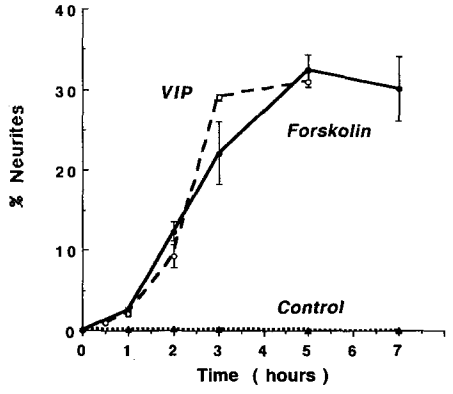

Fig.1. Time courses for the appearance of neurites in cultures of NB-OK1 cells following treatment with forskolin or VIP. Cells were incubated in DMEM containing 10 % fetal bovine serum. For determination of neurite outgrowth, the number of cells extending a neurite longer than two or more diameters of the cell body were counted and expressed as a percentage of the total number of cells counted. Each data point represents the average of four random fields from each plate of triplicate cultures. Bars, S.E.

Table 1. Effect of carbachol on forskolin-stimulated cAMP levels in NB-OK1 cells

Treatment	Cyclic AMP (pmol/mg protein)
Control	10 ± 1
Carbachol (100 μM)	22 ± 1
Forskolin (10 μM)	941 ± 37
Carbachol + Forskolin	2684 ± 242

NB-OK1 cells were pretreated with 100 μM IBMX for 10 min. Carbachol and forskolin were added and the cells were incubated for another 10 min. cAMP was assayed as described in Materials and Methods. Values are mean ± SEM on 5 replicate cultures.

It has been shown that forskolin potently stimulates adenylate cyclase in a variety of neural tissues and neuronal cell lines (11). The resulting increase in cAMP could serve as a second messenger for mechanisms involved in neurite outgrowth (12,13). To examine the possible effects of carbachol on cAMP levels in NB-OK1 cells, we tested forskolin-stimulated an increase in intracellular cAMP levels in the presence of carbachol, since we had previously (9) observed an enhancement of forskolin-stimulated cAMP levels elicited by carbachol in human neuroblastoma SH-SY5Y cells. Treatment with forskolin (10 μM) produced about 100-fold increase in cAMP levels in NB-OK1 cells. Addition of carbachol together with forskolin potentiated the cAMP response (Table 1).

In order to determine whether the changes in cAMP levels reflected a change in neurite outgrowth, we tested the effect of carbachol in forskolin-stimulated neurite outgrowth in NB-OK1 cells. Cells were exposed to carbachol, forskolin, or both compounds for 5 hr and photomicrographed (Fig.2A-F). In cultures of NB-OK1 cells, treatment with forskolin (10 μM) elicited the formation of many neurites, whereas the control cultures did not display any neurites. Carbachol suppressed forskolin-stimulated neurite outgrowth when compared to those treated with forskolin alone. Addition of carbachol (100 μM) showed no

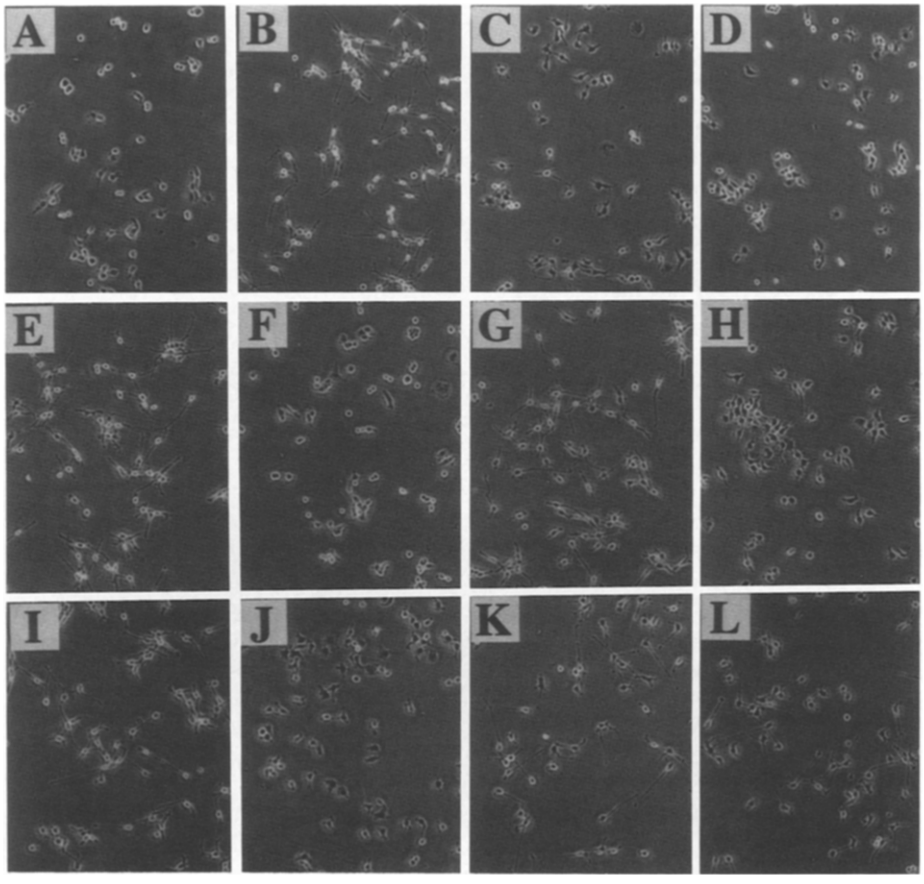

Fig.2. Phase-contrast photomicrographs of cultured NB-OK1 cells demonstrating carbachol-mediated suppression of neurite outgrowth induced by forskolin. NB-OK1 cells were cultured for 5 hr in DMEM containing 10 % fetal bovine serum and then photographed (X 100). **A**, control; **B**, forskolin (10 μM); **C**, carbachol (100 μM) + forskolin (10 μM); **D**, carbachol (100 μM); **E**, atropine (1 μM) + carbachol (100 μM) + forskolin (10 μM); **F**, atropine (1 μM); **G**, VIP (1 μM); **H**, carbachol (100 μM) + VIP (1 μM); **I**, atropine (1 μM) + carbachol (100 μM) + VIP (1 μM); **J**, PMA (100 nM); **K**, PMA (100 nM) + forskolin (10 μM); **L**, PMA (100 nM) + VIP (1 μM).

changes in the number of the neurites. The suppressive effect by 100 μM carbachol was abolished by classic muscarinic antagonist atropine (1 μM). Morphology obtained after treatment with atropine was similar to control culture. Similar results were obtained using VIP (1 μM) instead of forskolin (Fig.2G-I). These finding demonstrate that the suppression of forskolin-stimulated neurite outgrowth by carbachol is independent

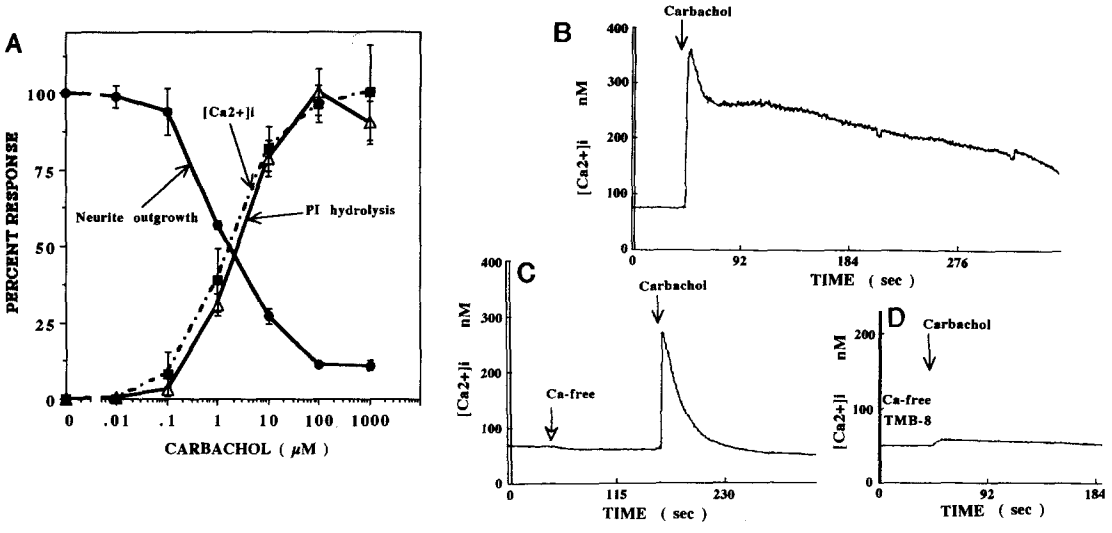

Fig.3. **A**) Dependence of forskolin (10 μM)-stimulated neurite outgrowth on carbachol concentration and relationship to PI hydrolysis or $[Ca^{2+}]i$ in NB-OK1 cells. Cells were exposed to carbachol at the indicated concentrations, and the proportion of cells with neurites was scored after 5 hr. Basal level of IP_1 formation is 1441±123 cpm/mg protein. Percentages are relative to the maximum response. The values are mean ± SEM of three cultures. **B**) Effect of carbachol on the $[Ca^{2+}]i$ in NB-OK1 cells. After stimulation with carbachol (100 μM), there was a sharp increase of $[Ca^{2+}]i$ followed by a sustained phase. **C**) Effect of carbachol on the $[Ca^{2+}]i$ in NB-OK1 cells in calcium-free medium (1mM EGTA). The addition of carbachol (100 μM) caused a rapid increase of $[Ca^{2+}]i$, but compared to the reaction in a calcium-containing medium the values reached baseline levels after 70 sec. **D**) Effect of TMB-8 on carbachol-mediated changes in $[Ca^{2+}]i$ in calcium-free medium. TMB-8 (100 μM) was added 30 min prior to carbachol (100 μM).

of the mechanism by which carbachol can enhance the intracellular levels of cAMP following direct stimulation of adenylate cyclase by forskolin.

Since carbachol that stimulates inositol triphosphate-triggered calcium system, we examined the effect of carbachol on PI hydrolysis and $[Ca^{2+}]i$. The relationship between neurite outgrowth and PI hydrolysis or $[Ca^{2+}]i$ is shown in Fig.3. The stimulation of IP_1 formation elicited by carbachol was dose-dependent. The maximal increase in IP_1 formation was about 20-fold with an EC_{50} of 2.4 μM. Whereas Carbachol (100 μM) added to the cells caused an increase in

$[Ca^{2+}]i$ in a dose-dependent manner with an EC_{50} of 1.5 μM. This increase slowly declined to a new steady state level above the original basal concentration (Fig.3B). Carbachol appied in the virtual absence of extracellular calcium was not significantly different between the calcium-free (264 ± 21 nM) and the calcium-containing medium (293 ± 20 nM). In contrast, the sustained phase was totally abolished (Fig.3C). In the absence of extracellular calcium, the addition of 100 μM TMB-8 (an inhibitor of calcium release from internal stores) prior to stimulation by 100 μM carbachol had inhibitory effect on carbachol response (Fig.3D). On the other hand, the suppressive effect in forskolin-stimulated neurite outgrowth was dose-dependent for carbachol concentrations from 0.1 μM to 1 mM. The EC_{50} for suppression was 1.6 μM.

The nature of the receptors responsible for suppressive neurite outgrowth was next investigated by using non-selective muscarinic antagonist atropine (ATR), M_1-selective antagonist pirenzepine (PZ), and M_2-selective antagonist AF-DX116. The suppression of neurite outgrowth caused by 100 μM carbachol addition was dose-dependently inhibited by the three antagonists (Fig.4A). The IC_{50} values were at 0.031, 0.27 and 10 μM for atropine, pirenzepine and AF-DX116, respectively. Atropine was 9-fold more potent than pirenzepine in the neurite outgrowth, whereas AF-DX116 was 37-fold less potent. The stimulation of IP_1 formation elicited by carbachol (100 μM) was also dose-dependently inhibited by the three antagonists (Fig.4B). The IC_{50} values were at 0.0065, 0.13 and 3.6 μM for atropine, pirenzepine and AF-DX116, respectively. Atropine was 20-fold more potent than pirenzepine in IP_1 formation, whereas AF-DX116 was 28-fold less potent. The initial rise of $[Ca^{2+}]i$ caused by 100 μM carbachol addition was dose-dependently inhibited by the three antagonist (Fig.4C). The IC_{50} values were at 0.014. 0.125 and 12 μM for atropine, pirenzepine and AF-DX116, respectively. Atropine was 9-fold more potent than

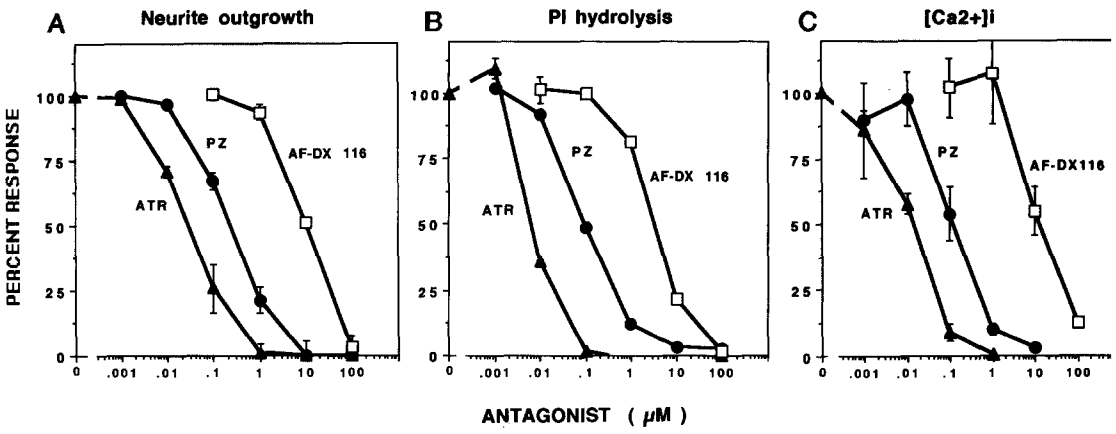

Fig.4. Muscarinic antagonist potency for carbachol-mediated changes in neurite outgrowth (**A**) induced by forskolin (10 μM), IP_1 formation (**B**) and $[Ca^{2+}]i$ (**C**). Carbachol (100 μM) was added with the indicated concentrations of antagonist. Values are mean ± SEM of three cultures. Percentages are relative to the maximal response.

pirenzepine in the initial rise of $[Ca^{2+}]i$, whereas AF-DX116 was 96-fold less potent. Thus, the competition curves for suppressed neurite outgrowth and increased IP_1 formation or increased $[Ca^{2+}]i$ are very similar. In addition, this differences in affinity for selective antagonists are characteristic of the activation of M_1 receptors.

The stimulation of PI hydrolysis causes the release of inositol 1,4,5-triphosphate (IP_3) which induces the release of intracellular calcium. To examine the possible effects of carbachol on neurite outgrowth in NB-OK1 cells, we tested the effect of TMB-8 on carbachol's ability to suppress forskolin-stimulated neurite outgrowth. TMB-8 blocked the suppressive effect of carbachol. The half-maximal inhibitory concentration for TMB-8 was 13 μM (Fig.5).

Since carbachol that stimulates the inositol phospholipid-PKC cascade mimic this response to phorbol ester (14), we examined the effect of PMA in mimicking the suppressive response on neurite outgrowth in NB-OK1 cells (Fig.2J-L). Addition of PMA (100 nM) showed no changes in the number of the neurites. PMA was without effect on forskolin- or VIP-stimulated neurite outgrowth. Long term

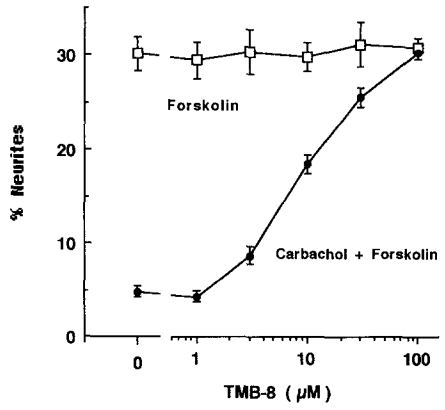

Fig.5. Effect of TMB-8 on carbachol's ability to suppress forskolin-stimulated neurite outgrowth in NB-OK1 cells. TMB-8 was added 15 min prior to carbachol (100 μM) and forskolin (10 μM). The proportion of cells with neurites were scored after 5 hr. Values are mean ± SEM of three cultures.

treatment with phorbol ester is known to desensitize PKC activity (15). Preincubation of NB-OK1 cells with 1 μM of PMA for 20 hr at 37°C did not interfere with the carbachol-induced suppression in forskolin-stimulated neurite outgrowth (data not shown).

The present results indicate clearly that carbachol suppresses forskolin-stimulated neurite outgrowth in NB-OK1 cells although the intracellular cAMP levels are more than doubled. Calcium has been suggested as having a growth inhibitory effect in neurite outgrowth of variety of cells (3). However, much of the evidence for this was based on manipulations expected to increase Ca^{2+} influx. We report here additional evidence in favor of the suppressive effect of calcium. In NB-OK1 cells, carbachol induces PI hydrolysis, generating diacylglycerol, which activates PKC. However, our results show that acute treatment with PMA does not mimic the suppressive effect of carbachol on neurite outgrowth. In addition, carbachol suppression of neurite outgrowth is unaffected by down regulation by prolonged treatment with PMA. Hence we conclude that PKC is not involved in the carbachol-induced suppression. On the other hand, carbachol also causes inositol triphosphate-triggered calcium release from intracellular organelles.

Inhibition of intracellular calcium release with TMB-8 blockes the suppressive effect of carbachol. Moreover, the competition curves for suppressed neurite outgrowth and increased IP_1 formation or increased $[Ca^{2+}]i$ are very similar. Hence we conclude that the mechanism for carbachol-induced suppression of forskolin-stimulated neurite outgrowth may act through mobilization of intracellular calcium in NB-OK1 cells. It is possible that an increase of intracellular calcium is involved in the assembly states of microtubules, which regulate neurite outgrowth (16). In future study it will be of interest to examine which Ca^{2+}-dependent processes of their responsiveness might be affected.

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