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PHORBOL ESTERS ALTER MUSCARINIC RECEPTOR BINDING AND INHIBIT POLYPHOSPHOINOSITIDE BREAKDOWN IN HUMAN NEUROBLASTOMA (SH-SY5Y) CELLS

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Many recent reports have indicated that the effect of the phorbol ester tumor promoters is mediated through the Ca^{2+}/phospholipid dependent protein kinase C. We have investigated the effect of two biologically active phorbol esters, 4 β-phorbol 12 β-myristate 13 α-acetate (PMA) and 4 β-phorbol 12 β,13 α-didecanoate (βPDD) on muscarinic agonist binding and receptor-stimulated phosphoinositide breakdown in cultured human neuroblastoma (SH-SY5Y) cells. Preincubation of these cells with phorbol esters significantly reduced the carbachol-stimulated breakdown of inositol phospholipids and caused a decrease of agonist affinity for [^{3}H](-)methyl quinuclidinyl benzilate ([^{3}H](-)MQNB) binding without affecting the affinity of antagonist to the muscarinic receptor. The nontumor promoting 4 α-phorbol 12^β,12 α-didecanoate (αPDD) was ineffective in our studies. These results suggest that the activation of protein kinase C may play an important role in regulating the muscarinic receptor system.

Activation of receptors by a variety of agonists, including muscarinic agonists (1-3), is associated with enhanced hydrolysis of inositol phospholipids. One of these phospholipids, phosphatidylinositol 4,5 biphosphate (PIP_2), which represents less than a few percent of the total inositol phospholipids (4), is hydrolysed to diacylglycerol (DAG) and inositol triphosphate (IP_3). Recent reports (5,6) have indicated that DAG stimulates the Ca-dependent phospholipid-dependent protein kinase C while IP_3 causes the release of Ca^{2+} from intracellular stores. Thus, the receptor-mediated turnover of inositol phospholipids represents a fundamental transducing mechanism for controlling a variety of cellular processes, such as secretion,

Abbreviations used: IC_{50}, concentration necessary to inhibit 50% of specific binding; $IC_{50(H)}$, value of high affinity site; $IC_{50(L)}$ value of low affinity site.

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metabolism, phototransduction and cell proliferation. Tumor-promoting phorbol esters appear to bind with high affinity to binding sites associated with protein kinase C in primary neuronal cultures (7) and their autoradiographic localization have been described in detail in rat brain (8). These agents seem to exert their actions by mimicing the stimulatory effect of DAG normally produced as a part of receptor activation (6,9). Protein kinase C phosphorylates cellular proteins when activated by DAG or phorbol esters (10). We have used cultured human neuroblastoma SH-SY5Y cells to study the effects of phorbol esters on phosphoinositide metabolism as well as agonist or antagonist binding to muscarinic receptors.

Materials and Methods

Cell Culture System

Human neuroblastoma SH-SY5Y cells, passages 60-90, were grown in tissue culture flasks (Falcon, 75 cm^2) in 20 ml growth medium consisted of 90% RMPI 1640 and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) supplemented with 100 U ml of penicillin and 100 g/ml of streptomycin (Eli Lilly, Indianapolis IN). Intact cells (60-80% confluency) were transferred into 2 cm^2 diameter wells (250,000 cells/well) and used for [^{3}H](-)methyl quinuclidinyl benzilate ([^{3}H](-)MQNB) binding and phosphatidylinositol turnover studies.

Radioligand Binding Assay

[^{3}H](-)MQNB binding to intact SH-SY5Y cells was performed as previously described for [^{3}H](-)QNB with minor modifications (11). Briefly, after removal of the culture medium, the cells were preincubated for 60 min at 37°C with the phorbol ester (Sigma, St. Louis, MO) and then incubated with approximately 500 pM [^{3}H](-)MQNB (70 Ci/mmol, NEN Boston, MA) at 37°C in 1 ml Iscove's modified Dulbecco's medium (IMDM, Irvine Scientific, Santa Ana, CA) in the absence (total binding) or presence (nonspecific binding) of 1 uM atropine sulfate and various concentrations of a muscarinic agonist or antagonist. The incubation was terminated by aspirating the medium and the tissue culture trays were placed on ice. The cells were rinsed for 10 min with 1 ml of ice-cold rinse buffer (NaCl 77.1 mM; KCl 4.4 nM; $MgSO_4$ 0.8 mM; $CaCl_2$ 1.5 mM; NaH_2PO_4 0.9 mM; D-Glucose 25 mM; Hepes 25 mM pH 7.4 at 4° C). The cells were then digested with 0.25 ml of 1% Triton X-100 and transferred to scintillation vials. Each well was then rinsed again with 0.25 ml Triton X-100. Scintillation fluid was added and the samples were counted at least 4 hr later by a liquid scintillation spectrophotometer (44% efficiency). Inhibition data were analyzed using nonlinear least square regression analysis from a program prepared for the Apple II+ microcomputer (S.H.M. Research, Tucson, AZ).

[^{3}H]Inositol Assay

The accumulation of [^{3}H]inositol phosphates was measured according to the method of Berridge et al. (12). Briefly, after removal of the culture medium the intact SH-SY5Y cells were prelabeled with myo-[2,^{3}H]inositol

(10.8 Ci/mmol, NEN Boston MA; final concentration=0.2 uM) in 0.5 ml IMDM medium and allowed to equilibrate with 90% air/10% CO_2 at 37° for 20-22 hr. The cells were washed once with IMDM medium and preincubated with phorbol ester solutions or medium (control). Lithium (10 mM) was added 10 min before the addition of carbachol (100 uM) which initiated the reaction; the incubation was carried out for 60 min at 37°C and terminated by the addition of 0.94 ml $CHCl_3$:CH_3OH (1:2). An additional 0.31 ml chloroform and 0.31 ml water was added to separate the phases. [^{3}H]inositol phosphates were separated by ion-exchange chromatography using the technique of Berridge et al. (12) with slight modification as previously described (11).

Phorbol esters were dissolved in ethanol. The final concentration of ethanol never exceeded 0.01%. Significant differences were established using t-test grouped or paired data (13). Partial F-test was performed to compare two-state with one-state model (14).

Results

We initially examined the effect of pretreatment with the tumor-promoting agent PMA on the intact SH-SY5Y cells, by examining the inhibition by the

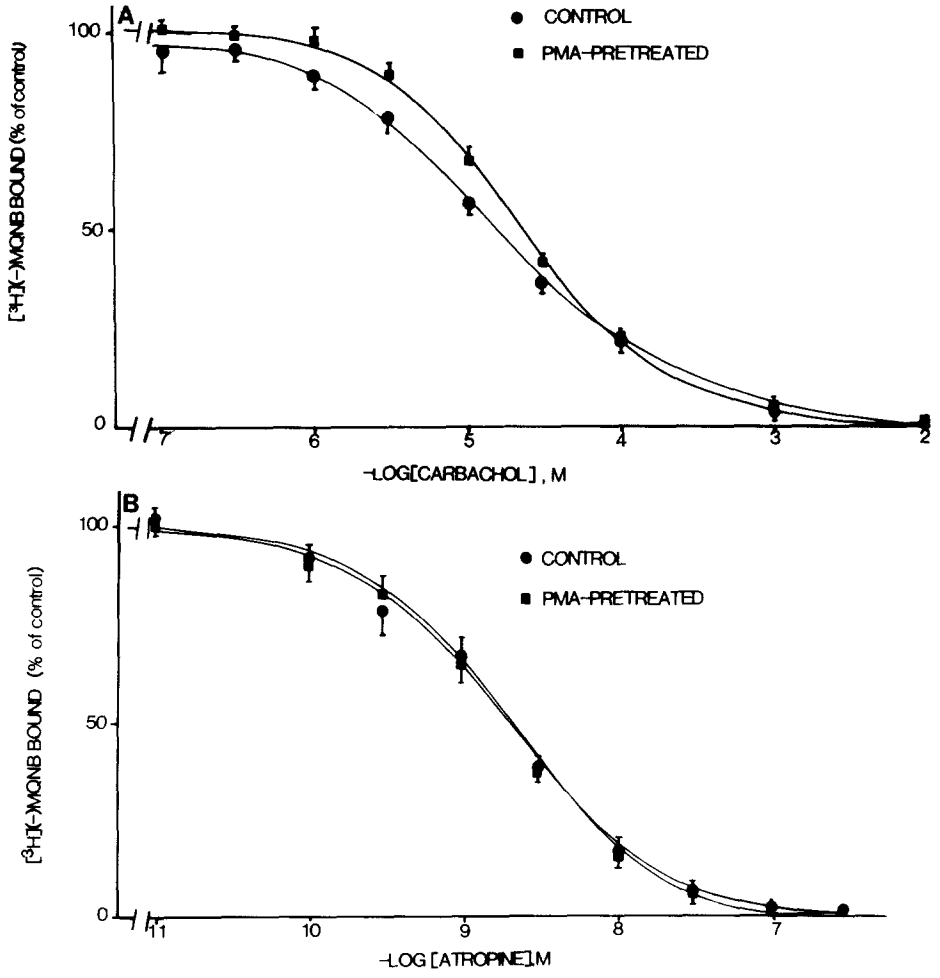

Table 1. Inhibition of [^{3}H](-)MQNB binding to control and PMA-pretreated SH-SY5Y cells by muscarinic drugs

	CONTROL		PMA-PRETREATED	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
ATROPINE	2.40 (2.0-2.9)	0.90±0.13	1.83 (1.8-1.8)	0.92±0.07
CARBACHOL				
one site fit	15,500 (12,900-18,600)	0.77±0.06	20,400 (17,000-24,500)	1.05±0.06
two site-fit*	8,467 77% (H) (6,240-11,500) 235,500 23% (L) (188,200-294,600)		-	-

Following 60 min pretreatment with or without 10 uM PMA the cells were incubated with various concentrations of unlabeled drugs in the presence of 500 pM [^{3}H](-)MQNB as described under "Materials and Methods". The IC_{50} values are the geometric mean with the range of values in parenthesis from 2-4 separate experiments done in duplicates. The Hill coefficients (n_H) are the arithmetic mean ± S.D. H and L denote high and low affinity states, respectively.

*Significantly improved fit to a two-site model ($p < 0.01$)

agonist carbachol and the antagonist atropine sulfate on [^{3}H](-)MQNB binding. Fig. 1 shows that pretreatment of the intact SH-SY5Y cells with 10 uM of PMA produced a decrease in the affinity of carbachol for the muscarinic receptor. This decrease in agonist affinity is expressed as a shift to the right of the inhibition curve, indicating an interconversion of the high affinity state of the receptor to a form which recognized the agonist with low affinity. The computer-assisted analysis of the inhibition curves (Table 1) gave a Hill coefficient for the control curve of less than one, indicating the presence of both high and low affinity agonist states ($IC_{50(H)}$ = 8.5 uM, 76.6%; $IC_{50(L)}$ = 236 uM, 23%), while in the PMA-pretreated system the inhibition curve had a Hill coefficient of one. Atropine sulfate competition curves were also performed in control studies and cells pretreated with PMA; as shown in Fig. 1 and Table 1, the IC_{50} for atropine was unchanged by PMA

Figure 1. Inhibition of [^{3}H](-)MQNB binding by carbachol (A) and atropine (B) in control and PMA-pretreated (10uM, 60 min) SH-SY5Y cells. The preincubation medium was removed and the cells were incubated with various concentrations of unlabeled drugs and 500 pM of [^{3}H](-)MQNB as described under "Materials and Methods". The data points represent the average values (± S.E.M.) obtained in four (A) and two (B) separate experiments carried out in duplicate.

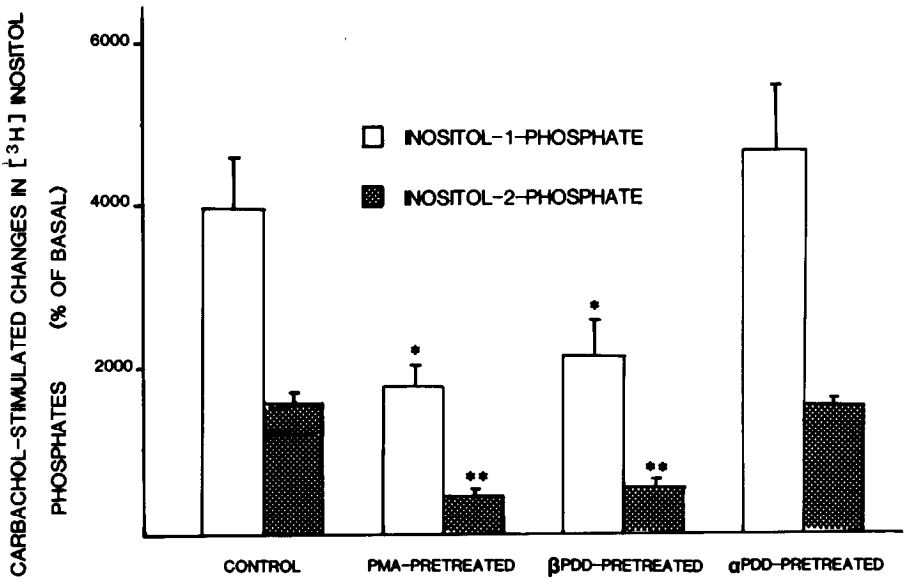

Figure 2. Effect of phorbol esters on carbachol-stimulated changes in [^{3}H]inositol phosphates. SH-SY5Y cells labeled with [^{3}H]inositol (0.2 uM) were washed, treated for 60 min with or without 10 uM phorbol esters and incubated for 1 h with 100 uM carbachol in the presence of 10 mM LiCl. Results shown are the mean $\pm$ S.E.M. of four separate experiments done in triplicates. Different from control by t-test grouped or paired data: ** $p < 0.01$, * $p < 0.05$.

pretreatment. Fig. 2 shows the effect of PMA, β PDD and its stereoisomer α PDD pretreatment on the carbachol-stimulated inositol phospholipid hydrolysis. The basal accumulation of inositol phosphates production was only slightly affected by the three phorbol esters (95-85% of control). These studies were done in the presence of 10 mM LiCl which inhibits the breakdown of inositol monophosphate to inositol (12). Carbachol (100 uM) caused a significant increase in inositol-1-phosphate (IP) and inositol-2-phosphate (IP_2) (40- and 16-fold increases, respectively). Under our conditions the level of inositol-3-phosphate (IP_3) was extremely low, therefore it was not routinely determined. Atropine sulfate (1 uM, final concentration) completely antagonized the stimulation of the PI turnover induced by carbachol (data not shown). PMA (10 uM) was more effective that β PDD (10 uM) in inhibiting the carbachol-induced response (54 and 46% decreases, respectively), while α PDD (10 uM) was completely ineffective.

Discussion

We have obtained data which show that activation of protein kinase C by phorbol esters produces a perturbation of the muscarinic receptor system. The mechanisms responsible for the perturbation are unclear at the present. Muscarinic agonists bind to receptors and stimulate the hydrolysis of PIP_2 which is catalyzed by a specific phosphodiesterase (phospholipase C). It has been proposed that a guanosine triphosphate (GTP) binding protein represents the link between the receptor and the enzyme with a mechanism resembling the role a GTP-binding protein in controlling cyclic AMP production (5). Evidence to support this hypothesis shows that GTP and its analogues activate PIP_2 phosphodiesterase in human neutrophil plasma membrane (15). These agents also stimulate the production of inositol-3-phosphate in rat cortical membranes (16) and pretreatment of rat mast cells with pertussis toxin produces an inhibition of inositol phospholipid breakdown (17). Pertussis toxin catalyses the ADP-ribosylation of the α-subunit of N_i involved in receptor-mediated inhibition of adenylate cyclase, thereby inactivating it. Furthermore, it has been recently shown that activated protein kinase C interferes with N_i-mediated adenylate cyclase inhibition in membranes of human platelets (18). It is feasible that the α-subunit of N_i, or an analogue protein material, represents the coupling between the receptor and the phosphodiesterase; thus the activation of the protein kinase C by phorbol esters could lead to the phosphorylation of the GTP-binding protein.

It has been shown that the tumor-promoting phorbol esters stimulate the phosphorylation of insulin, somatomedin C (19) and α_1-adrenergic receptors (20) and the internalization of the receptor protein has been hypothesized (20). Very recently (21) a significant decrease of the number of the muscarinic receptors after a 6 hour exposure to PMA was demonstrated in murine neuroblastoma cells. We performed [^{3}H](-)MQNB binding studies in PMA-pretreated and control SH-SY5Y cells and found no change in the maximum number of receptors or in its dissociation constant (data not shown). Our studies demonstrated that short-term (60 min) pretreatment with phorbol ester

does not lead to internalization of the muscarinic receptors. Conformational changes in the receptor protein may occur after short-term exposure to phorbol esters.

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