# The protein kinase C activator phorbol-12-myristate-13-acetate enhances cyclic AMP accumulation in pheochromocytoma cells

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The protein kinase C activator, phorbol-12-myristate-13-acetate (PMA), augments the cyclic AMP accumulation induced by forskolin in pheochromocytoma (PC 12) cells with an $EC_{50}$ value of 14 nM, while having no effect on basal values. At a concentration of 100 nM PMA markedly augmented the magnitude of the forskolin response and, in addition, caused a slight increase in the potency of forskolin. PMA also enhanced the maximal cyclic AMP accumulation produced by 2-chloroadenosine, and caused a slight increase in potency of the adenosine analog. Since PMA mimics the effect of diacylglycerols that form during the turnover of the membrane lipid, phosphatidylinositol, the results suggest an interrelationship between the systems involved in phosphatidylinositol turnover and cyclic AMP generation in PC 12 cells.

*cyclic AMP* *Phosphatidylinositol* *Phorbol ester* *Nerve growth factor* *Forskolin* *Adenosine*

## 1. INTRODUCTION

Phorbol esters induce a wide variety of functional and biochemical changes in many different tissues and cells [1,2]. Recently, it has been shown that phorbol-12-myristate-13-acetate (PMA) augments cyclic AMP (cAMP) accumulation in guinea pig cerebral cortical slices [3], lymphoma cells [4], pinealocytes [5] and smooth muscle [6].

Phorbol esters are activators of protein kinase C and thereby can mimic the action of diacylglycerols, which are a product of receptor-mediated phosphatidylinositol turnover [7]. Agents that initiate phosphatidylinositol metabolism do augment the accumulation of cAMP in brain tissue [3,8] and pinealocytes [5]. An augmentation of cAMP production linked to phosphatidylinositol turnover, thus, may represent a general functional correlate of the enhancement of cAMP accumulation by PMA, but this has been demonstrated as yet only in brain slices and pinealocytes.

This study extends the specific interaction of a protein kinase C activator with cAMP accumulation to pheochromocytoma (PC 12) cells. The presence of PMA results in an enhanced responsiveness of the cAMP generating systems to 2-chloroadenosine and to forskolin in such cells.

## 2. MATERIALS AND METHODS

### 2.1. *cAMP assay*

PC 12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were obtained from Dr G. Guroff (National Institutes of Health, Bethesda, MD). The cells were grown in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 6% fetal calf serum, 6% horse serum and a penicillin-streptomycin mixture. The cells were kept at 37°C in an atmosphere enriched in $CO_2$. The cells were washed twice with Hepes buffer which contains 25 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$,

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1.8 mM $CaCl_2$, 10 mM glucose, pH 7.4. After the last washing, cells were resuspended in Hepes buffer supplemented with 0.1% albumin and 0.5 ml portions of the cell suspension (about 2–3 × $10^6$ cells) were rapidly distributed into plastic vials prewarmed to 37°C. The cells were incubated at 37°C with shaking at 120 cycles/min in a total volume of 1 ml Hepes buffer, pH 7.4, containing 1 μg/ml adenosine deaminase. After 5 min preincubation the phosphodiesterase inhibitor rolipram (ZK 62,711, 30 μM) was added. Other agents were added 5 min later. The incubation was continued for 10 min. All assays were done in triplicate. The incubation was terminated by transferring 800 μl of the cell suspension into Eppendorf test tubes preheated at 95°C. After 3 min incubation at 95°C the suspension was centrifuged for 2 min at 12000 × *g*. The cAMP concentration in the supernatant was determined as described [9]. Materials and chemicals were from standard sources as described [3,8].

## 3. RESULTS

The phorbol ester PMA had no effect on basal cAMP accumulation in PC 12 cells at concentrations up to 300 nM (fig.1). Forskolin alone at a concentration of 3 μM increased cAMP accumulation about 8-fold over basal values and PMA augmented this forskolin-stimulated accumulation of cAMP in a concentration-dependent manner. At the maximally effective concentration of 100 nM PMA the cyclic AMP levels were up to 3-fold higher than in the absence of PMA. The $EC_{50}$ of PMA for this effect was 14 nM. Similar results were obtained after pretreatment of PC 12 cells with PMA for 5 min or 30 min before addition of forskolin (not shown). 4-*O*-Methyl-PMA (10 μM), a phorbol ester that has no effect on protein kinase C [10], did not affect forskolin-elicited accumulation of cyclic AMP (not shown).

Forskolin induced about a 20-fold increase in accumulation of cAMP in PC 12 cells with an $EC_{50}$ of 2.1 μM (fig.2). 100 nM PMA induced only a slight shift of the concentration-response curve of forskolin to the left, but markedly increased the magnitude of the maximal stimulation. In the presence of PMA, forskolin stimulated accumulation of cAMP about 30-fold with an $EC_{50}$ of 1.7 μM.

The phorbol ester also augmented receptor-mediated stimulation of cAMP accumulation in PC 12 cells. The adenosine analog 2-chloroadenosine increases cAMP levels in PC 12 cells via

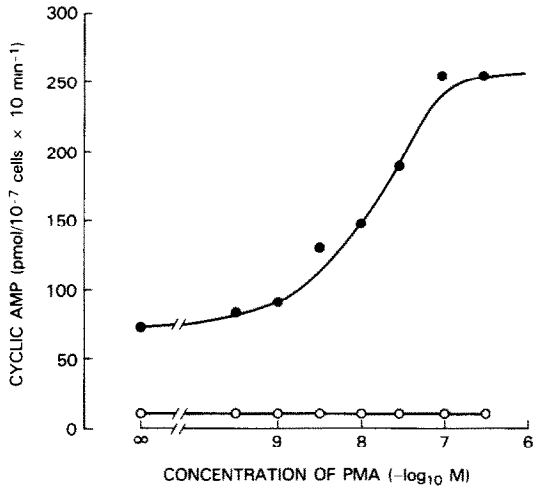


Fig.1. Effect of PMA on cAMP accumulation in PC 12 cells in the absence (○—○) and presence (●—●) of 3 μM forskolin. Values are means of a typical experiment done in triplicate.

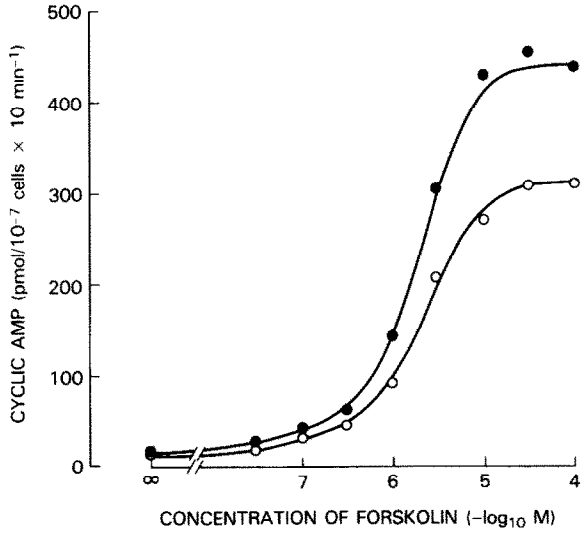


Fig.2. Effect of forskolin on cAMP accumulation in PC 12 cells in the absence (○—○) and presence (●—●) of 100 nM PMA. Values are means of a typical experiment done in triplicate.

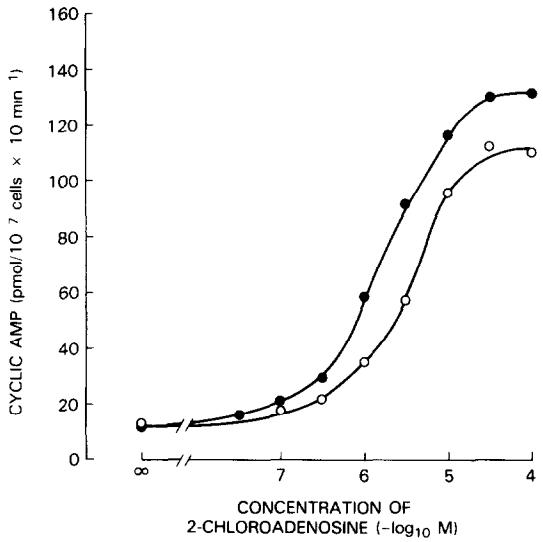


Fig.3. Stimulation of cAMP formation in PC 12 cells by 2-chloroadenosine in the absence (○—○) and presence (●—●) of 100 nM PMA. Values are means of a typical experiment done in triplicate.

$A_2$ adenosine receptors [11,12]. In the presence of 100 nM PMA, the $EC_{50}$ of 2-chloroadenosine decreased from 3.7 to 1.7 $\mu$M, whereas the degree of stimulation of cAMP formation increased from about 8.5-fold to about 11-fold (fig.3).

A variety of agents, including 6-fluoronorepinephrine (30 $\mu$M), histamine (100 $\mu$M), serotonin (30 $\mu$M), vasoactive intestinal peptide (1 $\mu$M) and nerve growth factor (50 ng/ml) did not augment the accumulation of cAMP elicited by 10 $\mu$M 2-chloroadenosine or 3 $\mu$M forskolin during 10 min incubations with PC 12 cells (not shown).

## 4. DISCUSSION

Phorbol esters, which activate protein kinase C, alone have no effect on cAMP accumulation in certain cells or tissues, but do augment receptor-mediated accumulation of cAMP [3–6]. In brain tissue and pinealocytes agents that initiate phosphatidylinositol turnover also augment receptor-mediated accumulation of cAMP [5,8] presumably through activation of protein kinase C by diacylglycerols [3]. The interrelationship of protein kinase C activation and cAMP generating systems may prove to be a general phenomenon, but as yet has been documented only in brain slices [3], lymphoma cells [4], pinealocytes [5] and smooth muscle [6]. The generality of the phenomenon has now been investigated in PC 12 cells.

Enhancement of the cAMP accumulation elicited by various agents is not the only effect reported for the phorbol esters concerning adenylate cyclase systems. In hepatocytes or $GH_3$ pituitary tumor cells, PMA decreases receptor-mediated cAMP accumulation [14,15]. In epidermal cells, PMA alone enhances cAMP accumulation [16]. Phorbol esters in certain systems have been reported to mediate a desensitization of stimulation of adenylate cyclase by $\beta$-adrenergic agonists [17,18].

In this study, PC 12 cells have been chosen because they have many neuronal characteristics, such as production and secretion of the neurotransmitters dopamine and norepinephrine, electrical excitability and formation of dendrites upon the addition of nerve growth factor [13]. PMA does augment cAMP in brain tissue, but the cell types involved have not been defined. In the homogeneous PC 12 system PMA increases forskolin- and 2-chloroadenosine-elicited cAMP accumulation (fig.1–3). The $EC_{50}$ of PMA for this effect is similar to the $EC_{50}$, of about 5 nM, for enhancement of $\beta$-adrenergic receptor-mediated increases in cAMP levels in pinealocytes [5] and to the $EC_{50}$, of about 4 nM, for the enhancement of $\beta$-adrenergic receptor-mediated increases of cAMP levels in S49 lymphoma cells [4] by 12-*O*-tetradecanoylphorbol-13-acetate. In contrast, the $EC_{50}$ of PMA for augmenting the 2-chloroadenosine response in cerebral cortex synaptoneurosomes is about 5 $\mu$M [3].

Initiation of phosphatidylinositol turnover in PC 12 cells presumably would also lead to increased responses of cAMP-generating systems to forskolin and 2-chloroadenosine, as seen in brain tissue [3,8]. Muscarinic stimulation has been reported to induce phosphatidylinositol turnover in PC 12 cells [19], but a muscarinic receptor-mediated inhibition of adenylate cyclase proved to be the only effect observed with combinations of forskolin plus carbamylcholine (not shown). Other agents including 6-fluoronorepinephrine, histamine, serotonin, vasoactive intestinal peptide and nerve growth factor did not affect 2-chloroadenosine or forskolin-induced accumulations of

cAMP in PC 12 cells (see section 3). The effects of these agents on phosphatidylinositol metabolism were not ascertained here. However, nerve growth factor has been reported to increase incorporation of radioactive phosphate or *myo*-inositol into phosphatidylinositols in PC 12 cells [20,21] and sympathetic ganglia [22]. Recently, nerve growth factor also was reported to enhance both cholera-toxin- and $N^6$-phenylisopropyladenosine-elicited accumulation of [$^3$H]cAMP in [$^3$H]adenine-labeled PC 12 cells [23]. Further studies will be required to establish whether or not there are correlations between receptor-mediated phosphatidylinositol breakdown and responsiveness of cAMP systems in PC 12 cells.

The site(s) of protein kinase C-catalyzed phosphorylation that results in enhanced responsiveness of cAMP-generating systems is unknown, but does, based on results with phorbol esters, appear relatively general in occurrence. It may represent a functional pathway whereby hormonal inputs to phosphatidylinositol and cAMP systems can modulate each other. Recently phorbol esters were shown to increase adenylate cyclase activity in frog erythrocyte lysates [24], representing the first instance of this phenomenon in a cell-free system.

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