differential tissue distribution of Aroclor 1254 but such data were not obtained in our study.

Another question left unanswered is whether PCBs present in Aroclor 1254 or contaminants such as chlorinated dibenzofurans [18] were responsible for the observed alterations in porphyrin metabolism. However, we failed to detect dibenzofurans in our Aroclor preparation by GC-mass spectrometric analysis. The effect of purified dibenzofurans at levels found in Aroclor 1254 [18] on enzymes of the heme pathway requires further investigation.

In summary, single oral administration of Aroclor 1254 produced porphyria in male Japanese quail characterized by excessive excretion of ALA and coproporphyrin I in feces, increased activity of ALA synthetase in liver and kidney, and a dramatic accumulation of uroporphyrins and 7-carboxyporphyrins in kidney. ALA-S activity was increased more in liver than in kidney, whereas URO-I-S activity was increased more in kidney than in liver. The activity of URO-D was decreased in kidney but not in liver. Further work is required to explain these tissue-dependent changes in porphyrin metabolism.

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Effects of phorbol esters and pertussis toxin on agonist-stimulated cyclic AMP production in rat osteosarcoma cells

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Tumor-promoting phorbol esters are thought to act through activation of protein kinase C and subsequent phosphorylation of specific substrates [1]. Treatment of intact cells with phorbol esters can alter cAMP production, but the effects are complex. Both decreased [2–5] and increased [6–10] cAMP production after phorbol ester treatment have been reported. The basis for the opposite effects of phorbol esters on cAMP production and the site(s) of action have not been defined.

We studied the effects of phorbol esters on cAMP accumulation in a well-characterized rat osteosarcoma cell line [11]. Parathyroid hormone (PTH*) and beta-adrenergic agonists stimulate cAMP production in these cells through distinct receptors interacting with the stimulatory guanine nucleotide regulatory protein, G_s [12]. These cells also possess a functional inhibitory regulatory protein, G_i [12]. We now report that phorbol esters can enhance agonist-stimulated cAMP accumulation in rat osteosarcoma cells, but that the effect is very rapid and transient. The effects of phorbol esters, moreover, are additive with those of pertussis toxin which inactivates G_i [13].

* Abbreviations: PTH, parathyroid hormone; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; 4-alpha-; PDD, 4-alpha-phorbol 12,13-didecanoate; ROS, rat osteosarcoma; G_s and G_i, the guanine nucleotide regulatory proteins associated with stimulation and inhibition, respectively, of adenylate cyclase.

Materials and methods

The 17/2.8 subclone of rat osteosarcoma (ROS) cells was originally obtained from Dr. Gideon Rodan. Phorbol esters and l-isoproterenol hydrochloride were from Sigma. Pertussis toxin was the gift of Dr. Ron Sekura. PTH (human 1-34 fragment) was from Bachem. The sources of other materials and the methods for culture of ROS cells and for cAMP measurement were as previously described [12, 14].

Incubations with phorbol esters, agonists, and pertussis toxin were performed with cells grown to confluence in 24 well plates. Agonist and phorbol ester incubations were done at room temperature for the times indicated in the figure legends. The final volume was 0.175 ml/well. Results are expressed as cAMP/mg of protein (determined on separate wells from each plate), and are the mean of triplicate wells from at least two separate experiments.

Results and discussion

Treatment of ROS cells with TPA (100 nM), in the presence of the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (0.5 mM), increased cAMP accumulation from 15 to 30 pmoles/mg protein. The peak response was reached by 10 min; longer TPA treatment or higher TPA concentrations (up to 500 nM) gave no further increase in cAMP. The non-tumor promoting phorbol ester, 4-alpha-phorbol 12,13-didecanoate (PDD), failed to increase cAMP production.

Brief exposure of cells (5 min or less) to TPA substantially enhanced the subsequent cAMP response to either PTH (Fig. 1A) or to the beta-adrenergic agonist isoproterenol (Fig. 1B). Again, 100 nM TPA was maximally effective. The effect of TPA was maximal at about 5 min; at longer incubation times, agonist stimulation of cAMP declined to values equal to those in cells unexposed to TPA, and after prolonged incubation (>30 min) agonist response was even lower than that of control cells (not shown). Incubation with 4-alpha-PDD caused no change in agonist stimulation of cAMP at any time point (Fig. 1).

Augmentation of agonist-stimulated cAMP response due to TPA was rapidly reversible. By 5 min after removal of TPA, enhancement of cAMP response was reduced by about 80%, and by 30 min after TPA removal cAMP response was equivalent to control (no TPA treatment).

Pertussis toxin has been shown to enhance agonist-stimulated production of cAMP production in ROS cells [12] (see also Fig. 2, 0 time point). The expected effect of brief exposure to TPA was observed in cells cultured without pertussis toxin (Fig. 2, lower curves), but note that treatment with TPA led to additive enhancement of agonist-stimulated cAMP accumulation in cells cultured with pertussis toxin (Fig. 2, upper curves).

Phorbol esters can affect cAMP production in multiple ways. Inhibition of agonist-stimulated cAMP accumulation by phorbols occurs in avian erythrocytes [2] and in rat C6 glioma cells [3] in association with phosphorylation [2] or sequestration [3] of the beta-adrenergic receptor. Phorbol-induced inhibition of cAMP production in Leydig cells [4] and hepatocytes [5], however, is not associated with altered receptor function. In murine S49 lymphoma cells [6], anterior pituitary cells [7], pinealocytes [8], cerebral cortex [9], and smooth muscle cells [10], phorbol esters increase agonist-stimulated cAMP production.

We found that the tumor promoting phorbol ester, TPA, had little effect on basal cAMP production in ROS cells, but that it substantially increased cAMP accumulation stimulated by agonists acting through distinct receptors. 4-alpha-PDD, which does not activate protein kinase C, was ineffective in augmenting cAMP production. This suggests that the effect of TPA is mediated by protein kinase C activation rather than nonspecific effects of phorbols on membrane lipids.

The time of exposure of cells to TPA was a critical determinant in observing enhancement of agonist-stimulated cAMP production. The effect was observed at the earliest time point measured (1 min), and generally peaked at 5 min. With prolonged (>30 min) exposure of cells to TPA, agonist-stimulated cAMP accumulation was often lower than in cells incubated with vehicle only.

Variation in time of incubation of cells with phorbol esters may account for the opposite effects on cAMP accumulation observed in different studies. With one exception [5], studies in which inhibitory effects were observed [2–4] involved incubation periods in excess of 1 hr. Augmentation of cAMP accumulation by phorbol esters has been observed in studies in which incubations with phorbols for shorter periods (<30 min) have been used [6–10].

The general ability of phorbols to enhance agonist-stimulated cAMP production presumably reflects an action on one or more components of the receptor–adenylate cyclase complex. cAMP phosphodiesterase is unlikely to be involved since TPA is effective under conditions of virtually complete phosphodiesterase inhibition. Receptor modification is also unlikely to account for the enhancement with TPA [2, 3].

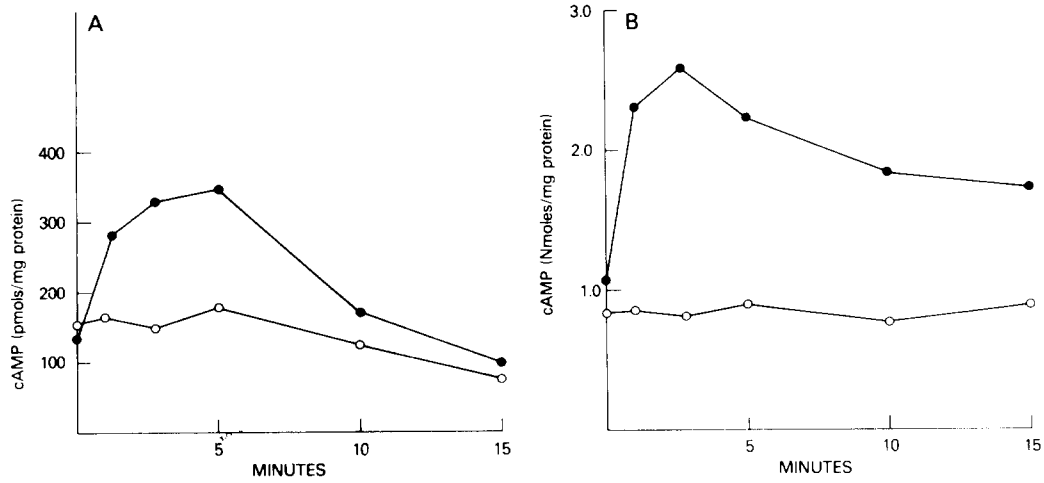

Fig. 1. Effect of phorbol esters on agonist-stimulated cAMP production. ROS cells were incubated with 100 nM TPA (●) or with 100 nM 4-alpha-PDD (○) for various times as indicated. After removal of the medium, fresh medium containing either 50 pg PTH (A) or 100 nM isoproterenol (B) was added, and incubation was continued for 10 min before measurement of cAMP accumulation.

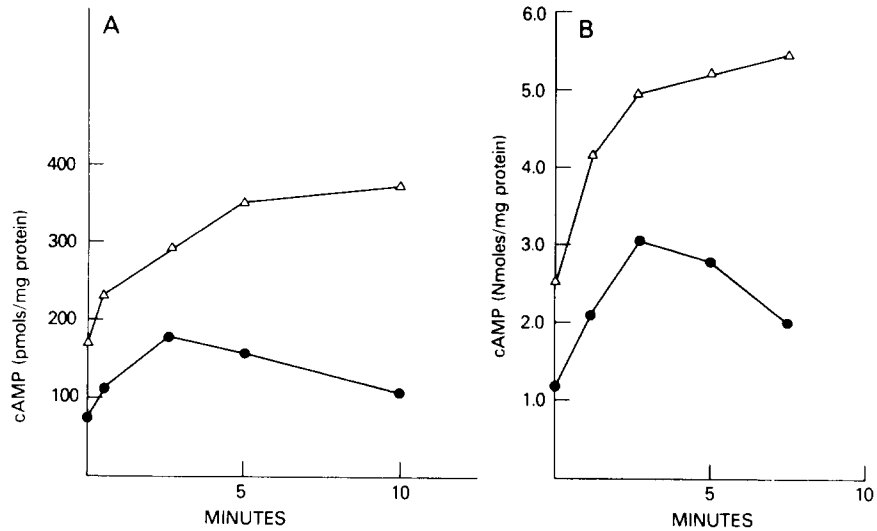

Fig. 2. Interaction of phorbol ester and pertussis toxin on agonist stimulation of cAMP. ROS cells were cultured with (△) or without (●) 50 ng/ml pertussis toxin for 24 hr. Cells were then incubated with TPA for the indicated times, after which the medium was replaced with fresh medium containing either 50 pg PTH (A) or 100 nM isoproterenol (B). cAMP was measured after 10 min of incubation with agonists.

G_s and G_i, the proteins that dually regulate adenylate cyclase activity, are potential targets for phorbol-induced modification. Recent studies show that the alpha subunit of G_i can serve as a protein kinase C substrate, and that brief exposure of platelets and S49 lymphoma cells to phorbols reduces the efficacy of agonists that inhibit adenylate cyclase via G_i [15, 16]. The authors suggest that phorbol esters modulate cAMP production through protein kinase C-catalyzed phosphorylation and inactivation of G_i.

Our data suggest that modification of G_i may not be the sole explanation for the enhancing effect of phorbol esters. Under conditions [12] that lead to maximal ADP-ribosylation of G_i by pertussis toxin, and to maximal augmentation of agonist-stimulated cAMP accumulation by the toxin [12], we nonetheless observed further increases in agonist-stimulated cAMP production by TPA. We cannot exclude the possibility that independent covalent modifications caused by pertussis toxin and phorbols are necessary to inactivate G_i fully.

In summary, TPA, the tumor-promoting phorbol ester, had a small effect on basal cAMP accumulation, but substantially increased agonist-stimulated cyclic AMP production in rat osteosarcoma cells. Enhancement of agonist-stimulated cyclic AMP production by TPA was maximal after brief (5 min or less) exposure to cells, was rapidly reversible, and was additive with the effect of pertussis toxin. The results suggest that phorbol esters can enhance agonist-stimulated cAMP production through protein kinase C phosphorylation of a receptor–adenylate cyclase component possibly other than the inhibitory guanine nucleotide regulatory protein.

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