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Cooperative interactions of protein kinase C and cAMP-dependent protein kinase systems in human promyelocytic leukemia HL60 cells

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Interactions of protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) systems were investigated in HL60 cells. It was found that the differentiating effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were potentiated by dibutyryl cAMP (dbcAMP) or prostaglandin E_2 (PGE_2). In addition, dbcAMP or PGE_2 inhibited TPA-induced binding of PKC to plasma membrane, leading to decreased protein phosphorylation, and promoted subsequent redistribution of enzyme to the nuclear membrane region. The findings are consistent with the hypothesis that PKC and PKA systems regulate cooperatively the phenotypical differentiation of leukemic cells.

12-*O*-Tetradecanoylphorbol-13-acetate; cAMP; Differentiation; Protein kinase C

1. INTRODUCTION

Human promyelocytic HL60 cells can be induced to differentiate either to monocytic macrophage-like cells or to granulocytes. Differentiation to monocytes is induced by phorbol esters [1], while acquisition of granulocytic properties can be achieved by dimethyl sulfoxide [2], retinoic acid [3] or cAMP derivatives [4]. Recent evidence supports a two-step model for the induction of terminal differentiation. It appears that the early events, which are associated with growth arrest and precommitment to differentiate, are not differentiation lineage-specific [5]. The choice of a specific differentiation lineage is regulated by late events. The existence of non-differentiation lineage specific mechanisms would suggest a certain degree of interaction between the two sets of differentiation inducers. Indeed, dimethyl sulfoxide and retinoic acid were shown to potentiate the effect of TPA, the most effective phorbol ester, on monocytic differentiation of HL60 cells [6].

In the present work, we investigated the possible effect of cAMP elevating agents on TPA-induced cell differentiation. We found that cAMP potentiated the monocytic differentiation of HL60 cells. Perhaps related to this effect, immunocytochemical studies also revealed a stimulatory effect of cAMP on the TPA-induced translocation of PKC to the nuclear membrane.

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Abbreviations: PKC, protein kinase C; PKA, cAMP-dependent protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; dbcAMP, dibutyryl cAMP; PGE_2, prostaglandin E_2

2. MATERIALS AND METHODS

2.1. *Materials*

TPA, PGE_2, dbcAMP and the diagnostic kits for the histochemical staining of specific and nonspecific esterases and acid phosphatase were purchased from Sigma; latex particles (diameter, 2.7 μm) were from Polyscience; the Vectastain biotin-avidin-peroxidase kit was from Vector Laboratories; fluorescein-conjugated goat anti-rabbit IgG was from Cooper

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Biomedical; ampholites (pH ranges of 3.5–10 and 5–7) were from LKB Instruments; [^{32}P]orthophosphate (carrier free, in water) was from ICN Radiochemicals.

2.2. *Cell culture*

Human promyelocytic leukemia cell line HL60 [7] was continuously cultured in RPMI 1640 medium (Gibco) supplemented with 20% heat-inactivated fetal calf serum, penicillin-streptomycin (50 units/ml and 50 μg/ml, respectively) and glutamine (2 mM). Cells (passage number: 26–41; doubling time: 27 h) were harvested for experiments at a density of 0.5–0.6 $\times$ 10^6/ml.

2.3. *Immunocytochemical localization of PKC*

Polyclonal antisera against PKC were raised in rabbits as described [8] and kindly donated by Peggy R. Girard in our laboratory. The cells were diluted into plastic dishes (4.5 $\times$ 10^5/ml) and were treated with TPA and/or dbcAMP as indicated in the legends to figs 1 and 2. The cells were pelleted on slides using cytospin (5 min), followed by fixation (30 min) with 4% (w/v) paraformaldehyde and 0.1% glutaraldehyde in 50 mM Tris-HCl (pH 7.5), and washing (7.5 min) with 0.2% (w/v) Triton X-100. The immunostaining procedures (fig.1) were essentially the same as originally described [9] and reported elsewhere [10]. Briefly, cells were successively incubated with 1% horse serum and 1% goat serum in Tris-buffered saline for 20 min each. Incubations with 1:500 dilutions of primary antisera were performed for 2 h at room temperature or overnight at 4°C. This step was followed by washing with Tris-buffered saline and incubating with 1% horse serum for 10 min. Finally, cells were incubated with diaminobenzidine (25 mg) and 8% hydrogen peroxide (10 μl) in 50 ml of ice-cold Tris-buffered saline (fig.1). For immunofluorescence visualization of PKC (fig.2), the cells were fixed, pretreated with the antisera and fluorescein-conjugated goat anti-rabbit IgG [9]. Preimmune serum was used as control for both methods, which yielded little or no immunostainings (micrographs not shown).

Table 1

Effects of TPA and cAMP on differentiation of HL60 cells

Treatment	Cell attachment	Marker of differentiation		
		Non-specific esterase	Acid phosphatase	Phagocytosis
None	2 ± 1	3 ± 1	1 ± 1	0 ± 0
TPA	10 ± 3	14 ± 4	7 ± 2	17 ± 5
dbcAMP	2 ± 2	7 ± 2	2 ± 1	0 ± 0
TPA + dbcAMP	55 ± 11	60 ± 7	72 ± 9	65 ± 12

For attachment studies, cells were cultured for 24 h in the absence or presence of 1 nM TPA and/or 0.6 mM dbcAMP, as indicated; dbcAMP was added 1 h prior to TPA. For the studies of differentiation marker enzymes and phagocytosis, cells were cultured as above for 7 days. The percentages of cells (mean ± SE) showing the positive characteristics in 3 Petri dishes from each treatment were presented

2.4. *Radiolabeling of cellular proteins and their separation by two-dimensional gel electrophoresis*

The cells (1 $\times$ 10^6/ml) were incubated in fresh RPMI 1640 medium with $^{32}P_i$ (0.5 mCi/ml) for 4 h, with 100 nM TPA or 100 nM TPA plus 1 mM dbcAMP for varying durations as indicated in the legend to fig.3. Incorporations of ^{32}P were terminated and proteins separated by the two-dimensional gel electrophoresis according to the procedure of O'Farrells [11] as described [12]. Aliquots, corresponding to about 35 μg protein and containing 2.3 $\times$ 10^5 cpm of acid-precipitable radioactivity, were loaded onto first-dimension (isoelectric focusing) gels. The second-dimension SDS gels contained 10% polyacrylamide. The ^{32}P-labeled proteins were detected by autoradiography and the radioactivity of specific proteins was measured as described [13].

2.5. *Other methods*

Cells attached to the plastic dish were estimated after shaking the dish gently to distinguish them from non-attached cells. Histochemical stains were used to evaluate the macrophage phenotypic properties of differentiated HL60 cells; Sigma diagnostic kits were used according to the instructions for the identifications of specific and nonspecific esterases and acid

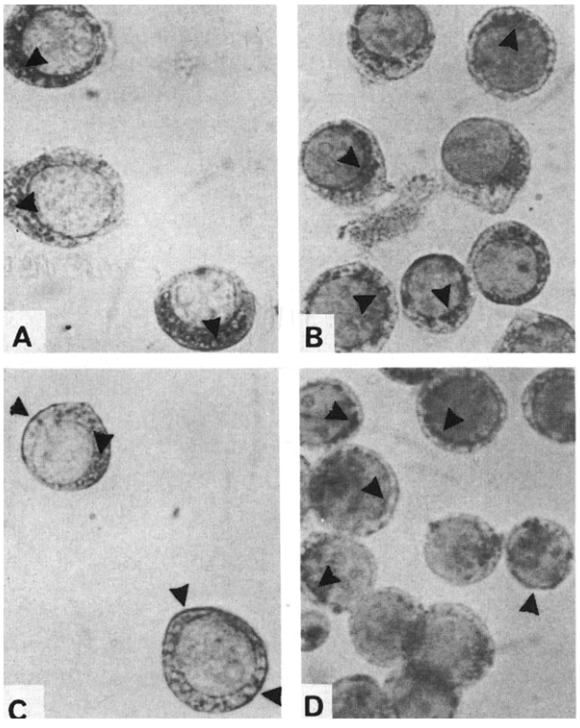

Fig.1. Effects of 1 nM TPA and dbcAMP, present singly or in combination, on immunocytochemical localization of PKC in HL60 cells. The cells were untreated (A), or treated with 0.6 mM dbcAMP plus 1 nM TPA for 4 h (B), or with 1 nM TPA for 4 h (C) or 8 h (D). Visualization of PKC was performed with the immunoperoxidase method. Arrowheads indicate accumulation of immunoproducts.

phosphatase. The activity of phagocytosis was determined by the uptake of latex particles. Briefly, cells (5×10^5) were washed and resuspended in 1 ml serum-free RPMI 1640 medium containing the particles (5×10^8/ml) in a polypropylene tube. After 1 h incubation at 37°C, cells were washed twice and resuspended in 0.5 ml medium. Cells ingesting more than 3 particles per cell were defined as phagocytosis positive and approx. 200 viable cells were examined microscopically.

3. RESULTS

3.1. *Potentiating effect of cAMP on TPA-induced cell differentiation*

A low concentration of TPA (1 nM) and dbcAMP (0.6 mM) had little or no effect on differentiation of HL60 cells when present individually. dbcAMP, however, acted synergistically to greatly increase the differentiating activity of TPA (table 1). It should be stressed here that the concentration of dbcAMP had to be diluted from 0.6 mM to at least 0.2 mM after an initial 4 h of incubation in order for its potentiating effect to be observed. 0.6 mM butyric acid, also diluted 3-fold after 4 h, was without effect (data not shown). A high concentration (100–200 nM) of PGE_2 similarly potentiated the differentiating effect of 1 nM TPA as dbcAMP did, provided the cells were washed free of this agent after 4 h of incubation (data not shown). Incubation of cells for 4 h with 200 nM PGE_2 increased cellular cAMP from 2.4 ± 0.2 to 23.1 ± 1.7 pmol/10^6 cells (triplicate determinations; $P < 0.001$). It appeared that a high concentration of cAMP stimulated initiation of cell differentiation, but inhibited the actual process of cell maturation.

A high (100 nM) concentration of TPA about

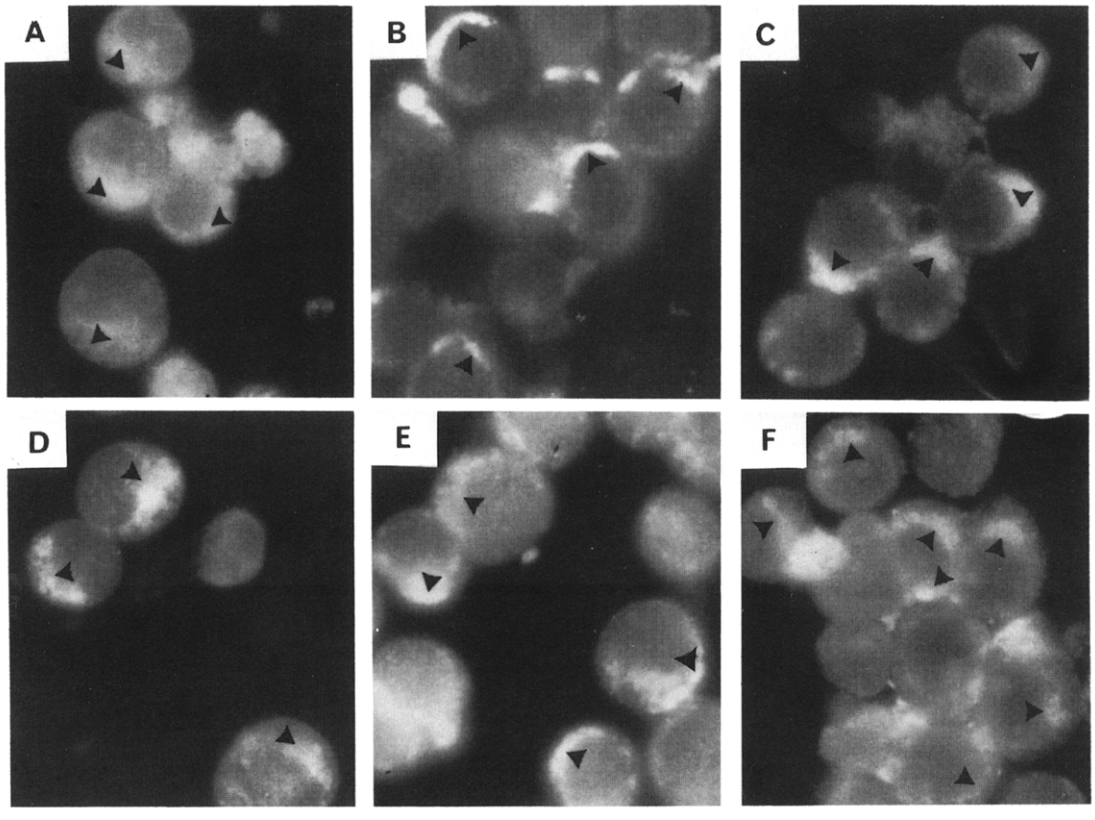

Fig.2. Effects of 100 nM TPA and dbcAMP, present singly or in combination, on immunocytochemical localization of PKC in HL60 cells. The cells were untreated (A), or treated with 100 nM TPA for 30 min (B) or 2 h (C); or with 0.6 mM dbcAMP in the absence of TPA for 2 h (D); or with 0.6 mM dbcAMP plus 100 nM TPA for 30 min (E) or 2 h (F). Visualization of PKC was performed using the immunofluorescence method. Arrowheads indicate accumulation of immunoproducts.

40–50 min treatment was sufficient to induce attachment of 70–80% of cells (which was observable 48 h later). In the co-presence of dbcAMP and 100 nM TPA, however, a similar rate of cell attachment required only 20–30 min treatment (data not shown).

3.2. *Effect of cAMP on TPA-induced PKC redistribution*

In a previous paper [14] we showed that in attached cells PKC molecules were associated predominantly with the nucleus. Since nuclear PKC could have a specific role in the regulation of differentiation, it was of interest to examine a possible cAMP effect on the TPA-induced redistribution of PKC. In the following experiments immunocytochemical methods were used to visualize intracellular localization of PKC. A highly specific antibody, frequently used in other studies [9,10,14–19], was employed. When cells were treated with 1 nM TPA plus dbcAMP (0.6 mM) for 4 h (fig.1B), PKC molecules were concentrated around the nuclear membrane. Direct measurement of enzyme activity revealed that only about 10–20% of total PKC activity was associated with isolated nuclei (data not shown) indicating that either (i) the association of enzyme with nuclear membrane was weak, or (ii) at this early stage most PKC molecules were associated with other structural (for example cytoskeletal) elements near the nucleus. However, as we have shown earlier [14], a major fraction of PKC molecules becomes tightly associated with the nucleus at later stages of differentiation. In the presence of 1 nM TPA alone, a similar accumulation of PKC around the nuclear membrane required 8 h treatment (fig.1D); after 4 h treatment (fig.1C) with TPA alone the enzyme was present mainly in the plasma membrane and cytoplasmic compartments.

Treatment of cells with high concentration of TPA (100 nM) induced translocation of PKC from the cytoplasm to the plasma membrane in 30 min (fig.2, compare A and B), while after 2 h treatment the enzyme was redistributed to the cytoplasm (fig.2C). Addition of dbcAMP to cells alone did not change PKC distribution (fig.2D); however, (i) after 30 min treatment it seemed to inhibit TPA-induced PKC binding to plasma membrane (fig. 2E), and (ii) after 2 h treatment it clearly promoted TPA-dependent redistribution of enzyme to the nuclear membrane region (fig.2F). These data were consistent with those obtained with low TPA concentration (fig.1), except that at high TPA concentration the effects of dbcAMP on PKC distribution were faster. PGE_2 mimicked the effect of dbcAMP on PKC distribution (data not shown).

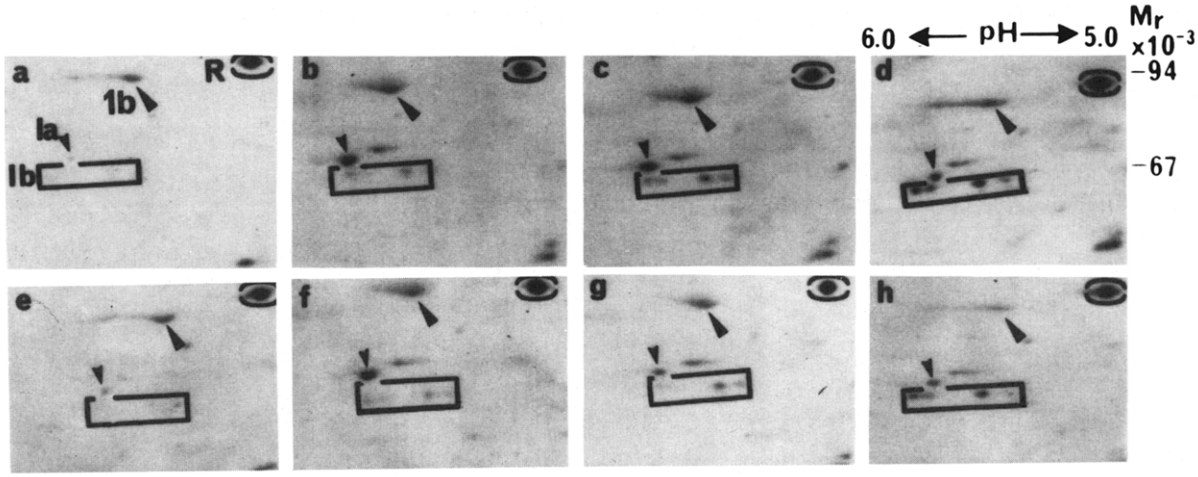

Fig.3. Effect of dbcAMP on the TPA-stimulated protein phosphorylation in HL60 cells. The cells were incubated with $^{32}P_i$ for 4 h and were untreated (a), or treated with 100 nM TPA for the final 5 (b), 30 (c) and 120 min (d); or with 100 nM TPA plus 0.6 mM dbcAMP for the final 5 (f), 30 (g) and 120 min (h) of the 4-h incubation period. dbcAMP was added 30 min prior to TPA in all cases. Cells were also treated with 0.6 mM dbcAMP alone for 150 min (e) or 5, 30 and 120 min (not shown). About 10^5 cpm of the acid-precipitable radioactivity (35 μg protein) was loaded onto each gel; autoradiographs were exposed for 3 days. The radioactivity of the reference protein (R) remained constant in each experiment.

3.3. *Effect of cAMP on TPA-induced protein phosphorylation and dephosphorylation*

In order to verify whether altered PKC distribution was accompanied by changes in protein phosphorylation pattern, cells were labelled with $^{32}P_i$ and treated with TPA (100 nM) and/or dbcAMP (1 mM) for 5–120 min. Protein Ia (M_r 66000, p*I* 5.6), previously referred to as protein 3 [20], is located in the plasma membrane [20] and is a particularly good substrate for PKC; its phosphorylation was maximally stimulated by TPA in 5–30 min (fig.3, comparing a–c), when a significant portion of PKC was translocated to the plasma membrane (fig.2B). After 2 h of the TPA treatment, phosphorylation of protein Ia was greatly reduced (by 70% as determined by measuring its actual radioactivity) (fig.3d), possibly reflecting a disappearance of the membrane-associated PKC (fig.2C). In the co-presence of TPA and 1 mM dbcAMP peak phosphorylation of protein Ia was observed after 5 min (fig.3f); however, this protein lost 65% of its ^{32}P activity after 30 min (fig.3g). dbcAMP had a similar effect when the concentration of TPA was 1 nM, except that dephosphorylation of protein Ia took place at a much later time (figures not shown). It is still worth noting that dbcAMP also inhibited, although less markedly, the TPA-stimulated ^{32}P labelling of proteins Ib (M_r 63000; p*I* 5.45–5.65) and 1b (M_r 80000; p*I* 5.0) (fig.3g,h).

4. DISCUSSION

Most receptor-mediated mechanisms utilize either the PKA or PKC messenger systems [21–24]. TPA has been known for some time to affect cAMP production in many cells [25–33]; however, only a few reports have dealt with effects of cAMP on the PKC system. A few years ago one of us reported multiple effects of cAMP on the TPA-mediated protein phosphorylation in S49 mouse lymphoma cells [34]. Since then, cAMP has been shown to modify TPA-induced protein phosphorylation in other cells too [35,36], and to promote PKC transfer to the nuclear compartment in lymphocytes [37]. The kinetics of phosphorylation-dephosphorylation of plasma membrane protein Ia, shown in the present paper, were consistent with a mechanism where cAMP promoted PKC dissociation from, rather than inhibited its initial association with, the plasma membrane. The absence of PKC from the plasma membrane would presumably increase the rate of dephosphorylation of its substrates. However, it was shown [38] that cAMP stimulated dephosphorylation of several proteins in S49 mouse lymphoma cells by a mechanism which was apparently unrelated to the activity state of PKC. This raises the possibility that in the present case as well the cAMP effect on protein dephosphorylation was unrelated to its effect on PKC distribution. More experiments will be needed to clarify this point.

The mechanism by which cAMP can modify PKC distribution is presently unknown. However, association of PKC with the nucleus, which becomes tight only at later stages of differentiation [14], is apparently an important mechanism by which certain steps of differentiation, along with the appearance of various differentiation markers, are regulated. Therefore, it is a significant observation that cAMP, which promoted the TPA-dependent accumulation of PKC around the nuclear membrane, also potentiated the TPA effect on cell differentiation.

In summary, these experiments show that cAMP, similarly to dimethyl sulfoxide and retinoic acid [6], also potentiates the effect of TPA on cell differentiation. We are presently investigating the possibility that these agents have similar effects on PKC distribution as well.

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