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COMPARATIVE EFFECTS OF POLYMYXIN B, PHORBOL ESTER AND BRYOSTATIN ON PROTEIN PHOSPHORYLATION, PROTEIN KINASE C TRANSLOCATION, PHOSPHOLIPID METABOLISM AND DIFFERENTIATION OF HL60 CELLS

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SUMMARY: The effects of protein kinase C (PKC) inhibitor polymyxin B (PMB) and PKC activators 12-O-tetradecanoylphorbol-13-acetate (TPA) and bryostatin on intact HL60 cells were examined. It was found that each of the three agents exhibited similar effects on phosphorylation of certain endogenous proteins, PKC translocation from cytoplasm to plasma membrane and formation of CDP-choline. TPA, however, was the only agent that stimulated phosphatidylcholine formation. Differentiation of HL60 cells was potently induced by TPA; in comparison bryostatin was a relatively weaker inducer and PMB was without effect. The data indicated that the effects of the PKC inhibitor PMB on intact cells could not be predicted by its in vitro activity, and that certain TPA-dependent but PKC-independent reactions might be crucial in HL60 cell differentiation.

The human promyelocytic leukemic cell line HL60 has been shown to differentiate to macrophage-like cells by TPA (1,2). Because TPA is a direct activator of PKC (3), it has been suggested that PKC may play a key role in cell differentiation (4). Diacylglycerols, such as 1-oleoyl-2-acetylglycerol and dioctanoylglycerol, also activators of PKC (5-9), however, had a relatively small or no effect on HL60 cell differentiation (9,10), perhaps due partly to their metabolic inactivation. Paradoxically, a macrocyclic lactone bryostatin, an effective PKC activator that is poorly metabolized in cells as is TPA (11,12), has been reported to be unable to induce HL60 cell differentiation (12) or even to be inhibitory to the TPA-induced cell differentiation (12). In the present studies, we examined and compared the effects of TPA, bryostatin and PMB, a polypeptide antibacterial antibiotic previously shown to be a potent and specific inhibitor of PKC (13),

Abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; PMB, polymyxin B; PGE_2, prostaglandin E_2; PC, phosphatidylcholine.

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on HL60 cells. It was noted, unexpectedly, that PMB exerted certain effects similar to those seen for the PKC activators in intact HL60 cells.

MATERIALS AND METHODS

Materials: TPA, PMB and PGE_2 were purchased from Sigma; fluorescein-conjugated goat anti-rabbit IgG was from Cooper Biomedical; [^{32}P]orthophosphate (carrierfree) was from ICN Radiochemical; [methyl-^{14}C]choline chloride (50 mCi/mmol) was from Amersham. The anti-PKC antisera were raised in rabbits (14) and bryostatin was purified from marine animal Bugula neritina (15) as described.

Radiolabeling of cellular proteins in HL60 cells: The cells (1×10^6) were incubated with ^{32}Pi for 8 h. Various agents were added to the incubation medium as indicated in the legend to Fig. 1. Incorporation of ^{32}P was terminated and proteins were separated by the two-dimensional gel electrophoresis (16). Aliquots of the acid-precipitable radioactivity (4.5×10^5 cpm; 45 µg protein) was loaded onto the first-dimension gel. The second-dimension sodium dodecyl sulfate gels contained 10% polyacrylamide. The ^{32}P-labeled proteins were then detected by autoradiography as described elsewhere (17).

Measurement of PC formation in HL60 cells: The cells (2×10^6/ml) were incubated with [methyl-^{14}C]choline (1 µCi/ml) for 2 h in the presence or absence of various agents as indicated in Table 1. At the end of incubation, cells (2×10^6/determination) were pelleted and the phospholipids were extracted with 3 ml of chloroform:methanol (2:1, v/v). Phospholipids were then separated by silica gel thin-layer chromatography and the radioactivity in PC was determined (18,19).

Measurement of CDP-choline formation in HL60 cells: After incubation of the cells (2×10^6/ml) with [methyl-^{14}C]choline for 2 h, the cells (5×10^6/determination) were extracted with 3 ml of chloroform:methanol (2:1, v/v), followed by further extraction of the cell residues with 20% ethanol (20). CDP-choline was separated from phosphocholine and choline on Whatman 3MM paper (20), using a solvent system of ethanol:1 M ammonium acetate at pH 7.1 (7:3, v/v). After the radioactive choline derivatives were detected by autoradiography, the spots of CDP-choline were cut and their radioactivity measured. In some experiments, CDP-choline was also separated on silica gel 60 plates (20), using a solvent system of 0.15 M NaCl:methanol:28-30% NH_4OH (50:50:5, v/v/v). The results obtained from the two methods were similar.

Immunocytochemical localization of PKC in HL60 cells: The cells (2.5×10^5/ml) were incubated in plastic dishes for 60 min in the absence or presence of the agents as indicated in Fig. 2. The cells were then pelleted on glass slides using a cytospin (5 min), fixed, treated with polyclonal anti-PKC antisera or preimmune sera (serving as control), and finally stained with fluoresceinconjugated goat anti-rabbit IgG, as described earlier (21).

Determination of differentiation and viability of HL60 cells: After incubating the cells (2×10^5/ml) for 48 h in the presence or absence of the agents indicated in Table 2, the nonadherent (suspended) cells were seperated by gentle shaking to distinguish them from the adherent (differentiating) cells. Viability of both nonadherent and adherent cells was determined by trypan blue exclusion. The cell numbers were quantitated hemocytometrically.

RESULTS

The effects of 50 nM TPA and 50 nM bryostatin on protein phosphorylation in HL60 cells were practically identical (Fig. 1). They both stimulated

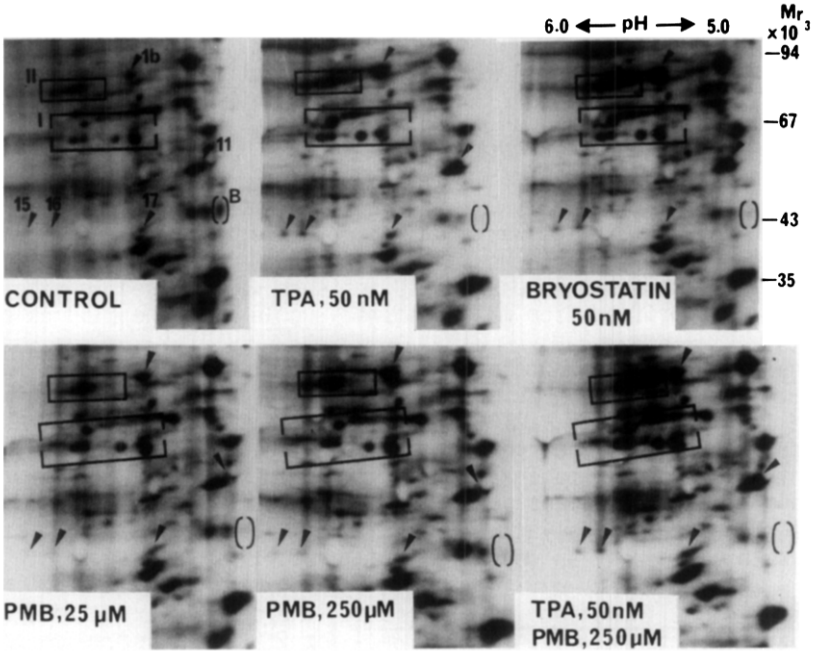

Fig. 1. Comparative effects of TPA, bryostatin and PMB on protein phosphorylation in intact HL60 cells. The cells were incubated with $^{32}P_i$ for 8 h. TPA and bryostatin were present during the final 90 min, whereas PMB was present during the final 45 min, of the entire 8-h incubation period. See Materials and Methods for further details.

phosphorylation of the same proteins, including proteins in blocks I and II, and proteins 1b, 11, 15, 16 and 17, and conversely promoted dephosphorylation of protein B. The PKC inhibitor PMB, at 25 and 250 μM, unexpectedly stimulated phosphorylation of proteins in blocks I and II and protein 1b, as well as dephosphorylation of protein B as did TPA and bryostatin (Fig. 1). PMB, however, unlike the PKC activators, was unable to stimulate phosphorylation of proteins 11, 15, 16 and 17. It was further noted that 250 μM PMB did not alter the phosphorylation states of cellular proteins elicited by 50 nM TPA (Fig. 1).

Immunocytochemical studies revealed that 100 nM TPA promoted a PKC translocation from cytoplasm to plasma membrane in HL60 cells (Fig. 2), as reported previously (21,22). Interestingly, both 100 nM bryostatin and 250 μM PMB also promoted a similar PKC redistribution. The overall immunofluorescence in cells treated with TPA, bryostatin or PMB, however, was lower compared to that seen in the untreated (control) cells, reflecting a down-regulation or depletion of the cytoplasmic enzyme in the treated cells. We have determined that phosphoproteins

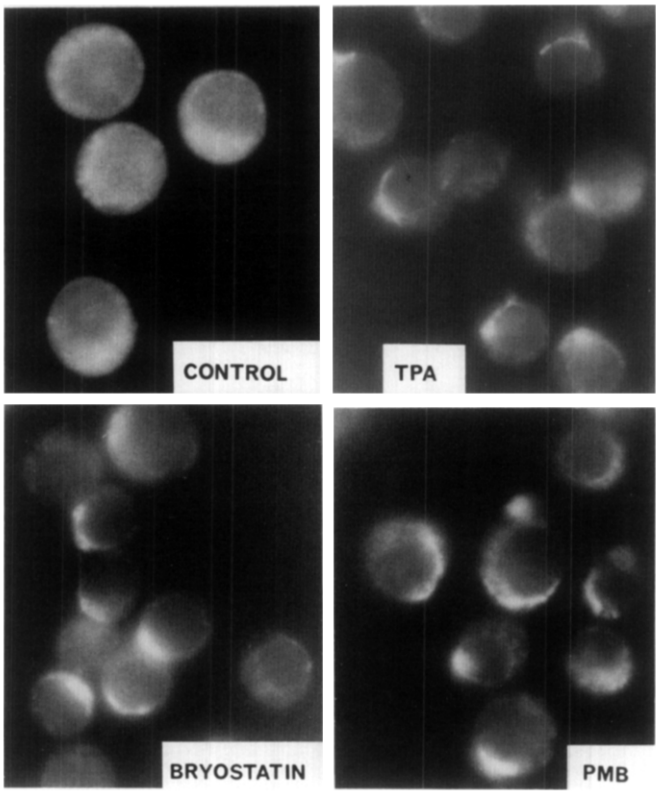

Fig. 2. Similar effects of TPA, bryostatin and PMB on immunocytochemical localization of PKC in HL60 cells. The cells were incubated with or without TPA (100 nM), bryostatin (100 nM) and PMB (250 μM) for 1 h before being fixed for immunostainings. See Material and Methods for further details.

in block I were associated with plasma membrane (unpublished), supporting the observations that the enhanced phosphorylation of these proteins in cells treated with TPA, bryostatin or PMB (Fig. 1) was likely correlated with the PKC redistribution to plasma membrane.

An increased PC synthesis has been implicated in the TPA-induced differentiation of HL60 cells (23). We found that TPA (200 nM), bryostatin (20 and 200 nM) and PMB (25 and 250 μM) increased formation of CDP-choline, an immediate precursor of PC synthesis (Table I). PGE_2 (200 nM) also stimulated CDP-choline formation and, moreover, its effect was additive to the stimulations seen with TPA, bryostatin or PMB. PC synthesis, on the other hand, was stimulated only by TPA; bryostatin and PMB were without effect (Table I). The findings indicated that an increased CDP-choline formation elicited by bryostatin and PMB did not necessarily lead to an increased PC synthesis, suggesting that formation of

TABLE I

Effects of TPA, bryostatin and PMB on CDP-choline and PC formation in HL60 cells

Addition	^{14}C-labeled CDP-choline (cpm/10^7 cells/2 h)		^{14}C-labeled PC (cpm/10^6 cells/2 h)	
	Basal	PGE_2 (200 nM)	Basal	PGE_2 (200 nM)
None (control)	770 ± 143	1,812 ± 133	5,106 ± 198	5,412 ± 198
TPA (200 nM)	1,427 ± 178[a]	2,482 ± 129[b]	7,811 ± 243[a]	10,810 ± 383[b]
Bryostatin (20 nM)	1,598 ± 127[a]	2,895 ± 156[b]	--	--
Bryostatin (200 nM)	1,940 ± 79[a]	3,345 ± 257[b]	5,877 ± 227	7,929 ± 148[b]
PMB (25 μM)	1,308 ± 73[a]	2,365 ± 231[b]	--	--
PMB (250 μM)	1,629 ± 130[a]	2,490 ± 297[b]	5,619 ± 150	7,605 ± 130[b]

Cells were incubated with [methyl-^{14}C]choline for 2 h, with or without the agents as indicated. The data presented are the means ± SE of three determinations.
[a]Significantly different from the control ($p < 0.05$).
[b]Significantly different from the respective values seen in the absence of PGE_2 ($p < 0.05$).

diacylglycerol, the other immediate precursor of PC synthesis, was likely a rate-limiting step. We have observed that TPA, but not bryostatin or PMB, stimulated the hydrolysis of PC by phospholipase C (unpublished). PGE_2, while without effect when present alone, increased PC synthesis in the presence of TPA, bryostatin or PMB (Table I). We observed recently that dibutyryl cyclic AMP, similarly without effect when present alone, also potentiated the effect of TPA on PC synthesis (24), and that PGE_2 elevated cyclic AMP levels 20- to 30-fold in HL60 cells (unpublished).

TPA (5 nM) promoted adhesion (an early indicator of differentiation) in 76% of HL60 cells within 48 h (Table II). Bryostatin (20, 50 and 200 nM) also induced differentiation in 20-25% of the cells. When the adherent cells, after being separated from the suspended cells and washed free of bryostatin, were cultured for additional 5 days, they matured to become macrophage-like cells, exhibiting the characteristic marker enzymes (non-specific esterase and acid phosphatase) and the ability of phagocytosis, as in the case of TPA-treated cells (data not shown). The present findings were in contrary to an earlier

TABLE II

Effects of TPA, bryostatin and PMB on Differentiation of HL60 cells

Addition	Adherent cells (% of total)	Cell viability (% of total)	
		Adherent	suspended
None (control)	<1	--	98 ± 1
TPA (5 nM)	76 ± 3	96 ± 2	94 ± 3
Bryostatin (20 nM)	25 ± 4	94 ± 4	93 ± 5
Bryostatin (50 nM)	21 ± 6	91 ± 6	90 ± 6
Bryostatin (200 nM)	20 ± 7	94 ± 3	61 ± 12
PMB (250 μM)	6 ± 3	95 ± 4	92 ± 4
TPA (5 nM) + PMB (250 μM)	71 ± 6	91 ± 5	91 ± 7

Cells (2×10^5/ml) were cultured for 48 h in the absence or presence of the agents as indicated. Cell differentiation was determined by adhesion and cell viability was judged by trypan blue exclusion. The data presented are the means ± SE of three determinations.

report by others that bryostatin was not an inducer of HL60 cell differentiation (12). We reported recently that bryostatin was nearly as effective as TPA in inducing differentiation of human leukemia KG-1 cells (25). PMB (250 μM) was practically ineffective and, furthermore, had no effect on the TPA-induced cell differentiation (Table II). The cell viability was greater than 90% under all incubation conditions except that 200 nM bryostatin was somewhat cytotoxic (Table II).

DISCUSSION

It has been shown that PKC inhibitors alkyllysophospholipid or sphingosine counteracted protein phosphorylation (26), differentiation (10,26) and PKC down-regulation (unpublished) in HL60 cells, and oxidative burst in neutrophils (27), mediated by TPA. It was quite unexpected to find in the present studies that PMB could not inhibit the TPA-induced protein phosphorylation and differentiation of HL60 cells, or could even mimic or partially mimic the TPA effects in stimulating protein phosphorylation, phospholipid metabolism and PKC translocation. Because TPA is an activator of PKC, it is possible that PMB could somehow

indirectly activate PKC in intact HL60 cells to phosphorylate, for example, proteins in block I (plasma membrane proteins) and protein 1b (shown to be a nuclear protein, unpublished). The unexpected "in vivo" activities of PMB perhaps could be explained by the fact that it is a surface-active polypeptide capable of extensively interacting with membranes and markedly altering their properties and functions. It should be noted here that, although PMB is a potent and specific PKC inhibitor in vitro (13), its in vivo effects should be interpreted with cautions.

Bryostatin, a PKC activator shown to be as potent as TPA (11,12,25), duplicated virtually all biochemical effects of TPA in HL60 cells, except that bryostatin was unable to stimulate PC formation (Table I). As PC synthesis has been linked to cell differentiation (23), the present findings might explain the inability (12) or low ability (Table II) of bryostatin to stimulate differentiation of HL60 cells. In this respect, TPA was unique in its effects, suggesting that both PKC-dependent and -independent events are likely involved in cell differentiation.

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