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Biochemistry

Phorbol ester induction of leukemic cell differentiation is a membrane-mediated process

(tumor promoter/membrane receptors)

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ABSTRACT **Phorbol esters are potent inducers of macrophage-like differentiation in the HL-60 promyelocytic leukemia cell line. The sequence of events by which they bring about this transition is poorly understood. However, it is known that phorbol esters bind to the surface membrane of HL-60 cells and to various other cells as well. Our studies were directed toward determining the biologic importance of this membrane association. [^{3}H]Phorbol dibutyrate (PBu_2) was specifically bound by HL-60 cells with a K_d of 23 nM and with 1.9×10^5 binding sites for [^{3}H]PBu_2 per cell. There was no internalization of bound [^{3}H]PBu_2. Specific binding was fully reversible upon washing in fresh medium, and [^{3}H]PBu_2 added thereafter bound normally to its receptor. Within 10 min of binding, PBu_2 stimulated [^{14}C]choline incorporation into phosphatidylcholine, with a rapid return to normal upon removal of the PBu_2. Membrane-bound PBu_2 progressively inhibited DNA synthesis, with 70% inhibition by 8 hr. This process was interrupted if the PBu_2 was removed, and little recovery of DNA synthesis occurred in previously inhibited cells. Between 8 and 16 hr, PBu_2 induced adherence of cells to plastic, but only in those cells in which phosphatidylcholine synthesis was stimulated, and this process was also interrupted if PBu_2 was removed prior to 16 hr. Similarly, nonspecific esterase, which develops after 72 hr of incubation, was induced in cells exposed to PBu_2 for the initial 16 hr but not in cells exposed for 5 hr. These studies demonstrate that phorbol esters exert their effects while retained at the cell surface. Inhibition of cell growth and the acquisition of surface and enzymatic properties that characterize macrophages are separable events, each of which proceeds through a receptor-mediated, transmembrane process. The stimulation of phosphatidylcholine synthesis appears to be a part of that process.**

A number of chemical agents are known to affect hematopoietic cell differentiation. This has been examined extensively with murine (1–3) and human (4–7) leukemia cell lines *in vitro* with chemicals as diverse as acetamide (3), dimethyl sulfoxide (2–4), retinoic acid (5), and the phorbol esters (6, 7). In the HL-60 human promyelocytic leukemia cell line, the first three agents induce differentiation toward mature granulocytes (4, 5), whereas phorbol esters induce differentiation toward macrophages (6, 7). Phorbol esters have also been shown to promote macrophage differentiation in normal granulocyte-monocyte colony-forming units obtained from murine (8) and human (9) bone marrow and in cells harvested from the bone marrow and peripheral blood of most patients with acute nonlymphocytic leukemia (10).

Phorbol esters produce a range of effects in mammalian cells. In addition to serving as tumor promoters in animals (11), they promote growth in various cells including human lymphocytes (12, 13). Their action on cell differentiation is complex, stimulating differentiation in certain cell lines and inhibiting it in others (14). Most studies of phorbol esters have utilized the very active agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA), also referred to as phorbol 12-myristate, 13-acetate (PMA). This hydrophobic molecule distributes throughout the hydrophobic areas of cells (15). It produces a large number of metabolic changes, some of which may relate to the mechanism whereby it causes phenotypic changes in cells and some of which are probably irrelevant. Phorbol 12,13-dibutyrate (PBu_2) is more hydrophilic and use of it has permitted a better characterization of phorbol ester action. Specific cell surface receptors for PBu_2 have been described in many cells, including fibroblasts, lymphocytes, and HL-60 cells (16–21). In several cell lines studied, PBu_2 binding has been reversible upon resuspension of cells in medium free of phorbol esters (16, 20, 21), although this property has not been examined in HL-60 cells.

What is the functional consequence of phorbol ester binding to the surface of cells? Must phorbol esters be internalized, with or without their receptors, for them to exert their action? To address these questions, we studied the binding of PBu_2 to HL-60 cells and analyzed the relationship between this binding and the induction of early lipid metabolic events, growth arrest, and late phenotypic changes in these cells. Our studies demonstrate that PBu_2 is reversibly bound to a specific surface receptor on HL-60 cells and that it is not internalized. While bound to its receptor, PBu_2 stimulates phosphatidylcholine synthesis. Inhibition of cell growth and the acquisition of properties that characterize macrophages depend upon the continued binding of PBu_2 to its receptor. Thus, the differentiation of HL-60 cells to cells that resemble macrophages proceeds through a receptor-mediated transmembrane process.

METHODS

Chemicals. [^{3}H]PBu_2 (6.4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and unlabeled PBu_2 were purchased from Life Systems (Newton, MA). [^{14}C]Choline and [^{3}H]dThd were obtained from New England Nuclear.

Cell Culture. HL-60 cells were obtained from Robert Gallo (National Cancer Institute). They were grown in plastic tissue culture flasks (Corning) in RPMI-1640 medium containing 15% (vol/vol) fetal calf serum and supplemented with penicillin (1 unit/ml) and streptomycin (100 μg/ml) (Flow Laboratories, Rockville, MD). This medium contains 0.33 μM choline. For studies of adherence, cells were grown in Corning flasks; for morphologic studies, including histochemical staining for nonspecific esterase (22), cells were grown in plastic wells (Costar, Cambridge, MA) with glass coverslips. In certain experiments, cells were grown in 50-ml polypropylene tubes obtained from Corning.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PBu_2, phorbol 12, 13-dibutyrate.

Measurement of [^{3}H]PBu$_2$ Binding. Cells were incubated in duplicate at a concentration of 5×10^6 cells per ml in a final volume of 1.0 ml containing a range of PBu$_2$ concentrations up to 200 nM. Incubation was terminated by pouring the incubation mixture over a 2-cm Whatman GFC glass-fiber filter and rapidly washing three times with cold phosphate-buffered saline at pH 7.4. Filters were placed in liquid scintillation vials containing ScintiVerse (Fisher Scientific, Fair Lawn, NJ), and their radioactivity was measured. Nonspecific binding was determined by incubating cells with [^{3}H]PBu$_2$ in the presence of 30 μM unlabeled PBu$_2$. Nonspecific counts represented 15–50% of the total counts. Duplicate determinations differed within ±6%.

Measurement of [^{14}C]Choline Incorporation. [^{14}C]Choline was added to cultures for periods of up to 2 hr, after which cells were sedimented and washed three times with phosphate-buffered saline, and the cell lipids were extracted with chloroform/methanol/water, 1:2:0.8 (vol/vol). The organic phase was dried in liquid scintillation vials, and, after the addition of ScintiVerse, the radioactivity was determined in a β counter. When examined by thin-layer chromatography, all of the radioactivity was recovered in phosphatidylcholine (23).

RESULTS

Reversible Binding of PBu$_2$ to HL-60 Cells. The binding of [^{3}H]PBu$_2$ to HL-60 cells is shown in Fig. 1. Binding was saturable at approximately 80 nM PBu$_2$. Scatchard analysis of these data indicates that the K_d for PBu$_2$ is 23 mM and that there are 1.9×10^5 molecules of PBu$_2$ bound per cell. This is in general agreement with the data of Solanki *et al.* (19) who found that the K_d of PBu$_2$ binding in HL-60 cells was 51 nM and that 0.8×10^5 molecules of PBu$_2$ were bound per cell.

The time course of binding was rapid at both 27°C (Fig. 2) and 37°C (not shown), with maximal binding achieved within 10 min. Although it had been reported that there is down-regulation of PBu$_2$ binding in HL-60 cells with the loss of two-thirds of the bound PBu$_2$ by 60 min (19), we observed only small downward drift in the amount of PBu$_2$ bound at both 27°C and 37°C, and in many experiments we observed no decrease at all.

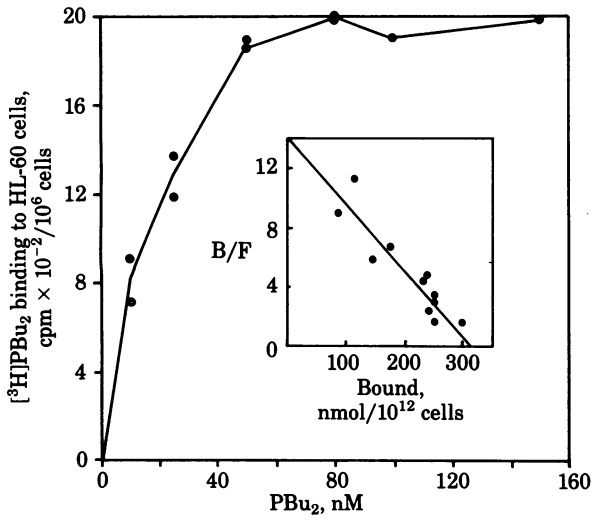

FIG. 1. Specific binding of PBu$_2$ to HL-60 cells. Cells were incubated at 37°C with [^{3}H]PBu$_2$. Nonspecific binding, determined in parallel incubations containing 30 μM unlabeled PBu$_2$, was subtracted from the total binding to yield values for specific binding. The specific binding was also analyzed by the Scatchard method (24). Each data point was obtained from duplicate samples, and data from two separate experiments are plotted together. (*Inset*) $r = 0.92$; $K_d = 23$ nM.

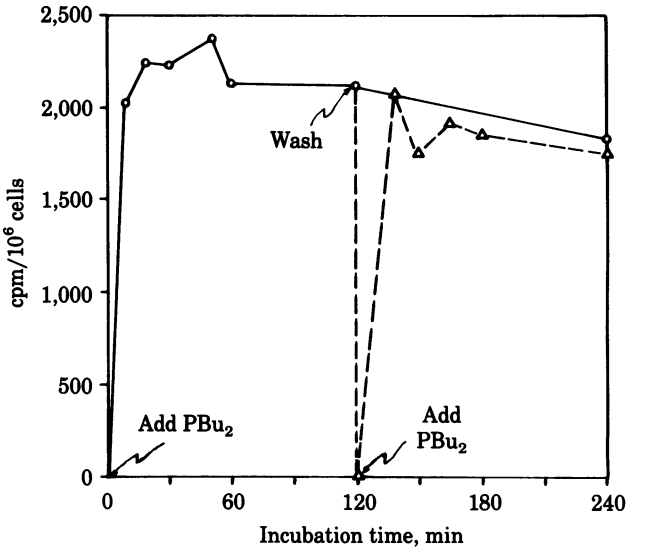

FIG. 2. Reversible binding of PBu$_2$ to HL-60 cells. Cells were incubated at 27°C with 100 nM [^{3}H]PBu$_2$. At 2 hr, an aliquot of cells (---) was washed three times in RPMI-1640 medium, and [^{3}H]PBu$_2$ was added again.

When cells incubated with [^{3}H]PBu$_2$ were centrifuged and resuspended, three times, in medium free of PBu$_2$, the specifically bound PBu$_2$ was quantitatively removed from the cells and [^{3}H]PBu$_2$ nonspecifically associated with cells decreased by 90%. Subsequent addition of [^{3}H]PBu$_2$ established a level of specific binding similar to that observed in cells continuously incubated with PBu$_2$. This was true both at 2 hr (Fig. 2) and after 24 hr of incubation with PBu$_2$ (not shown).

Relationship Between PBu$_2$ Binding and Phospholipid Synthesis. We have reported that the synthesis of phosphatidylcholine from diacylglycerol and choline increases during the first hour after exposure of HL-60 cells to TPA (25). Fig. 3 depicts the stimulation of phosphatidylcholine synthesis in the

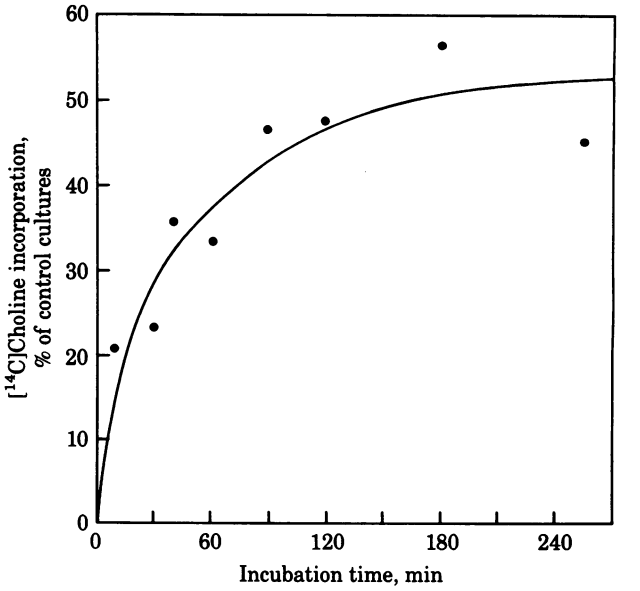

FIG. 3. PBu$_2$-induced stimulation of [^{14}C]choline incorporation into HL-60 cells. Cells were incubated at 37°C with [^{14}C]choline in the presence of 500 nM PBu$_2$. Cell lipids were extracted, and the radioactivity incorporated into phosphatidylcholine was determined. Data points are means of triplicate determinations from three similar experiments.

presence of 500 nM PBu_2. Stimulation was observed at the earliest time point examined, 10 min after addition of PBu_2. The rate of phosphatidylcholine synthesis increased to approximately 50% greater than that of control cells at 2–3 hr, and it reached levels of approximately twice that of control cells by 16 hr (Fig. 4).

When cells were incubated with PBu_2 for 16 hr and then washed, a process that removes PBu_2 from the cell surface, the incorporation of [^{14}C]choline returned to control levels (Fig. 4). Reexposure of cells to PBu_2 led to a rapid stimulation of phosphatidylcholine synthesis to levels approaching those observed prior to washing. Thus, this early change in phospholipid synthesis induced by phorbol esters depends on the continued presence of PBu_2 bound to specific surface receptors.

PBu_2 Binding and Cell Growth. Two of the changes that HL-60 cells undergo in the presence of phorbol esters are the cessation of DNA synthesis and adhesion to glass or plastic surfaces. Changes in DNA synthesis associated with PBu_2 binding and with removal of PBu_2 from its binding sites are shown in Fig. 5. [^{3}H]dThd incorporation into DNA progressively decreased during 16 hr of incubation with PBu_2. Removal of the PBu_2 at any time up to 16 hr halted this decrease in DNA synthesis. Upon washing, cells were able to recover DNA synthesis capacity at 4 hr but not thereafter. In a separate experiment (not shown), HL-60 cells were incubated with PBu_2 for 24 hr, washed, and incubated for a second period of 24 hr in medium free of PBu_2. There was only partial recovery of [^{3}H]dThd incorporation, to a level of 23% of control.

Rovera *et al.* (26) have shown that the decrease in DNA synthesis that occurs when HL-60 cells are treated with phorbol esters results from a progressive recruitment of cells into a no-growth differentiation pathway rather than from a decreased rate of DNA synthesis in all cells. Our data are consistent with that concept. The data in Fig. 5 indicate that removal of PBu_2 from cells yet to be recruited prevents their entry into the no-growth differentiation pathway. Conversely, the majority of cells recruited remain unable to synthesize DNA despite the subsequent removal of PBu_2. Viewed in this light, PBu_2 must remain bound to its receptor until cells are irreversibly recruited into a differentiation process.

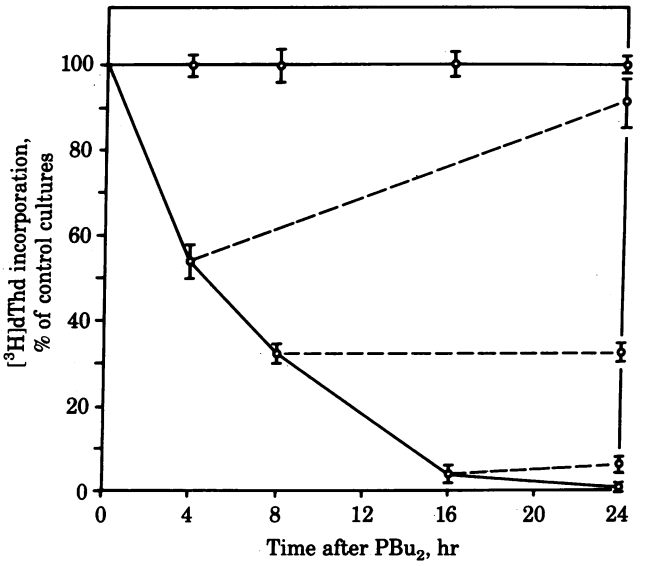

FIG. 5. Effect of PBu_2 on DNA synthesis. Cells were incubated for various times in 500 nM PBu_2. At the times shown, [^{3}H]dThd was added to cultures for 2 hr; then the cells were washed three times in phosphate-buffered saline and resuspended in buffered saline. An aliquot was added to ScintiVerse for determination of radioactivity. Cells from companion cultures were washed at the same times and incubated thereafter in medium free of PBu_2. Data points are means ± SEM of triplicate determinations from two separate experiments.

PBu_2 Binding and Cell Adherence. The progressive acquisition of adherent properties by HL-60 cells exposed to 500 nM PBu_2 is shown in Fig. 6. Relatively few cells became adherent during the first 8 hr. Thereafter, there was a rapid increase in adherence to 90% at 13 hr. In a separate experiment, nonad-

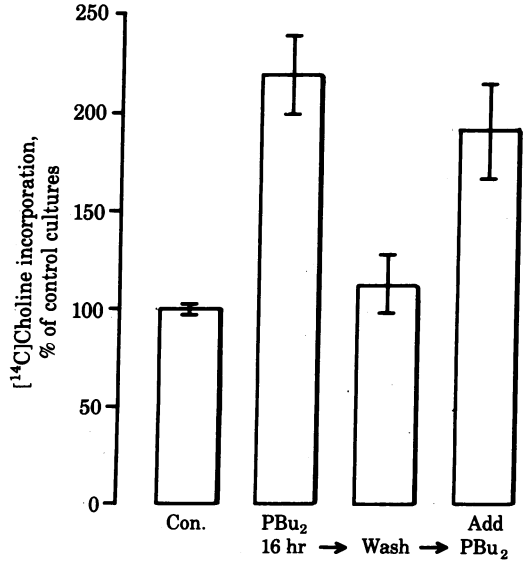

FIG. 4. Relationship between the presence of PBu_2 and the stimulation of phosphatidylcholine synthesis. Cells were incubated with 500 nM PBu_2 for 16 hr and then washed three times with RPMI medium. PBu_2 was added again, [^{14}C]choline was added, and cells were incubated for 2 hr, after which choline incorporation into lipid was determined. Data are the means ± SEM of triplicate determinations from three separate experiments. Con., control.

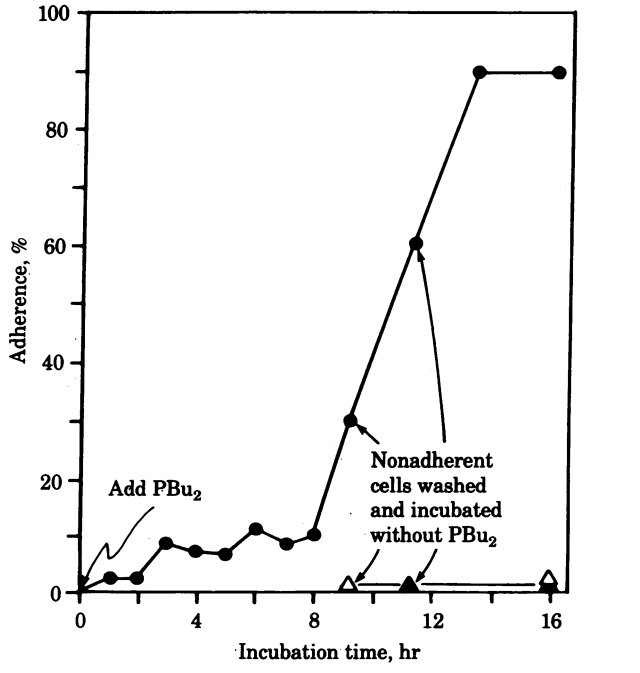

FIG. 6. Requirement for PBu_2 during the induction of adherence. Cells were incubated for up to 16 hr in Corning tissue culture flasks. At the times shown, nonadherent cells were removed from some flasks, washed three times in RPMI-1640 medium, and incubated for the remainder of the 16 hr in medium free of PBu_2.

herent cells were removed between 8 and 12 hr, washed, and incubated in medium free of PBu_2 for the full 16 hr. None of these cells subsequently became adherent (Fig. 6). When the adherent cells at 16 hr were washed and incubated for an additional 24 hr in medium without PBu_2, approximately 20% of the cells became nonadherent. However, within 5 min of reexposure to PBu_2, these cells again adhered to the plastic substrate, suggesting that they retained most of the surface qualities necessary for adherence. Thus, as suggested for the effect of PBu_2 on DNA synthesis, it appears that PBu_2 progressively recruits cells that acquire and retain adherent properties. The continued presence of PBu_2 on the cell surface is required for this recruitment.

To examine the interrelationships among the stimulation of phosphatidylcholine synthesis, the inhibition of DNA synthesis, and the acquisition of adherent properties, DNA and phosphatidylcholine synthesis were measured in the adherent and nonadherent cells at 16 hr (Table 1). In two separate experiments, approximately 75% of the cells were adherent at 16 hr. Whereas the adherent and nonadherent cells had equivalent inhibition of DNA synthesis, phosphatidylcholine synthesis was stimulated only in adherent cells. The rate of [^{14}C]choline incorporation into phosphatidylcholine in nonadherent cells was equivalent to that of control cells. This suggests a direct relationship between the stimulation of phosphatidylcholine synthesis and the subsequent acquisition of adherent cell properties. It also suggests that the inhibition of DNA synthesis does not depend on prior stimulation of phosphatidylcholine synthesis.

To examine the relationship between adherence and phosphatidylcholine synthesis further, cells were grown in polypropylene tubes, a surface to which they cannot adhere. The stimulation of phosphatidylcholine synthesis by PBu_2 under these conditions was equivalent to the stimulation seen with the usual tissue culture flasks. Thus, although adherence may depend on a sequence of events beginning with the stimulation of phosphatidylcholine synthesis, this stimulation is not dependent on the presence of the surface to which cells can adhere.

Development of α-Naphthyl Acetate Esterase (Nonspecific Esterase). The lysosomal enzyme, nonspecific esterase, is characteristic of monocytes and macrophages and is not found in HL-60 cells, myeloid leukemia cells, or HL-60 cells induced to undergo myeloid differentiation (7, 22). It is present after 72 hr of incubation in HL-60 cells induced along the macrophage pathway of differentiation (7). When HL-60 cells were incubated with PBu_2 for 5 hr, nonspecific esterase remained undetectable. After 72 hr, more than 95% of the cells were positive for this histochemical marker. When HL-60 cells were incubated with PBu_2 for 5 hr, washed, and incubated for the remainder of 72 hr in fresh medium, the esterase did not become expressed. In contrast, incubation of cells with PBu_2 for 16 hr permitted full development of esterase at 72 hr. Thus, exposure of cells to PBu_2 for a period of time sufficient to cause growth arrest in >50% of cells was insufficient for the induction of this macrophage-specific enzyme whereas exposure for a time sufficient to cause adherence led to its induction.

Table 1. Incorporation of [^{3}H]dThd and [^{14}C]choline into adherent and nonadherent HL-60 cells after 16 hr of incubation with 500 nM PBu_2

	Exp.	Nonadherent	Adherent
Cells, %	1	26	74
	2	23	77
[^{3}H]dThd incorporation, % of control cells	1	2.6	1.6
	2	12.4	12.2
[^{14}C]Choline incorporation, % of control cells	1	91	216
	2	133	380

DISCUSSION

These studies demonstrate that PBu_2 binds reversibly to the surface of HL-60 cells. The reversible nature of this binding has been described in mink lung cells, rat embryo fibroblasts, and human lymphocytes (16, 20, 21). While bound to the HL-60 cell, PBu_2 stimulates differentiation toward macrophages. Removal of PBu_2 from its binding sites halts the process with little tendency toward recovery and no evidence of progressive differentiation. There is no evidence of internalization of PBu_2. Rather, the ligand is retained at the cell surface, and its various actions appear to be mediated by transmembrane processes initiated by its surface membrane receptor.

How does PBu_2 on the cell surface mediate these various changes in HL-60 and other cells? One way may be through its effects on phospholipid metabolism. It has been reported that TPA stimulates phosphatidylcholine synthesis in HL-60 cells (25). In the present study with PBu_2, the stimulation of phosphatidylcholine synthesis was rapid, and it continued as long as PBu_2 was bound to the cell surface. An increased rate of phosphatidylcholine synthesis has also been observed in HeLa cells (27) and bovine lymphocytes (28) treated with phorbol esters. In HeLa cells it has been attributed to a stimulation of the enzymatic reaction catalyzed by CTP:phosphocholine cytidylyltransferase (29).

Stimulation of phosphatidylcholine synthesis via choline in the present study followed the same time course as the inhibition of phosphatidylcholine synthesis via the transmethylation of phosphatidylethanolamine (25). It also corresponded in time to an increase in $^{32}P_i$ incorporation into both phosphatidylcholine and phosphatidylethanolamine (30). It may also be related to changes in phospholipid metabolism in other cells, including the release of choline and arachidonic acid from phospholipids and an accelerated production of prostaglandin E (2, 31–33) and may represent a regeneration of phosphatidylcholine after its lipolysis.

The observation that cells that failed to display an accelerated rate of phosphatidylcholine synthesis also failed to adhere provides some circumstantial evidence for a causal association between these two phenomena. We recently reported that the acquisition of adherence by HL-60 cells coincides with a shift in the pattern of glycopeptide synthesis from one in which high molecular weight glycopeptides predominate to one in which low molecular weight glycopeptides predominate (34). Although phospholipid and glycoprotein synthesis generally have not been linked, an apparent relationship between these two membrane synthesis processes has been described recently in bacteria (24). Thus, the several membrane events—phosphatidylcholine synthesis, glycopeptide synthesis, and adherence—may be interrelated.

Unlike the induction of myeloid differentiation in HL-60 cells, which appears to proceed after one cell division (23), terminal differentiation induced by phorbol esters proceeds without cell division (26). Rovera *et al.* (26) have suggested that HL-60 cells are progressively recruited to differentiate as they enter a late stage in the G_1 phase of the cell cycle (26). Our results are consistent with that hypothesis, and they indicate that PBu_2 must be bound to its receptor as cells enter the recruitment stage. Very few recruited cells proceed to the S phase when PBu_2 is removed. However, cessation of DNA synthesis is not sufficient for the subsequent induction of phenotypic changes, such as adherence and nonspecific esterase.

The acquisition of adherent surface properties occurs approximately 8 hr after the decline in DNA synthesis. Among experiments, the maximal level of adherence varied from 70% to 90%. There are several indications that cessation of cell growth and induction of adherence are separately induced phenomena. First is the observation that removing PBu_2 at 8 hr is associated with a continued arrest of DNA synthesis in 70% of cells (Fig. 5), yet these cells do not become adherent in the absence of continued exposure to PBu_2 (Fig. 6). Second is the observation that DNA synthesis is inhibited in nonadherent cells 16 hr after exposure to PBu_2, whereas these cells fail to display an increased level of phosphatidylcholine synthesis (Table 1). This suggests that the effect of PBu_2 on phosphatidylcholine synthesis is unrelated to its effect on DNA synthesis. How events in the nucleus relate to the binding of PBu_2 on the cell surface is unclear. Possibly the effects on DNA and phosphatidylcholine synthesis are mediated by a transmembrane signal common to them both. Although the sequence of biochemical events that occurs after the binding of PBu_2 remains to be fully defined, the current study supports the concept that phorbol esters exert their effect at the cell surface.

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