Alterations of Glial Tumor Cell Ca$^{2+}$ Metabolism and Ca$^{2+}$-dependent cAMP Accumulation by Phorbol Myristate Acetate*

Margaret A. Brostrom, Charles O. Brostrom, Lori A. Brotman, Ching-shin Lee, Donald J. Wolff, and Herbert M. Geller

From the Department of Pharmacology, Rutgers Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

C6 glial tumor cells exposed to phorbol myristate acetate (PMA) possessed lowered cAMP content, reduced ability to accumulate cAMP in response to norepinephrine or cholera toxin, and a 3-fold increase in the concentration of norepinephrine producing 50% of the maximal rate of cAMP accumulation. Detectable effects on cAMP accumulation occurred within 10 min of exposure to PMA, and prominent effects by 2 h. PMA similarly affected cells pretreated with cycloheximide. In contrast, Ca$^{2+}$-depleted preparations of control and PMA-treated cells accumulated cAMP identically in response to norepinephrine or cholera toxin. Ca$^{2+}$ restoration, which increased the rate of cAMP accumulation in control cells severalfold, did not enhance cAMP accumulation in PMA-treated cells. Neither high catecholamine nor high extracellular Ca$^{2+}$ concentrations reversed the suppression of cAMP accumulation by PMA. Trifluoperazine, which inhibited the Ca$^{2+}$-dependent component of norepinephrine-stimulated cAMP accumulation in control cells, did not significantly reduce norepinephrine-stimulated cAMP accumulation in PMA-treated cells.

Cell-free preparations of control and PMA-treated cultures did not differ significantly in calmodulin content or in Ca$^{2+}$-stimulated adenylate cyclase, Ca$^{2+}$-dependent cAMP phosphodiesterase, and (Ca$^{2+}$-Mg$^{2+}$)-ATPase activities. The Ca$^{2+}$ content, however, of intact cells decreased with time of PMA treatment. Within minutes after exposure to PMA, the ability of Ca$^{2+}$-depleted cells to take up $^{45}$Ca was significantly reduced. Both $^{45}$Ca uptake and Ca$^{2+}$-dependent cAMP accumulation were reduced over the same PMA concentration range.

The mechanisms by which tumor promoters transforrino initiated, latent cells into tumor cells are incompletely understood. In cultured cells, the tumor-promoting phorbol esters produce numerous functional and biochemical changes closely resembling or mimicking those induced by hormones, retinoids, and transforming viruses (1, 2). Consequently, phorbol esters have frequently been employed in a broad variety of experimental models to study and define the biochemical events believed to occur in both oncogenic transformation and cell differentiation. Current hypotheses regarding the mechanisms through which phorbol esters exert their effects on cultured cells include actions at the cytoplasmic membrane and alterations in gene expression; the primary biochemical response to these agents, however, has not as yet been unequivocally identified.

Alterations in the cyclic nucleotide concentrations of mouse epidermal tissues are reported to occur following the application of phorbol myristate acetate (3). In addition, fibroblast (4) or epidermal (5) cells exposed to PMA exhibit a decreased ability to accumulate cAMP in response to hormones. The diminished responsiveness of PMA-treated epidermal tissues to catecholamines has been shown to involve RNA and protein synthesis-dependent uncoupling of the β-receptor from adenylate cyclase (6, 7). More recently, PMA was found to promote in myoblasts (8) rapid changes in Ca$^{2+}$ content and Ca$^{2+}$ fluxes which were hypothesized to alter cyclic nucleotide metabolism.

The C6 glial tumor cell line, which responds to β-adrenergic agonists with 100-fold increases in cAMP content, has been utilized as a model for the study of β-receptor-coupled adenylate cyclase systems. In addition, this cell type is useful for the investigation of potential interactions between Ca$^{2+}$ and cAMP. Accumulation of cAMP in intact cells in response to norepinephrine (9) or cholera toxin (10) has been shown to depend on intracellular Ca$^{2+}$, and cell-free preparations of C6 cells contain Ca$^{2+}$-stimulated forms of adenylate cyclase (11) and cyclic nucleotide phosphodiesterase (12). Since pretreatment of C6 cells with PMA was recently reported to produce a decreased cAMP response to isoproterenol (13), it was of interest to assess the role of Ca$^{2+}$ in this PMA-induced sub-sensitivity to hormone. The results obtained support the proposal that PMA promotes a Ca$^{2+}$ efflux from C6 cells following a reduction in the Ca$^{2+}$-dependent component of cAMP accumulation.

**EXPERIMENTAL PROCEDURES**

Materials—Phorbol myristate acetate, 4-a-d-phorbol, Tes, cycloheximide, and L-(−)-norepinephrine bitartrate were purchased from Sigma. Cholera toxin was purchased from Schwarz/Mann. Trifluoperazine was a gift of Smith, Kline and French Laboratories. $^{45}$CaCl$_2$, 40.5 mCi/mg, and [methoxy-3H]linulin, 515 Ci/mol, were obtained from New England Nuclear. Ham’s F-10 medium was purchased from Grand Island Biological Company. Calmodulin was purified from bovine brain by the procedure of Wolff et al (14), and calmodulin-deficient cyclic nucleotide phosphodiesterase was purified from beef brain extract by removal of endogenous calmodulin by column chromatography as previously described (15). Other reagents, enzymes, and isopes for assays of cAMP (9) adenylate cyclase (16).

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and phosphodiesterase and calmodulin (19) were as described. [32P] ATP was prepared by the method of Glynn and Chappell (17).

**Cell Culture and Preparation of Ca++-Depleted Cells—**Stock monolayer cultures of C6 cells were maintained (18) and experimental cultures grown (9) according to protocols previously described. Ca++-depleted cells were prepared from monolayer cultures grown in roller bottles (10) and suspended in Tes-buffered saline containing 1 mM EGTA and 1% L-glutamine. Ca++-depleted cells were prepared by adding CaCl2 to a concentration of 3 mM (2 mM in excess of EGTA) to portions of Ca++-depleted cells. Aliquots of cell suspensions were normally pretreated at 37 °C in glass or polyethylene tubes with or without CaCl2 (11) for 30 min. The Ca++ content of Ca++-depleted cells was routinely 4- to 6-fold greater than that of Ca++-depleted controls (9). All cell preparations were routinely 90-95% viable as assessed by exclusion of eosin Y. PMA and 4-a-phorbol was dissolved in dimethyl sulfoxide, and 5 μl of less of these stock solutions were added per ml to growing cultures or to Ca++-depleted and Ca++-restored cell preparations. Equivalent volumes of solvent were used in control preparations. Other test agents were dissolved in buffered saline and added in 10- to 20-μl volumes/ml of cell suspension.

**Determination of CAMP Content—**The CAMP contents of heated (10) extracts of cell suspensions or extracellular fluids alone were measured as previously described (9). Results were expressed as the mean ± standard deviation of triplicate or quadruplicate incubations from a single preparation of cells in a single experiment. Each experiment was performed at least three times to verify results, and experiments reported herein are considered representative.

**Adenylate Cyclase Assay—**Pellets of Ca++-depleted cells obtained by centrifugation at 1000 × g for 2 min were resuspended in 30 mM imidazole, pH 8.0, containing 9 mM MgCl2, 3 mM dithiothreitol, and 3 mM EGTA and homogenized by hand in a Potter-Elvehejm homogenizer fitted with a Teflon pestle. Aliquots (25 μl) of homogenate were assayed for enzyme activity as previously described (16) at pH 7.8 and in the presence of 5 mM MgCl2 and 500 μM EGTA. Results are expressed as the average of triplicate determinations ± the range of values obtained.

**ATPase Assay—**A modification of the method of Post and Sonn (19) was employed. Ca++-depleted cells were homogenized in 25 mM imidazole, pH 7.5, containing 7.5 mM MgCl2 and 1 mM EGTA. Aliquots of homogenate (150 μg of protein) were pretreated for 2 min at 37 °C in 120 μl containing 25 mM imidazole, pH 7.5, 5 mM MgCl2, 0.1 mM ouabain, and 1 mM EGTA either with or without 50 μM CaCl2. The reaction was initiated by the addition of 10 μl of 30 μM ATP containing 330 cpn/mmol of [32P]ATP. After 30 min the reaction was terminated with 100 μl of 0.6 N HCl containing 2% Norit A. Suspensions were centrifuged at 5000 × g for 5 min and aliquots of the supernatant fraction were assessed for radioactivity. Under these conditions, approximately 30-35% of the ATP was hydrolyzed. Zero time controls were subtracted as enzyme blank values.

**CAMP Phosphodiesterase Assay—**Control and PMA-treated cultures were washed with buffered saline and suspended by means of a plastic scraper in 10 mM imidazole, pH 7.5, containing 3 mM MgCl2. Suspensions were homogenized by sonic disruption and analyzed for cyclic nucleotide phosphodiesterase under standard conditions (12) at 0.1 mM EGTA with cAMP as substrate. Ca+++, calmodulin, and CAMP concentrations were as indicated in the text. Values were corrected for controls without enzyme.

**Determination of Ca++ Content and Ca++ Uptake—**For the measurement of the total Ca++ content of untreated and PMA-treated cells, a roller bottle culture was first equilibrated for 18 h at 37 °C with 250 μCi 45Ca in 50 ml of fresh Ham's F-10 medium containing 25 mM Tes, pH 7.5. The cells were then suspended in the radioactive medium with a plastic scraper and the suspension reequilibrated at 37 °C for 2 h. Suspensions were treated with PMA or solvent for varying periods. Aliquots (0.5 ml) were withdrawn from each experimental sample and added to 10 ml of ice-cold 150 mM NaCl containing 2.5 mM L-glutamate and 1.5% (w/v) serum albumin in conical glass centrifuge tubes. Equal volumes of 1000 × g for 6 min at 4 °C, supernatant fluids were removed and the tubes inverted. Traces of solution were removed from the sides of the tubes with cotton swabs and the cell pellets were dissolved in saline containing Triton X-100. Aliquots of dissolved cells were then analyzed for radioactivity. The contribution of extracellular 45Ca was estimated from the [methoxy-3H]inulin space of comparably treated unlabeled cells and subtracted. Results were expressed as the average ± range of values (6/experimental time point) obtained.

**RESULTS**

Inhibition of Ca++-dependent CAMP Accumulation by PMA—C6 cells were treated with 18 h with 0.1 μM PMA or solvent in complete growth medium. After harvesting, each culture was divided and used for the preparation of suspensions of (a) non-Ca++-depleted cells in fresh buffered Ham's F-10 medium, (b) Ca++-depleted cells in buffered saline, and (c) Ca++-restored cells in buffered saline. The three types of cell suspensions were assessed for cAMP content and for the ability to accumulate CAMP when challenged with either norepinephrine or cholera toxin (Table I). Unchallenged Ca++-depleted non-PMA-treated control cells were 25-30% lower in CAMP content than either non-Ca++-depleted or Ca++-restored controls, but of comparable content to all preparations of nonchallenged cells derived from PMA-pretreated cultures. CAMP accumulation in response to either 0.1 or 100 μM norepinephrine or to cholera toxin in Ca++-restored control cells was approximately 2.5 times greater than in Ca++-depleted control cells. Accumulation of CAMP in response to a saturating concentration (100 μM) of norepinephrine or to cholera toxin in control cells suspended directly in Ham's medium was not statistically different from that in Ca++-restored controls. When Ca++-depleted cells from the PMA-pretreated culture were challenged with either norepinephrine or cholera toxin, accumulation of CAMP was similar to that in control.

**TABLE I**

| Cell preparation | CAMP content (μM) | 0.1 μM norepinephrine | 100 μM norepinephrine | 6 μM cholera toxin | pmol-mg protein | 1
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<tbody>
<tr>
<td>Control, Ham's medium</td>
<td>42 ± 2</td>
<td>N.D.*</td>
<td>3370 ± 400</td>
<td>370 ± 120</td>
<td>1360 ± 100</td>
<td>100</td>
</tr>
<tr>
<td>Control, Ca++-depleted</td>
<td>44 ± 2</td>
<td>770 ± 50</td>
<td>1270 ± 40</td>
<td>440 ± 40</td>
<td>490 ± 10</td>
<td>100</td>
</tr>
<tr>
<td>Control, Ca++-restored</td>
<td>30 ± 6</td>
<td>N.D.*</td>
<td>2040 ± 200</td>
<td>520 ± 20</td>
<td>890 ± 10</td>
<td>100</td>
</tr>
<tr>
<td>PMA, Ham's medium</td>
<td>24 ± 6</td>
<td>550 ± 60</td>
<td>1340 ± 60</td>
<td>490 ± 10</td>
<td>380 ± 10</td>
<td>100</td>
</tr>
<tr>
<td>PMA, Ca++-depleted</td>
<td>28 ± 6</td>
<td>570 ± 90</td>
<td>1350 ± 60</td>
<td>380 ± 10</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

* N.D., not determined.
Ca²⁺-depleted control cells. However, restoration of Ca²⁺ to the PMA-pretreated cells did not enhance cAMP accumulation as it did in control cells. In addition, cAMP accumulation following 100 μM norepinephrine or cholera toxin in PMA-pretreated cells suspended directly into Ham's medium was reduced to 60% of that obtained in similarly prepared control cells. The norepinephrine concentration dependence of cAMP accumulation was examined for Ca²⁺-depleted and Ca²⁺-restored cell suspensions prepared from a solvent control and a PMA-pretreated culture (Fig. 1). Both preparations from the control culture (A) exhibited an apparent Kₐ₅ₐ of 80 nM. At saturating norepinephrine concentrations, however, cAMP accumulation in Ca²⁺-restored cells was 3-fold greater than that in Ca²⁺-depleted cells. As was observed with mouse epidermis (7), the apparent affinity for catecholamine was decreased following PMA treatment (B). For both Ca²⁺-depleted and Ca²⁺-restored preparations from the PMA-pretreated culture, a Kₐ₅ₐ of 250 nM for norepinephrine was obtained. In addition, accumulation of cAMP in Ca²⁺-restored cells was no greater than that observed in Ca²⁺-depleted cells at any norepinephrine concentration examined, and at saturating catecholamine the accumulation of cAMP in both preparations was equivalent to that obtained with Ca²⁺-depleted controls.

The extracellular CaCl₂ concentration dependence of cAMP accumulation in response to norepinephrine in PMA-treated and control cells is shown in Fig. 2. Accumulation of cAMP by control cells increased 3-fold as a function of increasing extracellular CaCl₂ concentration. Ca²⁺-depleted cells were prepared from cultures grown identically and pretreated for 18 h with either 0.1 μM PMA or solvent. Cells were treated for 30 min in medium containing the indicated concentrations of CaCl₂ and subsequently challenged with 10 μM norepinephrine for 20 min. cAMP measurements were performed on aliquots of cell suspensions. A, control cells; B, PMA-treated cells. Ca²⁺-depleted cells (○); Ca²⁺-restored cells (●).

![Fig. 1. Norepinephrine concentration-dependence of cAMP accumulation in Ca²⁺-depleted and Ca²⁺-restored preparations of control and PMA-treated C6 cells. Two roller bottle cultures of cells were seeded and grown identically from the same seed stock. Cultures were treated for 18 h prior to use with 0.1 μM PMA or with an equivalent volume (20 μl) of solvent. Ca²⁺-depleted and Ca²⁺-restored cells were then prepared from each culture and incubated with the indicated concentrations of norepinephrine for 20 min. cAMP measurements were performed on aliquots of cell suspensions. A, control cells; B, PMA-treated cells. Ca²⁺-depleted cells (○); Ca²⁺-restored cells (●).](http://www.jbc.org/)

![Fig. 2. Extracellular Ca²⁺ concentration-dependence of norepinephrine-stimulated cAMP accumulation in PMA-treated and control C6 cells. Ca²⁺-depleted cells were prepared from cultures grown identically and pretreated for 18 h with either 0.1 μM PMA or solvent. Cells were treated for 30 min in medium containing the indicated concentrations of CaCl₂ and subsequently challenged with 10 μM norepinephrine. After 20 min of incubation with hormone, cell suspensions were assessed for cAMP content. Control cells (○); PMA-treated cells (●).](http://www.jbc.org/)

![Fig. 3. Effect of PMA pretreatment on cAMP accumulation with time in Ca²⁺-depleted and Ca²⁺-restored C6 cells and the respective cAMP contents of their extracellular fluids following addition of norepinephrine. Ca²⁺-depleted (○, △) and Ca²⁺-restored (●, ▲) cells were prepared from cultures grown identically and pretreated for 18 h with either 0.1 μM PMA (△, ▲) or solvent (○, ●). Cell suspensions were challenged with 10 μM norepinephrine; at the times indicated, aliquots were removed and centrifuged immediately at 1000 × g for 2 min at 4 °C. Cell pellets and extracellular fluids were analyzed for cAMP content. A, cAMP contents of control cells; B, cAMP contents of PMA-treated cells; C, cAMP contents of extracellular media from control and PMA-treated cells.](http://www.jbc.org/)
external Ca²⁺; maximal nucleotide accumulation was obtained at 1 mM added CaCl₂. Since the EGTA concentration in the external medium was 1 mM, it was apparent that μM free Ca²⁺ concentrations were sufficient to restore maximal cAMP accumulation. In contrast, increasing external Ca²⁺ concentrations had relatively little effect on the ability of PMA-pretreated cells to accumulate cAMP.

The time dependences of norepinephrine-stimulated cAMP accumulation in Ca²⁺-depleted and Ca²⁺-restored cells prepared from a solvent control (Fig. 3A) and a PMA-pretreated culture (Fig. 3B) and of the respective cAMP contents of the extracellular medium from all cell preparations (Fig. 3C) were examined. The cAMP content of Ca²⁺-restored control cells increased rapidly during the first 20 min of incubation with hormone; the increase was then followed by a decline in nucleotide content which continued during the remaining 2½ h of incubation. Ca²⁺-depleted controls accumulated cAMP more slowly during the first 30 min of incubation, and the subsequent decline in cAMP content seen in Ca²⁺-restored controls was not as apparent. Ca²⁺-depleted and Ca²⁺-restored cells from the PMA-pretreated culture exhibited time courses of cAMP accumulation which were indistinguishable from each other and which were similar to that observed with Ca²⁺-depleted controls. The cAMP content of the extracellular medium from all cell preparations increased at a linear rate during the first 90 min of incubation and more slowly thereafter. The cAMP content of the medium from Ca²⁺-restored control cells was somewhat higher than that from Ca²⁺-depleted controls; the cAMP content of medium from both preparations of the PMA-pretreated culture was nearly identical to that from Ca²⁺-depleted controls.

Norepinephrine-stimulated cAMP accumulation in Ca²⁺-restored C6 cells is inhibited by trifluoperazine at micromolar concentrations, but nucleotide accumulation in Ca²⁺-depleted cells is unaffected by the drug (9). It was therefore of interest to ascertain whether this phenothiazine affected cAMP accumulation in PMA-pretreated preparations. Ca²⁺-depleted and Ca²⁺-restored cells were each prepared from solvent control and PMA-pretreated cultures. Each cell preparation was incubated for 10 min with varying trifluoperazine concentrations, then challenged with 100 μM norepinephrine for 20 min and subsequently assayed for cAMP content (Fig. 4). As was previously reported, cAMP accumulation in Ca²⁺-restored controls was inhibited by trifluoperazine concentrations between 10 and 30 μM; a 50% reduction in cAMP content was obtained with 30 μM drug. Accumulation of cAMP in Ca²⁺-restored PMA-pretreated cells was unaffected by low phenothiazine concentrations and reduced only 25% by 30 μM drug. Phenothiazine at these concentrations did not influence cAMP accumulation in Ca²⁺-depleted cells from control or PMA-pretreated cultures.

Accumulation of cAMP in intact C6 cells in response to cholera toxin is enhanced by Ca²⁺ (10), and the effects of Ca²⁺ on this process are in many respects comparable to effects of the cation on norepinephrine-stimulated cAMP accumulation in the same cell line. To determine whether PMA influenced cAMP formation in a manner which is agonist-specific, PMA-pretreated cultures were compared with controls with respect to enhancement by Ca²⁺ of cAMP accumulation in response to cholera toxin (Fig. 5). Ca²⁺-depleted and Ca²⁺-restored preparations of cells from control or PMA-pretreated cultures were challenged with varying concentrations of toxin, and the cAMP content of cell suspensions was determined after 45 min of incubation (Fig. 5A). Under these conditions with both preparations of control cells, maximal and half-maximal cAMP accumulation was obtained with 60 and 3 nm toxin, respectively. The cAMP content of Ca²⁺-restored controls, however, was 3-fold greater than that of Ca²⁺-depleted controls at all cholera toxin concentrations tested, with the exception of samples incubated without toxin. The cholera toxin concentration-dependence of cAMP accumulation in Ca²⁺-depleted preparations of PMA-pretreated cells resembled that observed in Ca²⁺-depleted control preparations. On the other
Ca\(^{2+}\) and cAMP in Phorbol Ester-treated Glial Tumor Cells

**Table II**

<table>
<thead>
<tr>
<th>PMA pretreatment period</th>
<th>−Norepinephrine cAMP</th>
<th>+Norepinephrine cAMP</th>
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<tbody>
<tr>
<td></td>
<td>Ca(^{2+})-depleted cells</td>
<td>Ca(^{2+})-restored cells</td>
</tr>
<tr>
<td>0 min</td>
<td>31 ± 2</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>10 min</td>
<td>31 ± 1</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>1 h</td>
<td>25 ± 1</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>2 h</td>
<td>24 ± 3</td>
<td>36 ± 3</td>
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<tr>
<td>5 days</td>
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</table>

**A.** Lack of effect of cycloheximide on the suppression by PMA of Ca\(^{2+}\)-dependent, norepinephrine-stimulated cAMP accumulation.

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>−Cycloheximide cAMP</th>
<th>+Cycloheximide cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, Ca(^{2+})-depleted</td>
<td>700 ± 10</td>
<td>710 ± 40</td>
</tr>
<tr>
<td>Control, Ca(^{2+})-restored</td>
<td>2140 ± 150</td>
<td>2100 ± 60</td>
</tr>
<tr>
<td>PMA, Ca(^{2+})-depleted</td>
<td>660 ± 30</td>
<td>510 ± 10</td>
</tr>
<tr>
<td>PMA, Ca(^{2+})-restored</td>
<td>660 ± 60</td>
<td>760 ± 40</td>
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</table>

Hand, Ca\(^{2+}\) did not enhance toxin-stimulated cAMP accumulation in these cell preparations. The time dependence of cAMP accumulation in response to cholera toxin in cell preparations from control and PMA-pretreated cultures was also investigated (Fig. 5). Ca\(^{2+}\)-restored control cells exhibited a time course of cAMP accumulation which typified the classical cellular response to cholera toxin; an initial lag phase of 15 min was observed and maximal cAMP accumulation was attained 90 min after addition of the agent. As previously reported, Ca\(^{2+}\)-depleted control cells accumulated cAMP much more slowly, with maximal nucleotide accumulation being attained after more than 2 h of incubation. While Ca\(^{2+}\)-restored cells from the PMA-pretreated culture accumulated cAMP somewhat more rapidly than did their Ca\(^{2+}\)-depleted counterparts, neither preparation of cells reached maximal nucleotide accumulation within 2 h of incubation, and the time courses of cAMP accumulation in these cells were remarkably similar to those of Ca\(^{2+}\)-depleted controls.

The experiments described above were conducted following 18 h of treatment with PMA. Consequently, the dependence of the suppression of Ca\(^{2+}\)-dependent cAMP accumulation on the duration of PMA exposure was determined, and evidence sought for or against a protein synthesis requirement of this inhibitory effect. Table IIA gives the cAMP contents of cell preparations following varying times of exposure to PMA. Unstimulated Ca\(^{2+}\)-restored cells showed small but significant decreases in cAMP content following 1-2 h of incubation with PMA. Norepinephrine-stimulated cAMP accumulation in Ca\(^{2+}\)-depleted cells was not altered by 2 h of PMA pretreatment; accumulation of cAMP following norepinephrine treatment in Ca\(^{2+}\)-restored cells, however, was reduced 25% by 10 min and 50% by 2 h of PMA pretreatment. Significantly greater reductions in the Ca\(^{2+}\)-dependent component of cAMP accumulation were not observed for longer (i.e. 5 days) pretreatments with PMA. To investigate the dependence of this PMA effect on protein synthesis, Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-restored preparations were pretreated for 70 min with or without cycloheximide and for 60 min with PMA or solvent, and were subsequently assessed for cAMP accumulation in response to norepinephrine (Table IIIB). Cycloheximide alone did not affect cAMP accumulation in control preparations, and the inhibition by PMA of the Ca\(^{2+}\)-dependent component of cAMP accumulation was not altered by pretreatment with this protein synthesis inhibitor. Cycloheximide pretreatment periods of up to 3 h also failed to alter the effect of PMA (data not shown).

Adenylate Cyclase Activity of PMA-treated Cells—The catecholamine-stimulated adenylate cyclase activity of homogenates of C6 cells is enhanced to a small extent (20-40%) by low free Ca\(^{2+}\) concentrations (11). Therefore, the enzyme activity of a PMA-treated culture was measured with or without Ca\(^{2+}\) at varying norepinephrine concentrations and was compared with activity from a solvent-treated control (Fig. 6). Adenylate cyclase activity in homogenates of control cells (Fig. 6A) was enhanced 5-fold by 0.1-1 mM norepinephrine, and an apparent K\(_{\text{act}}\) for norepinephrine of 8 \(\mu\)M was observed. Low free Ca\(^{2+}\) concentrations provided a small (25%) enhancement of activity at high catecholamine concentrations. Homogenates of PMA-pretreated cells contained the same amount of enzyme activity when assayed without catecholamine. One mM norepinephrine enhanced enzyme activity 5-fold and, in parallel with studies of cAMP accumulation in intact cells (Fig. 1), an increase in apparent K\(_{\text{act}}\) for hormone (20 \(\mu\)M) was observed. The degree of enhancement by Ca\(^{2+}\), however, of adenylate cyclase activity in homogenates of PMA-treated cells was similar to that observed in homogenates of control cells. Treatment of C6 cells with PMA, therefore, does not appear to result in a loss of adenylate cyclase catalytic component or in an altered sensitivity of the enzyme to Ca\(^{2+}\).

**Effects of PMA Pretreatment on Other Parameters**—The
**Table III**  
Calmodulin content and phosphodiesterase and ATPase activities of PMA-treated C6 cells  

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein mg ml⁻¹</th>
<th>Calmodulin ng calmodulin mg protein⁻¹</th>
<th>ATPase activity: cAMP pmol/min mg protein⁻¹</th>
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<td><strong>Control</strong></td>
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<tr>
<td><strong>PMA-treated</strong></td>
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**Table IV**  
Calcium content of PMA-treated C6 cells  

<table>
<thead>
<tr>
<th>Condition</th>
<th>Calcium content nmol mg protein⁻¹</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
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<tr>
<td><strong>PMA-treated</strong></td>
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</table>

**Table V**  
Effect of PMA on ⁴⁵Ca uptake by calcium-depleted C6 cells  

<table>
<thead>
<tr>
<th>Condition</th>
<th>Calcium content nmol mg protein⁻¹</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
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<tr>
<td><strong>PMA-treated</strong></td>
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Ca⁺ and cAMP in Phorbol Ester-treated Glial Tumor Cells  

Effects of treatment with PMA on other parameters recognized as influencing Ca⁺ and/or cAMP metabolism were examined (Table III). The calmodulin content (Table IIIA) of cells treated for 18 h with 0.1 μM PMA was identical with that of a solvent control. cAMP phosphodiesterase activity (Table IIIIB) was assessed at three substrate concentrations in the presence of EGTA, Ca⁺⁺, or Ca⁺ and calmodulin; the activities from control and PMA-treated cells were found to be comparable. Mg⁺⁺-ATPase activity (Table IIIIC) of control C6 cells was stimulated 30% by low Ca⁺⁺ concentrations. Higher Ca⁺⁺ was inhibitory (data not shown). PMA-treated cells were observed to contain the same amount of Mg⁺⁺-ATPase activity as untreated cells, and this activity was similarly enhanced by low Ca⁺⁺ concentrations.

Alterations in Ca⁺⁺ Content and Transport Following PMA Treatment—The total Ca⁺⁺ content of C6 cells was examined as a function of duration of treatment with PMA (Table IV). Cells were pre-equilibrated in buffered medium with ⁴⁵Ca for 18 h and subsequently treated with either PMA or an equivalent volume of solvent for the time periods indicated. Intracellular ⁴⁵Ca content of control cells was not altered during 5 h of incubation with solvent. However, the ⁴⁵Ca content of PMA-treated cells was observed to decrease slowly during the incubation period; 2 and 5 h of incubation with the agent resulted in losses of intracellular ⁴⁵Ca of 18 and 24%, respectively.

The abilities of Ca⁺⁺-depleted cell preparations from a control culture and from a culture pretreated for 18 h with 0.1 μM PMA to take up ⁴⁵Ca were compared (Table V). Uptake of ⁴⁵Ca by Ca⁺⁺-depleted control cells was rapid, with maximal uptake occurring within 2 min of incubation with labeled cation. Ca⁺⁺-depleted preparations of PMA-pretreated cells took up ⁴⁵Ca as rapidly as did control preparations but accumulated only half as much cation. In a separate experiment, cultures were treated for 18 h with solvent, with 0.1 μM PMA or with 0.1 μM 4-α-d-phorbol, a PMA analog believed to lack tumor-promoting properties (1, 2). Ca⁺⁺-depleted cell preparations from each of these cultures were then assessed for their ability to take up ⁴⁵Ca. PMA-pretreated cells accumulated only one-third as much ⁴⁵Ca as did control cells, whereas pretreatment with 4-α-d-phorbol did not affect the capacity of Ca⁺⁺-depleted cells to take up cation. The capacity of Ca⁺⁺-depleted cells to ⁴⁵Ca after varying times of pretreatment with PMA or solvent was also examined (Fig. 7). Cells treated with PMA for 1 min prior to addition of ⁴⁵Ca took up 25% less cation than did solvent-treated controls. Following PMA pretreatment periods of 10, 20, and 180 min, ⁴⁵Ca uptake was reduced to 50% of that observed with solvent controls.

The PMA concentration-dependence of inhibition of Ca⁺⁺-dependent cAMP accumulation was compared with that of inhibition of ⁴⁵Ca uptake (Fig. 8). Ca⁺⁺-depleted and Ca⁺⁺-restored cells were prepared from cultures which had been pretreated for 3 h in buffered Ham's medium with the indicated concentration of PMA. The effects of treatment with PMA on other parameters recognized as influencing Ca⁺⁺ and/or cAMP metabolism were examined (Table III). The calmodulin content (Table IIIA) of cells treated for 18 h with 0.1 μM PMA was identical with that of a solvent control. cAMP phosphodiesterase activity (Table IIIIB) was assessed at three substrate concentrations in the presence of EGTA, Ca⁺⁺, or Ca⁺ and calmodulin; the activities from control and PMA-treated cells were found to be comparable. Mg⁺⁺-ATPase activity (Table IIIIC) of control C6 cells was stimulated 30% by low Ca⁺⁺ concentrations. Higher Ca⁺⁺ was inhibitory (data not shown). PMA-treated cells were observed to contain the same amount of Mg⁺⁺-ATPase activity as untreated cells, and this activity was similarly enhanced by low Ca⁺⁺ concentrations.

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suspensions were then incubated for 3 h with the indicated concentrations of PMA and subsequently assessed for their capacity to take up 45Ca, the results shown in Fig. 8B were obtained. The greatest reduction (86%) in 45Ca uptake was obtained with 50 nM PMA, and a 28% reduction in cation uptake was observed with 10 nM PMA.

Morphology of PMA-treated C6 Cells—C6 cells treated with PMA assume many of the morphological changes noted in other cell types treated with this agent (1). When viewed by phase contrast microscopy, control monolayer cultures grown to late logarithmic phase contain cells which are relatively refractile to light and which tend to align in parallel arrays. Many rounded cells are present. PMA-treated cultures contain cells which are much less refractile to light and which assume a criss-crossed, disoriented arrangement. Few rounded cells are found. These PMA-induced changes in morphology are observable after 4-6 h and are maximal after 12-24 h of incubation with PMA. Alterations in Ca2+ and cAMP metabolism, therefore, are observed prior to the changes in morphology produced by this agent.

**DISCUSSION**

Accumulation of cAMP in intact C6 glial tumor cells in response to catecholamines (9) or to choleter toxin (10) was previously shown to be partially dependent on intracellular Ca2+. The results of the present study show that treatment of C6 cells with PMA specifically suppresses the Ca2+-dependent component of cAMP accumulation. The residual cAMP-accumulating ability shows little requirement for Ca2+ and occurs with an increased apparent K<sub>a</sub> for norepinephrine (Fig. 1). It appears, therefore, that PMA reduces cAMP accumulation through an effect which is independent of alterations in the β-receptor and which, furthermore, is independent of new protein synthesis (Table II).

Several findings provide support for proposing that PMA sponsors an early Ca2+ efflux from C6 cells with subsequent intracellular Ca2+ depletion, one manifestation of which is a decreased capacity to accumulate cAMP. First, PMA treatment was shown to lower total cell Ca2+ content (Table IV). Second, PMA-treated cells markedly resembled Ca2+-depleted controls in terms of basal cAMP content (Tables I and II) and in the ability to accumulate cAMP in response to (a) saturating norepinephrine concentrations (Table I; Figs. 1 and 3) or (b) choleter toxin at varying concentrations of times of incubation (Fig. 5). Accumulation behavior was also similar for both types of preparations in incubations containing trifluoperazine. Third, Ca2+-depleted cells prepared from PMA-treated cultures took up much less 45Ca than did Ca2+-depleted controls (Table V; Fig. 7) suggesting that, in the treated cells, mobilization of Ca2+ from intracellular to extracellular pools is favored. These reductions in 45Ca uptake were detectable at earlier times following PMA exposure (1 min, Fig. 7) than were reductions in Ca2+-dependent cAMP accumulation (10 min, Table II), and are consistent with a cause-effect relationship between the two PMA-mediated effects. Finally, it was observed that the capacities of C6 cells to take up 45Ca and to accumulate cAMP in a Ca2+-dependent manner were reduced over the same PMA concentration range (Fig. 8).

Two hours following addition of PMA, suppression of both 45Ca uptake and cAMP accumulation was nearly maximal, whereas total cell Ca2+ content was reduced only 20% (Table IV). Five hours of incubation with PMA reduced total Ca2+ content only an additional 5%. Such observations support the concept that PMA mobilizes a specific intracellular Ca2+ pool that functions in untreated cells in some manner, as yet undefined, to support maximal degrees of cAMP accumulation in C6 cells. While the location of this pool is in doubt, it may...
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conceivably be associated with the cell plasmalemma. The various components of adenylate cyclase are widely recognized to be associated with the cell membrane, and PMA is known to express a variety of actions at the cell surface (1, 2). Membrane-associated pools of Ca2+ in several cell types (21-23) have been postulated to be affected by hormones.

In erythrocytes, increased free intracellular Ca2+ concentrations are believed to activate a Ca2+-dependent ATPase resulting in active transport of Ca2+ across the plasmalemma into extracellular fluids with subsequent lowering of free intracellular Ca2+ concentrations and total cell Ca2+ content (24). Whether free intracellular Ca2+ concentrations initially increase in C6 cells in response to PMA cannot be determined from our studies. However, since most adenylate cyclase activities are inhibited by relatively high Ca2+ concentrations, it is plausible that reductions in cAMP accumulation following PMA reflect an inhibition of adenylate cyclase from the generation of high free Ca2+ concentrations intracellularly. Two observations rule against this proposal. First, accumulation of cAMP by PMA-treated cells exposed to a variety of incubation conditions was consistently similar to that by Ca2+-depleted controls. Second, supraphysiologic extracellular Ca2+ concentrations and total cell Ca2+ content in PMA-treated cells (Fig. 2). The dramatic alterations in cAMP accumulation within cells following either Ca2+ depletion or PMA treatment are not reflected in the cAMP contents of the extracellular medium (Fig. 3). Doore et al. (25), however, observed that cAMP in the medium of C6 cells treated with catecholamine did not increase in parallel with cell cAMP. While EGTA did not affect cAMP transport, inhibitors of mitochondrial function reduced both cellular ATP concentrations and cAMP efflux from cells. On the other hand, probenecid and prostaglandin A2 inhibited cAMP efflux without lowering the ATP content of the cells. It was concluded that cAMP transport across C6 cell membranes is energy-dependent and subject to regulation. Although the ATP contents of Ca2+-depleted and Ca2+-restored cells do not differ significantly (9), neither the ATP concentrations of PMA-treated cells nor the capacity of PMA to inhibit cAMP transport has been examined. However, it seems unlikely that the cAMP transport system is a major site of action of PMA since the intracellular to extracellular cAMP concentration ratio of PMA-treated cells during short term incubations with norepinephrine and at a given intracellular cAMP concentration did not differ markedly from that of untreated cells. In addition, intracellular and extracellular cAMP concentrations of PMA-treated cells exposed to norepinephrine were comparable to those of Ca2+-depleted controls at all incubation times examined.

In many cell types, PMA has been shown to mimic the action of hormones or other physiologic regulatory substances which exert their effects through Ca2+ transients. In these cell types, evidence in favor of a Ca2+ prerequisite for PMA effects is extensive (26-30). It is unclear whether C6 cells are similarly affected since catecholamines, prostaglandins, and cholera toxin do not appear to mobilize intracellular Ca2+ in these cells. Exposure to serum, however, acutely lowers the cAMP content of C6 cells (31); thus, it is possible that PMA mimics the action of factors, as yet unidentified, which regulate glial cell metabolism or function through Ca2+ fluxes.

Conclusive evidence is lacking to establish how closely disturbances of Ca2+ storage by PMA are associated with the primary actions of the ester. Nonetheless, it is attractive to speculate that Ca2+ is the effector of at least some of the secondary effects of PMA. Ca2+ has been proposed to mediate, in nontumored cells, certain of the PMA effects reputedly associated with the tumor-promoting capacity of this agent (32, 33). It should be recognized, of course, that the C6 cell line was originally cloned from a chemically induced rat tumor (34). Therefore, it is unclear how fully the findings reported here can be related to the effects of PMA on tumor production or Ca2+ metabolism in normal tissue. Nonetheless, PMA appears to be a potentially useful pharmacologic tool both in the identification of Ca2+-regulated processes in intact cells and in the investigation of interactions which occur between Ca2+ and cyclic nucleotides in vivo.

REFERENCES
5. Grimm, W., and Marks, F. (1974) Cancer Res. 34, 3128-3134

M. A. Brostrom, unpublished observations.
Alterations of glial tumor cell Ca2+ metabolism and Ca2+-dependent cAMP accumulation by phorbol myristate acetate.

M A Brostrom, C O Brostrom, L A Brotman, C Lee, D J Wolff and H M Geller


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