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Phorbol ester inhibits furosemide-sensitive potassium transport in BALB/c 3T3 preadipose cells

(phorbol 12-myristate 13-acetate/Na^+K^+/Cl^- cotransport/$^{22}Na^+$ uptake/K^+ content/cell volume)

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ABSTRACT **The tumor promoter phorbol 12-myristate 13-acetate (PMA) rapidly decreased the rate of $^{86}Rb^+$ uptake into BALB/c 3T3 preadipose cells. The component of total $^{86}Rb^+$ influx affected by PMA is insensitive to ouabain but sensitive to the diuretic furosemide. Experiments designed to investigate the characteristics of the K^+ transport system sensitive to PMA revealed that: (*i*) $^{86}Rb^+$ uptake is highly dependent on external Na^+, (*ii*) $^{86}Rb^+$ uptake is highly dependent on external Cl^-, (*iii*) $^{22}Na^+$ uptake is dependent on external K^+, and (*iv*) a major component of $^{86}Rb^+$ efflux that is sensitive to PMA and furosemide is not dependent on extracellular K^+. These features strongly implicate a Na^+K^+/Cl^- cotransport system as the target of PMA and furosemide in these experiments. PMA caused a net intracellular accumulation of K^+ within 15 min in these cells, presumably via its inhibitory effect on furosemide-sensitive K^+ transport. Within 30 min after PMA treatment, the mean cell volume was significantly reduced in treated compared to control cells, with a maximum decrease of 21% attained at 4 hr after PMA. The significance of these findings for biologic changes induced by PMA is discussed.**

In addition to their role in multistage carcinogenesis, the phorbol ester tumor promoters, exemplified by phorbol 12-myristate 13-acetate (PMA), cause a number of biologic and functional changes in diverse cell types in culture (for reviews, see refs. 1 and 2). To date, however, no experimental results to explain any of the actions of tumor promoters in molecular terms have been available. A key unanswered question is the identity in promoter-treated cells of early events that trigger later changes in cell function. Recently, specific binding sites for phorbol esters have been demonstrated in tissues and cells in culture (3, 4), but the relationship of these sites to subsequent promoter-induced events remains obscure.

Despite the importance of rapid changes in ion fluxes in many biologic processes, few previous studies have addressed the question of whether tumor promoters alter ion movements or intracellular concentrations. In chicken myoblast cultures, PMA has been reported to rapidly decrease Ca^{2+} fluxes and intracellular Ca^{2+} content (5). In some nonmuscle cell lines, PMA apparently can "sensitize" cells to Ca^{2+}, thereby allowing the synthesis of DNA to proceed under conditions (lower medium Ca^{2+} concentration) that usually block cell cycle progression at or near the G_1/S boundary (6, 7). Moroney *et al.* (8) reported that an early response of PMA-treated Swiss 3T3 cells was a stimulation of ouabain-sensitive $^{86}Rb^+$ uptake (i.e., Na^+/K^+ pump-mediated). Dicker and Rozengurt (9) later confirmed this finding and suggested that the stimulation of pump activity by PMA was caused by an increased Na^+ influx, although the mechanism of this effect was not specified.

In an attempt to relate early changes in ion movements or redistributions to subsequent promoter-induced biologic events, we have studied the effect of PMA on various modes of K^+ transport in BALB/c 3T3 preadipose cells (clones A3IT). This cell line is well suited for these studies because previous work has detailed numerous responses to PMA, including stimulation of sugar transport activity (10), increased rate of glycolysis (11), and inhibition of cell differentiation (12). Of particular importance to this study is the observation that PMA is mitogenic in quiescent cultures of this cell line (12), as it is for other 3T3 cell lines (8, 9). In contrast to the earlier work of others (8, 9) showing an increased rate of $^{86}Rb^+$ uptake caused by PMA, we found in these cells that inhibition of $^{86}Rb^+$ uptake was an early event after PMA treatment. The transport system inhibited by PMA in these cells is insensitive to ouabain and sensitive to the diuretic furosemide, and it apparently catalyzes the cotransport of Na^+, K^+, and Cl^-.

MATERIALS AND METHODS

Cells. BALB/c 3T3 cells (clone A3IT) were obtained from Leila Diamond (Wistar Institute) and cultured as described (12). The medium used was Eagle Auto-Pow minimal essential medium (Flow Laboratories) supplemented with 10% fetal bovine serum. For flux and ion content experiments, cells were plated from stocks into 35- or 60-mm plastic Petri dishes and used when growing logarithmically or just after reaching confluence. Routinely, cultures were refed with fresh medium every 2–3 days and always on the day prior to experiments.

Flux Experiments. The uptake of $^{86}Rb^+$ was measured in cells maintained in complete culture medium at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells in 35-mm dishes were washed with warm phosphate-buffered saline (unless otherwise stated) and uptake was started by the addition of 2 ml of fresh medium containing 10% fetal bovine serum (K^+, 5.4 mM) containing tracer $^{86}Rb^+$ (0.5–1 μCi/ml; 1 Ci = 3.7×10^{10} Bq) and other agents as stated in the figure legends. In some experiments, 2 mM ouabain was added to cultures 10 min prior to the start of the uptake period to ensure maximal inhibition of the Na^+/K^+ pump, but no difference was detected with this protocol compared to simultaneous addition of ouabain and $^{86}Rb^+$. Preliminary experiments established that 2 mM ouabain was optimal for inhibiting the Na^+/K^+ pump of these cells under the experimental conditions described. Uptake in the presence or absence of ouabain was linear for at least 20 min; routinely, a 5-min uptake period was used. Uptake was terminated by aspirating the $^{86}Rb^+$ medium and then rapid washing with ice-cold 0.1 M $MgCl_2$ as described (13). Cells were solubilized in 3% Na_2CO_3/0.1 M NaOH/1% sodium deoxycholate. Aliquots were taken for radioactivity determinations in a liquid scintillation counter using ACS II cocktail (Amersham/Searle)

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

and analyzed for protein content by the procedure of Lowry *et al.* (14).

$^{22}Na^+$ influx was measured essentially as described by Schuldiner and Rozengurt (15). Cells in 35-mm dishes were washed with 5 ml of uptake medium [38 mM choline HCO_3/70 mM choline Cl/38 mM Hepes/1.8 mM $CaCl_2$/0.9 mM $MgCl_2$/5 mM glucose/0.9 mM NaH_2PO_4/10% dialyzed (vs. 140 mM choline Cl) fetal bovine serum, pH 7.5] and incubated in 0.94 ml of this medium at 37°C in a humidified air atmosphere. After 30 min, ouabain was added to 2 mM final concentration. Ten minutes later, $^{22}NaCl$ (30 mM final concentration, 1–2 μCi per dish) was added with or without KCl (10 mM) and uptake was terminated 1 min later by the 0.1 M $MgCl_2$ washing procedure described above. Under these conditions, uptake rapidly becomes saturated (by 20 min) but approximately linear uptake rates were obtained at time points of 1 min or less. A blank value obtained by incubating precooled cells with $^{22}Na^+$-containing medium at 0–4°C for 5 sec followed by rapid washing was subtracted from each experimental value.

Intracellular K^+ and Cell Volume Determinations. For determination of cellular K^+ content, cells in 60-mm dishes were treated with PMA for various time periods and then rapidly rinsed with 12 ml of ice-cold 0.1 M $MgCl_2$. The dishes were drained and allowed to dry, and the cells were lysed by the addition of 1.0 ml of distilled H_2O. After scraping and transfer (with an additional wash with 0.5 ml of H_2O) to tubes, the lysates were sonicated and K^+ concentration was measured in an atomic absorption spectrometer (EEL, model 140) with standard solutions of known K^+ concentration used to calibrate the machine. Results were expressed as μmol of K^+ per mg of protein.

Cell volume was determined by using a Coulter electronic particle counter with C1000 Channelyzer attachment. Cell suspensions were prepared by aspirating the culture medium, washing once with phosphate-buffered saline, and then incubating cells in trypsin/EDTA solution until detached. Medium was added to stop trypsin action and the cells were diluted in Isoton solution for sizing in the Coulter Counter. The machine was calibrated with monosized polystyrene microspheres of known diameter.

Materials. PMA was purchased from Chemicals for Cancer Research (Eden Prairie, MN). $^{86}RbCl$ and $^{22}NaCl$ were obtained from Amersham. Furosemide and ouabain were from Sigma.

RESULTS

Effect of PMA on $^{86}Rb^+$ Influx. Unidirectional K^+ influx in confluent 3T3 cells treated for various time periods with 0.16 μM PMA was estimated by measuring $^{86}Rb^+$ uptake in the presence and absence of ouabain (Fig. 1). At all time points tested, total $^{86}Rb^+$ uptake was decreased by PMA. When ouabain was used to separate the total $^{86}Rb^+$ uptake into Na^+/K^+ pump-mediated and other pathways, it became apparent that the effect of PMA was to decrease uptake by ouabain-insensitive pathway(s) without affecting pump-mediated $^{86}Rb^+$ fluxes (Fig. 1 *Inset*). A similar inhibition by PMA of ouabain-insensitive $^{86}Rb^+$ uptake occurred in exponentially growing 3T3 cells (data not shown).

The diuretic furosemide is known to inhibit ouabain-insensitive K^+ fluxes in several cell types including BALB/c 3T3 cells (16–21). Fig. 2 compares the effects of PMA and furosemide on ouabain-insensitive $^{86}Rb^+$ uptake. It is apparent that both agents inhibit a major fraction of this mode of $^{86}Rb^+$ uptake. PMA and furosemide inhibited 74% and 70%, respectively, of the total ouabain-insensitive uptake; the combination of PMA plus furosemide inhibited uptake by 82%. The PMA effect on

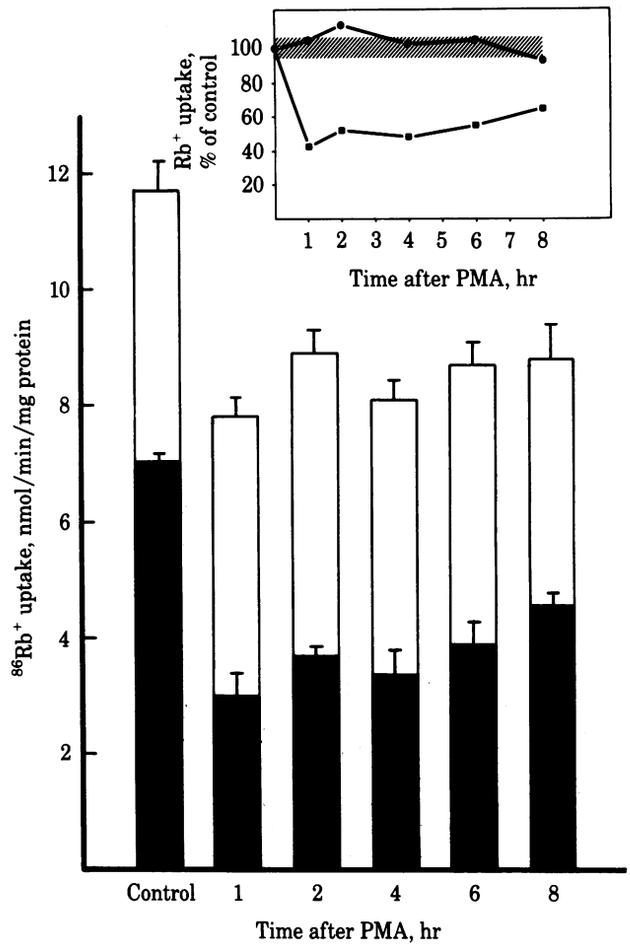

FIG. 1. Effect of PMA on $^{86}Rb^+$ uptake in confluent BALB/c 3T3 cells. PMA was added in a small volume of conditioned medium to a final concentration of 0.16 μM and $^{86}Rb^+$ uptake in the presence (solid bar) and absence (total height of bar) of 2 mM ouabain was measured at the indicated times after treatment. Results are shown as mean ± SEM of triplicate determinations. (*Inset*) Same data expressed as a percentage of the control (no PMA) uptake that occurred in the presence of ouabain (■) or that was inhibited by ouabain (●).

furosemide-sensitive uptake is virtually immediate because addition of PMA at the same time as the $^{86}Rb^+$ medium used to measure uptake (5 min time point in Fig. 2 *Inset*) produced a nearly maximal degree of inhibition. This uptake system is very sensitive to PMA; from dose–response curves, a half-maximal inhibition of ouabain-insensitive $^{86}Rb^+$ uptake occurred at 2 nM (data not shown). For comparison, this value for furosemide was 25 μM, or 12,500-fold greater than that for PMA.

Characteristics of the Furosemide-Sensitive Transport System. There are at least two possibilities for the nature of the K^+ transport system sensitive to PMA and furosemide in these cells: a Na^+K^+/Cl^- cotransport system (16–20) or a K^+/K^+ exchange diffusion system (21). To distinguish between these possibilities, four types of experiments were done. We measured: (*a*) the Na^+ dependence of ouabain-insensitive $^{86}Rb^+$ influx, (*b*) the Cl^- dependence of ouabain-insensitive $^{86}Rb^+$ influx, (*c*) the K^+ dependence of ^{22}Na influx in sodium-depleted cells, and (*d*) the effect of external K^+ concentration on $^{86}Rb^+$ efflux.

A major component of ouabain-insensitive $^{86}Rb^+$ influx in 3T3 cells was highly dependent on external Na^+, with a half-maximal stimulation occurring at approximately 33 mM Na (Fig. 3). This Na^+-dependent $^{86}Rb^+$ uptake was almost completely abolished at all Na^+ concentrations when PMA or furosemide was present in the external medium. Similarly, ouabain-insen-

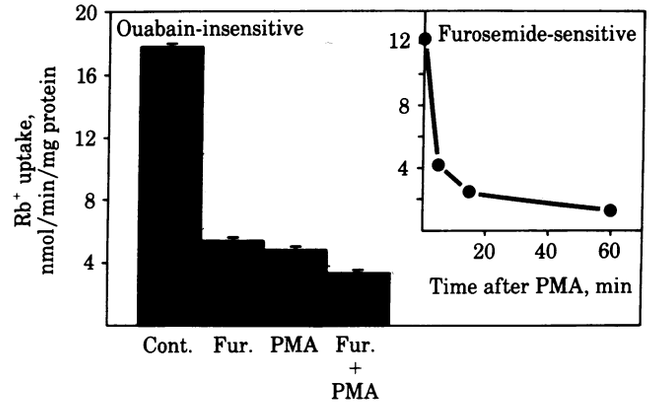

FIG. 2. Effect of furosemide (Fur.) and PMA on ouabain-insensitive $^{86}Rb^+$ uptake. Control (Cont.) or PMA-pretreated (0.16 μM for 1 hr) cells were assayed for $^{86}Rb^+$ uptake in the presence or absence of 1 mM furosemide. Ouabain was present during all uptake assays. Results are shown as mean ± SEM of triplicate dishes. (*Inset*) Time course of the PMA effect on furosemide-sensitive $^{86}Rb^+$ uptake. "Furosemide-sensitive $^{86}Rb^+$ uptake" is defined as uptake in the absence of furosemide minus uptake in the presence of the drug. Points are the means of triplicate determinations.

sitive $^{86}Rb^+$ uptake was dependent on external Cl^- (Fig. 4). As the concentration of medium Cl^- increased from 0 mM to 120 mM, uptake increased in a sigmoidal fashion. The half-maximally effective Cl^- concentration in this experiment was approximately 60 mM. This value and the sigmoidal dependence of Cl^--stimulated $^{86}Rb^+$ uptake are similar to published data on the Na^+K^+/Cl^- cotransport system in MDCK cells (22). As expected, Cl^--dependent $^{86}Rb^+$ uptake via this system was completely inhibited by furosemide and greatly decreased by PMA.

One explanation for the Na^+ dependence of $^{86}Rb^+$ uptake (Fig. 3) is that Na^+ is actually being transported by the system being studied. To address this question directly, we measured the ouabain-insensitive uptake of $^{22}Na^+$ in the presence and absence of external K^+ (Table 1). Although the presence of K^+ did not affect the final equilibrium value of $^{22}Na^+$ accumulated within the cells (data not shown), the 1-min uptake of isotope was significantly stimulated by 10 mM K^+ in the uptake medium. This K^+-dependent uptake, but not the basal K^+-independent uptake, was essentially abolished by PMA or furosemide.

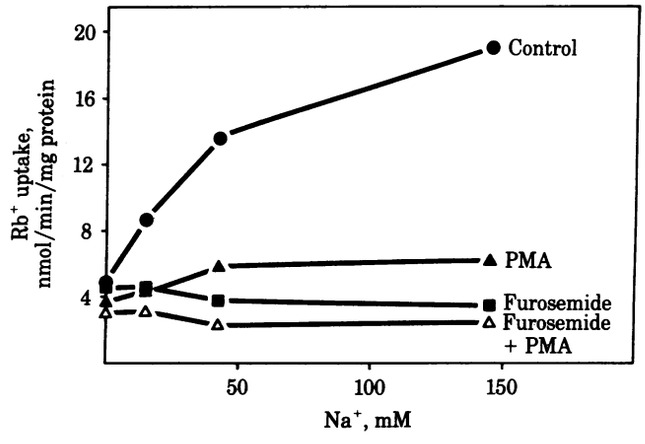

FIG. 3. Na^+ dependence of ouabain-insensitive $^{86}Rb^+$ uptake. Cells grown in standard medium were washed with 140 mM choline Cl and assayed for ouabain-insensitive $^{86}Rb^+$ uptake in culture medium of different Na^+ concentrations. Choline Cl was added to maintain osmolarity. When present, PMA was added to a final concentration of 0.16 μM 1 hr prior to assay and was present in the uptake medium. Furosemide (1 mM) was only present during the uptake period. Points are the means of duplicate dishes.

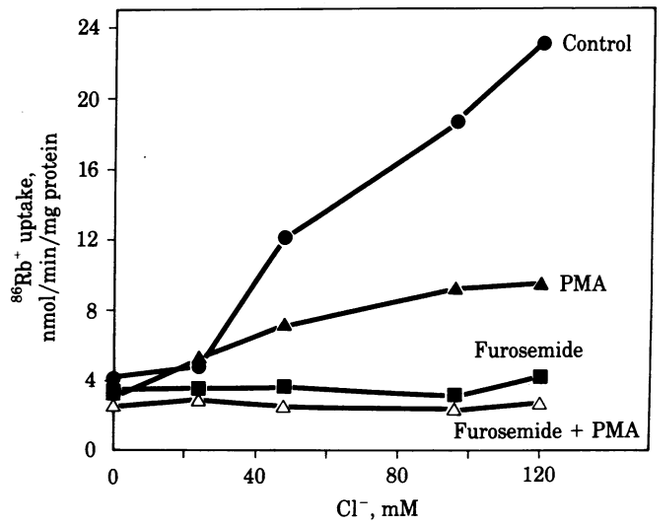

FIG. 4. Cl^- dependence of ouabain-insensitive $^{86}Rb^+$ uptake. Cells grown in standard medium were washed with 0.25 M sucrose and assayed for ouabain-insensitive $^{86}Rb^+$ uptake in culture media at different Cl^- concentrations. Sodium gluconate was added to maintain osmolarity when the Cl^- concentration was decreased. When present, PMA was added to a final concentration of 0.16 μM 1 hr prior to assay and was present during the uptake period. Furosemide (1 mM) was only present during the uptake period. Points are the means of duplicate dishes.

If furosemide-sensitive K^+/K^+ exchange were responsible for PMA-induced changes in $^{86}Rb^+$ fluxes, the efflux of $^{86}Rb^+$ should be affected by removal of external K^+. To test this hypothesis, the experiment shown in Fig. 5 was done. In the absence of external K^+, $^{86}Rb^+$ efflux was slowed somewhat, but it is apparent that furosemide still effectively inhibited this process. This result suggests that the major component of K^+ efflux in these cells does not depend on external K^+; i.e., it is not a K^+/K^+ exchange system. Similar experiments (not shown) indicated that PMA can also inhibit $^{86}Rb^+$ efflux into K^+-free medium as well as into medium of normal K^+ concentration. Taken together, the above series of experiments strongly suggests that the K^+ transport system sensitive to both PMA and furosemide

Table 1. K^+ Dependence of $^{22}Na^+$ uptake: Effect of PMA and furosemide

Treatment	K^+, mM	$^{22}Na^+$ uptake, nmol/min/mg protein: Total*	K^+ dependent
Control	0	65.1 ± 4.8	33.6
	10	98.7 ± 5.1†	
PMA	0	58.4 ± 10.0	0
	10	58.3 ± 10.7	
Furosemide	0	58.2 ± 4.3	3.3
	10	61.5 ± 2.9	

Exponentially growing cells were washed and assayed for $^{22}Na^+$ uptake over a 1-min period in the presence or absence of K^+ at 10 mM. Treatment with PMA (0.16 μM) was for 40 min prior to uptake. Furosemide was added to 1 mM (final concentration) just before addition of $^{22}Na^+$. Ouabain (2 mM) was present throughout.

*Mean ± SEM of triplicate dishes.

†$P < 0.01$ for difference from 0 mM K^+ control.

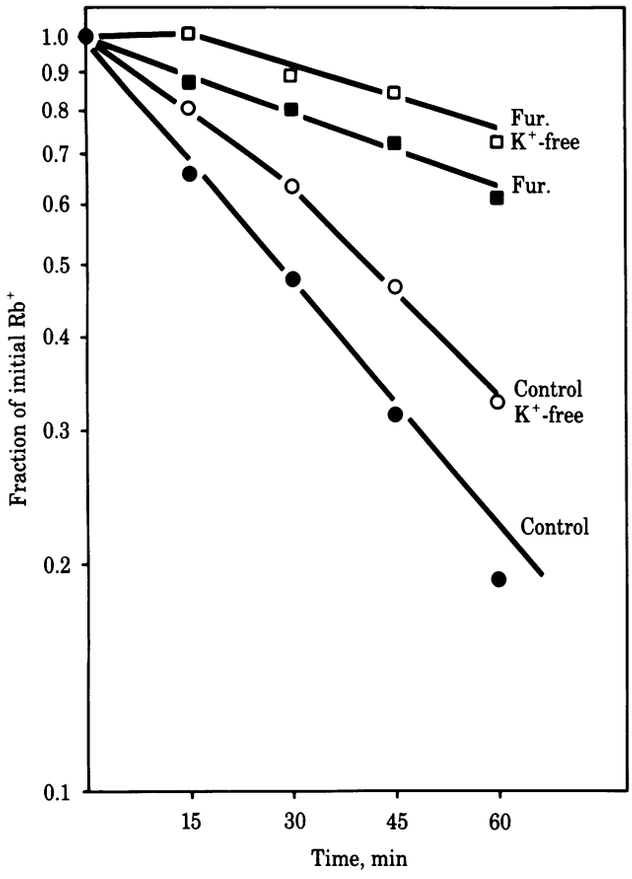

FIG. 5. Effect of furosemide (Fur.) on $^{86}Rb^+$ efflux into standard or K^+-free medium. Cells were preloaded with $^{86}Rb^+$ for 4 hr in regular medium and then washed and incubated in nonradioactive medium containing 5.4 mM K^+ (●, ■) or 0 mM K^+ (○, □). Furosemide-containing medium (□, ■) was added to one-half of each set of dishes at 0 time. In this experiment, all media were supplemented with 10% dialyzed fetal bovine serum to eliminate K^+ from this source. Points are the means of two dishes, expressed as the fraction of initial radioactivity remaining in the cells at the indicated times.

is a Na^+K^+/Cl^- cotransport system similar to that described in erythrocytes (18, 19), L cells (16), Ehrlich ascites cells (17), and epithelial cells (20, 22).

Effect of PMA on Intracellular K^+ Content and Cell Volume. In order to determine whether PMA affects the net flux of K^+ in these cells, the intracellular K^+ content was measured at various times after PMA treatment. Intracellular K^+ levels were significantly higher than control at all time points tested (Table 2). Because our $^{86}Rb^+$ flux data demonstrate that PMA had no early effect on inward K^+ movement mediated by the Na^+/K^+ pump but did inhibit a presumptive Na^+K^+/Cl^- cotransport system responsible for a major component of total K^+ efflux, the net effect of PMA treatment is to slow K^+ efflux, allowing intracellular levels to increase.

Table 2. Effect of PMA on intracellular K^+ content

Duration of PMA treatment, min	K^+ content, μmol/mg protein
Control	1.09 ± 0.13 (8)*
15	1.44 ± 0.20 (6)*
30	1.30 ± 0.20 (5)†
60	1.31 ± 0.24 (5)†
240	1.21 ± 0.11 (5)†

Exponentially growing cells were treated with 0.16 μM PMA for the indicated times and then washed and harvested for K^+ determination by atomic absorption spectroscopy. The data shown are the means ± SD pooled from two separate experiments. Numbers in parentheses indicate number of replicate dishes.
*Significantly different from control, $P < 0.005$.
†Significantly different from control, $P < 0.05$.

Table 3. Effect of PMA on cell volume

Duration of PMA treatment, hr	Cell volume, μm^3	% change
Control	4,088 ± 29	
0.5	3,693 ± 48	− 9.7
1	3,570 ± 11	−12.7
2	3,278 ± 22	−19.2
4	3,226 ± 33	−21.1
6	3,274 ± 70	−20.0

Exponentially growing cells were treated with PMA at 0.16 μM for the indicated times and then were trypsinized and cell volume was determined in a Coulter Counter with a Channelyzer attachment. Results are means ± SD of triplicate dishes.

Because the previous data demonstrated that PMA was a potent inhibitor of both unidirectional and net K^+ fluxes, it was necessary to determine whether this agent could effect changes in the volume of these cells. In the experiment summarized in Table 3, measurement of cell volume of BALB/c 3T3 cells with a Coulter Counter and multichannel analyzer indicated that PMA rapidly decreased cell volume over a 6-hr treatment period. Although the cell volume of PMA-treated cells was significantly smaller at all time points, a maximal decrease (19–21%) occurred between 2 and 6 hr. Therefore, the intracellular K^+ values shown in Table 2 underestimate the actual increases in K^+ concentrations that occur after PMA treatment, assuming that the reduction of cell volume is due to a loss of cell H_2O.

DISCUSSION

Rapid changes in monovalent cation fluxes have been implicated in the triggering of proliferation in quiescent cells exposed to various growth-promoting factors (23, 24). It has been suggested that a common feature of all mitogens is their ability to stimulate entry of Na^+ into cells, thereby activating the Na^+/K^+ pump from the cytoplasmic side of the cell membrane (25). The mitogens used for most of the previously reported studies were fetal bovine serum or purified peptides, used either alone or in combination. The phorbol ester tumor promoters are a class of low molecular weight nonpeptide mitogens active in many systems, including mouse keratinocytes *in vivo* and *in vitro*, human fibroblasts, Swiss 3T3 cells, and BALB/c 3T3 cells (reviewed in ref. 1). In view of the synergistic action of the phorbol ester PMA with most peptide mitogens (26, 27), we thought it possible that the early ionic events in PMA-treated cells might differ qualitatively from changes in ion fluxes and redistributions caused by peptide mitogens. As an initial test of this idea, the effect of PMA on both active and passive modes of K^+ transport, as measured by $^{86}Rb^+$ fluxes, was studied.

Our results demonstrate that a rapid, if not immediate, effect of PMA on BALB/c 3T3 cells is an inhibition of ouabain-insensitive, furosemide-sensitive K^+ transport. Both unidirectional influx and efflux of $^{86}Rb^+$ were inhibited by PMA, with the net result being a significant increase in intracellular K^+ content within 15 min after PMA treatment. The transport system affected by PMA in these cells has many characteristics of a Na^+K^+/Cl^- cotransport system. The evidence for this is the following: (*i*) the influx of $^{86}Rb^+$ is dependent on external Na^+ (Fig. 3); (*ii*) the influx of $^{86}Rb^+$ is dependent on external Cl^- (Fig. 4); (*iii*) the influx of $^{22}Na^+$ is greatly stimulated by external K^+ (Table 1); (*iv*) the efflux of $^{86}Rb^+$ is relatively unaffected by

the absence of external K^+ (Fig. 5). These results argue against a K^+/K^+ exchange system described earlier for BALB/c 3T3 cells (21) and fit most easily a Na^+K^+/Cl^- cotransport model as described in other cell types (16–20).

In contrast to the work of Moroney *et al.* (8) and Dicker and Rozengurt (9) with Swiss 3T3 cells, we did not observe any significant increase in Na^+/K^+ pump activity in the first 8 hr after PMA treatment of either confluent, nongrowing cells or exponentially growing cultures. Neither did these workers report any inhibition by PMA of ouabain-insensitive, furosemide-sensitive K^+ transport. This discrepancy could be due in large part to differences in the physiological state of the cells at the time of assay; the earlier work was done in postconfluent cultures depleted of serum growth factor whereas our studies were conducted for the most part in growing cultures fed the previous day with fresh serum-containing medium. However, our $^{86}Rb^+$ flux results were qualitatively similar when confluent quiescent cultures were used (unpublished data); one of the reasons for using subconfluent cultures was to avoid the possibility that an adipocyte-specific (as opposed to fibroblast-like) pattern of K^+ transport might be present after confluence as these preadipose cells enter a program of terminal differentiation. Another explanation for the divergent results in this compared to earlier studies may be the use of different cell lines (BALB/c vs. Swiss 3T3). As has been pointed out (28, 29), even the biochemical responses of similar cell lines to PMA treatment can be unpredictable.

During the course of these experiments we confirmed the earlier observations by Diamond *et al.* (30) and Driedger and Blumberg (31) that PMA causes a rapid decrease in cell volume. Although it is premature to discuss possible mechanisms for this effect, others have suggested a volume regulatory role for Na^+K^+/Cl^- cotransport systems similar to that described in this report (17, 19). The volume response of these cells to PMA may be unique in that the cellular K^+ level increases as volume decreases. However, until we have accurate data on how other osmotically active species change after PMA treatment, the exact mechanism of volume regulation in these cells and how it is interfered with by PMA remain open questions. By whatever mechanism, volume changes induced by PMA could have biologic significance. After an acute decrease in cell volume (and decreased cellular area in contact with the substratum), confluent density-regulated cell types such as BALB/c 3T3 cells could be temporarily released from growth arrest mediated by cell–cell contact. Whether the volume response of these cells to PMA is necessary or even sufficient to trigger the onset of cell proliferation in quiescent cells is not known but is amenable to experimental testing.

One of the major cellular targets of phorbol esters such as PMA is thought to be the cell membrane. However, despite many experimental observations, coherent hypotheses to explain how phorbol ester-induced membrane perturbations can effect changes in nuclear and cytoplasmic functions have not been forthcoming. As shown in this report, inhibition of a specific ion transport system by PMA can unbalance, at least temporarily, net ion fluxes between the cell and its external environment and result in significant changes in intracellular ion concentrations. Such changes might be important intracellular mediators of some of the biologic effects of phorbol esters in certain cell types.

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