Phorbol Esters and Gene Expression: The Role of Rapid Changes in K^+ Transport in the Induction of Ornithine Decarboxylase by 12-O-Tetradecanoylphorbol-13-acetate in BALB/c 3T3 Cells and a Mutant Cell Line Defective in $Na^+K^+Cl^-$ Cotransport

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ABSTRACT A BALB/c 3T3 preadipose cell line defective in $Na^+K^+Cl^-$ cotransport (3T3-E12a cells) has been used to study the relationship between phorbol ester-induced rapid changes in cation fluxes and changes in expression of a gene known to be modulated by this agent. In contrast to its effect on parental 3T3 cells, 12-O-tetradecanoylphorbol-13-acetate (TPA) did not inhibit either furosemide-sensitive $^{86}Rb^+$ influx or the rate of $^{86}Rb^+$ efflux from preloaded mutant cells. TPA-induced changes in intracellular K^+ content were diminished in 3T3-E12a cells as compared with parental cells. Thus, mutation of the $Na^+K^+Cl^-$ cotransport system renders overall potassium transport in mutant cells largely insensitive to modulation by TPA.

The morphological and functional responses of 3T3 and 3T3-E12a cells to TPA were also compared. In contrast to the extensive and long-lasting changes in morphology of 3T3 cells after 0.16 μM TPA addition, only slight and shorter-lived morphological effects of TPA were observed in 3T3-E12a cells. The transport properties of mutant cells were not totally unresponsive to TPA since hexose transport (2-deoxyglucose uptake) could be stimulated in both cell types. To establish a possible link between early changes in cation fluxes and activation of gene expression by TPA, the induction of the enzyme ornithine decarboxylase (ODC) was studied in detail. Addition of fresh medium containing serum or exposure to hypoosmotic conditions resulted in the induction of ODC in both 3T3 and 3T3-E12a cells. However, TPA failed to cause an increase in ODC activity in mutant cells, although a substantial induction of the enzyme was seen in parental cells. These results suggest that rapid changes in ion fluxes mediated by the $Na^+K^+Cl^-$ cotransport system are necessary for at least one of the phorbol ester-induced changes in gene expression in responsive cells.

Phorbol ester tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA)[1] are potent mitogens for some cell types in vivo and in vitro (for reviews see references 1 and 2). Under appropriate conditions repetitive treatment with TPA or other active tumor promoters enhances tumor formation in vivo (3) and cell transformation in vitro (4–6). The mechanisms responsible for the long-term biological effects of phorbol esters in any system are poorly understood. It has recently been demonstrated that phorbol esters can activate the enzyme protein kinase C in cell-free systems (7), which suggests that phosphorylation of cellular proteins might be involved in mediating some or all of the biological effects of these agents. However, the cellular substrate(s) for protein

[1] *Abbreviations used in this paper:* ODC, ornithine decarboxylase; PDBu, phorbol 12,13 dibutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 DECEMBER 1985 2316–2323

kinase C and the immediate postreceptor events involved in phorbol ester action have not yet been identified.

This laboratory and others have documented that some of the earliest functional changes in phorbol ester–treated cells are alterations in ion transport. Several different transport systems in a variety of cell types have been shown to be modulated by TPA including the Na^+K^+ ATPase (8, 9), Na^+/H^+ antiport (10–12), $Na^+K^+Cl^-$ cotransport (13), and epithelial Na^+ channels (14). The resultant changes in intracellular ion concentrations (K^+, Na^+, Ca^{++}, H^+; alone or in various combinations) may act as signals or second messengers for subsequent phorbol ester–induced changes in gene expression, cell proliferation, and/or differentiation. However, in view of the multitude of changes in cell metabolism that occur as a result of phorbol ester treatment, it has been difficult to establish the significance of changes in ion fluxes or intracellular ion concentrations as a mediator of any given phorbol ester–induced event.

The approach taken here toward resolving this question was to isolate cell mutants defective in an ion transport system that is an especially sensitive target of TPA. In BALB/c 3T3 preadipose cells it has been shown previously that a furosemide-sensitive $Na^+K^+Cl^-$ cotransport system is rapidly inhibited by TPA, resulting in changes in intracellular K^+ levels and cell volume (13). The isolation and characterization of a cell mutant defective in this transport system has been described recently (15). The purpose of these studies was to use a transport-defective cell line to determine if TPA alters K^+ (or $^{86}Rb^+$) fluxes as it does in parental cells and, furthermore, to ascertain whether functional responses to TPA are affected in the mutant cells. As was expected because of the mutation in $Na^+K^+Cl^-$ cotransport, K^+ fluxes were not significantly altered by TPA in mutant cells as compared with parental cells. In addition, in mutant cells TPA did not induce the enzyme ornithine decarboxylase (ODC), a gene product known to be induced in parental cells by the phorbol ester (16) as it is in many other cell types including mouse epidermis in vivo (17–20). However, this enzyme was inducible in both cell lines by other stimuli such as fresh serum-containing medium and addition of distilled H_2O to reduce the osmolarity of the medium. These results suggest that an intracellular ionic signal needed to trigger ODC induction after TPA exposure is not generated in $Na^+K^+Cl^-$ cotransport–defective cells.

MATERIALS AND METHODS

Cells: The parental BALB/c 3T3 (clone A31T) cells used and methods of culturing them have been described previously (13). The mutant cell line BALB/c 3T3-E12a used in this study is a subclone derived from the original 3T3-E12 line (15); it was cloned in standard growth medium and selected for these studies on the basis of its ability to maintain a low but stable saturation density at confluence, a property characteristic of parental 3T3 cells. The 3T3-E12a cell line is similar in all transport-related properties examined to the 3T3-E12 cell line from which it was subcloned (Sussman, I., R. Prettyman, and T. G. O'Brien, unpublished observations). Both parental and mutant cell lines were grown in Eagle's minimum essential medium (AutoPow, Flow Laboratories, McLean, VA) supplemented with 10% fetal bovine serum and subcultured when subconfluent once or twice weekly with 0.025% trypsin, 0.01% EDTA.

Measurement of $^{86}Rb^+$ Fluxes and Intracellular K^+: These procedures have been described in detail previously (13). All experiments were performed on exponentially growing cells refed the previous day with fresh growth medium. Flux experiments were performed at 37°C in culture medium in an atmosphere of 95% air, 5% CO_2. Influx of $^{86}Rb^+$ was performed for 5–10 min to ensure measurement of initial rates of uptake, while efflux experiments were done on cells preloaded to equilibrium with $^{86}Rb^+$ in standard culture medium for 4 h.

[^{3}H]Phorbol-12, 13-Dibutyrate (PDBu) Binding: Binding of radioactive phorbol ester to intact cells was performed essentially as described by Blumberg (21). Cells were plated into 16-mm wells of 24-well tissue culture dishes (~10^5 cells/well) 16–24 h before assay. Various concentrations of [^{3}H]-PDBu (17 Ci/mmol, New England Nuclear, Boston, MA) dissolved in growth medium in the presence or absence of 30 μM nonradioactive PDBu were added to the wells to initiate the binding assay. After incubation at 37°C for 15–30 min, the medium was aspirated, the cells rapidly washed four times with ice-cold growth medium, the wells drained thoroughly, and the cells solubilized in 3% Na_2CO_3, 0.1 M NaOH, 1% deoxycholate. Aliquots were taken for determination of radioactivity by liquid scintillation counting and for protein determination. Specific binding of [^{3}H]PDBu represents the amount of total binding (pmol/mg) minus the binding that occurred in the presence of 30 μM PDBu.

Other Procedures: Uptake of 2-deoxyglucose and ODC assays were performed as described previously (22, 23). For ODC assays, confluent cultures refed 2–3 d previously were treated as described in the legends to individual figures, and then were washed quickly three times with ice-cold phosphate-buffered saline (PBS) and quick-frozen in dry ice-ethanol. Then cell monolayers were lysed in hypotonic buffer (50 mM Tris-HCl, 2.5 mM dithiothreitol, 0.1 mM EDTA, pH 7.4), frozen and thawed twice, and the lysates centrifuged at 20,000 *g* for 15 min. The supernatants were assayed for ODC activity by release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described (23). Results are expressed as units of enzyme activity per milligram protein. A unit of enzyme activity corresponds to 1 nmol CO_2 liberated in 60 min.

RESULTS

Effect of TPA on $^{86}Rb^+$ Fluxes and Intracellular [K^+] in Parental Versus Mutant Cells

In a recent paper this laboratory has documented the greatly reduced activity of a furosemide-sensitive $Na^+K^+Cl^-$ cotransport system in 3T3-E12a cells compared with parental 3T3 cells (15). This mutant was selected by its ability to survive in medium containing low (0.14 mM) K^+, a condition that favors survival of cotransport–defective cells, presumably because they maintain a higher intracellular [K^+] than do parental cells that have normal levels of this major K^+ transport system. The $Na^+K^+Cl^-$ cotransporter has previously been shown to be the major K^+ transport system altered by TPA in these cells (13). The kinetic properties of the co-transporter in parental vs. mutant cells have been described in detail elsewhere (15). The salient characteristics of $^{86}Rb^+$ transport in mutant cells was the lack of detectable furosemide-sensitive $^{86}Rb^+$ efflux under standard assay conditions and greatly increased K_m values of the mutant cotransporter for Na^+ and K^+ determined by $^{86}Rb^+$ influx assays. Thus, at physiological extracellular ion concentrations, the mutant cells exhibit no furosemide-sensitive efflux and a reduced rate of furosemide-sensitive influx.

To determine what effect TPA treatment had on overall K^+ (or $^{86}Rb^+$) transport in parental vs. mutant cells, three kinds of experiments were performed. First, the effect of 0.16 μM TPA on $^{86}Rb^+$ efflux from preloaded cells was measured; as shown in Fig. 1, TPA slowed the rate of $^{86}Rb^+$ efflux from parental 3T3 cells, as expected, but did not do so from mutant cells. Our previous work (15) and that of others (24) demonstrate two components of $^{86}Rb^+$ efflux under these conditions, suggestive of efflux from both a rapidly emptying pool (presumably trapped extracellular $^{86}Rb^+$) and a slower-emptying pool (i.e., intracellular $^{86}Rb^+$). TPA increased the half-time of the slower component of $^{86}Rb^+$ efflux from parental 3T3 cells from 40 to 90 min. In contrast, TPA did not inhibit $^{86}Rb^+$ efflux from mutant 3T3-E12a cells; the times for $^{86}Rb^+$ efflux were 78 min in control versus 63 min in TPA-treated

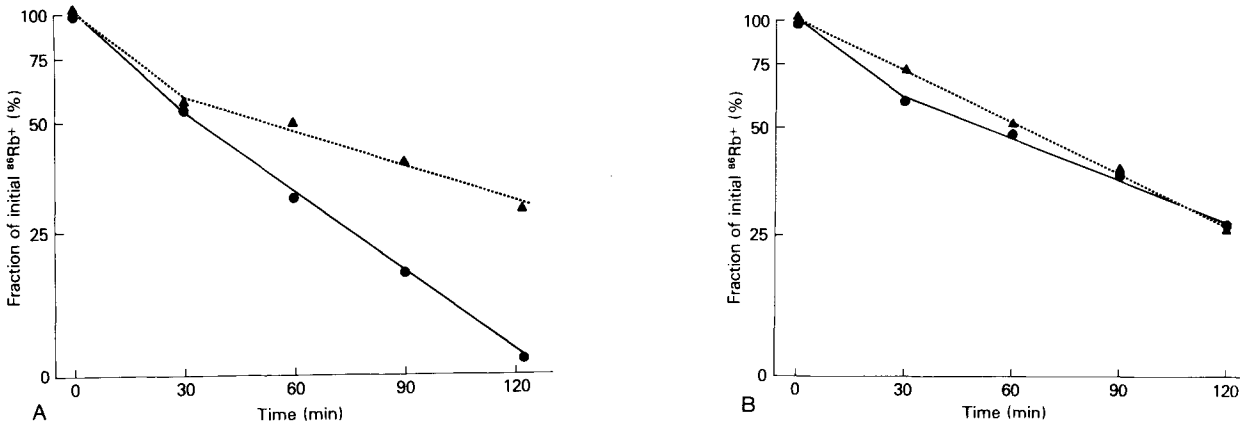

FIGURE 1 The effect of TPA on $^{86}Rb^+$ efflux from 3T3 cells (*A*) and from 3T3-E12a cells (*B*). Cells preloaded with $^{86}Rb^+$ (0.5 μCi/ml) in standard culture medium for 4 h at 37°C were washed and incubated in nonradioactive medium with (▲) or without (●) TPA at 0.16 μM. At the indicated times cells were rapidly washed with ice-cold 0.1 M $MgCl_2$, solubilized, and the fraction of initial radioactivity determined. From the slopes of the lines drawn between 30 and 120 min, $t_{1/2}$ values were calculated representing the time required for loss of 50% of intracellular $^{86}Rb^+$. Points are the average of duplicate dishes.

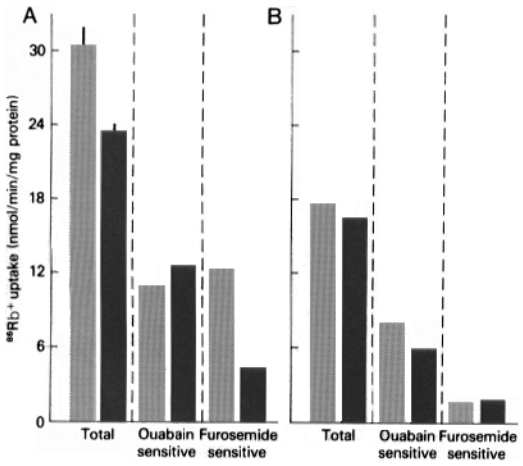

FIGURE 2 The effect of TPA on $^{86}Rb^+$ influx into 3T3 cells (*A*) and 3T3-E12a cells (*B*). Cells were treated with 0.16 μM TPA (■) or ethanol vehicle (▨) for 2 h, after which $^{86}Rb^+$ influx was measured as described in Materials and Methods. Fluxes sensitive to ouabain (2 mM) or furosemide (1 mM) were determined by subtracting the flux measured in the presence of these drugs from the total $^{86}Rb^+$ flux. The furosemide-sensitive flux was determined in the presence of ouabain. Results are the mean of triplicate dishes for each experimental condition.

TABLE I. *Effect of TPA on Intracellular K^+ Content in 3T3 and 3T3-E12a Cells*

Cell line	Time of treatment	K^+ content (μmol/mg protein) + Ethanol	K^+ content (μmol/mg protein) + TPA	% Change
	h			
3T3	0.0	1.40 ± 0.015		—
	0.5	1.39 ± 0.06	1.43 ± 0.03	2.5
	1.0	1.30 ± 0.045	1.40 ± 0.07	7.7
	2.0	1.24 ± 0.01	1.48 ± 0.03*	19.4
	4.0	1.20 ± 0.05	1.39 ± 0.02*	15.8
	6.0	1.33 ± 0.03	1.62 ± 0.02*	22.5
	8.0	1.34 ± 0.02	1.45 ± 0.04	8.2
3T3-E12a	0.0	1.14 ± 0.025		—
	0.5	1.09 ± 0.02	1.05 ± 0.04	−3.7
	1.0	1.11 ± 0.01	1.15 ± 0.02	3.6
	2.0	1.12 ± 0.02	1.23 ± 0.09	9.8
	4.0	1.25 ± 0.03	1.33 ± 0.01	6.4
	6.0	1.31 ± 0.03	1.47 ± 0.03*	12.6
	8.0	1.29 ± 0.05	1.37 ± 0.01	6.2

* Significantly different from ethanol control ($P < 0.05$) by *t*-test. Cells were treated with 0.16 μM TPA or the ethanol vehicle for the indicated times, then rapidly harvested for K^+ determinations as described in Materials and Methods. Results are the mean ± SE of triplicate dishes.

cells, a slightly increased rate of efflux that was not consistently observed. It is also worth noting that the control rate of efflux from mutant cells ($t_{1/2}$ = 78 min) is approximately double the efflux rate of $^{86}Rb^+$ from control cells ($t_{1/2}$ = 40 min), indicating a reduced activity in mutant cells of a major K^+ efflux system.

In a second set of experiments the effect of TPA on $^{86}Rb^+$ influx was measured in parental versus mutant cells. The $^{86}Rb^+$ fluxes in the experiments shown in Fig. 2 were measured at 2 h after 0.16 μM TPA treatment, a time when the inhibition of $^{86}Rb^+$ fluxes in parental cells was maximal (13). In parental cells, TPA reduced total $^{86}Rb^+$ fluxes by 23% and the furosemide-sensitive flux by 65%. In absolute terms, the reduction of total $^{86}Rb^+$ flux by TPA could be largely accounted for by the inhibition of furosemide-sensitive $^{86}Rb^+$ flux (7 vs. 8 nmol/min per mg). In 3T3-E12a cells, the magnitude of the total $^{86}Rb^+$ flux and the individual flux components were consistently smaller than in parental cells. As noted previously (15), the furosemide-sensitive $^{86}Rb^+$ flux component in these cells was very small in both absolute terms and as a percentage of the total $^{86}Rb^+$ flux. TPA treatment had only a slight inhibitory effect on overall $^{86}Rb^+$ fluxes, which could be accounted for by a 25% reduction in the ouabain-sensitive component.

Finally, the effect of TPA on the intracellular K^+ content of parental 3T3 and mutant cells was determined (Table I). Beginning at 1 h and persisting for up to 8 h after TPA treatment, elevated intracellular K^+ contents (8–20% greater than controls) were measured in 3T3 cells, confirming earlier results (13). In mutant cells the increase in intracellular K^+ content after TPA treatment was of a smaller magnitude (Table I). It is likely that relative changes in the parameter of interest, the intracellular K^+ concentrations, in the two cell types are even greater than indicated by the data in Table I

since there is a much larger reduction in cell volume (measured by a rapid electronic sizing method) after TPA treatment in the parental cells than in the mutant cells (13, 15). However, it has not been possible to reliably measure the rapid changes in intracellular H_2O space that presumably occur in situ after TPA treatment in these monolayer cells, since the time required to reach equilibrium of various labeled compounds (3-O-methylglucose, urea) with intracellular H_2O is long (1–2 h) compared with the rapid onset of changes in total cell volume that occur after TPA treatment (13). Thus, it is concluded from both the flux experiments and the measurement of intracellular K^+ content that TPA causes more extensive changes in cellular K^+ homeostasis in 3T3 cells than in 3T3-E12a cells.

Specific Binding of [^{3}H]PDBu in Parental Versus Mutant Cells

One explanation for the above results is that specific cellular binding sites for phorbol esters are necessary for effects on ion transport to occur, but these are absent from the 3T3-E12a cell line. To evaluate this possibility the specific binding of a radioactive phorbol ester [^{3}H]PDBu to intact 3T3 and 3T3-E12a cells was measured (Fig. 3). In both cell lines specific, saturable binding was observed in intact cells under normal culture conditions, although the number of these sites appeared to be somewhat lower in 3T3-E12a cells. The lower level of specific [^{3}H]PDBu binding at saturation in 3T3-E12a cells vs. 3T3 cells was consistently observed in several repeat experiments (data not shown). In the representative experiment shown in Fig. 3 half-maximal specific binding of [^{3}H]-PDBu occurred at 16 nM in 3T3 cells and at 24 nM in 3T3-E12a cells. This difference in apparent affinity is not significant since the $K_{1/2}$'s determined in several replicate experiments in both cell lines were identical (22 ± 4 nM for 3T3 cells, 22 ± 3 nM for 3T3-E12a cells, mean ± SEM, n = 3). Whether the apparent difference in total specific binding between 3T3 and 3T3-E12a cells is involved in the different functional responses of these two cell lines to TPA treatment is beyond the scope of this study; however, it is clear from these results that both cell lines have specific binding sites of similar apparent affinities for PDBu. Thus any differences in functional responses of these two cell lines to TPA cannot be explained by the total lack of specific phorbol ester binding sites, although more subtle receptor-associated defects cannot be ruled out at this time.

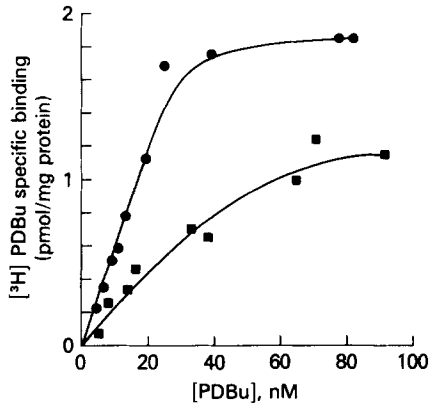

FIGURE 3 Specific binding of [^{3}H]PDBu to intact 3T3 (●) and 3T3-E12a (■) cells. Cells were incubated with the indicated concentration of [^{3}H]PDBu in the presence or absence of 30 μM PDBu and specific binding determined as described in Materials and Methods. Points are the average of duplicate wells. The experiment shown is representative of at least two other experiments in each cell line.

Comparison of the Effects of TPA on Morphology and 2-Deoxyglucose Uptake in 3T3 Versus 3T3-E12a Cells

As is true for many cell types in culture, TPA treatment of 3T3 cells causes pronounced and often striking changes in cell morphology (2, 25, 26). However, when 3T3-E12a cells were exposed to TPA, the morphological changes were considerably less pronounced. The results of a typical experiment are shown in Fig. 4, in which the TPA-induced changes in morphology of 3T3 and 3T3-E12a cells were monitored for up to 24 h. Confluent 3T3 presented a typical cobblestone appearance consisting of flat cuboidal cells with prominent nuclei (Fig. 4*A*). After treatment for 2 h with 0.16 μM TPA these cells rapidly became much more refractile with many cells assuming a more irregular shape with long processes (Fig. 4*B*). The cells appeared smaller as judged by the presence of obvious intercellular spaces in the monolayer. These morphological changes persisted and became even more prominent after 24 h (Fig. 4*C*). Confluent 3T3-E12a cells were more fibroblastic in appearance, and exposure to 0.16 μM TPA did not produce the extensive changes in morphology seen in 3T3 cells (compare Fig. 4*E* to 4*D*). The morphological response of 3T3-E12a cells that was observed is short-lived since by 24 h the cells appear very similar to untreated cells (Fig. 4*F*).

In addition to cation transport, TPA has been shown to influence the function of many other transport systems, membrane enzyme systems, and receptors (1, 2). To rule out the possibility of a generalized change in the membrane properties of 3T3-E12a that abrogates the response of several enzyme/transport systems to TPA, another transport system known to be affected by TPA was studied. Carrier-mediated sugar transport is stimulated by TPA in many cell lines, including the BALB/c 3T3 preadipose line used in this study (22). Therefore, the stimulation of 2-deoxyglucose uptake caused by TPA treatment in 3T3 and 3T3-E12a cells was compared (Fig. 5). In both cell types a large stimulation of uptake occurred with a similar time course, although the basal level of uptake was higher and the maximally stimulated levels slightly lower in mutant cells. Thus, the $Na^+K^+Cl^-$ cotransport mutation does not eliminate the ability of the hexose transport system to be modulated by TPA.

Induction of ODC in Mutant Versus Parental Cells

One of the earliest changes in gene expression caused by phorbol esters is the induction of ODC (1, 17, 20). In virtually every system studied the induction of this enzyme has been shown to be dependent on new protein and RNA synthesis. For this and other reasons (discussed below), ODC is an excellent example of a gene whose expression might be triggered by rapid perturbation in ion fluxes caused by TPA. To test this hypothesis the induction of this enzyme in confluent cultures of parental 3T3 and mutant 3T3-E12a cells by fresh medium or TPA was determined. Both treatments have previously been shown to be effective inducers of ODC in parental 3T3 cells (16). As demonstrated in Fig. 6, the induction of ODC by TPA in 3T3 cells was confirmed but there was no

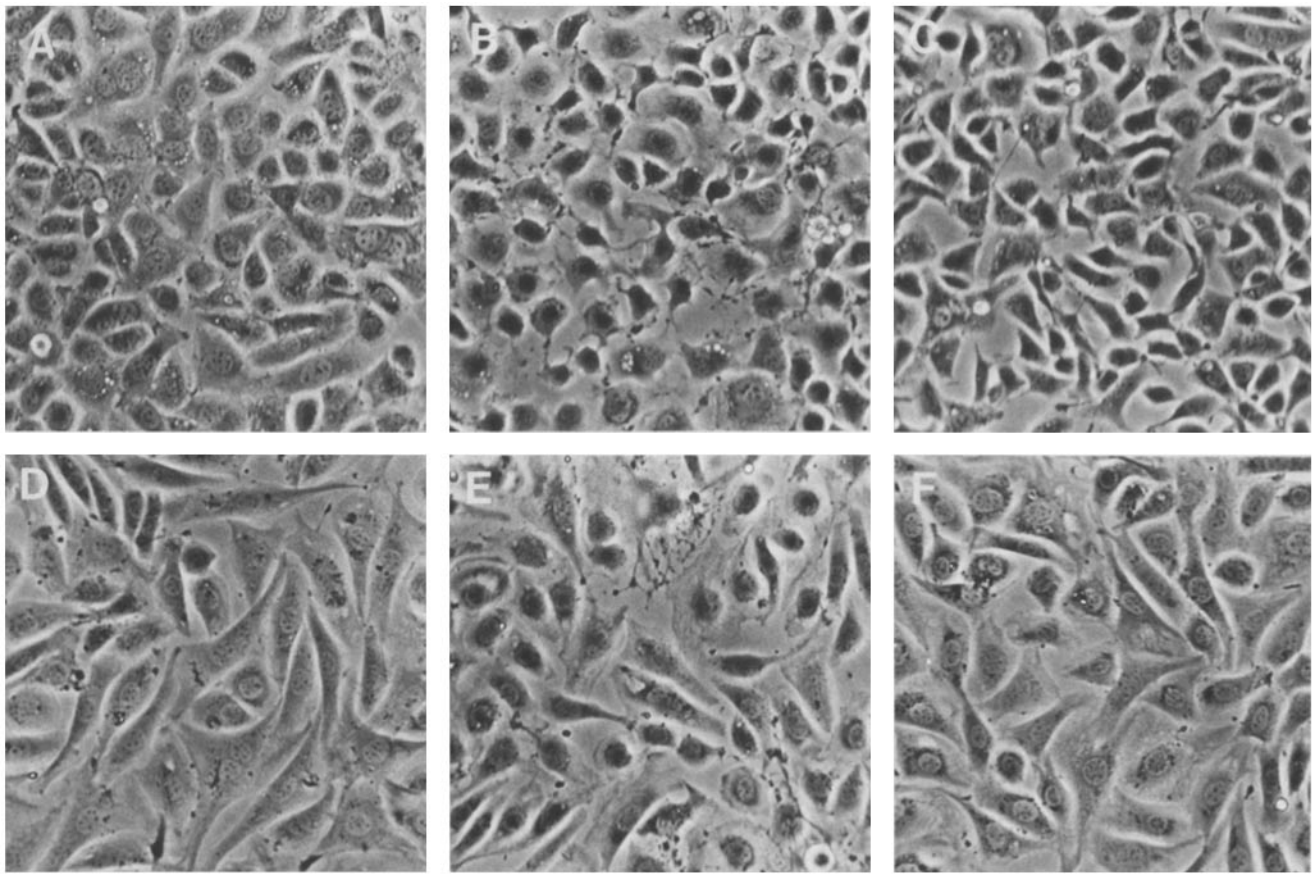

FIGURE 4 Effect of TPA on morphology of 3T3 and 3T3-E12a cells. Confluent cultures of 3T3 cells (*A–C*) or 3T3-E12a cells (*D–F*) were treated with 0.16 μM TPA at 37°C in standard culture medium. After various times at 37°C cultures were removed from the CO_2 incubator and representative areas photographed under phase contrast optics. (*A* and *D*) Untreated cultures; (*B* and *E*) cells 2 h after TPA addition: (*C* and *F*) cells 24 h after TPA addition. × 375.

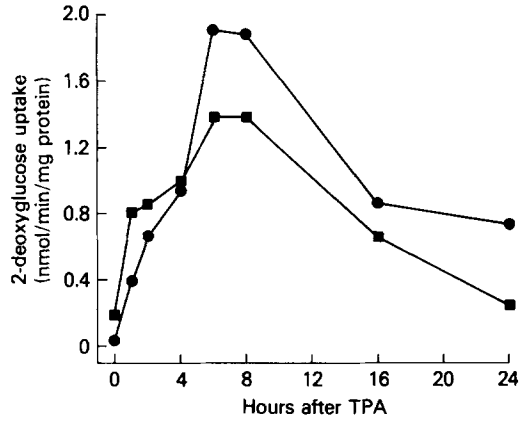

FIGURE 5 The effect of TPA on 2-deoxyglucose uptake. Postconfluent cultures of 3T3 (●) and 3T3-E12a (■) cells were treated with 0.16 μM TPA at 0 time and assayed for 2-deoxyglucose uptake at the indicated times thereafter as described in Materials and Methods. The basal (unstimulated) uptake values were 0.03 nmol/min per mg protein for 3T3 cells and 0.18 nmol/min per mg protein for 3T3-E12a cells. Points are the average of duplicate dishes from a representative experiment (of a total of three) with each cell line.

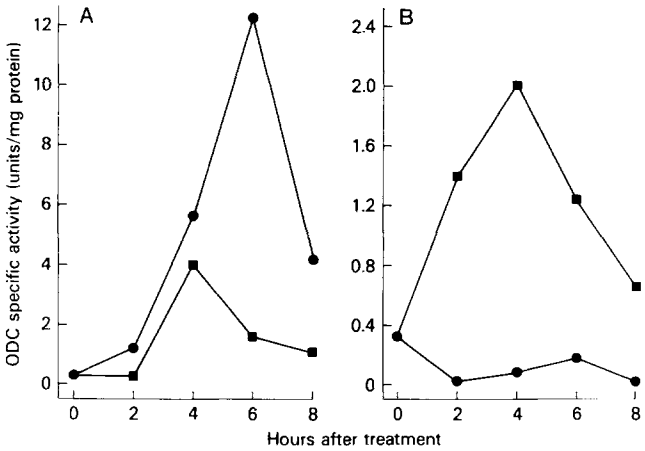

FIGURE 6 Induction of ODC activity in 3T3 (*A*) and 3T3-E12a (*B*) cells. Confluent cultures of each cell line were refed with fresh medium (■), or TPA was added in conditioned medium to a final concentration of 0.16 μM (●). Duplicate dishes were harvested for enzyme activity determination at the indicated times after treatment. Similar results were obtained in three additional experiments for each cell line.

increase in enzyme activity in the mutant 3T3-E12a cells after TPA treatment. Similar results have been obtained under a variety of different conditions that include various passage levels of the mutant cells, TPA concentrations from 0.016–1.6 μM, and use of growing rather than confluent cells (data not shown). It is important to emphasize that the induction of ODC by the growth factors present in fresh medium gave approximately the same level of induction in the two cell lines, with a similar time course. In a second independently isolated $Na^+K^+Cl^-$ cotransport-defective mutant cell line, TPA similarly failed to induce ODC activity, whereas fresh medium was an effective inducer of this enzyme (data not

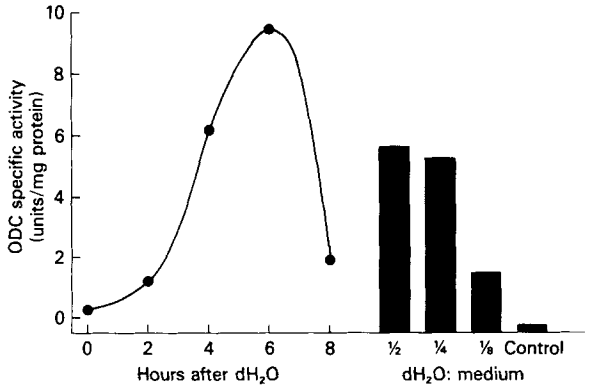

FIGURE 7 Induction of ODC activity by addition of distilled H_2O to 3T3 cell cultures. Distilled H_2O was added to confluent cultures to reduce the osmolarity by 33% (2.5 ml H_2O to 5 ml medium), and dishes were harvested for ODC activity measurements at the indicated times (left). In the same experiment, different volumes of distilled H_2O were added and cells harvested 4 h later for enzyme determinations. Results are the average of duplicate dishes from a representative experiment.

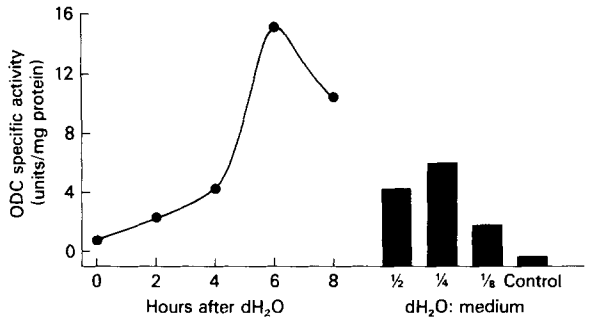

FIGURE 8 Induction of ODC activity by addition of distilled H_2O to 3T3-E12a cell cultures. Experimental conditions are as described in the legend to Fig. 7.

shown). Thus, a general defect in responsiveness of the ODC gene to inducing stimuli is not likely to be the cause of the failure of TPA to induce this enzyme in $Na^+K^+Cl^-$ cotransport-defective cells. To strengthen this conclusion, the effect of reducing culture medium osmolarity was studied. Changes in osmolarity have been shown by many workers to alter the level of ODC in mammalian cells (27–29). In general, hypoosmotic conditions induce ODC, whereas hyperosmotic conditions lower ODC levels. In Figs. 7 and 8, the effect of distilled H_2O addition to cultures of 3T3 or 3T3-E12a cells on ODC activity is compared. Two major points can be made: (*a*) a decrease in osmolarity is a very effective inducer of ODC in both cell lines (34-fold induction in 3T3 cells and 19-fold in 3T3-E12a cells 6 h after addition of ½ volume of H_2O), and (*b*) changes in ionic fluxes and/or intracellular ionic concentrations that are presumably triggered by dH_2O addition may be involved in the mechanism of ODC induction in both of these cell lines. By whatever mechanism, the mutant cells are clearly capable of responding to changes in their ionic environment with an ODC induction. As shown in Figs. 7 and 8, even rather small differences in medium osmolarity represented by addition of ⅛ volume of distilled H_2O (11% decrease in medium osmolarity) can cause a significant induction of this enzyme in both cell lines.

If phorbol ester–induced alterations in ion fluxes between the external medium and the cell interior are required for ODC gene expression, then reduction of the extracellular $[K^+]$

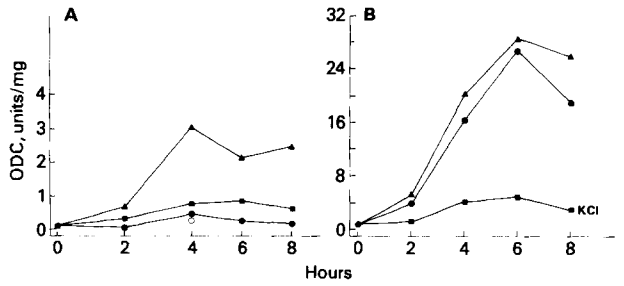

FIGURE 9 Induction of ODC activity in 3T3 cells maintained in low K^+ medium. (*A*) Confluent cells refed the previous day with fresh serum-containing medium of 0.18 mM K^+ were treated with either 0.16 μM TPA (●), KCl added to a 5 mM final concentration (■), or KCl (to 5 mM) plus 0.16 μM TPA (▲). Cells were harvested at the indicated times and ODC activity determined as described in Materials and Methods. (*B*) Confluent cultures, incubated in 5 ml low K^+ medium overnight as in *A*, were treated with either 2.5 ml distilled H_2O (●), KCl added to 5 mM (■), or KCl (to 5 mM) plus 2.5 ml distilled H_2O (▲). Cells were harvested at the indicated times and ODC activity measured. Points are the average of duplicate dishes per time point. The entire experiment has been repeated twice with identical results.

to a value well below the K_m for the major K^+ transport systems should exert an influence on ODC induction by TPA in transport-competent cells. As shown in Fig. 9*A*, this is indeed the case. TPA failed to significantly induce ODC in parental 3T3 cells where the K^+ concentration of the medium was 0.18 mM, well below the apparent K_m for both the Na^+K^+ ATPase and the $Na^+K^+Cl^-$ cotransport systems (15). Addition of KCl to a final concentration of 5 mM caused a slight but reproducible induction of the enzyme, whereas KCl (added to 5 mM) and TPA added simultaneously caused a substantial ODC induction, much greater than the sum of the inductions resulting from either TPA or KCl addition alone. A possible explanation for these results is that the overnight incubation in low K^+ medium made the cells generally incapable of new protein synthesis, by virtue of a low intracellular K^+ concentration or some other metabolic imbalance. This possibility appears unlikely, however, because distilled H_2O addition was an equally effective inducer whether added to cells maintained in low K^+ medium or medium in which the $[K^+]$ is raised to 5 mM (Fig. 9*B*). Based on what is known of cellular responses to hypoosmotic conditions, extracellular ion concentrations are thought to be relatively unimportant since cells generally recover from this form of stress by a net loss of intracellular ions (and H_2O). Thus it would be predicted that the external K^+ concentration would have little effect on the ODC-inducing ability of distilled H_2O. Regardless of the exact mechanism, the data in Fig. 9*B* demonstrate that cells incubated overnight in low K^+ medium are fully capable of responding to an appropriate ODC-inducing stimulus.

DISCUSSION

In recent years several cell lines in culture have been described that exhibit various degrees of nonresponsiveness to phorbol esters compared with the parental cell populations from which they were derived. These include mouse epidermal cell lines (30), human promyelocytic leukemia cells (31), Freund erythroleukemia cells (32), and Swiss 3T3 cells (33). While different in many respects, these variant cell lines share at least two common features: they exhibit apparently normal specific phorbol ester binding, and the genetic defect(s), if any, con-

ferring nonresponsiveness to phorbol esters is (are) unknown. Thus, such lines have not been able to provide much information on the identity of the immediate post-receptor events in phorbol ester–treated cells that trigger changes in membrane function, gene expression, growth regulation, or cell differentiation. In this study we have taken an alternative approach to this problem by first isolating a cell mutant altered in a known target of phorbol esters and then determining the responsiveness (or nonresponsiveness) to phorbol esters. Thus it should be emphasized that the mutant cell line described in this report, 3T3-E12a, was not subjected to any selection for resistance to phorbol esters during its isolation process. The particular phenotype that was selected for, a defect in furosemide-inhibitable $Na^+K^+Cl^-$ cotransport, was chosen because (*a*) it is rapidly inhibited by phorbol esters leading to changes in the intracellular K^+ concentration and cell volume, and (*b*) a simple selection system is available for isolation of such mutants that lack cotransport activity (33). Mutants altered in this system have been isolated in two other cell types (34, 35); the mutant cells in these previous studies have the ability to grow at lower extracellular K^+ concentrations than parental cells. In at least one cell type, however, mutant cell lines were judged to have normal volume regulatory behavior after hypoosmotic conditions (35). We have previously reported the growth characteristics, potassium transport properties, and volume regulatory behavior after TPA treatment of the mutant cells used in this study (15).

The results reported here and elsewhere (15) demonstrate that when compared with parental 3T3 cells, mutant cells are selectively nonresponsive to TPA treatment. While the stimulation of 2-deoxyglucose uptake was similar after TPA treatment in parental versus mutant cells, other well-characterized responses to TPA were altered or completely absent in mutant 3T3-E12a cells. Because of the reduced level of $Na^+K^+Cl^-$ cotransport activity under standard culture conditions, changes in $^{86}Rb^+$ fluxes and intracellular K^+ content are not observed or only slightly altered in mutant cells exposed to TPA, whereas large changes in these parameters are observed in parental 3T3 cells. As might be expected if $Na^+K^+Cl^-$ cotransport has a volume regulatory role under physiological conditions, TPA was shown to cause a large reduction in cell volume in 3T3 cells but a much smaller effect was observed in mutant cells (15). The prominent morphological changes induced by TPA in parental cells were considerably less noticeable in mutant cells. The induction of the enzyme ODC did not occur in mutant cells after TPA treatment, although it was induced after exposure to other stimuli. It is unlikely that the above results are due to the lack of TPA receptors in the mutant cell line, since specific binding sites for the radioactive phorbol ester [^{3}H]PDBu to intact cells were readily detected in both cell lines. More subtle receptor-associated differences, such as the absence of an important receptor subclass, cannot be ruled out as being responsible for the lack of responsiveness of mutant cells to phorbol esters.

In a related study by Butler-Gralla and Herschman (36), the ability of TPA to modulate two of the same parameters used here (2-deoxyglucose uptake, ODC induction) was studied using Swiss 3T3 cell variants selected for resistance to the mitogenic action of TPA. Even though a completely different selection system was used, results similar to those reported here were obtained: 2-deoxyglucose uptake was stimulated by TPA in both parental and each of two nonresponsive cell lines, whereas ODC was not induced or only weakly induced by TPA in the variant cells. However, the nature of the defect conferring resistance to the ODC-inducing and mitogenic effects of TPA in these variant cells is not known.

The lack of ODC inducibility by TPA in $Na^+K^+Cl^-$ cotransport-defective cells could be explained in several ways. For example, the 3T3-E12a cells could harbor a second mutation in a regulatory gene involved in ODC induction after exposure to various stimuli including TPA. This possibility is unlikely since the inductions of this enzyme caused by fresh medium and hypo-osmolar conditions were essentially the same in parental and mutant cells. It is only the TPA-mediated induction that is altered in mutant cells, suggesting that these cells do not lack any of the important cytoplasmic or nuclear regulatory elements involved in the expression of this gene. An important question for future analysis is whether other genes known to be expressed in 3T3 cells after TPA treatment such as c-myc (37), c-fos (38), and MEP (39) behave similarly to ODC expression in parental versus mutant cells. Because of the known influence of external osmolarity on ODC expression in many systems (27–29; Figs. 7–9), ODC might be a gene whose expression is uniquely regulated by intracellular ionic events, whereas other genes whose expression is induced by TPA might be regulated by entirely different mechanisms.

Another explanation for the observed noninducibility of ODC in transport-defective cells is that an ionic signal generated by TPA in parental cells is not generated in mutant cells because the transport system responsible for the signal is defective. Experimental support for this putative mechanism is the finding that parental 3T3 cells can be made functionally nonresponsive to TPA (with respect to ODC induction) by lowering the extracellular K^+ concentration of the medium well below the K_m values of the two major K^+ transport systems, the Na^+/K^+ pump and the $Na^+K^+Cl^-$ co-transport system (Fig. 9). Since these two transport systems operating in concert are responsible for overall cellular K^+ homeostasis, the ionic signal generated by TPA leading to ODC expression may be a change in intracellular K^+ concentration. In support of this hypothesis, intracellular K^+ levels are substantially increased by TPA in 3T3 cells but to a much smaller extent in mutant 3T3-E12a cells (13; Table I). However since the changes in intracellular levels of other ions of interest (Na^+, H^+, Ca^{++}, Cl^-) have not been determined, the exact identity of the ionic signal(s) generated by TPA in the system is still not known. Another large area of uncertainty is the exact mechanism by which ionic signals cause changes in gene expression such as ODC, although preliminary results using a highly inducible hamster fibroblast line show that induction of ODC activity is accompanied by a substantial accumulation of ODC mRNAs, indicating increased transcription of the ODC gene (O'Brien, T. G., and S. Gilmour, unpublished observations). Whatever the molecular mechanism, the use of transport-defective cell lines is a potentially powerful experimental system to study the relationship between early ionic redistributions triggered by hormones and mitogens and subsequent gene expression. In the particular case of ODC it is apparent from our results that the growth factors present in serum trigger the expression of this gene by a different mechanism than TPA since serum was an effective inducer of ODC in both parental and ion transport–defective cells. If ionic signals are involved they are presumably generated by activation (or inhibition) of different transport systems from those affected by TPA. A strong candidate for the target of peptide

mitogens is the amiloride-sensitive Na^+/H^+ transport system that is known to cause a rapid intracellular alkalinization in several cell types after mitogen treatment (40–43).

Despite much study over the last decade, very little is known of the intracellular second messengers of phorbol ester action. Given the increasingly strong evidence that protein kinase C is a major cellular receptor for these agents, it is conceivable that protein phosphorylation may play an important role in phorbol ester action. If so, the identity of the substrates for protein kinase C becomes critically important for understanding longer-term responses of cells to these agents, such as gene expression. Because changes in both ion transport and protein phosphorylation can be detected very rapidly after TPA treatment, we suggest that membrane transport systems may be important substrates for protein kinase C, especially after phorbol ester treatment. The idea that phorbol esters trigger biological changes via effects on ion transport is not new. The early studies of Moroney et al. (8) and Dicker and Rozengurt (9) demonstrated alterations in transport via the Na^+K^+ pump, perhaps mediated by rapid Na^+ influx. More recently, the activation by phorbol esters of an amiloride-sensitive Na^+/H^+ antiport system involved in intracellular pH control has been documented in several cell types, including Swiss 3T3 cells (10), a pre B-lymphocyte cell line (11), and a human leukemic cell line (12). In each of the latter cases a phorbol ester–dependent intracellular alkalinization has been postulated as a trigger of subsequent biological changes. However, in all of the previous studies, a firm cause-and-effect relationship between phorbol ester–triggered changes in ion transport and biological changes could not be established.

In this paper for the first time a genetic approach was used to provide more direct evidence that at least in this system early changes in ion transport mediated by the $Na^+K^+Cl^-$ cotransport can mediate subsequent changes in the expression of at least one gene. The mutant cells we have described, as well as revertants once they are obtained, should also prove useful in analyzing the mechanisms involved in other responses to phorbol esters such as mitogenesis and modulation of cell differentiation.

We thank Maria Stover for help with the ornithine decarboxylase assays.

This work was supported by grants CA-36353 and ES-01664 from the National Institutes of Health, Department of Health and Human Services.

Received for publication 13 May 1985, and in revised form 12 August 1985.

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