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Protein kinase C and membrane transport: divergent responses of $Na^+/K^+/Cl^-$ cotransport and sugar transport to exogenous diacylglycerol

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Even though the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is known to bind to and activate protein kinase C (PKC), it is still not certain that all cellular responses to phorbol esters are necessarily mediated by PKC. In BALB/c 3T3 preadipose cells, TPA has previously been shown to rapidly inhibit $Na^+K^+Cl^-$-cotransport activity, stimulate 2-deoxyglucose uptake and induce ornithine decarboxylase activity. The cell-permeable diacylglycerol *sn*-1,2-dioctanoylglycerol (DiC_8) was used in order to distinguish between PKC-dependent and -independent responses of BALB/c 3T3 cells. DiC_8 modulated $^{86}Rb^+$ fluxes in BALB/c 3T3 cells in the same manner as TPA: furosemide-sensitive $^{86}Rb^+$ influx and efflux was inhibited, while in cotransport-defective cells no effect was observed. In contrast, DiC_8 did not stimulate 2-deoxyglucose uptake in either parental or cotransport-defective cell lines, even though TPA is a very effective inducer of this transport system in both cell types. Pretreatment of cells with DiC_8 did not substantially alter the subsequent induction of 2-deoxyglucose uptake by TPA, although a slight but reproducible reduction in the magnitude of the response was observed in DiC_8-pretreated cells. The PKC-dependent phosphorylation of an acidic 80-kDa protein was stimulated by both TPA and DiC_8 in parental and cotransport-defective cell lines, suggesting that a gross defect in the primary effector system used by both TPA and diacylglycerols cannot explain any of our results. Ornithine decarboxylase was induced by DiC_8 and the $K_{1/2}$ was approximately the same as that for inhibition of $Na^+/K^+/Cl^-$ cotransport in these cells. Thus, our results suggest that PKC is clearly essential for some phorbol ester membrane transport responses (such as inhibition of $Na^+/K^+/Cl^-$ cotransport), but our results do not allow us to conclude that other responses (such as stimulation of 2-deoxyglucose uptake) necessarily require PKC activation.

Introduction

Increasing evidence implicates membrane transport systems as important targets of tumor-promoting phorbol esters [1–5]. It is widely assumed that binding of phorbol esters to the enzyme PKC is required for modulation of transport

Abbreviations: DiC_8, *sn*-1,2-dioctanoylglycerol; ODC, L-ornithine decarboxylase (EC 4.1.1.17); PKC, protein kinase C (EC 2.7.1.37); TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMSO, dimethylsulfoxide.

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activity to occur. Evidence supporting this idea comes from the observations in many systems that synthetic diacylglycerols known to activate PKC can mimic the effect of phorbol esters on some transport systems [6,7]. However, what is still not clear for any transport system are the precise biochemical mechanisms responsible for phorbol ester-induced modification of transport function.

Recent studies from our laboratory have suggested that phorbol esters may trigger changes in gene expression and cell proliferation in BALB/c 3T3 cells via modulation of a specific membrane ion transport system, the $Na^+/K^+/Cl^-$-cotransporter [8,9]. The experimental approach taken was to isolate, by positive selection from mutagenized cell populations, cloned cell lines that were defective in $Na^+/K^+/Cl^-$-cotransport activity, and compare the responses of parental cells and cotransport-defective cells to the phorbol ester TPA. This experimental system promises to be a useful model in which to examine the relationship between early ionic signals elicited by phorbol esters and longer-term responses, such as gene expression and DNA synthesis.

Because of the important role of PKC in transducing phorbol ester signals, as well as the relative lack of knowledge of the activated enzymes physiological substrates, we decided to investigate whether the phorbol ester inhibition of the $Na^+/K^+/Cl^-$-cotransport system was mediated via PKC activation. To answer this question, we used the synthetic, cell-permeable diacylglycerol *sn*-1,2-dioctanoylglycerol (DiC_8) which can activate PKC in intact cells [10]. If DiC_8 can alter ion transport in parental cells, in the same manner as does TPA, without affecting transport in $Na^+/K^+/Cl^-$-cotransport-defective cells, this would support the view that this membrane system is a physiological substrate of activated PKC. A related question is of whether all phorbol ester-modulated membrane functions are mediated by PKC. To attempt to answer this question, we have investigated whether DiC_8 can act in a similar manner as TPA in stimulating hexose transport activity, a membrane transport system known to be modulated by phorbol esters [1,11].

Materials and methods

Cells

The isolation and characterization of the $Na^+/K^+/Cl^-$-cotransport-defective BALB/c 3T3 cell lines 3T3-E12a and 3T3-C5 from parent BALB/c 3T3 (clone A31T) cells have been described [9,12]. Both parental and mutant cell lines were grown in Eagle's AutoPow minimum essential medium supplement with L-glutamine, $NaHCO_3$ and 10% fetal bovine serum. Tests for mycoplasma contamination of all cell lines were routinely negative.

Biochemical procedures

The influx and efflux of $^{86}Rb^+$ (as a tracer for K^+) was measured exactly as described previously [3]. For these assays, nearly confluent cultures refed the previous day with fresh growth medium were used. ODC activity and uptake of 2-deoxyglucose were measured as described previously [11,13] in confluent cultures last refed 3 days earlier. Results are expressed relative to the amount of protein, measured by the procedure of Lowry et al. [14]. For two-dimensional gel analysis of phosphoproteins, cells were grown to confluence in regular medium, then washed twice with phosphate-free medium and incubated with phosphate-free medium and 10% dialyzed (vs. 140 mM choline chloride) fetal bovine serum and 0.1 mCi/ml [^{32}P]orthophosphate for 3 h at 37°C in order to label intracellular ATP pools. At various times prior to harvest, TPA or DiC_8 was added from a concentrated stock solution (in phosphate-free medium) to a final concentration of 0.16 μM, or 0.2 mM, respectively. Controls received the equivalent concentration of ethanol or DMSO vehicle (0.1%). Cells were harvested by aspiration of the radioactive medium, washing with cold saline soln., extraction with 5% trichloroacetic acid, and solubilization with 2X sample buffer as described in Ref. 15. Two-dimensional gels were run essentially as described by O'Farrell [16] with isoelectric focusing (pH range 4–7) in the first dimension and SDS polyacrylamide gel electrophoresis (10% gel) as the second dimension. Gels

were fixed, stained with Coomassie blue, dried and exposed to X-ray film at −70°C using intensifying screens.

Chemicals

TPA was purchased from Chemicals for Cancer Research, Eden Prairie, MN. All radioisotopes used were supplied by Amersham/Searle. *sn*-1,2-Dioctanoylglycerol was obtained from Avanti Polar Lipids, Birmingham, AL and used in experiments as follows: just prior to use, the chloroform solvent was evaporated under a stream of nitrogen and either DMSO or ethanol vehicle added to bring the DiC_8 concentration to 0.2 M. Appropriate aliquots were then added to conditioned medium so that upon further dilution into cell cultures, the desired concentration was obtained.

Results

Effects of exogenous diacylglycerols on $Na^+/K^+/Cl^-$-cotransport activity

If the inhibition of $Na^+/K^+/Cl^-$-cotransport activity by TPA is mediated by PKC, activation of this enzyme by means other than TPA should yield similar effects on transport activity. As shown in Fig. 1, this is indeed the case. BALB/c 3T3 cells exposed for 30 min to the synthetic diacylglycerol DiC_8 at 0.2 mM had a reduced total influx of $^{86}Rb^+$ which could be accounted for by a 64% inhibition of the furosemide-sensitive component of $^{86}Rb^+$ influx, which, as we have previously shown, is almost exclusively due to $Na^+/K^+/Cl^-$ cotransport. No effect of DiC_8 was observed on the ouabain-sensitive component of $^{86}Rb^+$ influx due to Na^+/K^+ pump activity (Fig. 1). In other experiments (data not shown), the onset of inhibition of furosemide-sensitive $^{86}Rb^+$ influx by DiC_8, although quite rapid, still exhibited a noticeable lag period of approx. 5 min, suggesting that the diacylglycerol does not inhibit transport activity competitively (as diuretics do), but rather by an indirect mechanism. The concentration-dependence for inhibition of furosemide-sensitive $^{86}Rb^+$ influx (Fig. 2) indicates that the half-maximal inhibitory effect was obtained at approx. 50 μM. It should be emphasized that the extent of inhibition of cotransport activity by DiC_8 was as great as that caused by TPA [3], although the dose-response relationships are quite different (half-maximal inhibitory concentration for TPA is 2 nM (O'Brien T.G., unpublished data)). Presumably, this disparity in potency reflects differences in affinity for PKC as well as other factors such as solubility, cell permeability, and metabolism for TPA vs. DiC_8.

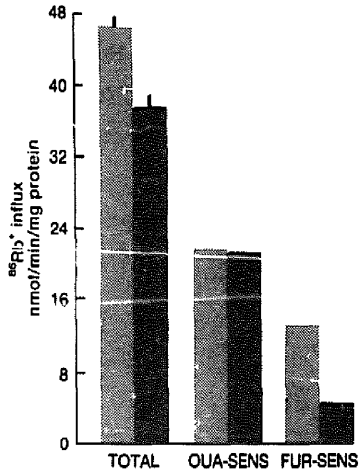

Fig. 1. Effect of DiC_8 on $^{86}Rb^+$ influx. Nearly confluent cultures of BALB/c 3T3 cells were treated with DiC_8 at 0.2 mM (solid bars) or vehicle control (hatched bars) for 30 min, then assayed for $^{86}Rb^+$ influx. In this experiment, DiC_8 was present in the radioactive solution used to measure the rate of influx. Results are the mean of triplicate dishes for each condition.

Exogenous diacylglycerol also inhibited $^{86}Rb^+$ efflux from 3T3 cells (Fig. 3). In the experiment shown, 0.1 mM DiC_8 clearly slowed the rate of $^{86}Rb^+$ efflux compared to the vehicle control. The calculated half-times for $^{86}Rb^+$ efflux were 41 min for control, 71 min for DiC_8-treated cells and 106 min for the diuretic furosemide (1 mM). Thus, in these cells, DiC_8 alters $^{86}Rb^+$ fluxes, in both an inward and an outward direction, in a manner similar to that of diuretics. Therefore, with respect to $^{86}Rb^+$ fluxes, DiC_8 acts in an identical manner to TPA.

It is likely that these effects of DiC_8 are a specific response of the $Na^+/K^+/Cl^-$-cotransport system rather than a generalized effect of the diacylglycerol on membrane permeability, for the following reasons: $^{86}Rb^+$ influx via the ouabain-sensitive Na^+/K^+-ATPase is unaffected by DiC_8 (Fig. 1); the diffusional component of total $^{86}Rb^+$

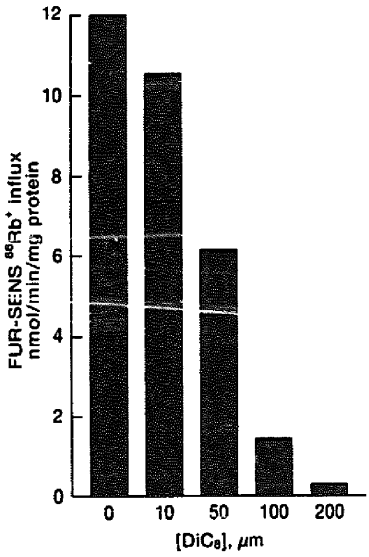

Fig. 2. Concentration-dependence for the effect of DiC_8 on furosemide-sensitive $^{86}Rb^+$ influx. Nearly confluent cultures of BALB/c 3T3 cells were treated with the indicated concentration of DiC_8 for 30 min, then assayed for furosemide-sensitive $^{86}Rb^+$ influx, as described in Materials and Methods. Results are the mean of triplicate determination of fluxes in the presence and absence of 1.0 mM furosemide, with 2 mM ouabain present in all dishes. Since the data presented represent the difference between two fluxes, error bars are not given. However, the mean S.D. of all groups was approx. 12%.

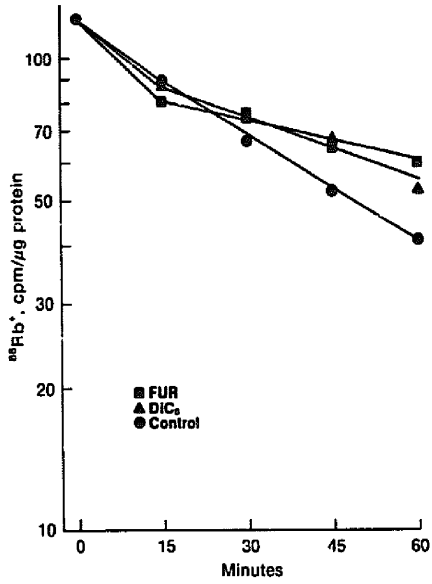

Fig. 3. Effect of DiC_8 on $^{86}Rb^+$ efflux. Nearly confluent cultures of BALB/c 3T3 cells were loaded with $^{86}Rb^+$ for 4 h at 37°C in growth medium, then washed and incubated in medium containing no addition (●), 100 mM DiC_8 (▲) or 0.1 mM furosemide (■). At the indicated times, the efflux was stopped, as described in Materials and Methods, and the $^{86}Rb^+$ content of the cells was determined. Points are the average of duplicate dishes per experimental point.

influx (insensitive to both furosemide and ouabain) is not altered by DiC_8, and the diacylglycerol does not affect any component of $^{86}Rb^+$ flux in BALB/c 3T3 mutant cells defective in $Na^+/K^+/Cl^-$-cotransport activity (data not shown).

Effects of diacylglycerols on 2-deoxyglucose uptake

Another membrane transport system of BALB/c 3T3 cells known to be modulated by TPA is the hexose transporter [1,11]. By analogy with the experiments testing the effect of diacylglycerol on $^{86}Rb^+$ fluxes, similar experiments were carried out in order to determine whether hexose transport could also be modulated by DiC_8. As shown in Fig. 4, DiC_8 at 0.1 mM did not stimulate 2-deoxyglucose uptake, at least within the first 16 h after treatment. Treatment with TPA (0.16 μM) resulted in a typical 20-fold stimulation at 6 h after addition. Concentrations of DiC_8 of 0.01–0.2 mM were ineffective when 2-deoxyglu-

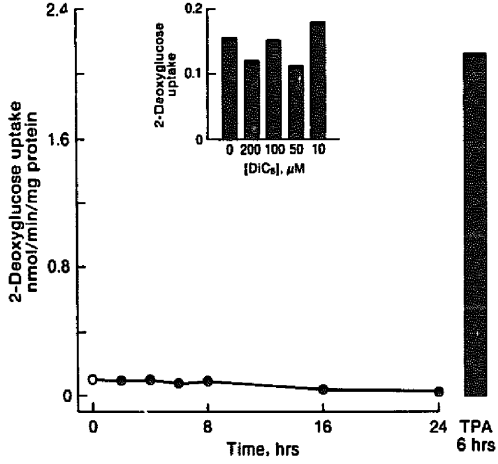

Fig. 4. Effect of DiC_8 on 2-deoxyglucose uptake. Confluent cultures of BALB/c 3T3 cells were treated with 0.1 mM DiC_8 for the indicated times or 0.16 μM TPA for 6 h and then assayed for 2-deoxyglucose uptake, as described in Materials and Methods. In a separate experiment, the cells were exposed to different concentrations of DiC_8 for 4 h (inset). Results are the average of duplicate dishes.

TABLE I

EFFECT OF DIACYLGLYCEROL PRETREATMENT ON TPA-INDUCED 2-DEOXYGLUCOSE UPTAKE

Cells were treated for 4 h as indicated prior to assay of 2-deoxyglucose uptake. In some cases, dishes were pretreated 15 min prior to TPA with DiC_8 in DMSO vehicle. Results are the mean (±S.E.) of triplicate dishes.

Pretreatment	Treatment	2-Deoxyglucose uptake (nmol/min per mg protein)
–	none	0.063±0.005
–	TPA, 0.16 μM	1.35 ±0.003
–	DiC_8, 0.2 mM	0.033±0.001
DiC_8, 0.2 mM	TPA, 0.16 μM	0.91 ±0.01
DMSO	TPA, 0.16 μM	1.21 ±0.09

cose uptake was measured at 4 h after addition (Fig. 4, inset). As illustrated in Table I, pretreatment of cells with a high concentration of DiC_8 (200 μM) also did not impair the ability of the cells to respond to subsequent TPA treatment. In this and repeat experiments, DiC_8 did cause a reproducible 25–30% inhibition of the TPA-stimulated level of 2-deoxyglucose uptake. The significance of this slight reduction is not known. In mutant cells lacking $Na^+/K^+/Cl^-$-cotransport activity, DiC_8 similarly did not stimulate hexose uptake (data not shown), even though TPA is capable of stimulating this transport system in these cells to approximately the same extent as in parental cells [8].

Because of the likelihood of rapid metabolism of DiC_8, it was considered possible that the inability of this compound to activate the hexose transport system was due to the transient nature of PKC activation by this agent. A more sustained activation of PKC may be necessary to achieve a significant increase in hexose transport activity. To test this possibility, DiC_8 was added at hourly intervals (up to eight additions, each to a final concentration of 200 μM) to BALB/c 3T3 cells and 2-deoxyglucose uptake measured over an 8-h period. As shown in Table II, no increase in 2-deoxyglucose uptake was observed after DiC_8 addition, while a single application of TPA resulted in the expected 35-fold increase. Rapid metabolism of DiC_8 does not appear to be responsible for the lack of effect of this compound on 2-deoxyglucose uptake.

Effect of TPA and DiC_8 on 80-kDa protein phosphorylation in parental vs. cotransport-defective cells

It was considered necessary to establish whether the PKC effector system was functional in both parental and $Na^+/K^+/Cl^-$-cotransport-defective cells and whether it could be modulated by DiC_8 as well as TPA. To address this question, two-dimensional gel analyses were performed of phosphoproteins labeled in vivo after brief (5–15 min) exposure of parental and cotransport-defective cells. For simplicity we restricted our analysis to the well-characterized PKC substrate of 80-kDa molecular mass and an acidic isoelectric point [15,17]. As shown in Fig. 5, a 5-min treatment of TPA stimulated phosphorylation of this protein in confluent cultures of both cell types. In the experiment shown, the basal level of phosphorylation of the 80-kDa protein was quite low or absent in parental cells, but substantially higher in cotransport-defective cells (compare A to C). This difference may relate to the relative degree of quiescence these cells reach at confluence, or the potential for adipocyte differentiation at postconfluence [9]. Nevertheless, in both cell lines, TPA caused a large increase in the phosphorylation of this protein relative to a reference protein whose phosphorylation status was unaffected by TPA (arrows). A similar experiment is shown in Fig. 6, except that here the cells were exposed to 200 μM

TABLE II

EFFECT OF HOURLY ADDITIONS OF DIACYLGLYCEROL ON 2-DEOXYGLUCOSE UPTAKE

Cells were treated with DMSO to a final concentration of 0.1% or DiC_8 to a final concentration of 0.2 mM every 60 min. At 60 min after one, three, five or seven such treatments, cultures were assayed for 2-deoxyglucose uptake. The dishes exposed to TPA received a single treatment (final concentration, 0.16 μM) 4 h before assay of uptake. Results are the average of two dishes per experimental treatment. The 2-deoxyglucose uptake activity of untreated cultures was 0.05 nmol/min per mg protein.

Treatment	2-Deoxyglucose uptake (nmol/min per mg protein)			
	2 h	4 h	6 h	8 h
DMSO	0.08	0.07	0.05	0.04
DiC_8	0.04	0.06	0.05	0.06
TPA		1.18		

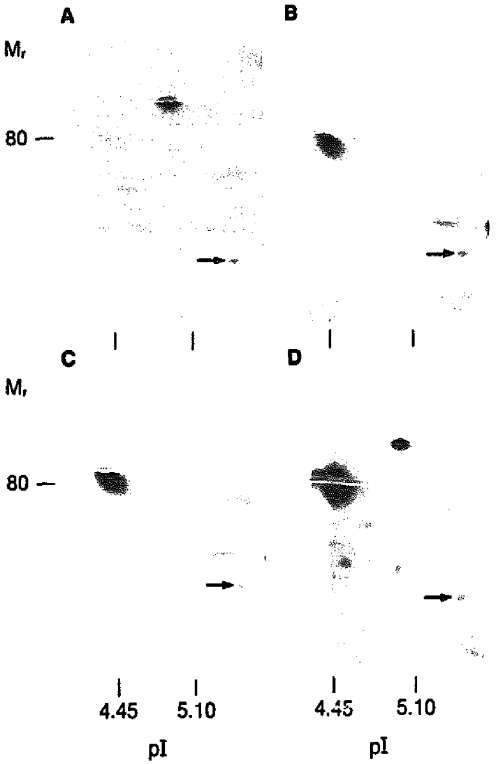

Fig. 5. Two-dimensional gel analysis of phosphoproteins from parental and cotransport-defective 3T3 cells exposed to TPA. Confluent cultures of parental (A, B) or the cotransport-defective cell line 3T3-C5 (C, D), were labeled with [^{32}P]P_i for 3 h at 37°C. Cultures were then treated with ethanol (A, C) or TPA (B, D) for 5 min, then harvested for two-dimensional gel electrophoresis, as described in Materials and Methods. Equal amounts of cpm (approx. $1.5 \cdot 10^5$) were run on isoelectric focusing gels and the final, dried two-dimensional gels were exposed to X-ray film with intensifying screens for an identical length of time. In A, no phosphorylation of the 80-kDa protein substrate was detected – the dark spot at the top middle of the panel represents a protein of higher molecular weight and more basic p*I* whose phosphorylation status is reduced by TPA. The arrow indicates a reference spot where phosphorylation is consistently unaffected by TPA. In other similar experiments, a variable level of labeling of the 80-kDa protein was observed in ethanol-treated parental cells, but in every case TPA markedly increased the level of phosphorylation.

DiC_8 for 15 min. In this experiment, the basal levels of phosphorylation of the 80-kDa protein were similar in parental and cotransport-defective cells, and DiC_8 caused a 2-fold stimulation of phosphorylation in each cell line (quantitated by densitometry). We have consistently found that while DiC_8 stimulated a phosphorylation of the 80-kDa protein, the peak response always occurred later (i.e., 15 min vs. 5 min for TPA) and the extent of phosphorylation was always less than with TPA when direct comparisons were made in the same experiment (data not shown). The important conclusion from this limited phosphoprotein analysis was that the PKC effector system was capable of being activated by TPA and DiC_8 in both parental and cotransport-defective cells.

Do diacylglycerols mimic TPA effect on ODC gene expression?

We have previously suggested, based on experiments in the same parental and cotransport-defective cell lines used in this study, that ionic signals produced by modulation of $Na^+/K^+/Cl^-$-

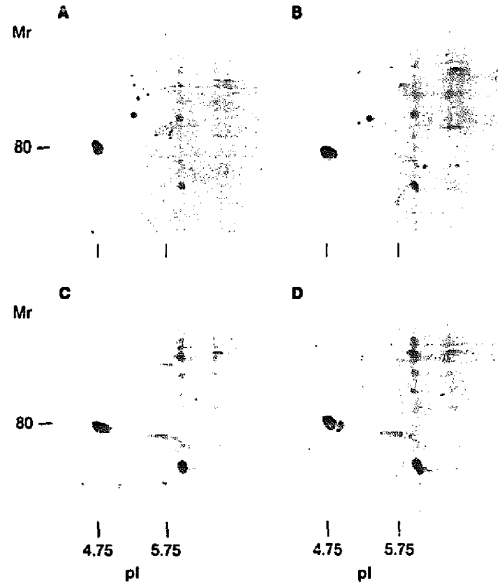

Fig. 6. Two-dimensional gel analysis of phosphoproteins extracts from parental and cotransport-defective 3T3 cells exposed to DiC_8. A similar experiment to that shown in Fig. 6 was done, except cells were treated with 0.2 mM DiC_8 (B, D) for 15 min, instead of 0.16 μM TPA for 5 min. Quantitation by laser densitometry indicated that the stimulation of phosphorylation by DiC_8 in this experiment relative to ethanol-treated cultures was 1.80-fold for parental cells (A, B) and 2.15 (C, D) for cotransport-defective cells.

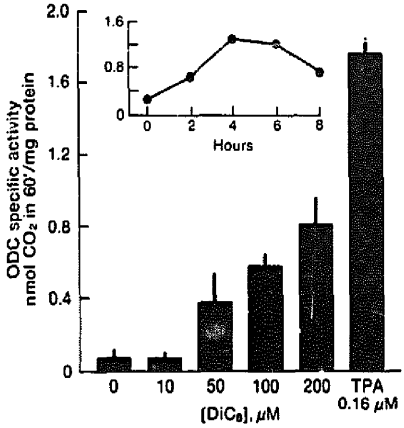

Fig. 7. Effect of DiC_8 or ornithine decarboxylase activity. Confluent cultures of BALB/c 3T3 cells were treated with the indicated concentration of DiC_8 for 4 h, or 0.16 μM TPA for the same time, then harvested for ODC measurements, as described in Materials and Methods. In a separate experiment, cells were treated with 0.1 mM DiC_8 for 2–8 h and harvested as above (inset). Results are the average of duplicate dishes.

cotransport activity by TPA are required for subsequent effects of the tumor promoter on gene expression [8]. If PKC is involved in the modulation of cotransport activity, as is suggested by the diacylglycerol experiments described above, then PKC activation by exogenous diacylglycerol should lead to changes in gene expression like those seen after TPA treatment. To address this question, we investigated whether the biologically active diacylglycerol DiC_8 induces ODC, an enzyme rapidly induced by TPA in many cell types including BALB/c 3T3 preadipose cells [18]. As shown in Fig. 7, DiC_8 caused a dose-dependent induction of ODC with a time-course very similar to the previously determined time-course for TPA in these cells [18], although the magnitude of the induction is not quite as large as that caused by TPA. Albeit much higher than that for TPA, the half-maximally effective concentration of DiC_8 for inducing ODC (approx. 50 μM) was the same as that for inhibiting $Na^+/K^+/Cl^-$-cotransport in these same cells (Fig. 2). The ability of DiC_8 to induce ODC in parental 3T3 cells, as has been reported for epithelial cells in vitro [19] and mouse epidermis in vivo [20], demonstrates that diacylglycerols can affect gene expression in the same manner as TPA. Because of the time-course of hexose transport activation by TPA [11], it is conceivable that one attribute of an effective inducer of hexose transport system activity in these cells would be the ability to influence gene expression, perhaps of the gene encoding the transport protein itself. While the mechanism of hexose transport activation by TPA or DiC_8 is not known, the lack of effect of DiC_8 on sugar transport cannot simply be ascribed to a general inability to influence gene expression, since ODC is effectively induced by DiC_8 under identical experimental conditions in which hexose transport is unaffected by this chemical.

Discussion

One of the major unanswered questions concerning the mechanism of action of phorbol ester tumor promoters is the identity of the critical substrates for PKC that generate intracellular signals leading to changes in cellular behavior. While numerous physiological substrates for this enzyme probably exist, it is not yet clear in any experimental system which of these substrates are involved in signal transduction. The approach we have taken to solving this problem in BALB/c 3T3 preadipose cells is a genetic one, involving the isolation of cell mutants defective in what is likely to be an important signal-generating target of phorbol esters, the $Na^+/K^+/Cl^-$ cotransporter. If this membrane protein is indeed an important target of phorbol esters, rendering it non-functional by mutation might make such cells unresponsive to phorbol esters. This has proved to be the case when biologic effects such as ODC induction [8] and mitogenesis [9] have served as indices of phorbol ester responsiveness. However, there has been no direct evidence that PKC, either activated or in its basal state, can regulate the activity of the $Na^+/K^+/Cl^-$-cotransport system. In this study, we provide evidence that activation of PKC by means other than TPA can lead to inhibition of $Na^+/K^+/Cl^-$-cotransport activity. The ability of exogenous diacylglycerols to mimic the effect of TPA on $Na^+/K^+/Cl^-$-cotransport activity (Figs. 1–3) and ODC induction (Fig. 5) reinforces the view that PKC-mediated phosphorylation can modulate cotransport activity, resulting in ionic signals that trigger ODC gene expression.

Further support for this hypothesis is the finding that neither TPA [12] nor exogenous diacylglycerol (this report) alter $^{86}Rb^+$ fluxes or induce ODC in mutant cell lines defective in $Na^+/K^+/Cl^-$ cotransport. This lack of responsiveness is apparently not due to a loss of specific phorbol ester binding [8] or the inability of TPA or DiC_8 to stimulate phosphorylation of specific PKC substrates such as the 80-kDa protein of acidic p*I* (Figs. 5 and 6). Together with the substantial literature on the ability of phorbol esters and/or exogenous diacylglycerols to modulate several membrane transport systems, our data suggests that these functional entities represent an important class of PKC substrates in the plasma membranes of mammalian cells. However, because of the difficulties involved in purifying and reconstituting many membrane transport proteins, the necessary direct biochemical evidence that PKC-mediated phosphorylation causes functional changes in transport activity has not yet been obtained.

While PKC is undoubtedly involved in modulating a variety of transport systems in many cell types after phorbol ester treatment, a related question would be of whether PKC always mediates the observed effect of phorbol esters on membrane transport. To address this question, it would be useful to have an experimental system in which more than one membrane transport system is known to be modulated by TPA. Such a situation exists in BALB/c 3T3 preadipose cells, wherein TPA inhibits $Na^+/K^+/Cl^-$-cotransport activity and stimulates hexose transport activity. In contrast to the similar effects of TPA and DiC_8 on $Na^+/K^+/Cl^-$-cotransport activity, divergent responses to these two agents are seen when the hexose transport system is considered. Under identical treatment conditions in which an effect on $Na^+/K^+/Cl^-$-cotransport activity can be observed, no effect of DiC_8 on 2-deoxyglucose uptake was measured in either parental or cotransport-defective cells (Fig. 4). At least three possible explanations for the divergent responses of the hexose transport system to TPA vs. DiC_8 can be offered. First, because of the different time-courses for peak responses of the $Na^+/K^+/Cl^-$-cotransport system and the hexose transport system to TPA (minutes vs. several hours), sustained activation and/for translocation of PKC may be required for modulation of hexose transport to occur. Diacylglycerols (but not TPA) may not be able to accomplish such sustained action due to its rapid metabolism and/or qualitative differences in PKC translocation triggered by DiC_8 vs. TPA. Secondly, the mechanisms involved in modulation may be quite different for these two transport systems and exhibit different sensitivities to exogenous diacylglycerol. Our previous results demonstrate a rapid effect of TPA on $Na^+/K^+/Cl^-$-cotransport activity suggesting functional modulation of existing transporters.

Activation of hexose transport by TPA or diacylglycerol may require PKC activation, but the important substrate(s) of PKC is not the transporter itself but proteins involved in the synthesis of turnover of the hexose transporter. It is known that depending on the cell type, the mechanism of hexose transport activation by TPA can apparently be quite different. In mesenchymal-derived cell lines, such as the BALB/c 3T3 preadipocyte cell line used here, the increase in hexose transport due to TPA occurs over a relatively long time-frame (several hours) and requires new protein and mRNA synthesis, presumably reflecting new transporter synthesis [21]. TPA treatment, however, can rapidly increase the rate of hexose uptake in isolated adipocytes, perhaps by causing the translocation of transporters from an inactive intracellular pool to an active configuration in the plasma membrane [22]. The observation reported here that the slower activation of hexose transport in undifferentiated preadipose cells by TPA was not mimiced by diacylglycerol treatment may reflect different mechanisms of transport regulation in differentiated vs. undifferentiated adipocytes. It is at least conceivable that diacylglycerols may rapidly activate hexose transport in adipocytes via PKC, but may have no effect on the same transport system in preadipose cells because the biochemical mechanisms involved are distinct. Finally, the third mechanism that can account for our results is the involvement of PKC in the modulation of $Na^+/K^+/Cl^-$-cotransport activity by TPA, but not in the activation of hexose transport. Unfortunately, such a hypothesis is difficult to prove in the absence of suitable reconstitution systems, such as using purified hexose transporter

incubated with TPA in the presence and absence of purified PKC and its necessary substrates and cofactors. It is at least theoretically possible that phorbol esters may regulate at least some membrane properties by PKC-independent mechanisms. Recently, on the basis of similar indirect evidence, Brooks et al. [23] have suggested that TPA may inhibit agonist-induced phosphoinositide hydrolysis via a PKC-independent mechanism.

The aforementioned concept that stimulation of hexose transport by TPA could involve two distinct mechanisms, one occurring rapidly and not involving new transporter synthesis and the other occurring more slowly and requiring new transporter synthesis, could explain some of the apparently conflicting reports in the literature concerning TPA/diacylglycerol effects on hexose transport. For instance, in human polymorphonuclear leukocytes, addition of an exogenous diacylglycerol was shown to stimulate 2-deoxyglucose uptake [24], while in Swiss 3T3 cells 'down-modulation' of PKC prevented subsequent restimulation of hexose transport by phorbol ester [25]. In human erythrocytes and 3T3-L1 adipocytes, it has been reported that TPA catalyzes the phosphorylation of the glucose transporter [26,27], presumably via PKC, although the functional significance of this phosphorylation has not been demonstrated. At this point it is not clear whether the differing conclusions on the involvement of PKC in regulating hexose transport reflect mechanistic differences, as proposed above, or differences in cell type, transport protein, or experimental design. Nevertheless, our data suggest that TPA-induced modulation of membrane transport and other membrane properties may exhibit different sensitivities to the presumed physiological activators of PKC (diacylglycerols). Although our previous results and the present data from BALB/c 3T3 preadipose cells strongly implicate the $Na^+/K^+/Cl^-$ cotransporter as both a substrate of PKC and an important component of the cellular signal transduction pathway used by phorbol esters, it is conceivable that PKC-independent mechanisms account for other transport effects of these agents, such as hexose transport activation, as well as some of the long-term changes in growth and differentiation [28] known to be affected by phorbol esters. The nature of such mechanisms is not known, but this question merits further study.

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