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Translocation of Diacylglycerol Kinase From the Cytosol to the Membrane in Phorbol Ester–Treated Swiss 3T3 Fibroblasts

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The tumor-promoting phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate, causes a rapid, partial redistribution of 1,2-diacylglycerol kinase from the cytosol to the particulate fraction of quiescent Swiss 3T3 fibroblasts. The inactive alpha form of the phorbol ester does not cause any change in diacylglycerol kinase localization, and depletion of protein kinase C by chronic administration of phorbol ester blocks the redistribution. Phorbol ester has no direct effect on membrane-bound diacylglycerol kinase in 3T3 cells. When phorbol ester is added to 3T3 membranes in the presence of ATP, Mg^{2+}, and Ca^{2+}, there is no activation of membrane-bound kinase, indicating that phorbol ester does not activate membrane-bound kinase through phosphorylation by protein kinase C. Stimulation of the cells with phorbol ester increases the total mass of diacylglycerol. In protein kinase C–depleted cells, addition of a cell-permeable synthetic diacylglycerol, dioctanoylglycerol, results in a partial redistribution of cytosolic diacylglycerol kinase to the membrane, also suggesting that the translocation of DAG kinase is regulated primarily by substrate concentration.

Key words: tumor promoters, protein kinase C, phosphatidylinositol, lipid, second messenger

1,2-Diacylglycerol (DAG) metabolism appears to be of critical importance in the control of cell growth and differentiation [1]. DAG is an endogenous activator of protein kinase C [2], the high-affinity phorbol-ester receptor [3]. Transformed cells possess significantly elevated DAG levels, even under conditions of exponential growth [4,5], and the induction of terminal differentiation of a transformed erythrocyte cell line is accompanied by a rapid decrease in DAG levels [6], suggesting that DAG may play an essential role in the maintenance of the transformed phenotype. A major route for removal of DAG is through its phosphorylation to phosphatidic acid, a metabolite that has recently been reported to possess mitogenic activity [7–9]. DAG kinase is therefore likely to prove a key element in the regulation of DAG metabolism.

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DAG kinase is an ubiquitous enzyme activity found mainly in the cytosol but also associated with membranes [10] and microtubules [11]. Whether the bound and free forms of the enzyme are of identical structure has not yet been clearly resolved. Soluble DAG kinase from pig brain can use endogenous membrane substrate [12], and Besterman et al. have reported that the soluble DAG kinase can bind to DAG-enriched membranes [13]. These results all suggest that the membrane-bound activity is a consequence at least in part of the association of cytosolic DAG kinase species with the membrane. They also suggest that changes in DAG concentration in intact cells may directly mediate the translocation of DAG kinase from the cytosol to the membrane.

12-O-tetradecanoyl-phorbol-13-acetate (PMA) induces the cytosolic-membrane translocation of some "ambiquitous" proteins, such as CTP-cytidyltransferase and protein kinase C. On the basis of these observations, we have asked whether phorbol ester induces the translocations of DAG kinase to the 3T3 cell membrane.

We have used a rapid method of separating cytosol from membrane to show that in serum-starved Swiss 3T3 fibroblasts, PMA decreases cytosolic DAG kinase activity as the membrane activity concurrently increases. The effect is abolished in cells depleted of protein kinase C but can be restored by addition of synthetic dioctanoylglycerol (diC8). We therefore propose that the redistribution of DAG kinase induced by PMA is at least in part a consequence of a protein kinase C–dependent increase in membrane DAG concentration.

MATERIALS AND METHODS

Materials

Phorbol esters were from LC Systems (Woburn, MA). Dioctanoylglycerol, phosphatidylserine, and cardiolipin were from Avanti Polar Lipids Inc. (Birmingham, AL). *E. coli* DAG kinase was from Lipidex Inc. (Westfield, NJ). Swiss 3T3 cells were kindly provided by Don Young (University of Rochester, NY) and were grown in Dulbecco's modified essential medium (DMEM) with 10% calf serum. They were serum starved in 0.33% calf serum for 24–48 h before use.

Digitonin-Mediated Release of DAG Kinase From Swiss 3T3 Fibroblasts

Cytosolic extract was obtained by digitonin permeabilization, as described by Pelech et al. [14,15] with the following modifications: cells were incubated at 0°C for 6–10 min in 0.5 ml digitonin buffer (0.25 mg/ml digitonin, 20 mM Hepes–NaOH, pH 7.4, 0.25 M sucrose, 2.5 mM EDTA, 10 mM sodium fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The cytosolic extract was then collected and spun in a microfuge for 10 min to remove debris, and sodium deoxycholate was added to 1 mM. The insoluble fractions were incubated in digitonin buffer containing 1 mM deoxycholate and scraped into microfuge tubes. Cytosolic and particulate fractions were then assayed for DAG kinase and lactate dehydrogenase (LDH) activity.

Enzyme Assays

DAG kinase activity was measured essentially as described by Kanoh et al. [16], with modifications detailed in Maroney and Macara [17]. Under these conditions the

assay was linear with time (0–20 min) and protein concentration (0–150 μg). LDH activity was measured by the method of Johnson [18].

Measurement of DAG Mass in Swiss 3T3 Membranes

DAG concentration was measured by equilibrium [^{3}H]-glycerol labelling [5] and by the mass assay of Preiss et al. [20].

RESULTS

To determine the distribution of DAG kinase between the cytosol and membrane, we have applied a rapid digitonin permeabilization technique, similar to that described by Pelech et al. [14,15]. To validate the procedure, the release of the cytosolic marker, lactate dehydrogenase (LDH), was monitored after addition of the detergent. As shown in Figure 1A, 0.025% digitonin causes a rapid release of LDH activity into the medium, with a half-time of approximately 3 min, at 4°C. More than 90% of total LDH activity is released, indicating a high efficiency of permeabilization. Assay of supernatant DAG kinase showed that about 65% of the total activity was released from the cells after permeabilization, with kinetics similar to those of the LDH release (Fig. 1B). Therefore, about one-third of the total 3T3 cell DAG kinase

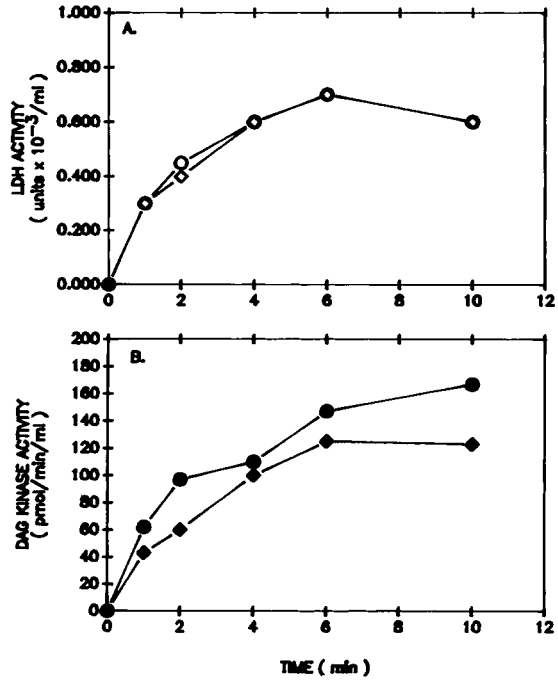

Fig. 1. Time course for digitonin-induced release of cytosolic enzymes from 3T3 fibroblasts. Duplicate plates were preincubated with either or 100 nM PMA in DMSO (diamonds) or vehicle alone (circles) for 2 min before permeabilization. The cells were placed on ice and permeabilized as described in Materials and Methods, and the cytosolic fractions were assayed for LDH activity (**A**) and DAG kinase activity (**B**).

appears to be associated with the particulate fraction. Validation of the DAG kinase assay on the cytosolic and particulate fractions is described elsewhere [17].

To determine whether the stimulation of protein kinase C affects DAG kinase distribution, 100 nM PMA was added 2 min before cooling and permeabilizing the cells. As shown in Figure 1B, this treatment caused a small but consistent decrease in the amount of activity released from the cells. To ensure that this effect was not a result of a change in the efficiency of permeabilization, LDH release was monitored simultaneously. As can be seen from Figure 1A, no difference in LDH release was caused by addition of PMA. To determine whether the decreased cytosolic DAG kinase activity was a consequence of inhibition by PMA, the phorbol ester was added directly to the cytosolic fraction before assay. No inhibition was observed at any concentration tested, indicating that PMA does not compete with DAG for binding to the kinase. This result is of some interest since phorbol esters and diacylglycerols do compete for binding to protein kinase C [19].

To determine whether PMA induces a translocation of cytosolic DAG kinase to the particulate fraction, the activities in the two fractions were measured in parallel, with the cells permeabilized at different times after addition of 100 nM PMA. As can be seen from Figure 2, phorbol ester produces a very rapid, but transient, increase in membrane-associated DAG kinase activity that parallels the decrease in cytosolic activity. The redistribution at 2 min post addition of PMA is highly significant ($P < .001$ for the cytosol and $P < .0005$ for the membrane) as determined by the Student t-test for paired samples. Measurements of LDH activity revealed that there was less than 8% cytosolic contamination in the particulate fraction, which represents only about 15% of the total membrane DAG kinase activity. This level of contamination did not change with PMA treatment and therefore can not account for the 54% increase in membrane activity after PMA addition. The sum of the cytosolic and

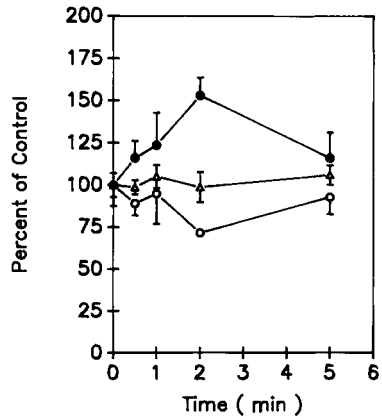

Fig. 2. Time course for PMA-induced translocation of DAG kinase from the cytosol to the particulate fraction in 3T3 fibroblasts. Cells were incubated with PMA (100 nM final concentration) for various times. Plates were then washed and permeabilized as described in Figure 1. DAG kinase activity is expressed as percentage control of triplicate samples in the cytosol (open circles), membrane (closed circles), and total activity (open triangles). Values are ± 1 S.E.M. (n = 3). Significant differences from control values were determined for the 2-min time point by the Student t-test for paired samples (cytosol: $P < .001$; membrane: $P < .0005$, n = 21).

TABLE I. Effect of PMA, α-PMA, and Protein Kinase C Depletion on the Distribution of DAG Kinase in 3T3 Fibroblasts*

	DAG kinase activity (pmol/min/ml)			
	Cytosol		Membrane	
Treatment	Control (T = 0 min)	Treated (T = 2 min)	Control (T = 0 min)	Treated (T = 2 min)
100 nM PMA	51.7 ± 8	37 ± 1.2	25.5 ± 0.7	42 ± 6
100 nM α-PMA	62.7 ± 9	78 ± 12	17.3 ± 2	17.4 ± 0.7
100 nM PMA*	82 ± 11	92 ± 10	41 ± 5	46 ± 3

*Cells were pretreated with 500 nM PMA for 24 h. Control shows the DAG kinase activity in the absence of drugs (time zero). Results are shown as the mean of triplicate samples ± one standard deviation.

membrane absolute activities is constant, implying that PMA does not directly stimulate the membrane activity.

To ascertain whether this effect of the phorbol ester is mediated by protein kinase C, two controls were performed. First, cells were treated with the inactive isomer, α-PMA, before permeabilization. Second, cells were depleted of kinase C before the experiment by chronic administration of PMA, and were then challenged with a further addition of the active (β) ester. No redistribution of DAG kinase was detected (Table I) in either case.

Finally, to be certain that PMA did not directly activate the membrane-bound DAG kinase, the phorbol ester was added to membrane fractions after the digitonin permeabilization. No increase in activity was observed (Table II). Moreover, when PMA was added to 3T3 membranes in the presence of MgATP, under conditions that activate membrane-bound protein kinase C, no stimulation of membrane DAG kinase activity (Table II) was observed. Rather, this treatment caused a substantial decrease in membrane-bound activity, regardless of the presence of PMA or its inactive isomer. We do not yet know whether this observation represents inhibition or release from the

TABLE II. Direct and Indirect Effects of PMA on Membrane-Bound DAG Kinase in Swiss 3T3 Cells*

	DAG kinase activity (ρmol/min/mg)				
	Control	+α-PMA	+PMA	+MgATP/Ca^{2+} +α-PMA	+MgATP/Ca^{2+} +PMA
Membrane	88 ± 4	75 ± 8	85 ± 13	5 ± 3	7 ± 2
Cytosol	138 ± 12	127 ± 30	153 ± 33	N.D.	N.D.

*Starved cells were permeabilized and the membrane fractions were scraped off the plates in 5 mM phosphate buffer pH 7.4. The cytosolic acid and membrane fractions were preincubated with 5 mM $MgCl_2$ and 5 mM $CaCl_2$ in the absence or presence of 4 mM ATP, at 30°C for 5 min. The membranes were then treated with 100 nM PMA or 100 nM α-PMA for 2 min. Cytosolic fractions received the same treatments, but in the absence of ATP. After treatment, the cytosol was placed on ice and the membrane fractions were spun in a microfuge for 10 min. The supernatant was discarded and the membrane pellet was suspended in digitonin buffer containing 1 mM deoxycholate. Results are shown as mean of triplicate samples ± 1 standard deviation.

membrane, because the buffer contains an unknown concentration of ATP, which interferes with the DAG kinase assay. The decrease in activity was not due to the presence of calcium [17], however, which suggests that there is an ATP-dependent increase in the off-rate of membrane-bound DAG kinase.

It has been previously shown that partially purified cytosolic DAG kinase from brain can bind to red cell membranes in a DAG-dependent fashion [13]. The results described here therefore could be interpreted in two ways: protein kinase C regulates the translocation of DAG kinase either directly, through phosphorylation, or indirectly, through the activation of a metabolic pathway that generates DAG, such as phosphatidylcholine turnover [21–24].

To test the hypothesis that PMA induces the transient translocation of 3T3 cell DAG kinase by increasing the concentration of membrane DAG, we first assayed cells for total DAG by isotopic and mass measurements. Although no significant increase in [^{3}H]-DAG was detected by [^{3}H]-glycerol labeling, mass measurements that used the procedure of Preiss et al. [20] showed a consistent 45% increase in cell DAG ($P < .1$, n = 4, by Student t-test for paired samples) within 2 min of PMA addition (Table III).

Finally, to demonstrate that a change in membrane DAG can directly induce a translocation of DAG kinase in intact 3T3 cells, exogenous synthetic diacylglycerol (diC8) was added to cells before digitonin permeabilization. To prevent the diC8 from activating protein kinase C, the cells were pretreated for 24 h with 500 nM PMA. As shown here and elsewhere [25], such treatment blocks response to further protein kinase C stimulation. The effect of 50 μM diC8 is shown in Figure 3. A significant redistribution of DAG kinase enzyme from the cytosol to the membrane occurs within 5 min of diC8 addition. These results strongly suggest that the PMA-induced translocation of DAG kinase is mediated by an increase in the membrane concentration of DAG.

DISCUSSION

A rapid, partial translocation of DAG kinase from the cytosol to the membrane of Swiss 3T3 cells has been observed in response to stimulation by phorbol ester. This translocation appears to be mediated at least in part by a PMA-dependent increase in cell DAG concentration. PMA has previously been reported to inhibit phosphatidylinositol turnover [26] but increases phosphatidylcholine breakdown [21–24]; it is

TABLE III. DAG Levels in PMA-Treated 3T3 Fibroblasts*

Addition	Percentage of ^{3}H-DAG	DAG/60-mm plate[a] (nmol)	DAG/60-mm plate[b] (nmol)
None	5.26 ± 0.15	16.7 ± 1.1	12.8 ± 0.6
100 nM PMA (2 min)	5.99 ± 0.47	25 ± 1	18.2 ± 1.3

*DAG was assayed either isotopically, using cells labeled to equilibrium with [^{3}H]glycerol [5] or by the mass measurement described in Preiss et al. [20], with *E. coli* DAG kinase. Results are shown as the mean of triplicate samples ± 1 standard deviation.

[a]Normalized to cell number.

[b]Normalized to total ^{3}H-glycerolipids.

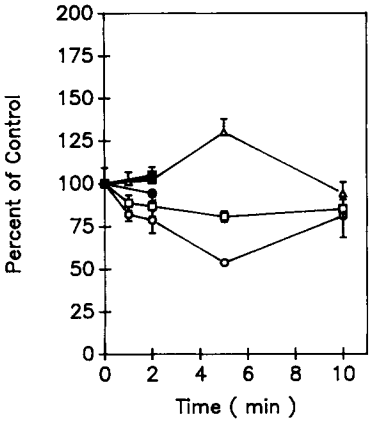

Fig. 3. DiC8-induced translocation of DAG kinase in PMA down-regulated 3T3 fibroblasts. PMA (500 nM) was added to deplete the cells of kinase C 24 h before addition of either 50 μM diC8 in DMSO (open symbols) or 100 nM PMA (closed symbols). The plates were then placed on ice at intervals and permeabilized as described in Figure 1. The cytosolic (circles), membrane (triangles), and total activity (squares) are expressed as percentages of control of triplicate samples. Values are ± 1 S.E.M. (n = 3).

likely therefore that the DAG originates from this latter process. PMA has no direct effect on DAG kinase activity, despite the fact that it competes with DAG for binding to protein kinase C [19]. This result indicates that phorbol esters recognize a very specific structure in kinase C rather than a consensus binding site, and DAG kinase therefore may provide a useful negative control in the search for pharmacologically valuable inhibitors of protein kinase C.

Our data do not address the issue of alternative mechanisms either for regulating the binding of DAG kinase to the membrane or for the reversal of binding, and it remains possible that other factors, such as direct phosphorylation of the enzyme by protein kinase C, play an important role. Kanoh and Ono [27] have reported that a DAG kinase from brain is phosphorylated on serine residues, although the kinase responsible for phosphorylation was not identified and it is not known whether the phosphorylation state changes with cell stimulation. Interestingly, another ambiquitous enzyme, CTP:phosphocholine cytidyltransferase, which also translocates to membranes on activation of protein kinase C by phorbol esters, has been shown to respond in the same way to the addition of exogenous diacylglycerol in kinase C-depleted cells [24]. Because DAG is not a substrate for this transferase, the mechanism controlling the translocation is not clear, but it suggests that the cellular concentration of DAG may control the distribution and activity of a wide variety of enzymes independently of the stimulation of protein kinase C. Whether the transient nature of the movement of DAG kinase to the membrane reflects a similarly brief change in DAG level is not yet known, although Besterman et al. [22] have provided evidence that the PMA induced production of DAG is transient. It is also possible that separate mechanisms control the on- and off-rates from the membrane. The loss of membrane-bound activity in the presence of MgATP, for instance, may indicate that catalytic activity and production of phosphatidic acid causes subsequent release from the membrane. A detailed investigation of this problem will await the availability of pure DAG kinase.

We have recently discovered that rat brain cytosol contains at least six distinct isoforms of DAG kinase (Maroney, A., and Macara, I.G., manuscript in preparation), and it is therefore possible that if 3T3 cells also contain multiple forms of the enzyme, the relatively small translocation in overall DAG kinase activity seen in the experiments described here may reflect a much more dramatic translocation by a single isoform or a subset of the isoforms present in the cytosol. The substrate specificities and tissue distribution of these DAG kinase isoforms is currently under investigation.

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