Phospholipase D-derived Products in the Regulation of 12-O-Tetradecanoylphorbol-13-acetate-stimulated Prostaglandin Synthesis in Madin-Darby Canine Kidney Cells*

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Madin-Darby canine kidney (MDCK) cells stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in the presence of ethanol synthesize phosphatidylethanol (PEt) instead of phosphatidic acid (PA) and diglyceride (DG). We have used ethanol to block the production of phospholipase D (PLD)-derived PA and DG (from PA hydrolysis) to study their role in signal transduction. In MDCK cells, TPA-stimulated prostaglandin E$_2$ (PGE$_2$) synthesis was inhibited by ethanol at concentrations which inhibit PA and DG formation. In addition, TPA elicited a prolonged increase in PGE$_2$ synthesis that is dependent upon continuous activation of PLD. The TPA-stimulated translocation of protein kinase C$_a$ (PKC$_a$) from cytosol to membrane was unaffected by ethanol. This suggests that PLD-derived products act downstream of PKC in TPA-stimulated prostaglandin synthesis. The calcium ionophore, A23187, did not activate PLD, and PGE$_2$ synthesis in response to A23187 was unaffected by ethanol. TPA increased prostaglandin endoperoxide H synthase (PGHS) activity and increased the amount of immunodetectable prostaglandin endoperoxide H synthase 2 (PGHS-2). A23187 did not induce PGHS-2 and A23187-stimulated PGE$_2$ synthesis appears to be due to the constitutively expressed PGHS-1. Blocking the formation of PLD-derived products, PA and DG, inhibited the induction of PGHS-2 by TPA. These results indicate that prolonged PGE$_2$ synthesis in response to TPA is due to the continuous induction of PGHS-2, which is dependent upon PLD activation. In contrast, induction of PGHS-2 by epidermal growth factor was not affected by ethanol. Epidermal growth factor did not induce PKC$_a$ translocation nor activate PLD. Taken together, these data suggest that PLD-derived PA or DG act as second messengers in the induction of PGHS-2 by PKC-dependent pathways. The demonstration that inhibition of TPA-induced PA formation inhibits Raf-1 translocation in MDCK cells (Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L. W., and Bell, R. M. (1996) J. Biol. Chem. 271, 8472-8480) suggests that PA is the active PLD metabolite in TPA-stimulated signaling.

The tumor promoter, TPA, stimulates PC turnover in MDCK cells, resulting in the generation of DG, the major endogenous activator of PKC. Agonist-induced hydrolysis of PI was once considered to be the sole mechanism by which DG is generated. However, hormones, growth factors, and serum stimulate PC turnover in a variety of cell types; including fibroblasts (3, 4), neutrophils (5–10), and HL-60 cells (11, 12). It is now recognized that PC degradation is an important source of lipid-derived second messengers in cell signaling (for review, see Ref. 13). Because PI-derived DG is transient, a function of PC-derived DG may be to maintain PKC activation (14, 15). However, some groups have reported that DG derived from PC turnover does not activate PKC (16, 17).

The generation of DG from PC has been attributed to the direct action of PLC (18, 19) or to the sequential actions of PLD and PAP (20–24). In neutrophils (5, 6), HL-60 granulocytes (25), platelets (26), endothelial cells (27), and hepatocytes (22, 28), diverse agents that cause rapid hydrolysis of PC by PLD to generate PA and subsequently DG. Huang and Cabot (29) have demonstrated that TPA induces a time-dependent increase in the formation of PA prior to DG formation as a result of PLD activation in MDCK cells. As a result of its rapid and transient formation during cell activation, PA is thought to have a biological function in cell signaling. PA has been shown to stimulate DNA synthesis and cell division in fibroblasts (30) and has been shown to have a role in the activation of NADPH oxidase in human neutrophils (31–33). More recently, Khan et al. (34) have identified a PA-activated protein kinase in human platelets. However, a specific physiological function for this PA-activated kinase has not been determined.

In addition to these potential biological functions, PA can be rapidly converted to DG by PAP. Unlike PI-derived DG, DGs generated from PC degradation contain both acyl and alkyl linkages. Recently, we have demonstrated that TPA-stimulated PC hydrolysis by PLD is selective for alkyl-PC in MDCK cells, thus generating both alkyl-PA and -DG (35). The alkyl-DG generated did not activate PKC derived from MDCK cells.

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; 20:4, arachidonic acid; DG, diglyceride; EGF, epidermal growth factor; MDCK, Madin-Darby canine kidney; PLAP, phospholipase A$_2$; PLC, phospholipase C; PLD, phospholipase D; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylincholine; PET, phosphatidylethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PGE$_2$, prostaglandin E$_2$; PGHS, prostaglandin endoperoxide H synthase; PKC, protein kinase C; Tes, N-tri(hydroxy-methyl)methyl-2-aminoethane sulfonic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PLP, pyridoxal phosphate.

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cells, suggesting an alternative role for alkyl-PC turnover. Alkyl-DG has been shown to activate HL-60 acute myelocytic leukemia cell differentiation (36). In addition, it has been demonstrated that acyl-DG and alkyl-DG have differential effects on the metabolism of released 20:4 (37) and on the activation of NADPH oxidase (38). However, the significance of agonist-induced PLD activation has been unclear.

To determine a role for PLD activation, we used ethanol to block the production of PA and DG derived from the PLD/PAP pathway. In the presence of ethanol, PLD catalyzes a transphosphatidylation reaction generating PEt at the expense of alkyl-DG. Wang et al. (25) first demonstrated that PEt is formed exclusively by PLD in cultured cells, and utilized PEt formation as an indication of PLD activation by a variety of agonists. Huang et al. (29) have shown that ethanol completely blocks the formation of PA and DG by PLD in TPA-treated MDCK cells. In order to identify potential PLD dependent signaling events, ethanol was used to block PA and DG formation. We report herein that the inhibition of PA and DG synthesis by ethanol blocks TPA-induced PGE2 production in MDCK cells. Prolonged PGE2 synthesis by TPA is due to the induction of PGHS-2. This induction is dependent upon TPA-induced PLD activation. Taken together, these findings indicate that the products of PC degradation via the PLD/PAP pathway, are required for downstream signaling events in TPA-stimulated prostaglandin synthesis.

**Experimental Procedures**

Materials—MDCK cells were purchased from the American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Life Technologies, Inc. [5,6,8,9,11,12,14,15-3H]Arachidonic acid (1 mCi/ml) and [3H]20:4 (50 mCi/mmol) were purchased from American Type Tissue Culture, Inc. All solvents were purchased from Fisher. Silica Gel 60 plates were from EM Science (Merck). 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from LC Services Corp., Woburn, MA. Calcium ionophore A23187 and epidermal growth factor were gifts from Dr. Robert L. Wylie and Dr. Peter B. Smith, respectively (Wake Forest University). PGHS-1 and -2 antibodies were a gift from Dr. David DeWitt (Michigan Science (Merck)). 12-[14C]-Tetradecanoylphorbol-13-acetate (TPA) was obtained from American Radiolabeled Chemicals, Inc. All chemicals were purchased from Sigma. 100,000 cells/ml were seeded into 6-well plates, and allowed to adhere overnight. The medium was then replaced with 1 ml of fresh DMEM containing 10% FBS. MDCK cells were grown for 2–3 days (or until 80% confluent) at 37°C. After TPA stimulation, cells were detached by trypsinization and washed with ice-cold buffer consisting of 5 mM Tris, 5 mM KCl, 2.5 mM MgCl2, 138 mM NaCl, and 5.5 mM glucose, pH 7.4. The cells were pelleted by centrifugation at 800 x g for 8 min at 4°C. The pellet was resuspended in 2 ml of buffer and sonicated on ice for 3 x 30-s bursts with a Branson probe-type sonicator set at 10% of maximum energy. The sonicates were assayed immediately. Protein content was determined by the method of Bradford (41) using bovine serum albumin as a standard. One mg of total protein from sonicates was incubated for 3 min at room temperature in 1 ml of assay buffer consisting of 100 mM Tris-HCl, pH 8.5, 2 mM reduced glutathione, 5 mM l-tryptophan, 1 mM hematin, 0.1% Triton X-100, and 10 μM 20:4 containing 0.5 μCi of [3H]20:4 per sample to be assayed as described by Beaudry et al. (42). The reaction was stopped by the addition of 2 ml of methanol, 10 mM HCl, and 2 M acetic acid. Lipids were extracted and quantitated as described previously. Specific activities were calculated by determining the percent conversion of [3H]20:4 to all stable prostaglandin products. Specific activities were expressed as pmol of 20:4 converted to prostaglandins/min/mg of total protein.

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Prostaglandin Endoperoxide H Synthase 1 and 2 Analysis—MDCK cells were grown for 2–3 days (or until 80% confluent) at 37°C. After stimulation, cells were washed with cold phosphate-buffered saline and lysed in 100 μl of lysis buffer consisting of 100 mM Tris, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 10 min. Cell pellets were centrifuged for 10 min at 4°C. Protein content was determined by the method of Bradford (41) using bovine serum albumin as a standard. 100 μg of protein from each cell lysate sample were separated by SDS-polyacrylamide gel electrophoresis (5% stacking, 7% running gel) according to Laemmli (43), and transferred onto nitrocellulose membranes (Schleicher and Schuell) at 100 mAmps, at room temperature overnight. Membranes were blocked with phosphate-buffered saline containing 5% non-fat dry milk and 1% Tween 20 for 1 h; and then hybridized with primary antibodies in phosphate-buffered saline containing 5% bovine serum albumin and 0.2% sodium azide for an additional hour. After washing, the membranes were incubated with goat anti-rabbit horseradish peroxidase (Transduction Labs) for 1 h. PGHS-1 and -2 were detected using enhanced chemiluminescence (Du Pont NEN) according to the instructions of the manufacturer.

Translocation of PKCα—MDCK cells were grown for 2–3 days (or until 80% confluent) at 37°C. Cells were incubated with serum-free DMEM overnight at 37°C prior to stimulation. Cells were then stimulated with 10 nM TPA or 100 nM EGF in the presence or absence of 1% ethanol. After stimulation, cells were washed with cold phosphate-buffered saline and scraped in buffer H (10 mM Hepes, pH 7.4, 2 mM EDTA, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride). Cells were pelleted and resuspended in 500 μl of buffer H plus 50 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were lysed by sonication on ice for 3 x 15-s bursts with a Branson probe-type sonicator set at 10% of maximum energy. Untouched cells were pelleted by centrifugation at 2,950 x g for 2 min at 4°C. Cell sonicates were then centrifuged (Bedman) at 120,000 x g for 80 min at 4°C. The cytosol was removed and saved. Membrane pellets were sonicated as before in buffer H plus 50 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cytosolic proteins were analyzed for PKCα by methods described above using a monoclonal mouse PKCα antibody (Upstate Biotechnology Inc.) as the primary antibody and a goat anti-mouse horseradish peroxidase antibody (Transduction Labs) for 1 h. The membranes were washed with 5% bovine serum albumin and 0.2% sodium azide for an additional hour. After washing, the membranes were incubated with goat anti-mouse horseradish peroxidase (Transduction Labs) for 1 h. PGHS-1 and -2 were detected using enhanced chemiluminescence (Du Pont NEN) according to the instructions of the manufacturer.

**Results**

When we used ethanol to block PA and DG formation by PLD, TPA (10 nM)-induced PET formation increased with increasing concentrations of ethanol (0–2%, v/v), while the formation of TPA-induced PGE2 was inversely proportional to the concentration of ethanol (Fig. 1). Inhibition of PGE2 synthesis was seen at pharmacological concentrations (0.1%) of ethanol. Similar results were obtained using butanol (0–50 mM, data not shown). From these data, we chose 1% ethanol to determine the time dependent effect of ethanol on PGE2 synthesis. TPA-stimulated PGE2 synthesis was inhibited by ethanol (Fig. 2) at
Similar results were obtained in three independent experiments.

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data provide additional evidence that ethanol is not causing
alterations in cellular lipid metabolism as a result of nonspe-
cific membrane or metabolic perturbations. In order to deter-
mine if ethanol is acting on a specific signaling pathway, we
compared ethanol’s effect on 20:4 release and PGE2 production
induced by the calcium ionophore, A23187, or TPA (Fig. 5).
PGE2 synthesis was inhibited by ethanol in response to TPA
(Fig. 5) but not in response to 10 μM A23187 (Fig. 5). Ethanol
carried a small reduction in TPA-mediated 20:4 release (Fig. 5);
however, the release of free 20:4 induced by A23187 was not
affected by the presence of ethanol (Fig. 5). In addition, A23187
is not a good activator of PC-PLD (data not shown) and does
not stimulate prolonged PGE2 synthesis (49). Ethanol had no
effect on PGE2 production nor 20:4 release in unstimulated
cells (Fig. 5).

To further characterize ethanol’s effect on PGE2 synthesis,
we determined if ethanol was inhibiting TPA-induced PGHS
activity (Fig. 6). MDCK cells were stimulated with 10 nM TPA
in the presence or absence of ethanol (1%) for 4 h. Sonicates
were prepared and assayed for PGHS activity as described
previously (42). These data show that there is a reduced stim-
ulation of PGHS activity in intact MDCK cells stimulated
with TPA in the presence of ethanol (Fig. 6A). In contrast, ethanol
had no effect on induced PGHS activity in response to 100 nM
EGF (Fig. 6B). When sonicates (control or TPA-treated) were
assayed in ethanol (1%), PGHS activity was not affected,
indicating that ethanol did not inhibit the conversion of 20:4 to
prostaglandins in vitro (data not shown).

Beaudry et al. (42) demonstrated that inhibition of protein
synthesis with cycloheximide prevented the induction of PGHS
activity by TPA. This indicated that the increased PGHS
activity in MDCK cells involves de novo synthesis of the enzyme.
To obtain direct evidence that TPA stimulates de novo synthesis
of PGHS in MDCK cells, immunodetection was employed
using polyclonal antibodies against PGHS-1 and PGHS-2.
MDCK cells were stimulated with TPA in the presence or
absence of ethanol for 6 h. Cell lysates were prepared and
analyzed for PGHS-1 and -2. TPA caused a marked stimulation
of PGHS-2 (Fig. 7, bottom). When PLD product formation was
blocked by ethanol, TPA-induced PGHS-2 was inhibited (Fig. 7,
bottom). MDCK cells express low levels of PGHS-1, and this
expression was not inhibited by the presence of ethanol (Fig. 7,
top). PGHS-1 was not detected in TPA-treated cells in the
presence or absence of ethanol. Increased PGE2 synthesis by
TPA (Fig. 2) correlated with the marked induction of PGHS-2
(Fig. 7, bottom, and Fig. 10, top) without any significant effect
on PGHS-1 (Fig. 7, top). By 4 h, PGHS-2 synthesis resumed in
cells stimulated with TPA in the presence of ethanol (Fig. 10,
top), which correlates with the rate of PGE₂ synthesis seen in
Fig. 2. When ethanol is added at the time of stimulation, by 4 h
the ethanol is apparently depleted; and PA and DG formation
as well as PGHS-2 synthesis resumes. In contrast, A23187 did
not cause induction of PGHS-2 (data not shown).

To determine if the induction of PGHS-2 seen in cells not
stimulated with TPA was due to serum stimulation (Fig. 7,
bottom), MDCK cells were made quiescent (1% FBS/DMEM for
48 h) followed by serum starvation for 3 h prior to stimulation.
PGHS-2 was induced by serum (10% FBS) and serum stimulation
was inhibited by the presence of ethanol (Fig. 8). We also
found that serum-stimulated PLD activity in MDCK cells made
quiescent by serum reduction.² In addition, ethanol did not
affect PGHS-2 in unstimulated cells (Fig. 8).

Previous work from this laboratory suggested that PKC ac-
tivation by TPA is involved in the increased prostaglandin
production (44). In addition, it has been reported that PKCα
mediates PLD activation and PGE₂ synthesis induced by TPA

² C. Huang, V. Sciorra, and L. Daniel, unpublished observations.

FIG. 3. Long-term activation of PLD and PGE₂ production by TPA. MDCK cells were incubated with 1 μCi/ml [³H]20:4 for 24 h at 37 °C.
Cells were stimulated with 10 nm TPA. Two h before each indicated time point, the media were removed and replaced with fresh DMEM (1 ml)
containing 1% ethanol. Untreated (no TPA, no ethanol) cells were harvested at time 0 to give background levels of PEt and PGE₂. Lipids were
analyzed as described in the legend of Fig. 1. Data are expressed as total disintegrations/min in lipid ± S.E. (n = 3). Similar results were obtained
in three independent experiments.

FIG. 4. The effect of ethanol on the incorporation of [³H]20:4
into phospholipids. MDCK cells were incubated with 1 μCi/ml
[³H]20:4 in the presence (open circles) or absence (filled circles) of
ethanol (1%) for the indicated times. Lipids in cells were extracted and
resolved on Silica Gel 60 plates developed in a solvent system consisting
of chloroform/methanol/acetic acid/water (50:25:8:2, v/v). NL, neutral
lipid. Data are expressed as total disintegrations/min in lipid ± S.E.
(n = 3). Similar results were obtained in two independent experiments.

FIG. 5. Ethanol inhibits PGE₂ production in response to TPA
but not in response to A23187. MDCK cells were incubated with 1
μCi/ml [³H]20:4 for 24 h at 37 °C. Cells were stimulated with 10 nm TPA
or 10 μm calcium ionophore A23187 in the presence or absence of 1%
ethanol for the indicated times. Lipids were extracted and quantitated
as described in the legend of Fig. 1. Data are expressed as total disin-
tegrations/min in lipid ± S.E. (n = 3). Similar results were obtained in
two independent experiments. ●, stimulated; ○, stimulated +EtOH; ◦,
unstimulated; Δ, unstimulated +EtOH.

DISCUSSION

The degradation of PC by the PLD/PAP pathway is a potential
source of lipid-derived second messengers; however, the role of PC-derived products has not been determined (13). It
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has been suggested that PC-derived DG acts to sustain PKC activation (48); however, alkyl-linked DGs are also generated by PC degradation, and neither stimulate PKC nor inhibit its activation by diacylglycerol (35). It is possible that PA generated directly from PLD serves as a second messenger in cellular signaling. In this report, we have used ethanol to examine if PLD-derived products, PA and DG, participate in TPA-stimulated PGE2 synthesis. In MDCK cells, TPA-stimulated PGE2 synthesis was inhibited by ethanol at concentrations which inhibit PA and DG formation. Previously, we reported that TPA stimulates prolonged PGE2 production in MDCK cells (49). We report here that TPA also stimulates prolonged activation of PLD. PGE2 synthesis in response to TPA is dependent on continuous activation of PLD. Thus, it appears that PA and/or DG derived from PC degradation by the PLD/PAP pathway act as second messengers in the regulation of TPA-stimulated PGE2 synthesis.

Regulation of prostaglandin synthesis involves the regulation of both 20:4 mobilization and PGHS synthesis. It is believed that phospholipase A2 (PLA2) is the primary enzyme involved in regulating 20:4 liberation. Balsinde et al. (50) showed that ethanol inhibited prostaglandin synthesis in zymosan-stimulated macrophages, but did not directly inhibit PLA2. This report suggested that ethanol inhibited a process leading to PLA2 activation. In addition, Fernández et al. (51) demonstrated that PA induces 20:4 mobilization in mouse peritoneal macrophages. The mechanisms by which PA potentiates 20:4 release remain in question. Ethanol caused a small reduction in TPA-mediated 20:4 release; however, this effect cannot fully explain the complete inhibition of PGE2 synthesis in response to TPA. In addition, 20:4 release in response to A23187 and 20:4 incorporation into phospholipids was not affected by ethanol. It is possible that ethanol inhibits a process leading to PLA2 activation, for example, alterations in cPLA2 phosphorylation may result in reduced activity.

In addition to our observations in MDCK cells, Diez et al. (52) have shown that ethanol has no effect on PGHS or lipoxygenase activities in intact macrophages or in a cell-free system. PGHS activity is inhibited in TPA-treated MDCK cells grown in the presence of ethanol. PGHS activity induced by TPA can also be blocked by protein synthesis inhibitors (42). This suggested that TPA stimulates the de novo synthesis of PGHS. Recently, two PGHS genes were identified (53). PGHS-1 was first purified from ovine seminal vesicles (54) and is present in virtually all mammalian tissues (55). In contrast, PGHS-2 was originally isolated as a v-src inducible gene product in chicken fibroblasts (56). PGHS isoforms from a single species are about 60% identical, with protein regions believed to be important for enzyme function conserved (57). A major difference between the two gene products lies in the factors that regulate expression (58). PGHS-2 is up-regulated in inflammation and mitogenesis (59). PGHS-1 was once considered to be constitutively expressed in mammalian cells (53); however, there have been recent reports that the expression of PGHS-1 and not PGHS-2 is modulated in some cell types (60–64).

We report that MDCK cells constitutively express PGHS-1, and that TPA stimulates the induction of PGHS-2 and not PGHS-1. PGHS-1 expression remains constant in unstimulated cells grown in the presence or absence of ethanol; while the induction of PGHS-2 in response to TPA is inhibited by ethanol. The increase in PGHS-2 correlated with the elevated PGE2 synthesis. Furthermore, prolonged PGE2 synthesis and the continuous induction of PGHS-2 are dependent on PLD-derived product formation, since both of these events are inhibited by blocking PLD product formation with ethanol.

Balboa et al. (45) have demonstrated that PKC mediates PLD activation by TPA in MDCK cells. It has been previously shown in this laboratory that PKC activation by TPA is required for TPA-stimulated 20:4 release and PGE2 synthesis (44). In addition, Godson et al. (46) have demonstrated that PKC is involved in TPA-mediated 20:4 release. We have demonstrated that blocking PLD-derived products with ethanol does not inhibit the translocation of PKC in response to TPA. These data indicate that PLD-derived products act further...
downstream from PKC. We also have shown that EGF did not cause significant translocation of PKCα (other isozymes have not been examined) nor cause activation of PLD. However, the effects of EGF on phospholipid metabolism appear to be cell-specific (65–67). EGF induces PGHS-2 synthesis, and this induction was not affected by ethanol. It appears that EGF stimulates PGHS-2 synthesis by a PKC-independent pathway not requiring PLD activation.

It is possible that the signaling pathways in response to both TPA and EGF converge downstream of PKCα activation, leading to the induction of PGHS-2. There is now evidence that activation of the mitogen-activated protein kinase pathway is required for PGHS-2 induction by v-src (68) and both TPA and EGF activate mitogen-activated protein kinase (69, 70). Therefore, the pathways may differ in their activation of upstream kinases (for example, Raf-1). Activation of Raf-1 can trigger a protein kinase cascade which results in mitogen-activated protein kinase activation (71). Once activated, mitogen-activated protein kinase can translocate to the nucleus and activate a variety of transcription factors (72).

We have recently shown that TPA stimulates Raf-1 translocation to the membrane in MDCK cells (73). Further studies found that ethanol, used under the same conditions as described herein, inhibits TPA-induced translocation of Raf-1 (73). In addition, PA, not diacylglycerol or PET, binds to Raf-1 and stimulates Raf-1 binding to model membranes (73). These data indicate that PA may be the active metabolite of PC-PLD-dependent signaling in TPA-stimulated MDCK cells.
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