Evidence That Protein Kinase C Is Involved in δ-Aminolevulinate Synthase Expression in Rat Hepatocytes

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There are many factors that regulate the rate of synthesis of δ-aminolevulinate synthase (ALA-S), the enzyme which governs the rate-limiting step in heme biosynthesis. In rat hepatocytes, phenobarbital increases ALA-S gene transcription and dibutyryl cAMP potentiates this induction, whereas insulin and glucose have the opposite effect. The present report provides evidence that protein kinase C (PKC) activation negatively influences ALA-S mRNA levels, as measured by Northern and slot-blot analysis. The addition of 1,2-dioctanoyl-sn-glycerol (DOG) or 12-O-tetradecanoylphorbol 13-acetate (TPA), a PKC activator that mimics diacylglycerol function, to cultures led to a significant decrease of both basal and phenobarbital-induced ALA-S mRNA levels in a dose-dependent manner. This TPA effect depends on the specific activation of PKC because the analog 4α-phorbol 12,13-diacetate, a nonstimulatory PKC phorbol ester, is unable to inhibit ALA-S mRNA. Furthermore, the effect of TPA is blocked by the PKC inhibitors staurosporine and calphostin C. Desensitization of the PKC pathway by prolonged exposure to TPA abolished the subsequent action of the phorbol ester. On the other hand, neither TPA nor DOG modified the half-life of ALA-S mRNA. The study of the combinatorial action of TPA and cAMP revealed that the inhibitory effect of TPA overcomes dibutyryl cAMP induction. Thus, these results indicate that PKC plays an essential role in regulating ALA-S expression, probably at transcriptional levels.

Key Words: aminolevulinic acid synthase; protein kinase C; phorbol esters; diacylglycerol; gene expression; hepatocytes; cAMP.
suggested that cAMP would exert its effect at the transcriptional level (12).

It is well known that the activated PKA appears to modulate the function of nuclear factors that bind to DNA sequences present in the promoter regions of cAMP-inducible genes. Most of these genes contain one or a few CRE (cAMP-responsive element). The consensus CRE is composed of the palindromic sequence TGA-CGTCA, which is strikingly similar to the binding site of the transcription factor AP-1 that mediates transcriptional induction in response to phorbol esters (13, 14). Another cAMP transcription element should be considered: this sequence, termed AP-2 binding site, confers transcriptional stimulation by cAMP and phorbol esters (15). Phorbol esters mimic the action of diacylglycerol, a physiologic mediator of cell surface signals, by activating PKC (16). Therefore, AP-2 seems to mediate transcriptional activation in response to these different signal transduction pathways, one activated by cAMP and involving PKA, the other activated by phorbol esters and diacylglycerol and involving PKC (15, 17).

The structure of the 5′-ALA-S gene sequences from rat liver has been elucidated recently by Brädött et al. (18) and Yomogida et al. (19). While the upstream region of the rat ALA-S gene does not contain a consensus sequence for AP-1 binding, there are sequences with approximately 75% homology to the CRE and 87% homology to the AP-2 element.

On the other hand, in a previous report we have demonstrated that insulin inhibits activity of ALA-S (20), although the mechanism by which this hormone exerts its effect is still unknown. Insulin increases the synthesis of phosphatidic acid, diacylglycerol, and many phospholipids in rat adipocytes (21). Likewise, insulin activates phospholipase C, which catalyzes the hydrolysis of phosphoinositide to diacylglycerol (22). These results might suggest that a number of insulin cellular effects may be attributed to the hormone’s ability to increase the formation of phospholipids and diacylglycerol. In this respect, some cases in which phorbol esters and insulin affect the expression of the same gene have been reported (23).

The presence of an AP-2 transcriptional factor consensus sequence in the promoter region of ALA-S gene and, on the other hand, the inhibitory effect of insulin on ALA-S mRNA and the well-established insulin-like effect of phorbol esters raise the possibility that PKC could be involved in the regulation of the ALA-S gene expression.

Due to the preceding observations, we decided to investigate the effects of the phorbol ester TPA—a potent tumor promoter—and of diacylglycerol—the physiologic activator of PKC—on the hepatic ALA-S mRNA. We have demonstrated that both compounds specifically decrease the quantity of ALA-S mRNA and that they operate at a transcriptional level. Their inhibitory effect seems to be mediated by PKC. Finally, TPA exhibits a dominant effect with respect to the cAMP inducer.

MATERIALS AND METHODS

Chemicals. Collagenase (type I), hyaluronidase (type I-S), DME/F12 medium, guanidine isothiocyanate, 12-O-tetradecanoylphorbol 13-acetate, 4α-phorbol 12,13-diacetate, 1,2-dioctanoyl-sn-glycerol, staurosporine, calphostin C, dibutyryl cAMP, random primer’s kit, salmon sperm DNA, Denhardt’s solution, agarose, and glyoxal were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-32P]ATP (sp act 6000 Ci/mmol) and [α-32P]dCTP (sp act 3000 Ci/mmol) were purchased from New England Nuclear–DuPont Co. (Wilmington, DE). T4polynucleotide kinase was purchased from Gibco BRL. All the other chemicals were of analytical grade.

Animals. Male chubb Thom albino rats (about 150–200 g wt) from Thomae Laboratories, (Boehringer–Ingelheim, Germany) were maintained under standardized conditions of light (from 0630 to 1830) and temperature (21°C). Animals were allowed free access to Purina Laboratory chow (41% carbohydrates, 23% proteins) and water. The rats were killed between 0700 and 0800 following 24 h of fasting.

Preparation and suspension of rat liver hepatocytes. Rats were killed by decapitation. Livers were removed, washed with ice-cold 0.9% saline, trimmed, and minced into small pieces. Cell suspensions were prepared according to Fry et al. (24). Seventy-eight percent of the cells were initially viable as judged by staining with trypan blue. The isolated liver cells were suspended to approximately 7 × 106 cells in 1 ml of DME/F12 medium, pH 7.4, devoid of glucose. The metabolic activity of this preparation was evaluated as described previously (25).

Treatments. Phenobarbital and dibutyryl cAMP were dissolved in 0.1 ml of DME/F12 medium. Phorbol esters, DOG, staurosporine, and calphostin C were dissolved in Me2SO and added to cell suspensions at zero time, unless otherwise indicated. Hepatocyte suspensions were gently shaken at 37°C in a Dubnoff water bath at 60 oscillations/min. Viability was determined at different times for each treatment. Usually cell viability fell by 1 to 2%/h as judged by trypan blue staining. At the time indicated in each experiment, samples containing about 5 × 107 cells were removed for RNA extraction as described below.

Total RNA extraction. Total RNA was extracted according to Chomczynski and Sacchi (26). RNA concentrations were determined spectrophotometrically assuming that A260 of 1.0 corresponds to 40 μg RNA/ml; its purity was routinely determined by measuring the 260:280 nm absorbance ratios, that were over 1.7 (27).

Northern and slot-blot analysis of ALA-S mRNA. Male chubb Thom albino rats (about 150–200 g wt) from Thomae Laboratories, (Boehringer–Ingelheim, Germany) were main-
polynucleotide kinase. The resulting probe had a specific activity of 4.5 x 10^6 cpm/mmol. Hybridization was carried out overnight at 70°C in the same prehybridization solution by adding a 32P-labeled oligonucleotide (2.7 x 10^6 cpm/mmol) probe. The filters were then serially washed three times with 1× SSC, 0.1% SDS for 2 min each at room temperature, and three times with 0.2× SSC, 0.1% SDS for 2 min each at 70°C (27). Autoradiographs were obtained by exposing these blots to Kodak films (X-OMAT, XAR 2) with an intensifying screen for 3–5 days at −70°C. Blots autoradiograms were quantitated with a densitometric scanner Bio-Rad Model GS-670.

Hybridization of G3PD mRNA. In order to detect G3PD mRNA, a cDNA was labeled by random priming using [α-32P]dCTP and Klenow, to a specific activity of 7.9 x 10^8 cpm/mg. Northern and slot blots were performed as described below. The membranes were prehybridized in 6× SSC, 0.01% SDS, 2× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 50% formamide for 1 h at 42°C. Hybridization was carried out overnight at 42°C in the same solution by adding the probe. Filters were then serially washed twice with 2× SSC, 0.1% SDS for 15 min each at room temperature, and twice with 0.2× SSC, 0.1% SDS for 15 min at 50°C. Autoradiographs were obtained by exposing these blots to Kodak films (X-OMAT, XAR 2) with a intensifying screen for 3–5 days at −70°C. Blot autoradiograms were quantitated with a densitometric scanner Bio-Rad Model GS-670.

RESULTS

In this work we present the results of ALA-S mRNA quantity obtained by Northern and slot-blot analysis in hepatocytes from normal rats. The probe specifically bound to a single RNA estimated to be 2.3 kb in length, consistent with the reported size of rat liver ALA-S mRNA (10, 12). As has been reported previously (12), there was an almost fourfold induction of ALA-S mRNA as a consequence of treating the hepatocytes with 0.6 mM phenobarbital for 3 h (Fig. 1).

Effect of TPA on ALA-S mRNA Level

Northern blots of total RNA extracted from normal hepatocytes maintained in culture for 3 h—with or without the addition of 1 μM TPA—were made to determine the effect of TPA on ALA-S mRNA.

Treatment with TPA reduces both basal and 0.6 mM phenobarbital-induced ALA-S mRNA levels approximately 75%, without altering the amount of the housekeeping G3PD mRNA (Fig. 1). Dimethyl sulfoxide—the TPA solvent—had no effect on either the basal or phenobarbital-induced levels of mRNA ALA-S (data not shown).

The TPA concentration–response curves for ALA-S mRNA inhibition in basal and phenobarbital-induced hepatocytes were examined in Fig. 2. These experiments revealed a clear dose-dependent effect. TPA was effective over a concentration range of 10^-8–10^-5 M. Maximal suppression of ALA-S mRNA was achieved with 10^-6 M and over, and the half-maximal concentration was approximately 10 nM for both basal and stimulated cells.

The time-course experiments of this response were examined using 1 μM TPA to achieve a maximal suppression of ALA-S mRNA (Fig. 3). Δ-Aminolevulinate synthase mRNA increased more than three times in cells preincubated for 2.5 h with 0.6 mM phenobarbital, and remained at that level for at least 2 h. Addition of TPA after the barbiturate preincubation resulted in a decrease of ALA-S mRNA. The t_{1/2} of this inhibition was approximately 20–25 min (Fig. 3B). Similar results were obtained with cells treated under basal conditions (Fig. 3A).

The action of 4αPDA—an analog of TPA that is ineffective in tumor promotion and PKC activation—was tested to exclude the possibility of a general inhibitory effect due to incubation with phorbol esters. The addition of 1 μM 4αPDA failed to diminish ALA-S mRNA in both basal and phenobarbital-treated hepatocytes (Fig. 1). These results were confirmed testing other concentrations (0.1 and 10 μM) (data not shown).

Effect of PKC Physiological Agonist DOG on ALA-S mRNA Level

The effect of phorbol ester suggested that diacylglycerol and PKC could be involved in the inhibition of ALA-S mRNA. In order to correlate TPA inhibitory ef-
treatment, respectively. No change was observed with TPA or DOG treatment following the addition of phenobarbital. In these cases, the ALA-S mRNA half-life was $20 \pm 1$ and $19 \pm 3$ min, respectively. Using the same approach, the ALA-S mRNA half-life was determined, both under basal conditions and in the phenobarbital-induction state. The results obtained in both cases were $20 \pm 3$ and $17 \pm 4$ min, respectively.

These results are similar to those reported previously (12) for the ALA-S mRNA half-life under basal conditions with physiologic PKC activators, similar experiments were carried out using DOG, a synthetic derivative that resists degradation and enters cells more readily than diacylglycerol. The effect of DOG was assayed at concentrations ranging from 50 to 1000 $\mu$M; it caused a concentration-dependent inhibition on both control and 0.6 mM phenobarbital-induced ALA-S mRNA. Conversely, G3PD mRNA was uninfluenced by the same range of DOG concentrations in the presence or in the absence of phenobarbital (data not shown).

The time courses of DOG effect on ALA-S mRNA are shown in Fig. 3. This effect is exerted rapidly on both control and phenobarbital-mediated induction of ALA-S mRNA with a $t_{1/2}$ of about 25–30 min.

Measurement of ALA-S mRNA Turnover

In order to determine if alterations in ALA-S mRNA levels following TPA or DOG treatments could be due to changes in its stability, the ALA-S mRNA degradation rate constant, $k$, was calculated under those conditions. Time course experiments shown in Fig. 3 were replotted in the integrated form (Fig. 3, inset). In this type of plot, the negative slope of the linear portion of this curve is equivalent to the degradation rate constant (28, 29). Half-life, $t_{1/2}$, was calculated from the relationship $t_{1/2} = \ln 2/k$. This analysis showed that—in the absence of phenobarbital—the ALA-S mRNA half-life was $18 \pm 2$ and $14 \pm 1$ min with TPA and DOG treatment, respectively. No change was observed with TPA or DOG treatment following the addition of phenobarbital. In these cases, the ALA-S mRNA half-life was $20 \pm 1$ and $19 \pm 3$ min, respectively. Using the same approach, the ALA-S mRNA half-life was determined, both under basal conditions and in the phenobarbital-induction state. The results obtained in both cases were $20 \pm 3$ and $17 \pm 4$ min, respectively.

These results are similar to those reported previously (12) for the ALA-S mRNA half-life under basal condi-

![FIG. 2. Effect of TPA concentration on basal and induced ALA-S mRNA. After a preincubation for 2.5 h in the absence (○) and presence (●) of 0.6 mM phenobarbital, different concentrations of TPA were added to cell suspensions and incubated for 1.5 h. ALA-S mRNA was determined by slot-blot analysis. Total RNA (20 $\mu$g/slot) was spotted to nitrocellulose filters and hybridized as described under Materials and Methods. Autoradiograph shows a representative experiment in which each of the successive slot blot represents each successive TPA concentration (left to right). Results are expressed as percentages of controls at zero time. Each point represents the average of two separate experiments in which individual values differed by less than 10% from the average.](image1)

![FIG. 3. Time curves of the effects of TPA and DOG on basal and induced ALA-S mRNA. Cell suspensions were preincubated for 2.5 h in the absence (A) and presence (B) of 0.6 mM phenobarbital. Subsequently, ALA-S mRNA was determined, at the indicated times, by slot-blot analysis. Total RNA (20 $\mu$g/slot) was spotted to nitrocellulose filters and hybridized as described under Materials and Methods. (●) TPA time courses, (○) without TPA addition, (●) with 1 $\mu$M TPA. (●●●●●●●●---------) DOG time courses, (○○○○○○○○) without DOG addition, (●●●●●●●●) with 500 $\mu$M DOG. Results are expressed as percentages of control at zero time and represent the mean ± SE of four separate experiments. (Inset) Kinetic determination of the half-life of ALA-S mRNA with 1 $\mu$M TPA or with 500 $\mu$M DOG in the absence (A) and presence (B) of 0.6 mM phenobarbital. Data from the time curves were replotted in the integrated form. $R_0$ is the level of ALA-S mRNA at each time, $R_0$ and $R_0*$ are initial and final levels, respectively, of ALA-S mRNA. (●●●●●●●●) from TPA time curves; (○○○○○○○○) from DOG time curves. Each point represents the mean ± SE of four separate determinations. The best fit lines have a correlation coefficient $>0.99$ in all cases. The negative slopes (equivalent to the degradation rate constant, $k$) of these curves were determined: A, (●●●●●●●●) $k = -0.039 \pm 0.003$ min$^{-1}$ and (○○○○○○○○) $k = -0.049 \pm 0.004$ min$^{-1}$; B, (●●●●●●●●) $k = -0.035 \pm 0.001$ min$^{-1}$ and (○○○○○○○○) $k = -0.036 \pm 0.005$ min$^{-1}$.](image2)
Prolonged Activation of PKC

In many cell types, prolonged treatment with phorbol esters resulted in almost complete depletion of cellular PKC. Because PKC activation led to ALA-S mRNA inhibition, the hepatocytes were pretreated with 1 μM TPA for 1.5 h (the time of maximal inhibition) to determine if PKC diminution by translocation to the cell membrane prevented this inhibition.

Figure 4 shows that prolonged PKC stimulation resulted in the restoration of ALA-S susceptibility to 0.6 mM phenobarbital. In this case, an almost 12-fold induction of ALA-S mRNA compared to that obtained with 0.6 mM phenobarbital plus 1 μM TPA incubation without any addition during the pretreatment was observed (compare lane 6 with lane 5). δ-Aminolevulinate synthase mRNA levels in control without any addition (lane 1) or with 1 μM TPA during the pretreatment (lane 3) are also shown in Fig. 4. As shown, 1 μM TPA inhibited ALA-S mRNA at 1.5 h, and this effect was maintained for the next 2.5 h (lanes 3 and 4).

Effect of PKC Inhibitors on ALA-S mRNA

Two PKC inhibitors, staurosporine and calphostin C, were used to fully understand the PKC influence on ALA-S mRNA (30). As shown in Fig. 5, the addition of 1 μM staurosporine or 1 μM calphostin C resulted in the blockage of the inhibitory effect of 1 μM TPA on both basal and 0.6 mM phenobarbital-induced ALA-S mRNA. In this way the levels of ALA-S mRNA remained unchanged in the presence of TPA and any of both PKC inhibitors when compared with the respective controls. Conversely, as can be seen in the same figure, staurosporine or calphostin C alone did not significantly modify the levels of ALA-S mRNA, either basal or induced by 0.6 mM phenobarbital.

Studies on the Combined Effect of cAMP and TPA on ALA-S mRNA

The relationship between the PKA and PKC pathways on the ALA-S mRNA level was studied. Cyclic

![Figure 4](image)

**FIG. 4.** The effect of prolonged activation of PKC on ALA-S mRNA. Cell suspensions were incubated for 1.5 h in the presence or in the absence of TPA as it is shown. At this time sample 3 was harvested and the other samples were incubated for an additional 2.5 h with the additions as indicated. 0 and 1.5 h represent the times when the additions were made. ALA-S mRNA was determined by slot-blot analysis. Total RNA (20 μg/slot) was spotted to nitrocellulose filters and hybridized as described under Materials and Methods. The autoradiograph shows a representative experiment. Autoradiographic signals were quantitated by densitometric scanning and are presented as bar graphs directly above each respective track. Results are expressed relative to control without any addition and represent the mean ± SD of four separate experiments. The paired Student t test was used to compare samples incubated in the presence of TPA with control (*P < 0.01), and samples incubated with TPA + PB with PB alone (**P < 0.001). Treatments: TPA, 1 μM TPA; PB, 0.6 mM phenobarbital.

![Figure 5](image)

**FIG. 5.** The effect of staurosporine and calphostin C on ALA-S mRNA. Cell suspensions were incubated for 3 h with the additions indicated. ALA-S mRNA was determined by slot-blot analysis. Total RNA (20 μg/slot) was spotted to nitrocellulose filters and hybridized as described under Materials and Methods. Hybridization to a probe for G3PD (bottom bands) was used to standardize results. Autoradiograph shows a representative experiment. Autoradiographic signals were quantitated by densitometric scanning and are presented as bar graphs directly above each respective track. Results are expressed relative to control without any addition and represent the mean ± SD of four separate experiments. The paired Student t test was used to compare samples incubated with different additions in the absence of PB with control (P < 0.01) and samples incubated with different additions in the presence of PB with PB alone (**P < 0.001). PB, phenobarbital; St, staurosporine; Cpn C, calphostin C.
diacylglycerol. Several lines of evidence indicate that PKC acts as a receptor for phorbol esters and diacylglycerol (32). Activation of PKC, as has been observed after treatment with several growth factors or phorbol esters, may influence biological events, such as the induction of DNA synthesis and cell division, and it may also alter the expression of specific genes in rat hepatic cells. In this respect, the inhibitory effect of TPA on the phosphoenolpyruvate carboxykinase expression was described in rat hepatoma H4IIE cells by Chu and Granner (33). On the other hand, Messina and Weinstock (34) have reported that β-actin gene transcription is induced by phorbol esters.

The aim of this work was to determine the possible role of PKC in the regulation of ALA-S gene expression according to previous observations from our laboratory (12, 20). For this reason, we determined the effects of a phorbol ester and a diacylglycerol analog—known to be direct stimulators of PKC activity—on ALA-S mRNA in rat hepatocytes.

The results presented here indicate that TPA rapidly reduced the amount of ALA-S mRNA on both control and phenobarbital-pretreated cells. The inhibitory effect of TPA was concentration- and time-dependent. Similar effects on ALA-S mRNA levels were observed with DOG, although a higher concentration was needed to achieve a full responsiveness, probably due to the lower lipophilicity of the diacylglycerol analog. Alterations in mRNA levels might reflect changes in its rate of synthesis, processing, or degradation. Therefore, ALA-S mRNA turnover was estimated in the presence of TPA or DOG. The results show that the rate of ALA-S mRNA degradation was not modified by TPA or DOG, suggesting that the observed decrease in the amount of ALA-S mRNA was the result of a reduction in its transcription rate or processing. In view of the close correlation observed between activity and the amount of ALA-S mRNA time courses in the presence of TPA or DOG (unpublished data), the transcription rate seems to be the primary site of phorbol ester and diacylglycerol action.

The well-documented interaction of phorbol esters and diacylglycerol with PKC suggests that protein phosphorylation is the mechanism by which TPA suppresses ALA-S mRNA transcription. Furthermore, the concentration of TPA required to inhibit the synthesis of ALA-S mRNA is similar to the concentrations of TPA needed to induce the phosphorylation of several cellular proteins (32, 35). Three lines of evidence support that PKC is involved in TPA- and DOG-mediated inhibition of ALA-S mRNA in rat hepatocytes. First, the effect of TPA is specific because phorbol ester 4αPDA has no effect on ALA-S mRNA. 4α-Phorbol 12,13-diace-
tate has been used as a negative control to distinguish between receptor-mediated activities of phorbol esters and nonspecific effects possibly due to their detergent-

**FIG. 6.** The effect of Bt2cAMP and TPA on ALA-S mRNA. Cell suspensions were incubated for 3 h with the additions indicated. ALA-S mRNA was determined by slot-blot analysis. Total RNA (20 μg/slot) was spotted to nitrocellulose filters and hybridized as described under Materials and Methods. Hybridization to a probe for G3PD (bottom bands) was used to standardize results. The autoradiograph shows a representative experiment. Autoradiographic signals were quantitated by densitometric scanning and are presented as bar graphs directly above each respective track. Results are expressed relative to control without any addition and represent the mean ± SD of four separate experiments. The paired Student’s t test was used to compare samples incubated in the presence of PB + TPA with PB alone (**P < 0.01), and samples incubated with PB + TPA + Bt2cAMP with PB + Bt2cAMP (***P < 0.01). PB, phenobarbital.

AMP potentiates phenobarbital-mediated induction of ALA-S mRNA in rat hepatocytes (12). Previous studies suggest that this action would be exerted by PKA activation (unpublished results). The aim of the present work was to determine the combined effect of both cAMP and TPA on ALA-S mRNA.

While 100 μM Bt2cAMP exerted an almost twofold potentiation of 0.6 mM phenobarbital induction, the addition of 1 μM TPA showed an 80% inhibition of the effect of Bt2cAMP. Likewise, 1 μM TPA seemed to overcome the cAMP effect, and the ALA-S mRNA inhibition mediated by PKC activation was dominant.

**DISCUSSION**

Protein kinase C, which is involved in intracellular signaling pathways in a variety of cell types and tissues, belongs to a family of serine/threonine protein kinases (31). The activation of one or more of these enzymes plays an important role in the second messenger system, which is necessary in many cellular functions. In addition to its physiologic activation by diacylglycerol, PKC is activated by tumor-promoting phorbol esters, which share some structural similarities with diacylglycerol. Several lines of evidence indicate that PKC acts as a receptor for phorbol esters and diacylglycerol (32). Activation of PKC, as has been observed after treatment with several growth factors or phorbol esters, may influence biological events, such as the induction of DNA synthesis and cell division, and it may also alter the expression of specific genes in rat hepatic cells. In this respect, the inhibitory effect of TPA on the phosphoenolpyruvate carboxykinase expression was described in rat hepatoma H4IIE cells by Chu and Granner (33). On the other hand, Messina and Weinstock (34) have reported that β-actin gene transcription is induced by phorbol esters.

The aim of this work was to determine the possible role of PKC in the regulation of ALA-S gene expression according to previous observations from our laboratory (12, 20). For this reason, we determined the effects of a phorbol ester and a diacylglycerol analog—known to be direct stimulators of PKC activity—on ALA-S mRNA in rat hepatocytes.

The results presented here indicate that TPA rapidly reduced the amount of ALA-S mRNA on both control and phenobarbital-pretreated cells. The inhibitory effect of TPA was concentration- and time-dependent. Similar effects on ALA-S mRNA levels were observed with DOG, although a higher concentration was needed to achieve a full responsiveness, probably due to the lower lipophilicity of the diacylglycerol analog. Alterations in mRNA levels might reflect changes in its rate of synthesis, processing, or degradation. Therefore, ALA-S mRNA turnover was estimated in the presence of TPA or DOG. The results show that the rate of ALA-S mRNA degradation was not modified by TPA or DOG, suggesting that the observed decrease in the amount of ALA-S mRNA was the result of a reduction in its transcription rate or processing. In view of the close correlation observed between activity and the amount of ALA-S mRNA time courses in the presence of TPA or DOG (unpublished data), the transcription rate seems to be the primary site of phorbol ester and diacylglycerol action.

The well-documented interaction of phorbol esters and diacylglycerol with PKC suggests that protein phosphorylation is the mechanism by which TPA suppresses ALA-S mRNA transcription. Furthermore, the concentration of TPA required to inhibit the synthesis of ALA-S mRNA is similar to the concentrations of TPA needed to induce the phosphorylation of several cellular proteins (32, 35). Three lines of evidence support that PKC is involved in TPA- and DOG-mediated inhibition of ALA-S mRNA in rat hepatocytes. First, the effect of TPA is specific because phorbol ester 4αPDA has no effect on ALA-S mRNA. 4α-Phorbol 12,13-diace-
tate has been used as a negative control to distinguish between receptor-mediated activities of phorbol esters and nonspecific effects possibly due to their detergent-
like structures. Thus, inactive phorbol ester does not bind to or activate PKC was not effective in diminishing ALA-S mRNA, in contrast with the marked inhibitory effect displayed by TPA. Second, experiments performed in the presence of staurosporine or calphostin C resulted in the blockage of the inhibitory effect of TPA on ALA-S mRNA. Staurosporine and calphostin C are demonstrated inhibitors of PKC (30); however, the use of inhibitors can be problematic. There have been several reports questioning the specificity of staurosporine. Additional data showed that it may have effects similar to those of phorbol esters (36). The effects of calphostin C on PKC activity were not studied in depth, but it has been documented as a highly specific PKC inhibitor (37). In spite of this, and assuming specificity by PKC, the data presented here illustrating the addition of staurosporine or calphostin C suggest that inhibition of ALA-S mRNA synthesis by TPA is dependent on PKC activation. However, we do not exclude the possibility that other protein kinases are being inhibited, and that these kinases are involved in ALA-S mRNA regulation. Third, in the present study both TPA and diacylglycerol rapidly decrease the amount of ALA-S mRNA; nevertheless, when cells were pretreated with TPA, the ability of this phorbol ester to decrease the level of ALA-S mRNA disappeared. Moreover, the capacity of phorbol to induce ALA-S mRNA was restored despite the presence of TPA. In this regard, it is well known that, in many cell types, prolonged treatment with phorbol esters results in almost complete depletion of cellular PKC (so-called down-regulation), which would be a prerequisite for proteolytic cleavage and would act in the prevention of permanent kinase activity (31). Consequently, the desensitization developed after pretreatment of rat hepatocytes with TPA observed in our experiments could be related to this down-regulation mechanism of PKC. Taken as a whole, these results strongly suggest that PKC is involved in TPA and DOG inhibitory effect on ALA-S transcription.

In a recent report we have presented data demonstrating that cAMP induces the synthesis of ALA-S mRNA in rat hepatocytes (12). This effect would be mediated by the catalytic subunit of cAMP-dependent protein kinase. Phorbol action in the presence of the inducer Bt$_2$cAMP was studied in order to obtain a clearer insight about the nature of the mechanism involved in the inhibitory effect exerted by TPA. The present data show that the effect of TPA in reducing the amount of ALA-S mRNA was dominant over the stimulatory effect of the cAMP derivative in combination with phenobarbital. In contrast to what happens with the action of TPA, cAMP requires the presence of a "heme-consuming drug," like phenobarbital, to exert its effect. Moreover, TPA seems to be equally effective for inhibiting phenobarbital-mediated induction of ALA-S mRNA in the presence or absence of Bt$_2$cAMP. These observations suggest that, in this case, TPA acts independently of the action of cAMP.

It is worth noting that PKC stimulation could be triggered through distinct pathways. An important insight into the molecular mechanisms underlying the regulation of ALA-S gene expression would be given by conducting studies on the relationship between PKC inhibition and insulin action. It has been reported that insulin increases diacylglycerol concentrations and PKC activity in cultured rat skeletal muscle (38). In other reports, the addition of insulin to H4 cells resulted in a decrease in cytosolic PKC activity, suggesting a translocation to the membrane response (39). Our previous work showed that insulin inhibits ALA-S mRNA; it is therefore possible that PKC had a role in insulin’s action.

In conclusion, this work provides evidence that TPA and DOG regulate ALA-S mRNA probably by inhibiting its synthesis. The identification of PKC as the intracellular target of TPA in many cellular systems (32) and the evidence presented here point to the involvement of PKC in this response. Our hypothesis is that PKC activation transmits a signal to the nucleus that results in suppressed ALA-S mRNA synthesis. This could occur via the phosphorylation of one or more of the proteins that regulate transcription of the ALA-S gene.

REFERENCES