Tumor-Promoting Phorbol Ester Amplifies the Inductions of Tyrosine Aminotransferase and Ornithine Decarboxylase by Glucocorticoid†

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ABSTRACT: In adrenalectomized rats, the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) markedly enhanced the inductions of tyrosine aminotransferase (TAT) and ornithine decarboxylase by glucocorticoids, even with sufficient concentration of glucocorticoids to have a maximal effect, whereas it had no effect on TAT activity and increased ornithine decarboxylase activity only slightly in the absence of glucocorticoids. Phorbol derivatives and components of TPA such as 4β-phorbol, phorbol 12-tetradecanoate, phorbol 13-acetate, and 4-*O*-methylphorbol 12-tetradecanoate 13-acetate, which have no tumor-promoting activity or ability to activate protein kinase C, did not have any effect on TAT induction by glucocorticoid. TPA enhanced the induction of TAT by various glucocorticoids but had no effect on induction of TAT by glucagon or insulin and did not enhance the induction of glucose-6-phosphate dehydrogenase by 17β-estradiol. These results suggest that TPA specifically enhances the induction of TAT and ornithine decarboxylase by glucocorticoids. Similar effects of TPA on TAT induction by glucocorticoid were observed in primary cultures of adult rat hepatocytes. Another activator of protein kinase C, *rac*-1,2-dioctanoylglycerol, was also found to have similar effects on the cells.

Recently the findings that many compounds, such as interferon, muramyl dipeptide, thymosin α_1, and various lymphokines, modulate immunological responses have led to the concept of "biological responses modulators" (Ivarie & O'-Farrell, 1978). In the case of hormonal responses, we have long proposed the existence of modulators that specifically enhance the actions of glucocorticoids in target organs or cells (Katunuma et al., 1971, 1977; Kido et al., 1977), and these modulators have recently been summarized and termed "glucocorticoid action biomodulators" (Kido et al., 1986).

We have found that *rac*-1,2-dioctanoylglycerol, a potent activator of Ca^{2+}/phospholipid-dependent protein kinase C, acts as a glucocorticoid action biomodulator: it markedly enhances the induction of liver enzymes by glucocorticoid in adrenalectomized rats (Kido et al., 1986). The properties of steroid hormone receptors are known to be modulated by phosphorylation and dephosphorylation (Sando et al., 1979; Leach et al., 1982), and the glucocorticoid receptor was shown to be phosphorylated by cAMP-dependent protein kinase in vitro (Singh & Mondgil, 1985). But the physiological role of phosphorylation in receptor function and the nature of the protein kinase that is involved in phosphorylation of glucocorticoid receptor under physiological conditions are unknown. From our findings, we speculate that protein kinase C has some roles in the action of glucocorticoid and that modulators of the activity of protein kinase C may regulate the action of glucocorticoid. For examination of these possibilities, we studied the effects of other potent activators of protein kinase C, such as TPA[1] and related compounds, on the inductions of tyrosine aminotransferase (TAT) (EC 2.6.1.5) and ornithine decarboxylase (EC 4.1.1.17) by glucocorticoids in vivo and in primary cultures of adult rat hepatocytes.

Here we show that TPA enhanced the inductions of liver enzymes by glucocorticoid in vivo and in vitro but that derivatives of TPA that have no ability to activate protein kinase C did not affect the action of glucocorticoid. TPA specifically enhanced the actions of various glucocorticoids but did not enhance the action of 17β-estradiol or the induction of TAT by insulin or glucagon. The mechanisms of the amplifying effects of TPA and *rac*-1,2-dioctanoylglycerol on the actions of glucocorticoids are discussed.

Materials and Methods

Materials. Male Wistar strain rats weighing 190–220 g were used. All animals were adrenalectomized 7–8 days before experiments and then given laboratory chow and saline ad libitum unless otherwise noted. Dexamethasone sodium phosphate was obtained from Merck & Co. Insulin, glucagon, TPA, phorbol 13-acetate, phorbol 12-tetradecanoate, 4β-phorbol, and 4-*O*-methylphorbol 12-tetradecanoate 13-acetate were from Sigma Chemical Co. DL-[1-^{14}C]Ornithine monohydrate (sp act. 61 mCi/mmol) was from Amersham. *rac*-1,2-Dioctanoylglycerol was a gift from Dr. Y. Nishizuka, Kobe University, School of Medicine, Kobe, Japan.

Assay of Biological Activity in Vivo. The biological activities of TPA and other phorbol esters were determined by measuring their amplifications of the inductions of TAT and ODC by glucocorticoids, insulin, and glucagon. Phorbol derivatives in saline containing 20% dimethyl sulfoxide and/or dexamethasone in saline or insulin in saline or glucagon in saline containing 20% dimethyl sulfoxide were injected intraperitoneally into adrenalectomized rats. The rats were killed by cervical dislocation 5 h after dexamethasone treatment or 3 h after treatment with insulin or glucagon. In experiments with insulin, rats also received 2 mL of 10% glucose at hourly intervals to prevent hypoglycemic shock. The livers were homogenized in 6 volumes of 0.25 M sucrose in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM 2-oxo-

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[1] Abbreviations: GPA, glucocorticoid potency amplifier; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TAT, tyrosine aminotransferase; ODC, ornithine decarboxylase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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Table I: Effects of TPA and Its Derivatives on TAT Induction by Dexamethasone[a]

compd (μg/100 g of body weight)	TAT activity (milliunits/ mg of protein)
saline	7.6 ± 1.2
dex[b] (0.5)	14.8 ± 2.8
TPA (20)	7.4 ± 1.4
Dex (0.5) + TPA (20)	42.3 ± 6.8
phorbol 13-acetate (20)	7.6 ± 1.3
Dex (0.5) + phorbol 13-acetate (20)	16.3 ± 2.7
phorbol 12-tetradecanoate (20)	7.4 ± 1.1
Dex (0.5) + phorbol 12-tetradecanoate (20)	13.0 ± 2.9
4β-Phorbol (20)	7.5 ± 1.2
Dex (0.5) + 4β-phorbol (20)	15.8 ± 2.8
4-*O*-methylphorbol 12-tetradecanoate 13-acetate (20)	7.4 ± 1.2
Dex (0.5) + 4-*O*-methylphorbol 12-tetradecanoate 13-acetate (20)	14.8 ± 2.7

[a] Dexamethasone in saline and/or phorbol derivatives in saline containing 20% dimethyl sulfoxide were injected intraperitoneally into adrenalectomized rats, and TAT activity in the liver was measured 5 h later. Values are means ± SD for 10 adrenalectomized rats. [b] Dex, dexamethasone.

glutarate and 48 μM pyridoxal phosphate for assay of TAT or in 5 volumes of 0.25 M sucrose in 0.1 M Tris-HCl buffer, pH 7.2, containing 5 mM dithiothreitol, 0.2 mM pyridoxal phosphate, and 1 mM EDTA for assay of ODC. For assay of ODC, the homogenate was centrifuged at 100000*g* for 30 min, and the resulting supernatant was used. For studies on the influence of TPA on the effect of 17β-estradiol, female rats weighing 160–180 g were ovarectomized and used for experiments 3–4 weeks later. 17β-Estradiol in 0.5 mL of saline containing 10% ethanol and TPA in 0.5 mL of saline containing 20% dimethyl sulfoxide were injected intramuscularly into separate sites in the femur 3 times at 12-h intervals, and the rats were killed 12 h after the last injection. The uterus was removed, homogenized in 10 volumes of 0.1 M triethanolamine buffer, pH 7.6, and centrifuged at 100000*g* for 30 min, and the resulting supernatant was used for assay of glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Assay of Biological Activity in Vitro. Hepatocytes were isolated from adult male Wistar strain rats (130–160 g) by perfusion of the liver with collagenase (Tanaka et al., 1978) and were suspended at a density of 5×10^5 cells/mL in Williams medium E containing 10% fetal calf serum (Flow Laboratories), 1×10^{-6} M dexamethasone, and 1×10^{-7} M insulin at 37 °C under 5% CO_2 in air. After 48 h, the medium was replaced by hormone-free medium containing 2% Ultroser G serum substitute (LKB). Dexamethasone and/or TPA or *rac*-1,2-dioctanoylglycerol were added to 1-day cultures in fresh medium, and the cells were cultured further for 10 h. Hepatocytes in 6-cm dishes were washed with ice-cold phosphate-buffered saline and harvested with a rubber policeman in 2.0 mL of 0.25 M sucrose in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM 2-oxoglutarate and 48 μM pyridoxal phosphate and then sonicated for 15 s. The sonicate was centrifuged at 100000*g* for 30 min, and the resulting supernatant was used for assay of TAT.

Enzyme and Protein Assays. TAT activities in the liver homogenate and the supernatant of hepatocytes were measured by the methods of Rosen et al. (1963) and Granner and Tomkins (1970), respectively. ODC activity was determined by measuring release of CO_2 as described by O'Brien and Diamond (1979). Glucose-6-phosphate dehydrogenase was assayed by the method of Kornberg and Horecker (1955). Protein concentrations were determined by the method of Lowry et al. (1951).

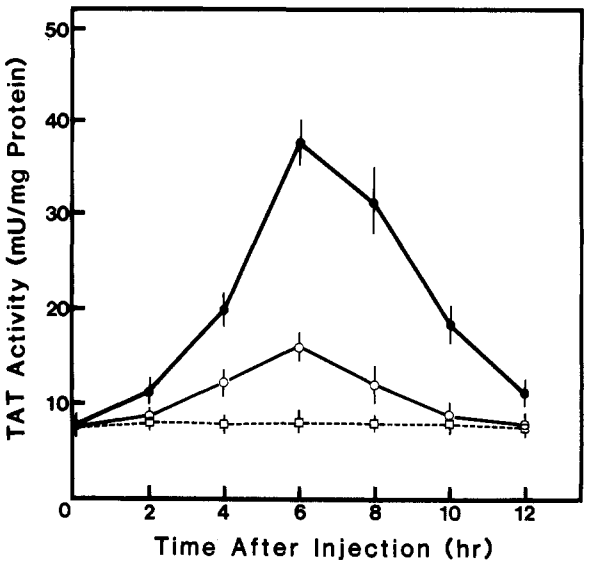

FIGURE 1: Time course of amplification of TAT induction. Curves show TAT activities in adrenalectomized rats after administration of dexamethasone (0.5 μg/100 g of body weight) and/or TPA (20 μg/100 g of body weight) at zero time: dexamethasone (○); dexamethasone plus TPA (●); TPA (□). Values are means for four adrenalectomized rats. Bars indicate SD.

RESULTS

Effects of Various Phorbol Derivatives on the Induction of TAT by Dexamethasone. The effects of various phorbol derivatives on the induction of TAT by dexamethasone in adrenalectomized rats are shown in Table I. TPA at a dose of 20 μg/100 g of body weight markedly enhanced the induction of TAT by dexamethasone. In contrast, phorbol 12-tetradecanoate, phorbol 13-acetate, 4-*O*-methylphorbol 12-tetradecanoate 13-acetate, and 4β-phorbol, which do not activate protein kinase C in vitro (Kikkawa et al., 1983) or promote tumor growth in vivo (Hecker, 1978), did not enhance the induction. TPA and phorbol derivatives themselves had no effect on the enzyme activity in adrenalectomized rats in the absence of dexamethasone. The activity of TAT was not increased by direct addition of TPA or phorbol derivatives in vitro (data not shown).

Mode of Amplification of Enzyme Inductions. The time course of amplification of TAT induction by dexamethasone in adrenalectomized rats was studied. As shown in Figure 1, when dexamethasone (0.5 μg/100 g of body weight) was injected intraperitoneally, the activity began to increase after a lag period of 1 h, reached a peak after 6 h, and decreased to the basal level after 10 h. When TPA was injected with the same amount of dexamethasone, the induction was markedly enhanced but showed a similar time course to that with dexamethasone alone. TPA alone had no effect on the activity in adrenalectomized rats.

The dose dependence of amplification of TAT induction by TPA is shown in Figure 2. The effect of TPA on TAT induction by dexamethasone was dose dependent in both adrenalectomized and conventional rats. In adrenalectomized rats, TPA was effective at doses of above 5 μg in the presence of 0.5 μg of dexamethasone and was maximally effective at doses of 20–40 μg. In the absence of dexamethasone, TPA had no effect. In conventional rats, TPA alone induced TAT, and its effect was also dose dependent. The effect of TPA in conventional rats may have been the result of its enhancement of the action of endogenous glucocorticoid or of this enhancement and the consequent stimulation of secretion of adrenocorticotropin (Abou Samra et al., 1986).

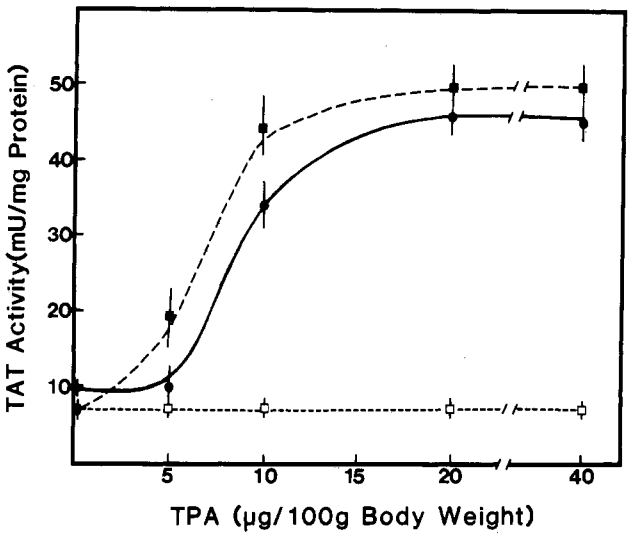

FIGURE 2: Dose–response curves for amplification of TAT induction by TPA in adrenalectomized and conventional rats. Dexamethasone (0.5 μg/100 g of body weight) and/or various doses of TPA were injected into adrenalectomized and conventional rats, and liver TAT activities were measured 5 h later as described under Materials and Methods. Values for TAT activity with (●) and without (□) dexamethasone in adrenalectomized rats and those for TAT activity in conventional rats without administration of dexamethasone (■) are shown as means for four rats. Bars indicate SD.

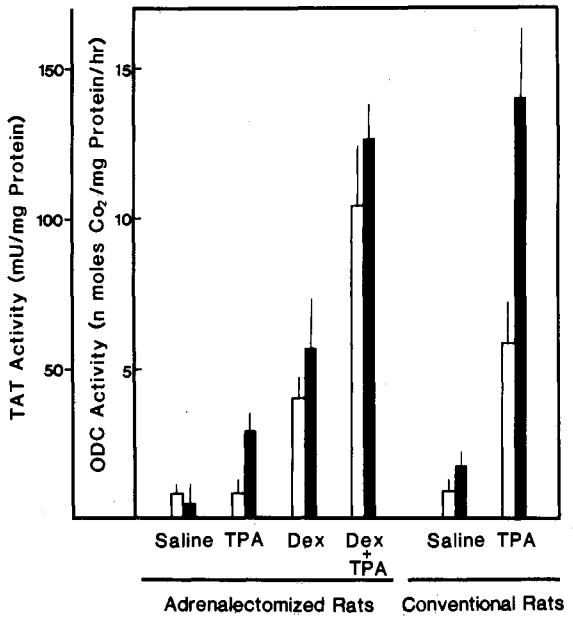

FIGURE 3: Effects of TPA on inductions of TAT and ODC by dexamethasone. Dexamethasone (Dex) (2 μg/100 g of body weight) and/or TPA (20 μg/100 g of body weight) was injected into adrenalectomized and conventional rats, and the activities of liver TAT (open columns) and ODC (closed columns) were measured 5 h later as described under Materials and Methods. Saline was injected into controls. Values are means for five rats. Bars indicate SD.

TPA also amplified the induction of ODC by dexamethasone in adrenalectomized rats, as shown in Figure 3. In conventional rats, injection of TPA without dexamethasone also induced both TAT and ODC. In adrenalectomized rats, TPA alone did not induce TAT but slightly induced ODC for some unknown reason. ODC was not activated directly by added TPA in vitro.

The effect of TPA on induction of TAT was studied as a function of the dose of dexamethasone. As shown in Figure 4, TPA markedly amplified the induction of TAT by not only low doses (0.5–10 μg) of dexamethasone but also doses of

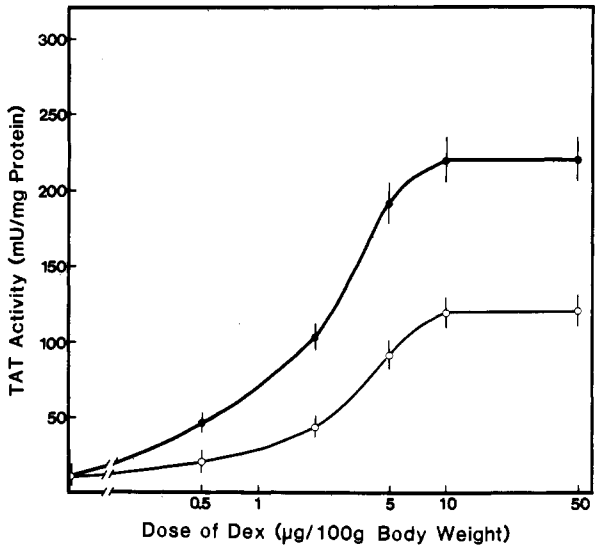

FIGURE 4: Dose–response curves for effect of dexamethasone on TAT induction with and without TPA. Adrenalectomized rats were treated intraperitoneally with various doses of dexamethasone (Dex) with (●) or without (○) TPA (20 μg/100 g of body weight). The rats were killed 5 h later, and the TAT activity of their liver homogenates was measured. Values are means for four adrenalectomized rats. Bars indicate SD.

Table II: Effects of TPA on TAT Inductions by Insulin and Glucagon[a]

treatment (μg/100 g of body weight)	TAT activity (milliunits/mg of protein)
saline	7.6 ± 2.3
TPA (20)	7.5 ± 2.1
glucagon (200)	33.3 ± 6.2
glucagon (200) + TPA (20)	27.4 ± 4.3
insulin (1 unit)	14.2 ± 2.5
insulin (1 unit) + TPA (20)	13.7 ± 2.6

[a] Insulin or glucagon and/or TPA was injected intraperitoneally into adrenalectomized rats, and TAT activity in the liver was measured 3 h later as described under Materials and Methods. Values are means ± SD for 10 adrenalectomized rats.

above 10 μg, which had a maximal effect. A similar mode of amplification by TPA of ODC induction by glucocorticoid was observed in adrenalectomized rats (data not shown).

Hormonal Specificity of the Effect of TPA. Glucagon and insulin are also reported to induce TAT in adrenalectomized rats (Holten & Kenney, 1967). As shown in Table II, insulin and glucagon caused 2- and 4-fold increase, respectively, of TAT in adrenalectomized rats, and TPA did not have any effect on these inductions. These results suggest that TPA specifically amplifies the induction of TAT without affecting the actions of insulin and glucagon.

The effects of TPA on the actions of various steroids are shown in Table III. TPA markedly enhanced the induction of TAT by glucocorticoids—not only by potent inducers of TAT, such as dexamethasone and triamcinolone acetonide, but also by weak inducers of TAT, such as corticosterone and hydrocortisone. In contrast, it had little effect on the induction of glucose-6-phosphate dehydrogenase in the uterus by 17β-estradiol. These results suggest that TPA has a specific effect on enzyme inductions by glucocorticoids but not by other steroids. The results also suggest that the effect of TPA in conventional rats, shown in Figure 2, was due to its amplification of the action of corticosterone, which is a major endogenous glucocorticoid in rats (Bush, 1953).

Amplification of TAT Induction in Primary Cultures of Adult Rat Hepatocytes. We examined the effect of TPA on

Table III: Effects of TPA on Enzyme Inductions by Various Steroids[a]

treatment (μg/100 g of body weight)	TAT activity (milliunits/mg of protein)	glucose-6-phosphate dehydrogenase activity (millunits/mg of protein)
saline	7.5 ± 1.2	2.6 ± 0.6
TPA (20)	7.4 ± 1.3	2.4 ± 0.3
corticosterone (4000)	35.0 ± 6.8	
corticosterone (4000) + TPA (20)	156.6 ± 21.2	
hydrocortisone (4000)	33.2 ± 6.5	
hydrocortisone (4000) + TPA (20)	189.1 ± 22.8	
Dex[b] (20)	81.2 ± 16.5	
Dex (20) + TPA (20)	189.6 ± 24.3	
triamcinolone acetonide (10)	116.6 ± 22.1	
triamcinolone acetonide (10) + TPA (20)	225.0 ± 40.3	
17β-estradiol (1.0)		7.2 ± 1.0
17β-estradiol (1.0) + TPA (20)		7.9 ± 1.1

[a] The effects of TPA on the actions of glucocorticoids were determined as amplifications of TAT induction in the liver by glucocorticoids. The effect of TPA on the action of 17β-estradiol was measured as its effect in amplifying the induction of glucose-6-phosphate dehydrogenase by 17β-estradiol in the uterus. For details of procedures, see Materials and Methods. Values are means ± SD for 10 adrenalectomized rats. [b] Dex, dexamethasone.

TAT induction in cultured hepatocytes to exclude the possibility that its effect was mediated by other organs or neural or hormonal factors. When dexamethasone was added to the medium, the specific activity of TAT increased rapidly to a plateau after 4–6 h and then remained constant unless the dexamethasone was removed (data not shown). Figure 5 shows that when hepatocytes were incubated with 10^{-7} M dexamethasone for 10 h, the induction of TAT was enhanced by TPA at concentrations of above 25 ng/mL and maximal at 100–200 ng/mL but that, in the absence of dexamethasone, TPA had no effect. A similar amplification of TAT induction by *rac*-1,2-dioctanoylglycerol was observed in primary cultures of rat hepatocytes, as shown in Figure 5. The induction was enhanced by *rac*-1,2-dioctanoylglycerol at concentrations of above 100 ng/mL and was maximal at 800 ng/mL.

Discussion

We conclude from this study that TPA specifically enhances the effect of glucocorticoids, even at concentrations of the latter that had maximal effects, without itself having any glucocorticoid-like action on TAT and thus that TPA seems to be a glucocorticoid action biomodulator. For this action, TPA was effective at very low concentrations (above 10–20 nmol/100 g of body weight).

The striking effects of TPA on cell functions, such as those of enabling various normal cells to proliferate like tumor cells in Ca^{2+}-deficient medium (Whitfield et al., 1973; Boynton & Whitfield, 1980; Jones et al., 1982) and of promoting neoplastic transformation (Berenblum, 1941; Boutwell, 1964; Hecker, 1978), have been suggested to be due to the ability of TPA to stimulate protein kinase C (Nishizuka et al., 1979; Kishimoto et al., 1980; Kikkawa et al., 1983). 4β-Phorbol, phorbol 12-tetradecanoate, 4-*O*-methylphorbol 12-tetradecanoate 13-acetate, and phorbol 13-acetate, which do not activate protein kinase C (Kikkawa et al., 1983), also did not enhance the action of glucocorticoids, as shown in Table I. These findings suggest that the effect of TPA on the action of glucocorticoid is also due to activation of protein kinase C. In addition, we have recently reported that *rac*-1,2-dioctanoylglycerol also enhances the induction by glucocorticoid in

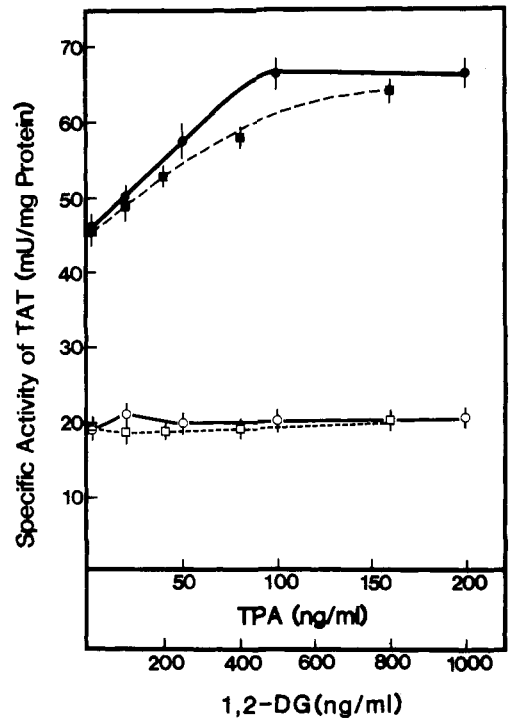

FIGURE 5: Effects of TPA and *rac*-1,2-dioctanoylglycerol on induction of TAT by dexamethasone in primary cultures of rat hepatocytes. Culture conditions were as described under Materials and Methods. TAT activity was measured 10 h after addition of the indicated concentrations of TPA (●) or *rac*-1,2-dioctanoylglycerol (■) with 10^{-7} M dexamethasone and also after addition of the indicated concentration of TPA (○) or *rac*-1,2-dioctanoylglycerol (□) without 10^{-7} M dexamethasone. Values are means from four dishes. Bars indicate SD.

adrenalectomized rats (Kido et al., 1986), and in this work we confirmed this action using primary cultures of adult rat hepatocytes (Figure 5). Though there is no direct evidence on this point, the glucocorticoid receptor would be a logical target for such phosphorylation, and the resulting phosphoprotein(s) may specifically enhance the action of glucocorticoid in target organs or cells. But TPA and *rac*-1,2-dioctanoylglycerol themselves have no effect on TAT induction in the absence of glucocorticoid in adrenalectomized rats, whereas they stimulate protein kinase C in vivo and in vitro (Nishizuka et al., 1979; Kishimoto et al., 1980; Kikkawa et al., 1983). These findings suggest that in the absence of glucocorticoid the phosphorylation of some specific protein(s) necessary for amplification or further steps in the mechanism of action of glucocorticoid after the phosphorylation do not take place.

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Registry No. TPA, 16561-29-8; TAT, 9014-55-5; ODC, 9024-60-6; 4β-phorbol, 17673-25-5; phorbol 12-tetradecanoate, 20839-06-9; phorbol 13-acetate, 32752-29-7; 4-*O*-methylphorbol 12-tetradecanoate 13-acetate, 57716-89-9; protein kinase C, 9026-43-1; dexamethasone, 50-02-2; corticosterone, 50-22-6; hydrocortisone, 50-23-7; triamcinolone acetonide, 76-25-5.

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In Vitro Synthesis of 16S Ribosomal RNA Containing Single Base Changes and Assembly into a Functional 30S Ribosome

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ABSTRACT: Functional 30S ribosomes were reconstructed from total *Escherichia coli* 30S ribosomal proteins and 16S ribosomal RNA synthesized in vitro by T_7 RNA polymerase. Up to 700 mol of RNA/mol of template could be obtained. The transcript lacked all ten normally modified bases and had three additional 5′ G residues, an A → G change at position 2, and, in 22% of the molecules, one or two extra 3′ residues. The synthetic 16S RNA could be assembled into a particle that cosedimented with authentic 30S and was indistinguishable from 30S by electron microscopy. When supplemented with the 50S subunit, the particles bound tRNA to the 70S P site in a codon- and Mg^{2+}-dependent manner. The specific binding activity was 94% that of particles reconstituted with natural rRNA and 52% that of native 30S. Cross-linking to P site bound tRNA was also preserved. Changing C-1400, the residue known to be close to the anticodon of P site bound tRNA, to A had little effect on reconstitution, but the C → G substitution caused a marked inhibition of assembly. tRNA could bind to both reconstituted mutants, but cross-linking was greatly reduced. These results show that none of the modified bases of 16S RNA are essential for P site binding and that position 1400 may be more important for ribosome assembly than for tRNA binding. Base-specific in vitro mutagenesis can now be used to explore in detail the functional properties of individual residues in ribosomal RNA.

The ribosome is a complex subcellular organelle consisting of numerous proteins, two large rRNAs, and one small rRNA divided between two unequally sized subunits. The primary structure of the three RNAs and 52 proteins of the *Escherichia coli* ribosome is known (Wittman, 1982), and detailed models for the secondary structure of the RNAs (Gutell et al., 1985; Brimacombe & Stiege, 1985) and some of the proteins have been proposed (Wittman, 1982; Liljas, 1982). The location of many of the proteins on and in the ribosome has been determined (Stöffler & Stöffler-Meilicke, 1984; Lake, 1985; Ramakrishnan et al., 1984) as well as the topographical location of certain of the RNA residues (Brimacombe & Stiege, 1985). The tertiary structure of the RNA within the ribosome is also beginning to be understood (Noller & Lake, 1984; Brimacombe & Stiege, 1985; Ofengand et al., 1985; Expert-Bezancon & Wollenzien, 1985; Hui & Cantor, 1985; Atmadja et al., 1986; Gutell et al., 1986; Brimacombe et al.,

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