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Biphasic Regulation by N^6,2′-*O*-Dibutyryl Adenosine 3′,5′-Cyclic Monophosphate (dbcAMP) of Steroid 21-Hydroxylase Activity in Rat Hepatocytes

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Steroid 21-hydroxylase activity has been identified in many tissues, including liver. But it is possible that the enzyme found in the liver is different from adrenal 21-hydroxylase. In the adrenal cortex, steroid 21-hydroxylase activity is increased by corticotropin (ACTH); the effect of ACTH is mediated by cyclic AMP (cAMP), and presumably involves a cAMP-dependent protein kinase (PKA). It is not yet clear, however, how extra-adrenal steroid 21-hydroxylase activity is regulated. In the present study, we examined the effect of N^6,2′-*O*-dibutyryl adenosine 3′,5′-cyclic monophosphate (dbcAMP), forskolin, *N*-[2-(methylamino)ethyl]5-isoquinolinesulfonamide (H-8) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on steroid 21-hydroxylase activity in primary cultures of rat hepatocytes to determine the nature of regulation of extra-adrenal steroid 21-hydroxylase activity. Steroid 21-hydroxylase activity in hepatocytes incubated with 10^{-11} M dbcAMP for 24 h was 1.6 times higher than that in control hepatocytes untreated with dbcAMP. On the other hand, steroid 21-hydroxylase activity decreased by 20 and 50% when the cells were incubated with 10^{-5} and 10^{-3} M dbcAMP, respectively. The stimulatory effect of 10^{-11} M dbcAMP was not blocked by 10^{-5} M H-8 (PKA inhibitor), but the inhibitory effect of 10^{-5} or 10^{-3} M cAMP was. TPA did not alter the activity of steroid 21-hydroxylase. These findings indicate that the steroid 21-hydroxylase in rat liver is regulated by mechanisms different from those in the adrenal glands.

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INTRODUCTION

The 21-hydroxylation of progesterone (P4) to deoxycorticosterone (DOC) and of 17-hydroxyprogesterone to 11-deoxycortisol in human adrenal cortex is mediated by *P*450c21, which is a member of the cytochrome *P*450 supergene family [1, 2].

Extra-adrenal production of DOC from plasma P4 has previously been reported to occur in a wide variety of species, including man [3–11]. In recent studies, extra-adrenal expression of *P*450c21 mRNA has been investigated in various organs of animals of numerous species. However, no *P*450c21 mRNA has been detected in any extra-adrenal human, bovine or rat tissue with the use of probes for human or bovine *P*450c21 cDNA [12–14]. Rat hepatic steroid 21-hydroxylation is mediated by *P*450PBI (*P*450k), which is structurally unrelated to *P*450c21 [15, 16]. *P*450PBI and *P*450k have now been designated as cytochrome *P*450IIC6 in a system proposed to make the nomenclature uniform [17]. The studies noted above demonstrated that extra-adrenal tissue might contain steroid 21-hydroxylase which differs from adrenal steroid 21-hydroxylase. However, the biochemical role and regulation of such an enzyme remains unknown. Few studies have shown how extra-adrenal steroid 21-hydroxylase is regulated [12, 18, 19].

In the present study, we examined the effect of N^6,2′-*O*-dibutyryl adenosine 3′,5′-cyclic monophosphate (dbcAMP), forskolin (FK), *N*-[2-(methylamino)ethyl]5-isoquinolinesulfonamide (H-8), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on steroid 21-hydroxylase activity in rat hepatocytes, to determine how extra-adrenal steroid 21-hydroxylase is regulated.

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EXPERIMENTAL

Materials

Eight-week-old male Sprague–Dawley rats were obtained from Nippon SLC (Shizuoka, Japan), William's medium E from Flow Labs (Irvine, Scotland), Collagenase S-1 from Nitta Gelatin (Osaka, Japan), and fetal calf serum, penicillin G and streptomycin from GIBCO Labs (Grand Island, NY). Amphotericin B, dexamethasone, insulin, dbcAMP, FK, H-8, TPA, P4, DOC and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO), 24-multiwell chambers from Sumitomo Bakelite (Tokyo, Japan), and Hanks Balanced Salt Solution (HBSS) from Nissui Pharmaceutical Co. (Tokyo, Japan). The Inertsil OSD-2 column for HPLC was obtained from GL Sciences (Tokyo, Japan). Bio-Rad protein Assay kits were purchased from Bio-Rad Labs (Richmond, CA).

Primary culture of rat hepatocytes

Hepatocytes were isolated from 8-week-old male Sprague–Dawley rats by collagenase perfusion. Briefly, the animals were subjected to laparotomy under ether anesthesia, and a cannula was inserted into the portal vein and superior vena cava to collect free hepatocytes by the collagenase recirculating method [12]. The collagenase solution, which consisted of HBSS containing 0.5% (w/v) collagenase, was circulated for 15 min to isolate hepatocytes. The cells were then suspended in William's medium E containing 10% (v/v) dextran-coated charcoal-treated fetal calf serum, 100 U/ml penicillin G, 100 U/ml streptomycin, 0.25 μg/ml amphotericin B, 10^{-9} M dexamethasone (DEX) and 10^{-9} M insulin. The cells were counted and their viability was assessed by trypan blue dye exclusion. A portion of the free hepatocytes was seeded into 24-multiwell chambers at a density of 2×10^{-5} cells/0.5 ml/well. After plating, the cells were incubated at 37°C for 4 h under 95% air–5% CO_2, the medium was then replaced with serum-free William's medium E and cells were further incubated for 24 h. On day 1, the medium was changed to serum-free medium containing dbcAMP, FK, H-8 or TPA. On day 2, the medium was discarded by aspiration and the cells were washed with HBSS prior to the assay for steroid 21-hydroxylase activity.

Assay for steroid 21-hydroxylase activity

The hepatocytes were incubated for 2 h at 37°C in 0.5 ml of William's medium E containing 50 μM P4 as the substrate. They were then placed on ice to stop the enzymatic reaction. The medium was then collected and frozen at −20°C until assay for DOC. The cells were solubilized in 0.5% NaOH solution and stored at −20°C until assayed to determine the protein content. Steroid 21-hydroxylase activity was measured from the amount of conversion of P4 to DOC in the 2 h incubation period, and expressed as pM/mg protein/h. Steroids in culture medium after the addition of DEX (1 μg) as an internal recovery standard were extracted twice with 5 vol diethyl ether (HPLC grade), and the extract was evaporated to dryness under nitrogen. The residue was dissolved in a mixture of acetonitrile–water (1:1; v/v) and subjected to HPLC on an Inertsil OSD-2 column in an isocratic system of acetonitrile–water (1:1; v/v) at a flow rate of 1.4 ml/min. The HPLC-fraction corresponding to DOC in each sample was collected, and DOC was measured by EIA.

The protein content was measured using the Bio-Rad Protein Assay and BSA as the standard.

Experimental results were expressed as the means ± SD.

Statistical analysis

The statistical significance of differences was assessed using the unpaired Student's t-test.

RESULTS

Effect of dbcAMP on 21-hydroxylase activity

dbcAMP is the membrane-permeable analog of cAMP. To determine the direct effect of cAMP on steroid 21-hydroxylase activity in rat hepatocytes, rat hepatocytes were maintained in culture with various concentrations of dbcAMP for 24 h.

The effect of dbcAMP on steroid 21-hydroxylase activity in rat hepatocytes is biphasic (Fig. 1). In the absence of dbcAMP, 21-hydroxylase activity was 10.9 ± 1.5 pM/mg protein/h (control). In hepatocytes treated with 10^{-11} M dbcAMP, steroid 21-hydroxylase activity was 1.6 times that of the control. However, when the concentration of dbcAMP was increased, an inhibitory effect was observed. In cells incubated with 10^{-5} and 10^{-3} M dbcAMP, 21-hydroxylase activity decreased by 20 and 50% of the control, respectively. The reduction in activity in medium with 10^{-5} and 10^{-3} M dbcAMP was statistically significant ($P < 0.05$ and $P < 0.01$).

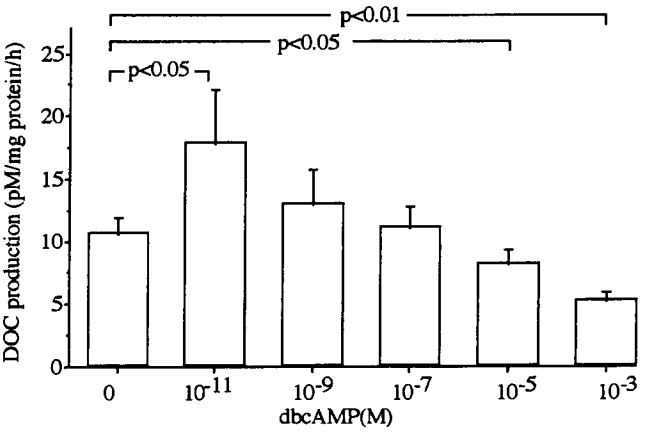

Fig. 1. Effect of dbcAMP on steroid 21-hydroxylase activity in rat hepatocytes. Rat hepatocytes were cultured as described in Experimental, and incubated with various concentrations of dbcAMP. Steroid 21-hydroxylase activity was measured from the conversion of P4 to DOC. Values are the means ± SD of three or four wells.

exclusion and cell counts (data not shown). Several investigators have demonstrated that dbcAMP exerts both stimulatory and inhibitory effects on rat hepatocyte DNA synthesis when added to primary monolayer cultures, and that stimulation occurs at low concentrations of dbcAMP while inhibition of DNA synthesis occurs at high concentrations [30–32]. We are unfamiliar with other models for the biphasic response to cAMP.

It is well established that cAMP activates PKA, which plays important roles in cellular regulation phosphorylating certain proteins. Houge *et al.* [33] found that cAMP induced down-regulation of PKA. At high concentrations, cAMP decreased the catalytic subunit of PKA and increased the regulatory subunit/catalytic subunit, and therefore reduced the activity of PKA. The system employed in our study might be considered a good model for this type of cAMP-induced down-regulation. However, inhibitory effect at high concentrations of cAMP is blocked by H-8, which inhibits the catalytic subunit of PKA. Since the stimulatory effect at low concentrations of cAMP was not blocked by H-8, it appears that it was not mediated by the catalytic subunit of PKA. Thus cAMP regulates steroid 21-hydroxylation in rat hepatocytes via two independent mechanisms.

The regulatory subunit of PKA may influence gene expression directly in the presence of low concentrations of cAMP. Nagamine and Reich [34] reported that the regulatory subunit may have cellular regulatory functions independent of the catalytic subunit. Consistent with these hypotheses, the regulatory subunit was found to have a sequence similar to that of catabolite activator protein of certain bacteria, which binds cAMP and then the nucleus to regulate gene expression. Alternatively, a different protein kinase activated by cAMP or calcium ion might play an important role in the activation of steroid 21-hydroxylase [29, 35]. However, in the present study PKC showed no effects on steroid 21-hydroxylase activity.

Regarding the inhibitory effect of cAMP at high concentrations there are various possible explanations. Jones *et al.* [36] found that the mouse $P_1$450 gene is regulated by a negative regulatory element. Furthermore, in mouse alpha 1-fetoprotein (AFP)-producing hepatoma cells, evidence has been obtained for the presence of negative elements in the AFP gene [37, 38]. We speculate that the inhibitory effect of dbcAMP detected in our experiments may have been due to the presence of a negative regulatory element for the steroid 21-hydroxylase gene. Alternatively, a negative regulatory factor mediated by PKA may exist. It also seems possible that a positive regulatory factor was reduced by indirect inhibition by PKA. Certain phosphatases are known to be indirectly inhibited by PKA. In glycogenolysis, for example, protein phosphate-1 (PP-1) is inhibited by Inhibitor-1. PKA activated by cAMP phosphorylates Inhibitor-1 and then binds to PP-1 [39].

Tukey *et al.* [40] demonstrated that *P*450 1, a rabbit liver steroid 21-hydroxylase, is produced from a multigene family. It is not known what type of regulation occurs in that gene of subfamily. Taken together, our findings appear to reflect the complex activity of various genes with differing promoters.

Characterization of the structure of steroid 21-hydroxylase gene and determination of the mechanisms that may be involved in the biphasic regulation of steroid 21-hydroxylase activity by cAMP are necessary to elucidate the role of steroid 21-hydroxylase in hepatocytes.

Recent studies have shown that steroid 21-hydroxylase in adrenal cells is also regulated by PKC induced by phorbol ester [28, 41]. Induction of steroid 21-hydroxylase by TPA was seen only in the non-quiescent stage in bovine adrenocortical cell cultures [41]. We tested TPA at a wide range of concentrations ($10^{-9} \sim 10^{-3}$ M) in this study, however, treatment with TPA had no effect on steroid 21-hydroxylation in rat hepatocytes. These findings suggest that PKC activation by itself is insufficient and additional factor(s) may be needed to regulate steroid 21-hydroxylase activity in hepatocytes.

In conclusion, we found that at low concentrations dbcAMP stimulated and at high concentrations it inhibited steroid 21-hydroxylase activity in rat hepatocytes. The inhibitory effect of cAMP on steroid 21-hydroxylase was mediated by the catalytic subunit of PKA pathway, but the stimulatory effect may have been mediated by a pathway other than the catalytic subunit of PKA and PKC pathways. We propose the hypothesis that steroid 21-hydroxylase in rat liver is regulated by mechanisms different from those in the adrenal glands.

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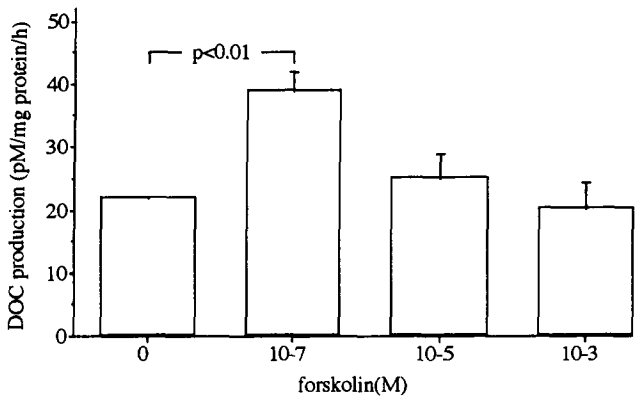

Fig. 2. Effect of FK on steroid 21-hydroxylase activity in rat hepatocytes. Rat hepatocytes were incubated with various concentrations of FK. Steroid 21-hydroxylase activity was measured from the conversion of P4 to DOC. Values are the mean ± SD of three or four wells.

Effect of FK on 21-hydroxylase activity

When the hepatocytes were incubated with FK, which is an adenylate cyclase activator, an increase in DOC production similar to that observed with dbcAMP was obtained (10^{-7} M concentration) (Fig. 2). With higher concentrations of FK steroid 21-hydroxylase activity did not change.

Effect of H-8 on 21-hydroxylase activity

To determine whether the biphasic effect of dbcAMP on steroid 21-hydroxylase activity is mediated by cyclic AMP-dependent protein kinase (PKA) activation, hepatocytes were incubated with various concentrations of dbcAMP together with 10^{-5} M H-8. H-8 is a protein kinase inhibitor with higher specificity for PKA and cyclic GMP-dependent protein kinase than for other kinases [20].

As shown in Fig. 3, H-8 blocked the inhibitory effect of dbcAMP on 21-hydroxylase activity, but had no effect on the stimulatory effect of dbcAMP on the activity. H-8 itself had no effect on 21-hydroxylase activity.

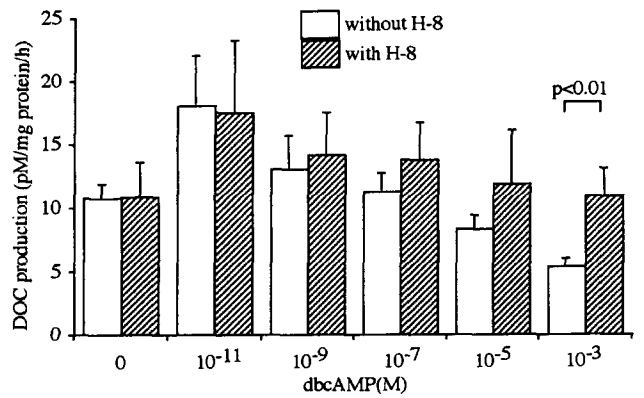

Fig. 3. Effects of dbcAMP on steroid 21-hydroxylase activity in rat hepatocytes with or without H-8. Rat hepatocytes were incubated with various concentrations of dbcAMP with or without 10^{-5} M H-8. Steroid 21-hydroxylase activity was measured from the conversion of P4 to DOC. Values are the mean ± SD of three or four wells.

Effect of TPA on 21-hydroxylase activity

To determine the effect of activated protein kinase C (PKC) on steroid 21-hydroxylase activity, rat hepatocytes were treated with various concentrations of TPA, which directly activates PKC [21].

Steroid 21-hydroxylase activity was not altered after treatment of hepatocytes with TPA for a 24 h at any of the concentrations tested (from 10^{-9} to 10^{-3} M). No alteration of the activity was seen after similar treatment for 6 h (data not shown).

DISCUSSION

In general, the expression of steroid 21-hydroxylase is induced by stimulation of adrenal cortex by ACTH [13, 22, 23]; this effect of ACTH is mediated by cAMP, and presumably involves PKA.

Recent studies indicate that adrenal steroid hydroxylase is regulated by other agents beside cAMP [24]. PKC has also been proposed as a regulation system for adrenal steroid hydroxylation. Phorbol esters, which can activate PKC, have been shown to modulate cortisol production in bovine adrenocortical, guinea pig adrenal and cultured human adrenocortical cells [25–28]. Calcium ion, on the other hand, plays an important role in the regulation of steroidogenesis in rat adrenocortical cells [29]. However, under physiological conditions cells are exposed to a variety of hormones that simultaneously activate different signalling pathways. Therefore, interaction between these pathways is of considerable importance for the regulation of cell function. In this fashion, adrenal steroid hydroxylase is regulated by cAMP, PKC, calcium ion and by the network of these intracellular signals. It has not yet been determined, however, how extra-adrenal steroid 21-hydroxylase is regulated. Therefore we examined the effects of dbcAMP, FK, H-8 and TPA on steroid 21-hydroxylase activity in rat hepatocytes to clarify regulation pathways for this enzyme.

We found that dbcAMP and FK regulated steroid 21-hydroxylase activity in rat hepatocytes, but that TPA had no effect on such activity. At low concentrations dbcAMP stimulated steroid 21-hydroxylase activity, and this stimulatory effect was not affected in the presence of H-8. On the other hand, at high concentrations dbcAMP reduced steroid 21-hydroxylase activity, and this inhibitory effect was almost completely blocked by H-8.

The exact mechanism by which dbcAMP is able to exert a biphasic effect on steroid 21-hydroxylation in rat hepatocytes is not known but several mechanisms may be involved. Since the inhibitory effect of dbcAMP at high concentrations was blocked by H-8, non-specific toxic effects of dbcAMP on hepatocytes can be excluded. Moreover, the addition of 10^{-3} M dbcAMP for 48 h had no effect on the viability of cultured hepatocytes as determined by trypan blue dye

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