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Biochemistry

Diazepam-binding inhibitor (DBI)-processing products, acting at the mitochondrial DBI receptor, mediate adrenocorticotropic hormone-induced steroidogenesis in rat adrenal gland

(mitochondrial diazepam-binding inhibitor receptor)

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ABSTRACT **Diazepam-binding inhibitor (DBI) is a 9-kDa polypeptide that colocalizes in glial, adrenocortical, and Leydig cells with the mitochondrial DBI receptor (MDR). By binding with high affinity to the MDR, DBI and one of its processing products—DBI-(17–50)—regulate pregnenolone synthesis and have been suggested to participate in the immediate activation of adrenal steroidogenesis by adrenocorticotropic hormone (ACTH). In adrenals of hypophysectomized rats (1 day after surgery), ACTH failed to acutely affect the amount of adrenal DBI and the density of MDR but increased the rate of DBI processing, as determined by the HPLC profile of DBI-(17–50)-like immunoreactivity. The similar latency times for this effect and for ACTH stimulation of adrenal steroidogenesis suggest that the two processes are related. The ACTH-induced increase in both adrenal steroidogenesis and rate of DBI processing were completely inhibited by cycloheximide; this result suggests the requirement for the *de novo* synthesis of a protein with a short half-life, probably an endopeptidase. This enzyme, under the influence of ACTH, may activate formation of a DBI-processing product that stimulates steroidogenesis via the MDR. In support of this hypothesis is the demonstration that in hypophysectomized rats the MDR antagonist PK 11195 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide completely inhibited the adrenal steroidogenesis stimulated by ACTH and by the high-affinity MDR ligand 4′-chlorodiazepam.**

The rate-limiting step in steroid biosynthesis is the conversion of cholesterol to pregnenolone. This reaction is catalyzed by the side-chain cleavage enzyme cytochrome P-450 [P-450_{scc}; cholesterol, reduced-adrenal-ferredoxin:oxygen oxidoreductase (side-chain-cleaving), EC 1.14.15.6], which is located on the inner mitochondrial membrane (1). Several laboratories have established that it is the rate at which cholesterol is transported to the inner mitochondrial membrane, and not P-450_{scc} activity itself, that limits the rate of pregnenolone synthesis (2–5). The transfer of cholesterol to P-450_{scc}, therefore, must be facilitated to achieve maximal rates of pregnenolone synthesis. The protein synthesis inhibitor cycloheximide has been shown to block the induction of steroidogenesis by adrenocorticotropic hormone (ACTH) (6–8), and this block appears to occur at the level of cholesterol transport from the outer to the inner mitochondrial membrane. It is believed that one or more proteins with a rapid turnover rate are required to effect cholesterol translocation within mitochondria and that ACTH may either increase the concentration of these proteins or activate them posttranslationally.

A receptor that is located on the outer mitochondrial membrane and that is enriched in the mitochondria of steroidogenic cells participates in the regulation of intramitochondrial cholesterol transport and may mediate the immediate action of steroidogenic hormones, such as ACTH and human chorionic gonadotropin (9–12). Because diazepam-binding inhibitor (DBI), an endogenous 9-kDa peptide, binds to this receptor and stimulates steroidogenesis by facilitating cholesterol transport to the inner mitochondrial membrane (13–19), the receptor has been termed the mitochondrial DBI receptor (MDR) (17).

DBI is an 86-amino acid peptide that was initially isolated from rat brain and subsequently found to be abundant in steroidogenic tissues, such as adrenal glands and testes (19). DBI and two known brain-derived processing products—DBI-(33–50) (octadecaneuropeptide; ODN) and DBI-(17–50) (triakontatetraneuropeptide; TTN) (Fig. 1)—have been shown to interact with the MDR and to stimulate steroidogenesis in adrenocortical, Leydig, and glial cell mitochondria (13–17). These results suggest that DBI or its processing products may mediate, via the MDR, the steroidogenic action of pituitary hormones on their target cells.

We now show that ACTH stimulates the rate of DBI processing in the adrenal gland and suggest that the blockade of ACTH-induced steroidogenesis by cycloheximide may result from an inhibition of specific DBI processing regulated by ACTH. Because the MDR antagonist PK 11195 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (20) inhibits the steroidogenesis stimulated by ACTH and by the high-affinity MDR ligand 4′-chlorodiazepam, we conclude that the steroidogenic action of ACTH requires both MDR availability and the synthesis of a specific DBI-processing product by an enzyme that is regulated by ACTH-receptor activation.

METHODS

Animals. Hypophysectomized male Sprague–Dawley rats (200–250 g) were obtained from Zivic–Miller. The animals were housed in groups of five per cage, supplied with food and water ad libitum, and maintained on a 12-hr light/dark cycle. Animal care and use were in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (21).

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Abbreviations: DBI, diazepam-binding inhibitor; MDR, mitochondrial DBI receptor; TTN, triakontatetraneuropeptide; ODN, DBI-(33–50) or octadecaneuropeptide; ACTH, adrenocorticotropic hormone; P-450_{scc} cholesterol, reduced-adrenal-ferredoxin:oxygen oxidoreductase (side-chain-cleaving); PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; DBI-LI, DBI-like immunoreactivity; TTN-LI, TTN-like immunoreactivity; AU, arbitrary units.

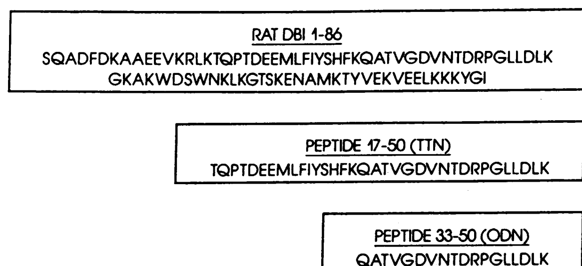

FIG. 1. Amino acid sequence of DBI-(1–86) (19) and its processing products in rat brain.

Drugs. ACTH (residues 1–39) was obtained from Parke–Davis. Diazepam was from Hoffman–La Roche. 4′-Chlorodiazepam (Ro5-4864) was purchased from Fluka. 4′-Chloro[^{3}H]diazepam was obtained from DuPont/NEN. Trilostane was from Sterling–Winthrop Research Institute. Cycloheximide and PK 11195 were obtained from Sigma.

Experimental Procedures. Hypophysectomized rats, 1 day after surgery, received a single i.v. injection of 0.9% NaCl (saline vehicle), ACTH (200 milliunits/kg) diluted in saline, or 4′-chlorodiazepam (18.8 μmol/kg). When indicated, hypophysectomized rats were injected with cycloheximide (142 μmol/kg i.p.) 10 min before saline, or ACTH (20 milliunits/kg i.v.), or with PK 11195 (28.3 μmol/kg i.v.) 5 min before saline, ACTH (1 milliunits/kg i.v.), or 4′-chlorodiazepam (18.8 μmol/kg i.v.). Animals were killed with a guillotine, and the adrenals were immediately frozen on dry ice and then stored at −70°C until the day of assay.

RIA for DBI and TTN. Frozen adrenals were homogenized for 20 sec in 1 M acetic acid (20 vol) with a Polytron homogenizer. The pH of the homogenate was adjusted to 5 by addition of 10 M NaOH, and the suspension was centrifuged at 48,000 × *g* for 20 min at 4°C. The pellet was discarded, and the clear supernatant was diluted 10-, 25-, 50-, and 100-fold with 50 mM sodium phosphate buffer (pH 7.2) containing 5% (wt/vol) bovine serum albumin. Two 50-μl aliquots of each dilution were assayed by a previously described DBI RIA procedure (22, 23). The assay of DBI-like immunoreactivity (DBI-LI) with the previously characterized antiserum to DBI (23) allows for authentic DBI measurement because analysis of adrenal extracts by reverse-phase HPLC revealed a distinct symmetrical DBI-LI peak that emerged at the position of standard DBI.

The RIA for TTN was done with a rabbit antiserum to synthetic rat TTN (24), and synthetic TTN iodinated with Na^{125}I by mild oxidation with *N*-chlorobenzene sulfonamide was adsorbed on polystyrene beads (Iodo-Beads; Pierce). The iodinated peptide was purified immediately after preparation on a Sep-Pack C_{18} column (Waters). The antiserum to TTN (1:1500 dilution) detected as little as 0.1 pmol of ^{125}I-labeled TTN. Because of DBI cross-reactivity with the antiserum to TTN, for tissue extracts the TTN RIA was performed on fractions eluted from a reverse-phase μBondapak C_{18} column (30 cm × 7.5 mm; Waters). TTN recovery through HPLC analysis was monitored by adding a trace amount of radioactive TTN (2000 cpm) to each tissue sample. The HPLC fractions were lyophilized and then suspended in 50 μl of H_2O and 100 μl of ^{125}I-labeled TTN (30,000 cpm per 0.1 pmol) diluted in 0.05 M sodium phosphate buffer (pH 7.4) containing 5% (wt/vol) bovine serum albumin. Subsequently, 100 μl of the antiserum to TTN diluted 1:1500 in the same phosphate buffer was added, and the RIA samples were incubated for ≈18 hr at 4°C. After addition of 250 μl of protein A (2.5 mg/ml, in 0.05 M Tris/2 mM $MgCl_2$, pH 8.0), the samples were incubated for a further 2 hr at 4°C. The samples were then centrifuged at 5000 × *g*, and the amount of radioactivity in the solid residues was determined.

Assay of MDR with 4′-Chloro[^{3}H]diazepam. The binding of 4′-chloro[^{3}H]diazepam to crude adrenal homogenate was determined as described (25); diazepam (5 μM) was used as displacing agent. The concentration of 4′-chloro[^{3}H]-diazepam was 1 nM, which is in the linear range of the binding curve (under our conditions, the K_d was ≈2 nM).

Plasma Corticosterone Determination. Blood samples were collected from the trunk immediately after decapitation. Heparin (100 units) was added to 1 ml of blood, which was then centrifuged at 900 × *g* for 15 min at room temperature. Corticosterone was assayed in plasma with an RIA kit from ICN.

Adrenal Steroid Biosynthesis. Frozen adrenals were exposed to microwave radiation (to denature enzymes), weighed, and homogenized with an Omni homogenizer at 20,000 rpm for 40 sec in 5 vol of water containing 7000 cpm of [^{3}H]dihydroepiandrosterone (DHEA) as internal standard. The homogenate was extracted with 3 vol of ethyl acetate by shaking for 10 min and was then centrifuged at 4000 × *g* for 5 min at room temperature; supernatants were collected. This procedure was repeated three times, and the combined supernatants were filtered through a Sep-Pack C_{18} minicolumn (Waters) that had been equilibrated with 10 ml of methanol and 10 ml of ethyl acetate. After filtration, the column was washed with 5 ml of ethyl acetate, and the eluate was combined with the initial filtrate. This combined sample was evaporated under vacuum, and the residue was dissolved in 500 μl of 5% (vol/vol) ethyl acetate in hexane, filtered through a 13-mm diameter Gelman poly(tetrafluoroethylene) 0.2-μm-pre-size filter, and applied to an HPLC column. HPLC separation was done with a modified version of the procedure of Schoneshofer *et al.* (26). The Lichrospher 100 sialic diol, 10-μm column (4 mm × 250 mm) (EM Separations, Gibstown, NJ) was equilibrated with hexane. After sample application, steroids were eluted with a one-step gradient of ethyl acetate from 0% to 15% (vol/vol) in 50 min; the flow rate was 1 ml/min. Fractions (1 min) corresponding to previously determined positions of corticosterone, pregnenolone, and internal standard [^{3}H]dihydroepiandrosterone were evaporated in a Speed-Vac evaporator, and the residues then dissolved in steroid diluent (ICN). Corticosterone, pregnenolone, and [^{3}H]dihydroepiandrosterone were assayed with RIA kits from ICN. Recovery of corticosterone, pregnenolone, and radioactive internal standard ([^{3}H]dihydroepiandrosterone) ranged from 65% to 75%.

RNA Blot Analysis. Total RNA was isolated from adrenals according to the guanidinium thiocyanate method described by Chirgwin *et al.* (27). RNA was subjected to electrophoresis on a 1.1% agarose gel containing 6% formaldehyde and then transferred to nitrocellulose paper as described by Thomas (28). The probe for DBI mRNA was a 450-base-pair (bp) fragment of rat DBI cDNA (29). The 781-bp probe for MDR mRNA contained the entire rat MDR cDNA (9). The probes were nick-translated with [α-^{32}P]dNTPs (DuPont/NEN) (800 Ci/mmol; 1 Ci = 37 GBq) to a specific activity of 5×10^8 cpm/μg and purified on a Sephadex G-50 column by the method of Maniatis *et al.* (30). Hybridization with the nick-translated cDNA probes was done in 50% (vol/vol) formamide/5× Denhardt's solution/4× SSPE (1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.5% SDS/salmon sperm DNA at 100 μg/ml at 42°C for 18 hr. The nitrocellulose filters were washed at 65°C in 0.2× standard saline citrate (SSC)/0.1% SDS. Subsequent hybridization of filters with pAc18.1 (β-actin) cDNA (31) and washing were done according to Milner and Sutcliff (32). Filters were exposed to Kodak X-Omat film with an intensifying screen at −70°C. The amounts of DBI and MDR mRNAs were expressed in arbitrary units (AU) that were defined as the ratio of the densitometric area of the DBI and MDR mRNA band to that of the β-actin mRNA band in the same sample.

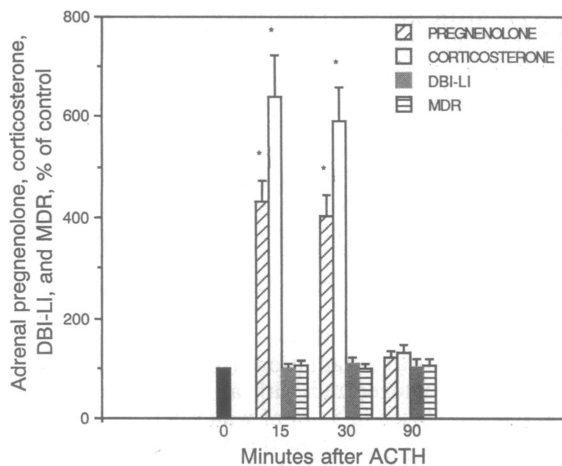

FIG. 2. Effects of ACTH on adrenal steroid biosynthesis and amounts of DBI-LI and MDR. The amounts of adrenal pregnenolone, corticosterone, DBI-LI, and MDR were measured in adrenal glands of hypophysectomized rats treated 1 day after surgery with ACTH (200 milliunits/kg i.v.). The control value (solid bar) (100%) refers to the amounts of pregnenolone (1.9 ± 0.15 ng/mg of tissue), corticosterone (1.2 ± 0.24 ng/mg of tissue), DBI-LI (77 ± 5.1 ng/mg of tissue), and MDR (5.1 ± 0.41 pmol/mg of protein) in the adrenals of untreated hypophysectomized rats. Similar results were obtained in vehicle-treated rats. Each value represents the mean ± SEM ($n = 8$). *, $P < 0.001$ (Student t test).

Adrenal cAMP Determination. Frozen adrenals were homogenized in cold 6% (wt/vol) trichloroacetic acid at 4°C to give a 10% (wt/vol) homogenate. The homogenate was then centrifuged at 2000 × g for 15 min at 4°C, and the supernatant was washed four times with 5 vol of water-saturated diethyl ether. The aqueous extract remaining was lyophilized and assayed for cAMP with a RIA kit from Amersham.

Statistical Analysis. The statistical significance of the differences between the results from various treatments was determined by the Student t test or two-way ANOVA.

RESULTS

Relation Between Immediate Steroidogenic Action of ACTH and Amounts of Adrenal DBI-LI and MDR in Hypophysectomized Rats. To investigate whether the immediate steroidogenic action of ACTH was associated with changes in the amount of adrenal DBI or MDR, we treated hypophysectomized rats, 1 day after surgery, with ACTH. The concentrations of adrenal pregnenolone and corticosterone increased rapidly after ACTH administration, reaching a peak within 15 min and thereafter declined to basal values by 90 min (Fig. 2). Neither adrenal DBI-LI nor MDR concentration was affected by injection of the pituitary hormone (Fig. 2). Furthermore, the concentrations of adrenal DBI and MDR mRNAs remained unchanged 30 min after injection of ACTH in hypophysectomized rats [DBI mRNA: control, 1.2 ± 0.15 AU; and ACTH treated, 1.3 ± 0.22 AU; MDR mRNA: control, 2.3 ± 0.14 AU, and ACTH treated, 2.1 ± 0.25 AU ($n = 3$)].

Effect of ACTH on Rate of DBI Processing in Adrenals of Hypophysectomized Rats. When the TTN-like immunoreactivity (TTN-LI) in adrenal extracts of hypophysectomized rats (1 day after surgery) was measured in various fractions eluted from the reversed-phase HPLC column, it partitioned into two major peaks (Fig. 3*A*). In addition to other DBI-processing products, the first broad peak (fractions 10–13) probably contains ODN because standard ODN eluted in fraction 12. The second peak (fraction 23) was immunoreactive with the antiserum to TTN but eluted before authentic TTN (fraction 27). Standard DBI eluted in fraction 32, as determined by DBI RIA (data not shown).

When hypophysectomized rats were treated with ACTH, the TTN-LI peak eluting in fraction 23 increased by more

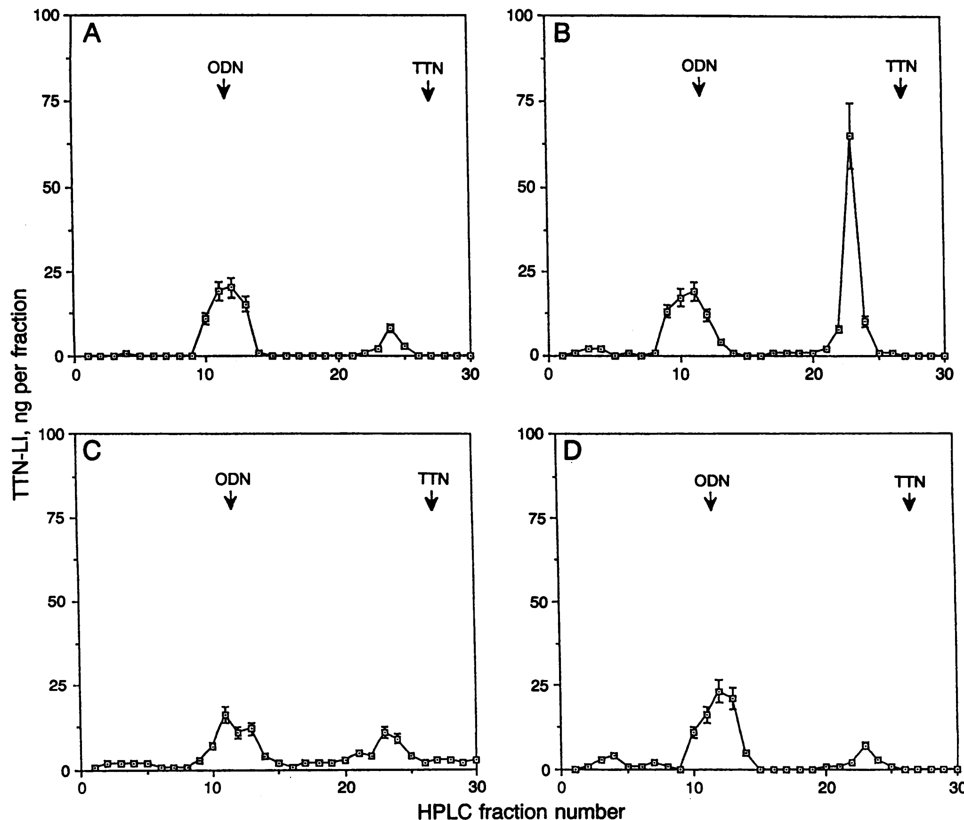

FIG. 3. Reverse-phase HPLC profiles of TTN-LI in adrenal glands of hypophysectomized rats. Adrenal extracts (equivalent to 10 mg of wet tissue) were subjected to chromatography on a μBondapak C_{18} (30 cm × 7.5 mm) HPLC column equilibrated with 0.1% trifluoroacetic acid. The column was developed with a gradient (30–45% in 30 min) of 0.1% trifluoroacetic acid in acetonitrile at a flow rate of 1 ml/min. Fractions (1 ml) were collected, lyophilized, and analyzed for TTN-LI. Synthetic ODN and TTN diluted in hot (90°C) 1 M acetic acid and then applied to the HPLC column produced only single peaks with retention times indicated by the arrows. Recoveries of ODN and DBI from the column ranged from 50% to 60%. (*A*) Hypophysectomized rats receiving vehicle only. (*B*) Hypophysectomized rats 15 min after ACTH injection (200 milliunits/kg i.v.). (*C*) Hypophysectomized rats 90 min after ACTH injection. (*D*) Hypophysectomized rats pretreated with cycloheximide (142 μmol/kg i.p.) 10 min before receiving ACTH for 15 min. Each value is the mean ± SEM ($n = 15$).

Table 1. Effect of cycloheximide on ACTH-induced steroidogenesis and the amounts of DBI-LI and MDR in adrenals of hypophysectomized rats

	Hypophysectomized rat				
	Adrenal				Plasma
	Pregnenolone, ng/mg of tissue	Corticosterone, ng/mg of tissue	DBI-LI, ng/mg of tissue	MDR, pmol/mg of protein	Corticosterone, ng/ml of plasma
Control	1.9 ± 0.15	1.2 ± 0.24	77 ± 5.1	5.1 ± 0.41	3.7 ± 0.88
ACTH	8.2 ± 0.60*	7.7 ± 0.85*	74 ± 7.8	5.3 ± 0.25	125 ± 15*
Cyclo	1.7 ± 0.19	1.1 ± 0.18	81 ± 7.5	4.8 ± 0.63	2.5 ± 0.44
Cyclo + ACTH	1.8 ± 0.14	1.5 ± 0.19	75 ± 8.8	4.6 ± 0.55	3.3 ± 0.42

The amounts of adrenal pregnenolone, corticosterone, DBI-LI, and MDR, as well as plasma corticosterone, were measured in hypophysectomized rats treated for 15 min with saline (control) or ACTH (200 milliunits/kg i.v.). Cycloheximide (Cyclo) (142 μmol/kg i.p.) was injected 10 min before saline or ACTH. Each value is the mean ± SEM ($n = 15$). *, $P < 0.001$ (Student t test).

than threefold 15 min after treatment (Fig. 3*B*), returning to levels equivalent to those of untreated rats after 90 min (Fig. 3*C*).

Effect of Cycloheximide on ACTH-Induced Adrenal Steroidogenesis and TTN-LI Formation. Administration of cycloheximide 10 min before ACTH completely inhibited the effect of the hormone on adrenal steroidogenesis, as determined by the adrenal pregnenolone concentration and the corticosterone concentration in adrenal and plasma (Table 1). Although cycloheximide did not significantly affect the adrenal DBI-LI concentration or the density of MDR-binding sites (Table 1), it completely inhibited the ACTH-elicited increase in TTN-LI in HPLC fraction 23 (Fig. 3*D*).

Effect of PK 11195 on 4′-Chlorodiazepam- and ACTH-Induced Adrenal Steroidogenesis. To investigate the *in vivo* role of the MDR in ACTH-induced adrenal steroidogenesis, we treated hypophysectomized rats (1 day after surgery) with 4′-chlorodiazepam and measured the concentration of plasma corticosterone. 4′-Chlorodiazepam induced a dose-related (3.1–28.2 μmol/kg i.v.) increase in plasma corticosterone that peaked 7 min after injection (data not shown). Intravenous injection of 4′-chlorodiazepam at a dose of 18.8 μmol/kg increased adrenal and plasma corticosterone by ≈300% (Fig. 4). This effect was antagonized by the injection, 5 min before 4′-chlorodiazepam, of PK 11195 (28.3 μmol/kg i.v.), which alone was devoid of any steroidogenic effect (Fig. 4). Similarly, PK 11195 inhibited the increase in adrenal and plasma corticosterone apparent 15 min after the injection of ACTH (1 milliunit/kg i.v.) (Fig. 4). The effect of PK 11195 on ACTH-induced steroidogenesis could be reduced by increasing the dose of ACTH to 10–25 milliunits/kg and was not from a blockade of ACTH binding to its receptor because PK 11195 failed to abolish the increase in adrenal cAMP elicited by ACTH [cAMP content of: ACTH 1 milliunit/kg i.v., 8.0 ± 0.92 pmol/mg of tissue; PK 11195 28.3 μmol/kg i.v., 2.5 ± 0.30 pmol/mg of tissue; PK 11195 plus ACTH, 7.5 ± 0.85 pmol/mg of tissue ($n = 3$)].

DISCUSSION

The intramitochondrial transport of cholesterol is believed to be both the rate-limiting step of adrenal steroid biosynthesis and the main target for regulation by physiological stimuli that induce steroidogenesis (1–5). In rat adrenocortical cells, cycloheximide and other protein synthesis inhibitors rapidly block ACTH-induced steroidogenesis, presumably at the level of intramitochondrial cholesterol transport (6–8). Thus, it has been proposed that one or more labile proteins are required for ACTH stimulation of adrenal steroidogenesis. Although a number of adrenal peptides have been proposed as regulators of mitochondrial cholesterol translocation (33–35), the biological significance of some of these factors is still unclear. For example, despite an initial report on the ability of the steroidogenesis activator polypeptide (SAP) to stimulate cholesterol side-chain cleavage in isolated adrenal mitochondria (33), the magnitude of this effect was relatively small (36). The mitochondrial phosphoprotein pp30 and its two precursor proteins, pp37 and pp32, have been shown to be produced in response to trophic hormones (34); however, no evidence is available that these polypeptides may function in the regulation of adrenal steroidogenesis. The sterol carrier protein 2 (SCP_2) is known to stimulate side-chain cleavage activity in adrenal mitochondria (35), but its adrenal level fails to change after ACTH stimulation (37). Finally, although the biological significance of these peptides cannot be completely disregarded, no interaction between them and a high-affinity mitochondrial receptor has been demonstrated.

In contrast, a role for DBI and the MDR in the action of pituitary hormones such as ACTH and human chorionic gonadotropin has been recently demonstrated (9–18). Flunitrazepam—a benzodiazepine that binds to the MDR with high

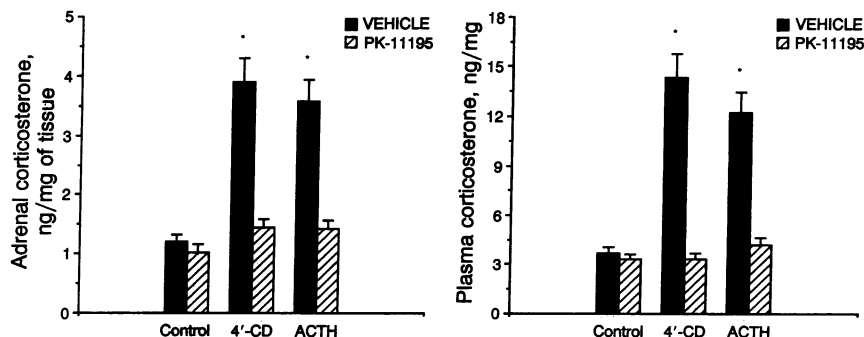

FIG. 4. Effect of PK 11195 on 4′-chlorodiazepam- and ACTH-induced steroidogenesis. Amounts of adrenal (*Left*) and plasma (*Right*) corticosterone were measured in hypophysectomized rats injected with vehicle or PK 11195 (28.3 μmol/kg i.v.) 5 min before receiving saline (control), 4′-chlorodiazepam (4′-CD) (18.8 μmol/kg i.v.), or ACTH (1 milliunit/kg i.v.). Animals were sacrificed 7 min after injection of 4′-chlorodiazepam and 15 min after injection of ACTH. Each value represents the mean ± SEM ($n = 12$). *, $P < 0.001$ (ANOVA test).

affinity but is endowed with little or no steroidogenic activity—can inhibit both the activation of the MDR by DBI and hormone-stimulated steroid biosynthesis (12).

The experiments described here were designed to investigate some of the possible mechanisms by which DBI and the MDR may mediate the immediate steroidogenic action of ACTH. Initially, we determined whether ACTH could rapidly increase DBI or MDR expression in adrenal cortex. Our results, in agreement with those already described (38, 39), provide clear evidence that in the rat adrenal gland, the immediate steroidogenic action of ACTH is not associated with changes in DBI or MDR expression. Treatment of hypophysectomized rats, 1 day after surgery, with ACTH failed to affect immediately (up to 1.5 hr) the amounts of adrenal DBI-LI or MDR or the concentrations of their mRNAs.

In contrast, ACTH rapidly increased the rate of DBI processing in adrenals of hypophysectomized rats, as demonstrated by the increase in a TTN-LI peak that emerged four fractions before authentic TTN on reverse-phase HPLC. These data—together with the demonstration that specific DBI-processing products (TTN) displace high-affinity synthetic ligands from the MDR (40, 41), can be cross-linked to the MDR (A. Berkovich, personal communication), and potently stimulate mitochondrial steroidogenesis in adrenocortical, Leydig, and glial cells (15–17)—suggest that the rapid increase in DBI processing elicited by ACTH may mediate the immediate steroidogenic action of ACTH. This suggestion is supported by the observation that PK 11195, which is endowed with an antagonistic activity at the MDR (20), antagonizes the effects of ACTH on adrenal steroidogenesis *in vivo*. PK 11195 might inhibit ACTH-induced steroidogenesis by antagonizing a specific DBI-processing product(s) capable of eliciting steroidogenesis by functioning as an endogenous MDR ligand.

The demonstration that the ACTH-induced increases in both adrenal steroidogenesis and the rate of DBI processing are completely inhibited by cycloheximide suggests that the putative labile protein proposed by others to participate in ACTH action (6–8) is required to activate DBI processing rather than to act directly in the steroidogenic process. Thus, ACTH may regulate the expression of an adrenal endopeptidase that catalyzes the formation of a specific DBI-processing product(s), which, like TTN, potently stimulates mitochondrial steroidogenesis but which binds to the MDR with an affinity greater than that of TTN.

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