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ON THE SITE OF ACTION OF THE ANTI-ADRENAL STEROIDOGENIC EFFECT OF CYPROTERONE ACETATE

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Abstract—Cyproterone acetate (CA) inhibited 1–24 ACTH (50 ng/l)-stimulated cortisol production by dispersed guinea-pig adrenal cells in a dose-related manner. Inhibition occurred over the range 10^{-6} to 5×10^{-5} moles/l. The concentration of drug which induced 50% inhibition was 4.6×10^{-6} moles/l. The sites of action of this anti-steroidogenic effect have been established. Dispersed adrenal cells were challenged with the cortisol precursor steroids (all at 10^{-5} moles/l) pregnenolone (Pe), 17α hydroxypregnenolone (17-OH Pe), 17α-hydroxyprogesterone (17-Po), and 11 deoxycortisol or 1–24 ACTH (100 ng/l) in the absence and presence of increasing concentrations of CA (10^{-5} to 10^{-4} moles/l). In the absence of drug, the steroid precursors or ACTH provoked cortisol secretion greater than 10-fold that secreted by cells incubated in their absence. ACTH-stimulated cortisol secretion was inhibited >68% at concentrations of CA $>10^{-5}$ moles/l. CA (10^{-5} moles/l) had no significant effect on steroid-stimulated cortisol production when the Δ^4, 3 ketosteroids 17-OH Po and 11-deoxycortisol were used, but depressed secretion by >61% ($P < 0.001$) when the Δ^5 3β hydroxysteroids (Pe, 17-OH Pe) were employed. Increasing CA concentrations to 10^{-4} moles/l had little effect on cortisol secretion provoked by 11-deoxycortisol, but significantly ($P < 0.05$) depressed cortisol secretion stimulated by 17-OH Po. These results suggest that the major site of action of CA is Δ^5, 3β hydroxysteroid dehydrogenase (3β-HSD) with a secondary effect on 21-hydroxylase activity. To confirm these findings cortisol secretion provoked by 17, 21, dihydroxypregnenolone (17, 21 diOH Pe) and 21 deoxycortisol (21-DOC) was measured in the absence and presence of increasing concentrations of CA. At the lowest concentration of CA (5×10^{-6} moles/l), cortisol secretion provoked by 17, 21 diOH Pe was inhibited by 28% ($P < 0.01$) whereas secretion provoked by 21-DOC was not significantly affected. At the highest concentration of CA (10^{-4} moles/l), the relative inhibition was 80% for 17, 21 diOH Pe and 38% for 21-DOC.

We conclude that cyproterone acetate inhibits adrenal steroidogenesis at both 3β-HSD and 21-hydroxylase, the degree of inhibition being more pronounced at 3β-HSD.

Cyproterone acetate (1, 2α-methylene-6-chloro-17α-acetoxy-4,6-pregnadiene-3, 20-dione) ("Androcur") is a powerful inhibitor of androgen action as well as an inhibitor of gonadotrophin release [1]. It is used clinically for the treatment of various androgen-dependent disorders such as hypersexuality, precocious puberty and hirsutism. The drug has also been shown to have a pronounced and long-lasting ACTH-suppressive effect [2, 3] and this probably explains the reported atrophic effects of CA on the adrenal glands of animals [4–7]. Furthermore, a direct inhibitory action of CA on adrenal steroidogenesis *in vivo* has been reported [8], and work by Panesar *et al.* [9], using a rat adrenal homogenate preparation, has indicated that this effect may be mediated by inhibition of 3β hydroxysteroid dehydrogenase.

We have recently described two systems [10–13] using guinea-pig adrenal cells maintained in primary cell culture [14, 15] which have allowed us to assess the *in vitro* anti-steroidogenic potency of different drugs and to delineate the enzymic sites of action of this inhibitory effect. We now report on similar studies performed with CA.

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MATERIALS AND METHODS

Dispersed guinea-pig adrenal cells were prepared essentially as described previously [14, 15], except that collagenase was used in place of trypsin. Briefly, the adrenals from two male guinea-pigs were chopped into 1-mm cubes using a McIlwain tissue chopper, the tissue pieces washed with Eagle's minimum essential medium (EMEM) and dispersed by mechanical agitation in EMEM containing collagenase (2 mg/ml). The cells were collected by centrifugation (300 *g* for 5 min) and washed twice with Eagle's medium containing bovine serum albumin (BSA, 0.5%), calcium (8×10^{-3} moles/l) and ascorbate (2×10^{-3} moles/l). The above mixture served as incubation medium. Finally the cell suspension was filtered through nylon (100 μ) mesh. The cells (1×10^6 cells/ml) were then pre-incubated for 2 hr at 37° in an atmosphere of 100% O_2. After pre-incubation the cell suspension was centrifuged to remove any secreted cortisol and resuspended in fresh incubation medium.

In all experiments aliquots (40 μl) of cell suspension were dispensed into a 96-well tissue culture plate, the total volume of incubate in each well was 100 μl and the final cell concentration was 0.75×10^6 cells/ml. Drugs and steroids were incorporated into

the cell suspension in dimethyl sulphoxide (DMSO) such that the final concentration of solvent was 2.5%. DMSO (2.5%) was also included in the control (no added ACTH) and ACTH-stimulated wells in the absence of drug or exogenous steroid. In the first set of experiments the cells were stimulated for 90 min at 37° with 50 ng/l ACTH (1–24) either alone or in combination with increasing concentrations of cyproterone acetate or trilostane. Trilostane was included as it is a proven inhibitor of 3β hydroxysteroid dehydrogenase and its anti-steroidogenic effect in this dispersed cell system has been studied in detail [13]. After 90 min incubation, duplicate 10-μl samples were taken and assayed for cortisol by radioimmunoassay.

In the second set of experiments the cells were challenged with pregnenolone (Pe), 17α hydroxypregnenolone (17-OH Pe), 17α hydroxyprogesterone (17-OH Po), 11-deoxycortisol, 17, 21 dihydroxypregnenolone, and 21-deoxycortisol (21-DOC) (all at 10^{-5} moles/l) or 1-24 ACTH (100 ng/l) alone or in the presence of cyproterone acetate (5×10^{-6}–10^{-4} moles/l). After 90 min incubation, duplicate 10-μl samples were taken for estimation of cortisol. Each experimental point was performed in at least duplicate wells: intra and inter-well coefficients of variation for this system were <6 and <10% respectively (N = > 100 in both cases) [15]. Neither trilostane nor cyproterone acetate interfere with the cortisol radioimmunoassay at concentrations up to 10^{-4} moles/l.

Source of chemicals. ACTH (1–24) (Synacthen) was a gift from Dr. C. McMartin (Ciba Geigy, Horsham, West Sussex). Cyproterone acetate was supplied by Schering Pharmaceuticals, Burgess Hill, West Sussex and trilostane by Sterling–Winthrop Research and Development Laboratories, Alnwick, Northumbria. Pregnenolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and 11-deoxycortisol were supplied by Sigma Chemical Co., Poole, Dorset. 21-deoxycortisol was obtained from Steraloids Ltd., Croydon, London. 17, 21 dihydroxypregnenolone was a gift from Professor Kirk, MRC Steroid Reference Centre, Westerfield College, London NW3. Dimethyl sulphoxide (AR) was purchased from Fisons p.l.c., Loughborough.

RESULTS

The effect of CA on cortisol secretion from dispersed guinea-pig adrenal cells

The effect of increasing concentrations of CA and trilostane on ACTH (50 ng/l)-stimulated cortisol secretion is shown in Fig. 1. Inhibition occurred over the ranges 10^{-6}–5×10^{-5} and 5×10^{-7}–10^{-5} moles/l for CA and trilostane respectively. The concentrations of drug which produced 50% inhibition (ED_{50}) was 4.6 ± 0.3 and 0.9 ± 0.2 μmoles/l (mean ± S.D., N = 2) for CA and trilostane respectively.

Investigation of the site of action of CA

Figure 2 shows ACTH stimulated, basal and steroid-provoked cortisol secretion by the cells in the presence and absence of CA (10^{-5}, 5×10^{-5} and 10^{-4} moles/l). ACTH-stimulated cortisol secretion was inhibited by 83%, 97% and 98%, with 10^{-5}, 5×10^{-5} and 10^{-4} moles/l CA respectively. The presence of the steroid precursors or ACTH in the absence of drug provoked cortisol secretion at least

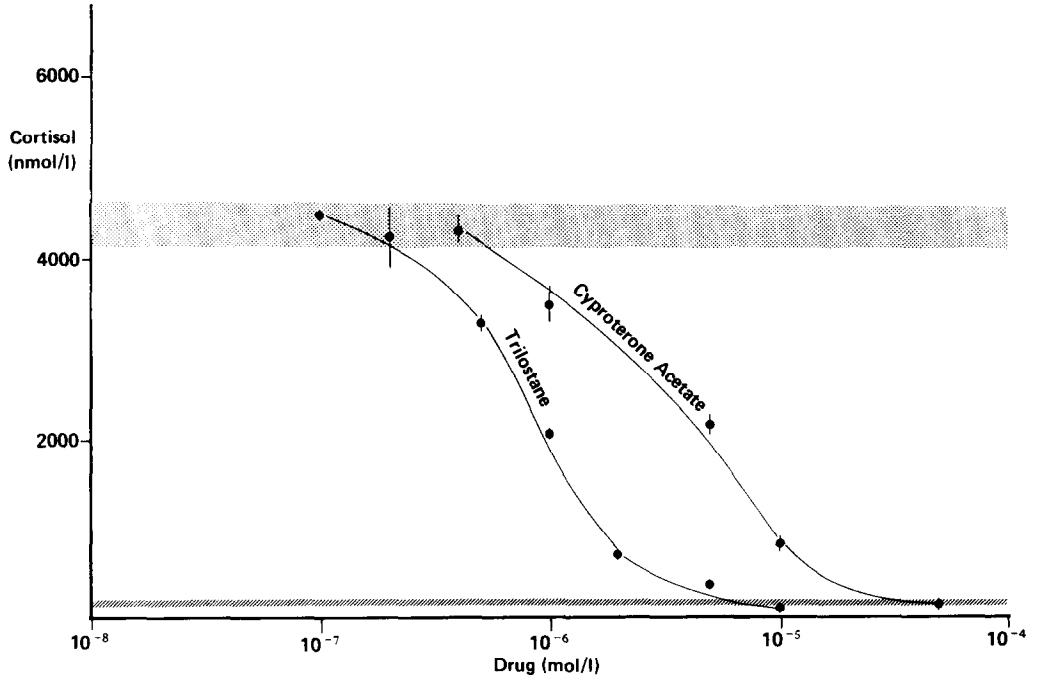

Fig. 1. Inhibition of ACTH (50 ng/l)-stimulated cortisol secretion by trilostane and cyproterone acetate. Each point is the mean ± S.D. (N = 4). The hatched and stippled areas represent the cortisol secretion (± S.D) in response to no added ACTH and 50 ng/l ACTH 1-24, respectively.

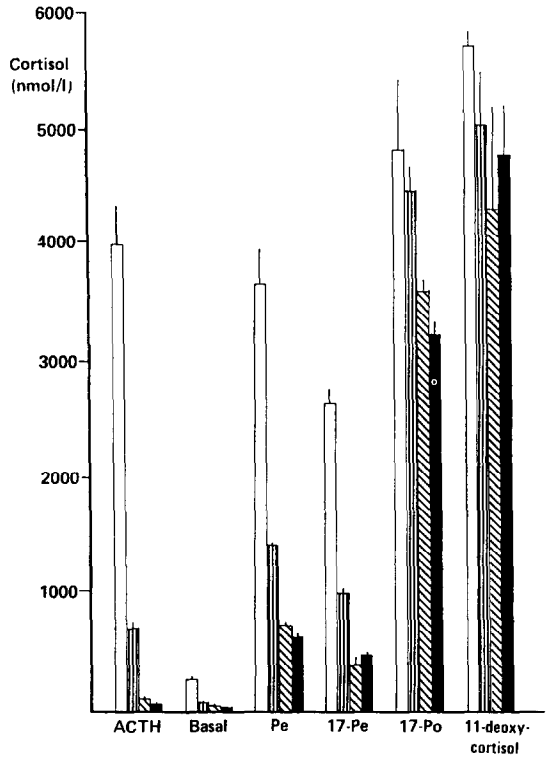

Fig. 2. Cortisol secretion (nmoles/l) is shown in response to 1–24 ACTH (100 ng/l), no added ACTH (basal) and the cortisol precursor molecules (all at 10^{-5} moles/l) pregnenolone (Pe), 17α hydroxypregnenolone (17-OH Pe), 17α-hydroxyprogesterone (17-OH Po) and 11-deoxycortisol in the absence (open columns) and presence of cyproterone acetate 10^{-5} moles/l (vertical hatched column), 5×10^{-5} moles/l (cross-hatched column) and 10^{-4} moles/l (solid column). Columns represent the mean ± S.D. (N = 4).

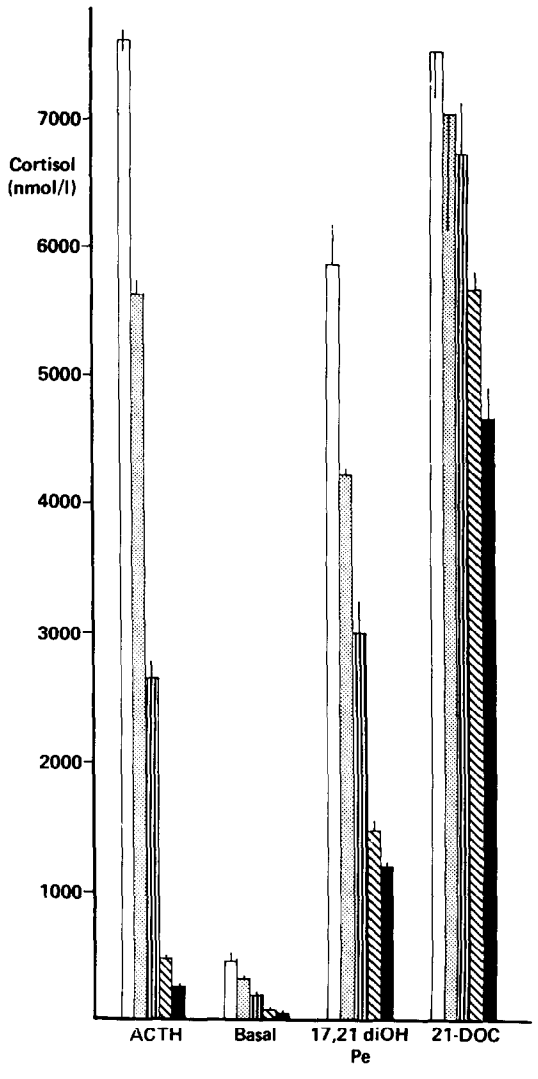

Fig. 3. Cortisol secretion (nmoles/l) is shown in response to 1–24 ACTH (100 ng/l), no added ACTH (basal) and the cortisol precursor molecules (at 10^{-5} moles/l) 17–21 dihydroxypregnenolone (17, 21 diOH Pe) and 21-deoxycortisol (21-DOC) in the absence (open columns) and presence of cyproterone acetate 5×10^{-6} moles/l (stippled column), 10^{-5} moles/l (vertical hatched column) 5×10^{-5} moles/l (cross-hatched column) and 10^{-4} moles/l (solid column). Columns represent the mean ± S.D. (N = 4).

10 times greater than that found in the absence of exogenous steroid or hormone. For example, 11-deoxycortisol 10^{-5} moles/l provoked a cortisol secretion of 5714 ± 333 nmoles/l (N = 4) when basal secretion was 275 ± 20 nmoles/l.

CA (10^{-5} moles/l) had no significant effect on steroid-stimulated cortisol secretion when the Δ^4, 3 keto steroids 17-OH Po and 11-deoxycortisol were used, but depressed secretion by >61% when the Δ^5 3β hydroxysteroids, (Pe, 17-OH Pe) were employed. Increasing the concentrations of drug to 10^{-4} moles/l had little effect on cortisol secretion provoked by 11-deoxycortisol but depressed cortisol secretion stimulated by 17-OH Po by 34% ($P < 0.05$). These results suggest that the major site of action of CA is 3β-HSD with a secondary site at 21-hydroxylase.

To confirm these findings cortisol secretion provoked by 17, 21-diOH Pe and 21-DOC was measured in the absence and presence of increasing concentrations of cyproterone acetate (5×10^{-6}–10^{-4} moles/l (Fig. 3). 17, 21 diOH Pe is converted to cortisol via 3β-HSD and 11β-hydroxylase whereas 21-deoxycortisol is transformed directly via 21-hydroxylase. At the lowest concentration of drug (5×10^{-6} moles/l) cortisol secretion provoked by 17, 21 diOH Pe was inhibited by 28% whereas the secretion provoked by 21-DOC was not significantly affected. At the highest concentration of drug (10^{-4} moles/l) the inhibition was 80% with 17, 21 di OH Pe and 38% with 21-DOC.

DISCUSSION

Direct anti-adrenal steroidogenic effects have been demonstrated for CA using a novel *in vitro* cell system which has been described in detail elsewhere [10, 11, 13]. This confirms and extends the earlier work of Panesar *et al.* [9] and confirms the very recent work of Pham-Huu-Trung *et al.* [16] using

an independent *in vitro* technique. The ED_{50} for inhibition of cortisol secretion from adrenal cells by CA was 4.6×10^{-6} moles/l which is of the same order as the value of 2.4×10^{-6} moles/l reported by Pham-Huu-Trung *et al.* (value derived from data given in a figure) and is also similar to the value of 3.5×10^{-6} moles/l reported for the established anti-steroidogenic drug, metyrapone [11, 13]. Trilostane, a proven inhibitor of 3β-HSD was included in this study as an internal standard since its anti-steroidogenic effects in an identical dispersed cell system have been studied in detail [10, 11, 13]. The ED_{50} for trilostane 0.9 μmoles/l agreed well with the value reported previously [10, 11, 13].

In adults receiving 200 mg cyproterone acetate per day, plasma levels reach approx. 600 ng/ml (1.4×10^{-6} moles/l) within 20 days of treatment [17]. This concentration would lead to < 25% inhibition of adrenal steroidogenesis in our *in vitro* system. However, to our knowledge, there are no data available on the concentration of cyproterone acetate reached within the adrenal gland and this information may be of greater importance. For example, etomidate, an anaesthetic which is known to inhibit adrenal steroidogenesis both *in vivo* [18] and *in vitro* [12], leads to a persistent adrenocortical suppression (for at least 3 hr) after an induction dose [19] despite having a plasma half life of < 5 min. This effect is probably due to the maintenance of high intra-adrenal levels and in this case, the plasma concentration of etomidate bears little relevance to its adrenal effect. In general, the relevance of the anti-adrenal steroidogenic potency of a drug measured in any *in vitro* system to its pharmacologic effects *in vivo* must be viewed with some caution, but in the absence of clinical or other evidence from animal studies, it may give some indication as to possible *in vivo* activity.

The sites of action of the anti-steroidogenic effect of cyproterone acetate have been established using an *in vitro* approach based upon the inhibition of cortisol secretion provoked by the various precursors on the biosynthetic route for the glucocorticoids [13]. The inhibitory effect of a drug will only be observed if the steroid precursor is before its site of action. By challenging the adrenal cells with the cortisol precursor steroids (Pe, 17-OH Pe, 17-OH Po and 11-deoxycortisol) in the absence and presence of increasing concentrations of drug, we have demonstrated that CA is a potent inhibitor of Δ^5, 3β hydroxysteroid dehydrogenase and a somewhat less potent inhibitor of 21-hydroxylase. This finding was confirmed in a second series of experiments in which the cells were challenged with two further steroids, 17, 21 diOH Pe, a steroid whose conversion to cortisol involves two enzymatic steps, 3β-HSD and 11β hydroxylase, and 21-DOC which is transformed to cortisol directly via 21-hydroxylase. As 11β hydroxylase has been shown to be only marginally affected by CA, this experiment investigated the relative efficacy of CA as an inhibitor of 3β-HSD and 21-hydroxylase. Our previous observation of a more marked CA-induced inhibition of 3β-HSD as compared with 21-hydroxylase was confirmed.

While this study was being prepared for publication, Pham-Huu-Trung *et al.* [16] reported the results of their work on the effects of CA on adrenal steroidogenesis using a similar dispersed guinea-pig adrenal cell system. They concluded that at a concentration of 4.8×10^{-6} moles/l, CA inhibited 21-hydroxylase but above this concentration 3β-HSD was affected. The apparent discrepancy in the relative potency of CA on the two enzymes may be explained by the different experimental approaches used in the two studies. Pham-Huu-Trung *et al.* investigated the effect of CA on maximal ACTH (250 pg/ml) stimulated steroidogenesis by measuring the production of cortisol, its precursors 11-deoxycortisol and 17-OH Pe and the adrenal androgens, Δ^4-androstenedione and dehydroepiandrosterone by the cells. Thus, in their work the effects of CA were studied when the cortisol biosynthetic pathway was under maximum ACTH drive. The experimental approach adopted in this study is more direct in that it allows investigation of the differential effects of CA on each enzymatic step in the pathway. Nevertheless, despite differences in the relative potency of CA on the two enzymes, the present study, which was performed using a completely independent method, confirmed the findings of Pham-Huu-Trung *et al.* in defining two sites of action of CA, 3β-HSD and 21-hydroxylase.

A parallel can be drawn between the work of Pham-Huu-Trung *et al.* and ourselves on the site of the anti-steroidogenic effect of CA and that of Kenyon *et al.* [20] and ourselves on the site of action of the anaesthetic agent, etomidate. Using an identical experimental system we have recently established that etomidate is a clear-cut inhibitor of 11β hydroxylase. Kenyon *et al.*, using an approach similar to Pham-Huu-Trung *et al.*, reached the same conclusion but interpretation of their data was complicated due to the system being under maximum ACTH drive. The method used in the present study is quick and simple to perform and gives unequivocal results on the site of anti-steroidogenic action of a drug *in vitro*.

In conclusion we have demonstrated that cyproterone acetate inhibits adrenal steroidogenesis primarily at the level of 3β-HSD and to a lesser extent 21-hydroxylase.

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