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DANTROLENE INHIBITS ADRENAL STEROIDOGENESIS BY A MECHANISM INDEPENDENT OF EFFECTS ON STORED CALCIUM RELEASE

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Summary—The muscle relaxant dantrolene has been widely used in signal transduction studies as an inhibitor of intracellular calcium release. However, *in vivo* studies have shown that the drug may inhibit steroidogenesis by a mechanism which is distinct from its effects on calcium mobilization. Using freshly isolated cells and mitochondria from the outermost regions of bovine adrenal cortex we have shown that dantrolene (0.2 mM) significantly inhibits steroid synthesis stimulated by either angiotensin II (AII) or by addition of various precursors. Our results suggest that dantrolene inhibits the rate-limiting steps of adrenocortical steroidogenesis, i.e. the intramitochondrial conversion of cholesterol to pregnenolone (for both aldosterone and cortisol) and the conversion of corticosterone to aldosterone (for aldosterone), by a mechanism independent from its known effects on calcium release. A possible alternative mechanism may involve direct inhibition of cytochrome *P*450-dependent hydroxylation reactions.

INTRODUCTION

Dantrolene (1-{[5-(*p*-nitrophenyl)furfurylidine]-amino}hydantoin) is a direct acting skeletal muscle relaxant widely used in the treatment of muscle spasticity and associated disorders [1–3]. It appears to exert its therapeutic effect by inhibiting the release of calcium from the terminal cisternae of the sarcoplasmic reticulum [4–6]. In recent years this property has made dantrolene an obvious tool to study the role of calcium release in mediating hormone action [7–9]. However, adverse clinical effects, most notably hepatic injury (which could be fatal), have been reported, especially following treatment with a high daily dose of the drug [10]. Francis and Hamrick have shown that liver damage may be associated with direct inhibition of hepatic, cytochrome *P*450-dependent, mixed function oxidase enzymes [11, 12]. The same investigators also showed that, *in vivo*, dantrolene inhibits glucocorticoid secretion [13]. Since steroidogenesis depends on various cytochrome *P*450 enzymes [14] dantrolene may exert an adrenal effect similar to that in the liver. With this hepatic cytochrome *P*450 effect in mind, the following study has focused on whether dantrolene directly affects steroidogenesis other than by inhibiting calcium release from intracellular stores and, if so, whether key mitochondrial steroid hydroxylation steps are affected.

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EXPERIMENTAL

Isolated cells and mitochondria were prepared from the adrenal glands of freshly slaughtered cattle according to the following protocols.

Cells

Briefly, as described by Simpson *et al.* [15]; thin slices of tissue were taken from the outer regions of defatted adrenal glands and minced before being subjected to collagenase digestion (1.5 mg/ml; Worthington Biochemical Corp., NJ, U.S.A.) in Krebs bicarbonate Ringer's solution containing 0.01 M glucose and bovine serum albumin (20 g/l). The cells were then filtered twice, firstly through a tea strainer and then through 100 μm gauze (Henry Simon Ltd, Stockport, U.K.) before being applied to a discontinuous gradient of isotonic Percol. Following centrifugation (10 min, 2800 *g*) the cells with sp. gr. > 1.039 and < 1.052 were removed and resuspended in medium 199 (modified so that the ionic composition was the same as that of the Ringer's solution) containing 0.01 M glucose and BSA (2 g/l). We have shown

previously that, although these cells are enriched with aldosterone-producing zona glomerulosa cells, they also synthesize significant amounts of cortisol [16]. The final concentration of cells used in the following experiments was approx. 3×10^5 cells/ml.

Mitochondria

Thin slices of tissue were again removed from the outermost region of bovine adrenal glands. Adrenocortical tissue was scraped from the capsule and homogenized (3×10 s) using a Polytron PTA20S homogenizer (Kinematic, Denmark) in ice-cold 0.25 M sucrose, pH 7.2, containing triethanolamine hydrochloride (10 mM), ethylenediaminotetra-acetic acid (0.1 mM) and BSA (10 g/l). This homogenate was then centrifuged at 600 *g* for 10 min to remove cellular debris and the supernatant centrifuged at 10,000 *g* for 10 min. The pellet was then washed twice by resuspending in sucrose and recentrifuging. Finally, prior to incubation, the mitochondria were suspended in 0.25 M sucrose, pH 7.1, containing KCl (20 mM), triethanolamine hydrochloride (15 mM), $MgCl_2$ (5 mM) and BSA (10 g/l) at a concentration of 3–15 mg protein/ml [17].

Incubations

Cell and mitochondrial suspensions (0.5 ml) were incubated in sextuplicate in 1.5 ml microcentrifuge tubes at 37°C in an atmosphere of 95% (O_2), 5% (CO_2) in medium 199 (cells) or sucrose solution (mitochondria) containing dantrolene and/or various steroids (final concentration 5 μmol/l). Steroids, dissolved in 10 μl methanol, were added to the appropriate tubes and the methanol was allowed to evaporate prior to the addition of incubation medium. Steroids used in these experiments (20α-hydroxycholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxy-corticosterone and 11-deoxycortisol) were all supplied by Sigma (Poole, Dorset, U.K.). The reactions were initiated by the addition of cells or for mitochondrial incubations by the addition of a solution composed of sodium succinate, isocitrate and $NADP^+$ (2.5 mM, 5 mM and 160 μM). Reactions were stopped after 1 h for cells by transferring tubes to ice-cold water. The cell incubations were then centrifuged at 800 *g* for 10 min at 4°C and the supernatant stored at −20°C for later analysis. Mitochondrial reactions were stopped after 15 min by dilution with methanol (1:20) prior to storage.

Steroid assays

Pregnenolone, cortisol and aldosterone were measured by RIA according to methods previously described [18, 19, 15, respectively].

Results were compared by analysis of variance using the Newman–Keuls multiple range test [20] or unpaired *t*-tests where indicated.

RESULTS

Figure 1 shows aldosterone output in basal and AII-stimulated cells in the presence of increasing concentrations of dantrolene; 0.02 mM reduced and 0.2 mM abolished the aldosterone response to AII ($P < 0.01$). The effects of the same concentrations of dantrolene on aldosterone synthesis in cells incubated in the presence and absence of hydroxycholesterol is shown in Fig. 2. In the absence of hydroxycholesterol, inhibition of aldosterone synthesis was slight and only reached significance ($P < 0.05$) at the highest concentration of dantrolene (0.2 mM). In the presence of hydroxycholesterol, aldosterone synthesis was inhibited at all concentrations of dantrolene in a dose-dependent manner ($P < 0.01$; IC_{50} value approx. 0.02 mM). However, aldosterone synthesis from added hydroxycholesterol was still greater than controls when cells were treated with the highest concentration of dantrolene (0.2 mM).

The effects of dantrolene (0.2 mM) on aldosterone production in cells which were incubated

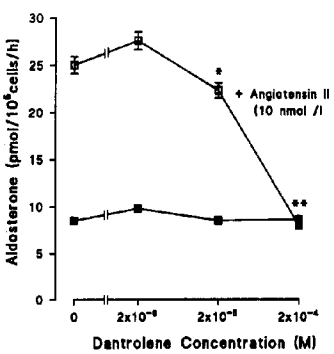

Fig. 1. Effect of dantrolene on aldosterone synthesis by bovine adrenocortical cells incubated in the absence or presence of AII (10 nmol/l). Values shown are means ± SE, $n = 6$. * And ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively (Newman–Keul's multiple range test), for aldosterone output from dantrolene-treated cells, compared with that from cells exposed to vehicle.

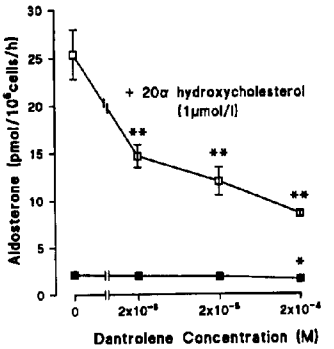

Fig. 2. Effect of dantrolene on aldosterone synthesis by bovine adrenocortical cells incubated in the presence and absence of 20α hydroxycholesterol. Values shown are means ± SE, $n = 6$. **Indicates significant inhibition by dantrolene ($P < 0.01$) using Newman–Keuls multiple range test for aldosterone output from dantrolene-treated cells is compared with that from vehicle-exposed controls.

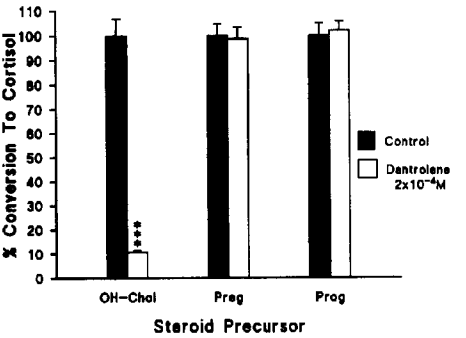

Fig. 4. Effect of dantrolene on cortisol synthesis from precursors added to bovine adrenocortical cells. Values shown are means ± SE, $n = 6$, and are expressed as a percentage of control, vehicle-exposed values (see text for absolute values). Steroid precursors were all added at a concentration of 5 μmol/l. Statistically significant inhibition of cortisol synthesis is indicated by ***($P < 0.001$) (unpaired t-test).

with various biosynthetic intermediates are shown in Fig. 3. Results are expressed as a percentage of controls (i.e. aldosterone synthesized in the absence of dantrolene). Control values (pmol/10^6 cells/h) were 70 ± 5, 250 ± 13, 356 ± 19, 942 ± 39, 667 ± 36 and 65 ± 3 for cells incubated with hydroxycholesterol, pregnenolone, progesterone, deoxycorticosterone, corticosterone and 18-hydroxycorticosterone, respectively. In all cases dantrolene significantly inhibited aldosterone synthesis ($P < 0.01$). The greatest percentage inhibition by dantrolene was in cells exposed to hydroxycholesterol (89%) and the least inhibition was with 18-hydroxycorticosterone (28%).

Figure 4 shows the effect of dantrolene on cortisol synthesis. The cells used in this experiment were the same as those in Fig. 3. Cortisol production was 2.27 ± 0.16, 3.99 ± 0.19 and 6.5 ± 0.32 nmol/10^6 cells/h for control cells incubated with hydroxycholesterol, pregnenolone and progesterone, respectively. Dantrolene only inhibited cortisol production in cells exposed to hydroxycholesterol (89%, $P < 0.01$).

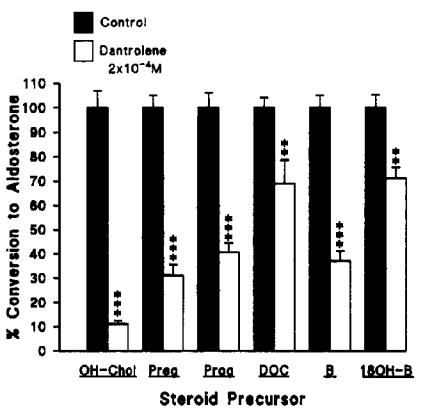

Fig. 3. Effect of dantrolene on aldosterone formation from added precursors in bovine adrenocortical cells. Values shown are means ± SE, $n = 6$, and are expressed as a percentage of control, vehicle-exposed values (see text for absolute values). Steroid precursors were all added at a concentration of 5 μmol/l. Statistically significant inhibition of aldosterone synthesis is indicated by **($P < 0.01$) and ***($P < 0.001$) (unpaired t-test). DOC, deoxycorticosterone; B, corticosterone; and 18OH-B, 18-hydroxycorticosterone.

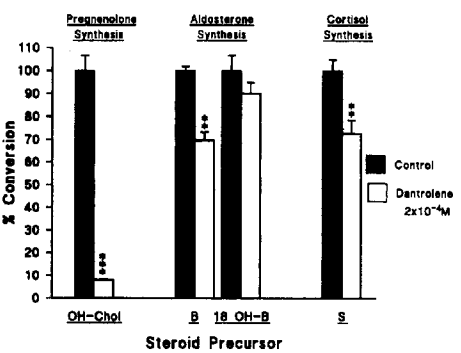

Fig. 5. Effect of dantrolene on pregnenolone, cortisol and aldosterone generation from added precursors by bovine adrenocortical mitochondria. Values shown are means ± SE, $n = 6$, and are expressed as a percentage of control, vehicle-exposed values (see text for absolute values). Steroid precursors were all added at a concentration of 5 μmol/l. Statistically significant inhibition of synthesis is indicated by **($P < 0.01$) and ***($P < 0.001$) (unpaired t-test). S, deoxycortisol; other abbreviations as in legend to Fig. 3.

Mitochondrial conversion of pregnenolone from added hydroxycholesterol and of aldosterone and cortisol from added corticosterone and deoxycortisol were inhibited by dantrolene ($P < 0.01$; Fig. 5). Control steroid production values (nmol steroid/mg protein/15 min) from mitochondria not treated with dantrolene were 1.66 ± 0.15 for pregnenolone, 0.057 ± 0.001 and 0.005 ± 0.0003 for aldosterone with added corticosterone and 18-hydroxycorticosterone, respectively and 12.88 ± 0.55 for cortisol.

DISCUSSION

As others have previously described [21], our studies show that dantrolene inhibits AII-stimulated aldosterone synthesis. The question that we have raised is whether this inhibition is due to prevention of calcium release from intracellular stores or some other calcium-independent process. In this regard, there are precedents suggesting that dantrolene does not always block intracellular calcium release. For example, Joseph *et al.* [22] report that IP_3-induced calcium release from hepatocytes is not affected by dantrolene. Similarly, not all of the actions of the principal agonists of adrenal catecholamine release are inhibited by dantrolene despite the fact that intracellular calcium mobilization is considered in each case to mediate the response [23].

In the case of adrenal steroidogenesis, a dantrolene-sensitive component of calcium mobilization in response to AII has been identified [24, 25]. It has also been noted that glucocorticoid synthesis *in vivo* is blocked by dantrolene [13]. Glucocorticoid synthesis is predominantly controlled by ACTH via an intracellular mechanism which does not involve phospholipase C activation and IP_3-induced calcium release [26]. Our present studies confirm *in vivo* findings that dantrolene inhibits calcium-independent steroidogenesis. Whether the mechanisms of *in vivo* and *in vitro* inhibition are the same is not clear. Therapeutic plasma concentrations of dantrolene are in the range 75–530 nM [27]. These concentrations would not inhibit steroidogenesis using the protocol of the present *in vitro* studies. However, it is not known whether, with long-term treatment, dantrolene is accumulated by adrenocortical tissue. It is clear though, that dantrolene effects *in vitro* represent more than a block of agonist-induced intracellular calcium release. Both aldosterone and cortisol formation from added hydroxycholesterol were inhibited. There were also differences in the patterns of aldosterone inhibition between AII- and hydroxycholesterol-treated cells. The threshold concentration for dantrolene's effects appeared lower in hydroxycholesterol-treated cells. In contrast, the highest concentration of dantrolene (0.2 mM) used here, completely inhibited the effects of AII but not those of hydroxycholesterol. These results are compatible with our hypothesis that dantrolene interferes with two steroidogenic processes: calcium mobilization and inhibition of one or more of the enzymes in the steroidogenic pathway.

To determine the site of this secondary direct action we incubated cells with different steroid precursors. Expressed in relative terms the greatest effect of dantrolene was on cells incubated with hydroxycholesterol. It is perhaps significant that aldosterone synthesis, which is regulated at two sites (cholesterol side-chain cleavage and conversion from corticosterone) [28], was inhibited in cells incubated with a variety of precursors whereas cortisol synthesis, which depends acutely on cholesterol side-chain cleavage, was inhibited only when cells were incubated with hydroxycholesterol. Taken together, these results suggest that dantrolene selectively inhibits two steroidogenic control steps (cholesterol side-chain cleavage and conversion of corticosterone to aldosterone) both of which are mitochondrial and calcium-dependent [29]. It seemed possible therefore that dantrolene blocks both aldosterone and cortisol synthesis by preventing calcium influx across the mitochondrial membrane. Accordingly, we tested dantrolene on isolated adrenocortical mitochondria. In the absence of added calcium, conversion of hydroxycholesterol to pregnenolone in mitochondria was inhibited, as was the synthesis of aldosterone from corticosterone. Although, in intact cells, we found that dantrolene did not affect cortisol synthesis from deoxycortisol, 11β hydroxylase activity in isolated mitochondria was profoundly inhibited. These data point at a direct action of dantrolene which is independent of acknowledged calcium-dependent, signal-response coupling processes. Direct inhibitory actions of several drugs on cytochrome *P*450-dependent, steroid hydroxylation reactions have been described in various tissues [18, 30, 31]. Invariably these drugs are also metabolized by cytochrome *P*450 enzymes in the liver. Since dantrolene is known to be a competitive inhibitor of hepatic cytochrome

*P*450 enzymes [11], it may be that the drug binds in a similar way to adrenal steroidogenic enzymes.

In summary, we have demonstrated a direct inhibitory effect of dantrolene on steroid synthesis in adrenocortical cells and in isolated mitochondria which is independent of its effects on intracellular calcium. The most likely explanation of this effect is competitive inhibition by the drug of cytochrome *P*450-dependent hydroxylase enzymes of the steroid biosynthetic pathway. In particular, cholesterol side-chain cleavage activity, which catalyses the important rate-limiting conversion of cholesterol to pregnenolone, is sensitive to dantrolene. These data do not deny that dantrolene blocks AII-stimulated, inositol trisphosphate calcium mobilization. However, caution is needed in interpreting experiments in which steroid measurements are used as an endpoint when dantrolene is used specifically to study signal transduction in steroidogenic tissues.

REFERENCES

1. Ward A., Chaffman M. O. and Sorkin E. M.: Dantrolene: a review. *Drugs* **32** (1986) 130–168.
2. Harrison G. G.: Dantrolene: dynamics and kinetics. *Br. J. Anaesth.* **60** (1988) 279–286.
3. Tidyman M., Prichard J. G., Deamer R. L. and Mac N.: Adjunctive use of dantrolene in severe tetanus. *Anesth. Analg.* **64** (1985) 538–540.
4. Danko S., Kim D. H., Streter F. A. and Ikemoto N.: Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. The effects of dantrolene on Ca^{2+} release induced by caffeine, Ca^{2+} and depolarisation. *Biochim. Biophys. Acta* **816** (1985) 18–24.
5. Oba T. and Hotta K.: Similar inhibitory effects of dantrolene sodium on twitch tension and on silver ion-induced contracture in skeletal muscle. *Eur. J. Pharmac.* **143** (1987) 275–278.
6. Durant N. N., Lee C. and Katz R. L.: The action of dantrolene on transmitter mobilization at the rat neuromuscular junction. *Eur. J. Pharmac.* **68** (1980) 403–408.
7. Lihrmann I. H., Delarue C., Homo-Delarche F., Feuilloley M., Belanger A. and Vaudry H.: Effects of TMB-8 and dantrolene on ACTH and AII induced steroidogenesis by frog interrenal gland; evidence for a role of intracellular calcium in angiotensin action. *Cell Calc.* **8** (1987) 269–282.
8. Mine T., Kojima I., Kimura S. and Ogata E.: Assessment of the role of calcium mobilization from intracellular pool(s), using dantrolene, in the glycogenolytic action of α-adrenergic stimulation in perfused rat liver. *Biochim. Biophys. Acta* **927** (1987) 229–234.
9. Kojima K., Kojima I. and Rasmussen H.: Dihydropyridine calcium agonist and antagonist effects on aldosterone secretion. *Am. J. Physiol.* **247** (1984) E645–E650.
10. Utili R., Boitnott J. K. and Zimmermann H. J.: Dantrolene-associated hepatic injury. *Gastroenterology* **72** (1977) 610–616.
11. Francis K. T. and Hamrick M. E.: Dantrolene inhibition of the hepatic mixed function oxidase system. *Res. Commun. Chem. Path. Pharmac.* **23** (1979) 69–80.
12. Roy S., Francis K. T., Born C. K. and Hamrick M. E.: Interaction of dantrolene with the hepatic mixed function oxidase system. *Res. Commun. Chem. Path. Pharmac.* **27** (1980) 507–520.
13. Francis K. T. and Hamrick M. E.: Effects of dantrolene on adrenal cortical function. *Biochem. Pharmac.* **29** (1980) 1669–1672.
14. Ogishima T., Mitani F. and Ishimura Y.: Isolation of aldosterone synthase cytochrome *P*450 from zona glomerulosa mitochondria of rat adrenal cortex. *J. Biol. Chem.* **264** (1989) 10935–10938.
15. Simpson H. D., Shepherd R. M., Shepherd J., Fraser R., Lever A. F. and Kenyon C. J.: Effects of cholesterol and lipoproteins on aldosterone secretion by zona glomerulosa cells. *J. Endocr.* **121** (1989) 125–131.
16. Shepherd R. M.: The role of potassium ions in angiotensin II stimulated aldosterone secretion. Ph.D. Thesis, Univ. of Glasgow (1989).
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
18. Holloway C. D., Kenyon C. J., Dowie L. J., Corrie J. E. T., Gray C. E. and Fraser R.: Effect of the benzodiazepines diazepam, des-*n*-methyldiazepam and midazolam on corticosteroid biosynthesis in bovine adrenocortical cells *in vitro*; location of site of action. *J. Steroid Biochem.* **33** (1989) 219–225.
19. Davies E., Kenyon C. J. and Fraser R.: The role of calcium ions in the mechanism of ACTH stimulation of cortisol synthesis. *Steroids* **45** (1985) 551–560.
20. Newman D.: The distribution of range in samples from a normal population expressed in terms of an independant estimate of standard deviation. *Biometrika* **31** (1939) 20–30.
21. Kojima I., Kojima K. and Rasmussen H.: Effects of Ang II and K^+ on Ca efflux and aldosterone production in adrenal glomerulosa cells. *Am. J. Physiol.* **248** (1985) E36–E43.
22. Joseph S. K. and Williamson J. R.: Inositol trisphosphate and calcium release. *J. Biol. Chem.* **261** (1986) 14658–14664.
23. Sumikawa K., Hayashi Y., Fumitsu K. and Yoshigi I.: Selective inhibition by dantrolene of caffeine-induced catecholamine release from perfused dog adrenals. *Res. Commun. Chem. Path. Pharmac.* **57** (1987) 45–53.
24. Kojima I., Kojima K. and Rasmussen H.: Role of calcium fluxes in the sustained phase of angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells. *J. Biol. Chem.* **260** (1985) 9177–9184.
25. Kojima I., Kojima K., Kreutter K. and Rasmussen H.: The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. *J. Biol. Chem.* **259** (1984) 14448–14457.
26. Bird I. M., Walker S. W. and Williams B. C.: Agonist stimulation of phosphoinositide metabolism in the adrenal cortex and its relationship to steroidogenesis. *J. Molec. Endocr.* **5** (1990) 191–209.
27. Vallner J. J., Honibger I. L., Stewart J. T. and Peyton A. B.: Dantrolene and metabolite levels in 6 patients on chronic therapy. *Curr. Ther. Res.* **25** (1979) 79–91.
28. Schiebinger R. J., Braley L. M., Menachery A. M. and Williams G. H.: Unique calcium dependencies of the activating mechanism of the early and late aldosterone biosynthetic pathways in the rat. *J. Endocr.* **110** (1986) 315–325.
29. Simpson E. R. and Waters J.: Effect of calcium on pregnenolone formation and cytochrome-*P*450 in rat adrenal mitochondria. *J. Steroid Biochem.* **6** (1975) 395–400.
30. Loose D. S., Ken P. B., Hirst M. A., Marcus R. A. and Feldman D.: Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome *P*450-dependent enzymes. *J. Clin. Invest.* **71** (1983) 1495–1499.
31. Lambert A., Mitchell R. and Robertson W. R.: Biopotency and site of action of drugs affecting testicular steroidogenesis. *J. Endocr.* **113** (1987) 457–461.