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Regulation of Adrenocortical Steroidogenesis by Benzodiazepines

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Benzodiazepines affect steroidogenesis in at least four ways depending on concentration and adrenocortical cell type. Firstly, at micromolar concentrations, they inhibit steroidogenic enzymes. Competition for microsomal 17- and 21-hydroxylase activity explains the inhibition of ACTH-stimulated aldosterone and cortisol synthesis by diazepam and midazolam. At slightly higher concentrations, we have evidence that 11β-hydroxylase activity is also inhibited. Secondly, at sub-micromolar concentrations, calcium influx is inhibited. T-type and L-type calcium channels appear to be blocked, this impairs signal response coupling and, in particular, decreases angiotensin- and K^+-stimulated aldosterone synthesis in zona glomerulosa cells. Thirdly, the mitochondrion of steroidogenic tissues is a sensitive site for the stimulatory effects of benzodiazepines. Aldosterone synthesis from added HDL-cholesterol by cultured bovine zona glomerulosa cells is stimulated by diazepam, RO5-4864 and PK11195. The fourth site of benzodiazepine's effect on steroidogenesis is particular to zona glomerulosa cells. In addition to cholesterol side chain cleavage, the final part of the aldosterone biosynthetic pathway, the conversion from deoxycorticosterone is controlled. Although high micromolar concentrations of diazepam appear to be inhibitory, lower nanomolar concentrations stimulate the synthesis of aldosterone from added deoxycorticosterone. *In vivo*, a fifth site of benzodiazepine activity may influence plasma steroid concentrations. Competition between steroids and benzodiazepines for hepatic clearance enzymes may affect half lives of both drugs and hormones.

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INTRODUCTION

The peripheral benzodiazepine receptor is pharmacologically distinct from receptors in the central nervous system. Central receptors are associated with GABA-gated chloride channels whereas peripheral receptors are linked to various activities including alterations of immune function, cell proliferation and cardiac action potential. Notably, high densities of peripheral receptors have been found in steroidogenic tissue [1]. Within adrenocortical cells these peripheral receptor sites are found in the outer mitochondrial membrane [2] and also the plasma membrane [3]. The mitochondrial receptor in steroidogenic tissues appears to regulate the translocation of cholesterol from the outer to the inner membrane [4]. Thus a variety of peripheral receptor ligands have been shown to stimulate steroidogenesis in isolated mitochondria and whole cells from various tissues including [5–8] the adrenal cortex.

Paradoxically, the effects of benzodiazepines on aldosterone synthesis have invariably been shown to be inhibitory [9–13]. We have investigated this paradox more closely by considering the following possibilities.

(i) Cholesterol stores in zona glomerulosa cells are significantly less than in zona fasciculata/reticularis cells [14]. This may limit the stimulatory effects of benzodiazepines.

(ii) ACTH-stimulated cortisol synthesis by zona fasciculata/reticularis cells is blocked by diazepam because of inhibition of 17- and 21-hydroxylase enzyme activities [10]. Enzymes specific to the aldosterone biosynthetic pathway may be particularly sensitive to benzodiazepines. The dose related effects of benzodiazepines on the key final steps in the aldosterone biosynthetic pathway are not known.

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(iii) Plasma membrane calcium and potassium ion movements are perhaps more important in the regulation of aldosterone than cortisol synthesis. One might expect that a peripheral effect of benzodiazepines to block calcium currents in adrenocortical cells, similar to that described in cardiac cells [15], would affect stimulated aldosterone synthesis.

MATERIALS AND METHODS

Cell preparation

Cells from the zona fasciculata/reticularis and zona glomerulosa regions of bovine adrenal glands were isolated as previously described [10, 16]. Experiments to test the effects of benzodiazepine on 21-hydroxylase activity and calcium uptake were carried out with freshly isolated cells. The possible stimulatory effects of benzodiazepines were investigated in zoma glomerulosa cells cultured for 6 days in 96-well plates.

21-hydroxylase activity

To avoid possible effects of benzodiazepines on cholesterol side chain cleavage and 11β-hydroxylase activity, 11β-hydroxyprogesterone (2.5 μM; Sigma Chemical Co., Dorset, U.K.) was added to cells as substrate for 21-hydroxylation. Cells were incubated with various concentrations of Midazolam (Roche Products, Welwyn Garden City, U.K.) for 1 h at 37°C in Hams' F-12 medium containing 0.2% glucose and BSA and 2.5 mM $CaCl_2$. For K^+-stimulated aldosterone synthesis, the potassium concentration in medium was increased from 3.8 to 11 mM.

^{45}Ca uptake. Cell suspensions were preincubated at 37°C for 30 min in Krebs'–bicarbonate–Ringers containing glucose, albumin and 1 mM $CaCl_2$, according to the method of Mauger *et al.* [17]. The incubation was continued for a further 30 min with added midazolam or vehicle. Then, following the addition of ^{45}Ca (S.A. of incubation was 170 MBq/mmol) aliquots of suspension were sampled. Cells were harvested, with washing, on to GF/C filters. Radioactivity associated with cells was counted in a liquid scintillation counter. For cells stimulated with K^+ (final concentration 10.8 nM), Ringers containing additional potassium was added together with ^{45}Ca.

Cell culture. 96-well tissue culture plates were seeded with zona glomerulosa cells at a density of 20,000 cells/well. After 6 day culture in a modified Hams' F-12 medium [18] spent medium was replaced with unmodified Hams' F-12 containing 2.5 mM Ca^{2+} and 0.2% BSA. The effects of diazepam were investigated in several experiments: (i) basally; (ii) with Ang II, ACTH and K^+ stimulation; (iii) with human HDL-cholesterol (prepared as described previously [19]); (iv) with deoxycorticosterone. The effects of PK11195 and RO5-4864 at various concentrations were also tested.

RESULTS

21-hydroxylase

Figure 1 shows the dose-related inhibitory effects of midazolam on the conversion of 11β-hydroxyprogesterone to aldosterone. The K_i value, 54 μM, was similar to that for the conversion of 21-deoxycortisol to cortisol (35 μM) in fasciculata/reticularis cells (data not shown).

Inhibition of K^+-stimulated aldosterone synthesis and ^{45}Ca uptake

Midazolam and diazepam inhibited K^+-stimulated aldosterone synthesis with IC_{50} values of approx. 1 μM (data not shown). Midazolam but not diazepam completely blocked the K^+-response.

Midazolam appeared to be more effective at blocking ^{45}Ca uptake into zona glomerulosa cells than zona fasciculata/reticularis cells. For example 0.85 μM midazolam reduced uptake in glomerulosa cells by 85% ($P < 0.002$) whereas the same concentration had no significant effect in fasciculata cells. Figure 2 compares the effects of midazolam on basal and K^+-stimulated ^{45}Ca uptake. Although both were reduced, the effects of K^+ were not completely abolished by midazolam.

Stimulatory effects of benzodiazepines

Basal aldosterone synthesis was reduced to very low levels after 6 days in culture. These low levels were increased 2–3-fold by concentrations of diazepam ranging from 1 nM to 10 μM, a modest increase compared with that achieved by maximal stimulation with Ang II (40–50-fold). The effect of diazepam on basal synthesis was apparent whether cells were pre-exposed to diazepam or when cells were treated with diazepam only during the 3 h when measuring steroidogenesis.

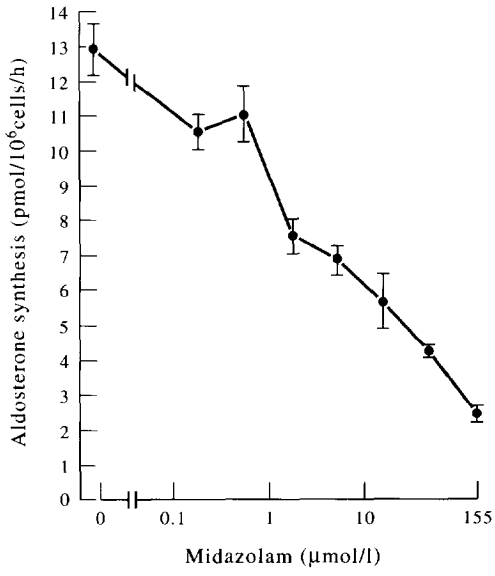

Fig. 1. Concentration-dependent effects of midazolam on aldosterone synthesis from added 11β-hydroxyprogesterone (2.5 μmol/l) in freshly isolated bovine zona glomerulosa cells. Mean ± SE, $n = 6$. From [12].

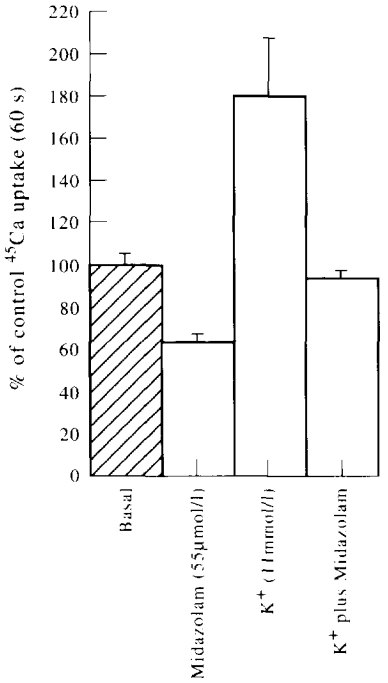

Fig. 2. Effect of midazolam on initial rates of ^{45}Ca uptake in glomerulosa cells under basal conditions (3.8 mM K^+) and when stimulated with 11 mM K^+. Means ± SE, $n = 6$. From [12].

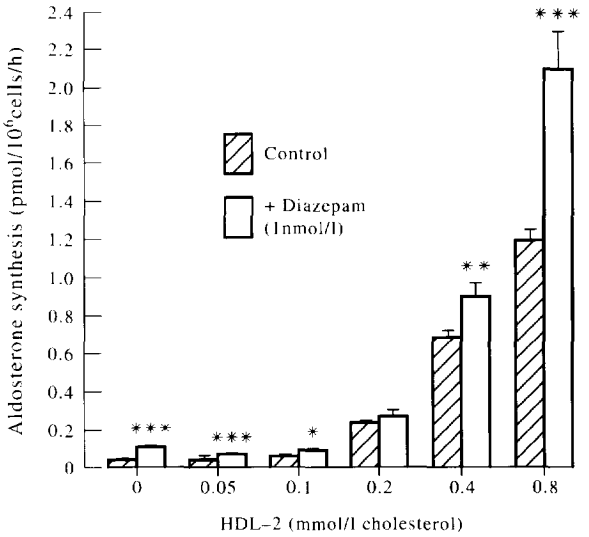

Fig. 4. Effects of diazepam (1 nM) on aldosterone synthesis in cultured bovine zona glomerulosa cells incubated with various concentrations of HDL-2. Values shown are means ± SE, $n = 8$. Significant differences (unpaired t-test) compared with controls are indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 3 compares the effects of diazepam and midazolam on cells stimulated with Ang II, ACTH and K^+. Note that diazepam is more effective than midazolam at enhancing Ang II and ACTH-stimulated aldosterone synthesis but that both drugs inhibit K^+-stimulated aldosterone synthesis.

Figure 4 shows that diazepam further enhances the effects of HDL-2 cholesterol. Similar effects were seen in cells treated with HDL-3. High concentrations of diazepam (0.1 mM) were inhibitory ($P < 0.001$). RO5-4864 and PK11195 (1 nM–0.1 mM) also enhanced aldosterone synthesis 2–5-fold ($P < 0.001$) in glomerulosa cells treated with HDL-2 or HDL-3 cholesterol.

Final steps of aldosterone synthesis

Diazepam increased the conversion of added deoxycorticosterone to aldosterone (Fig. 5). The effect was less than that achieved in the presence of HDL-cholesterol and was not clearly concentration dependent. A maximum 60% increase was observed with 10 μM diazepam ($P < 0.001$). RO5-4864, but not PK11195,

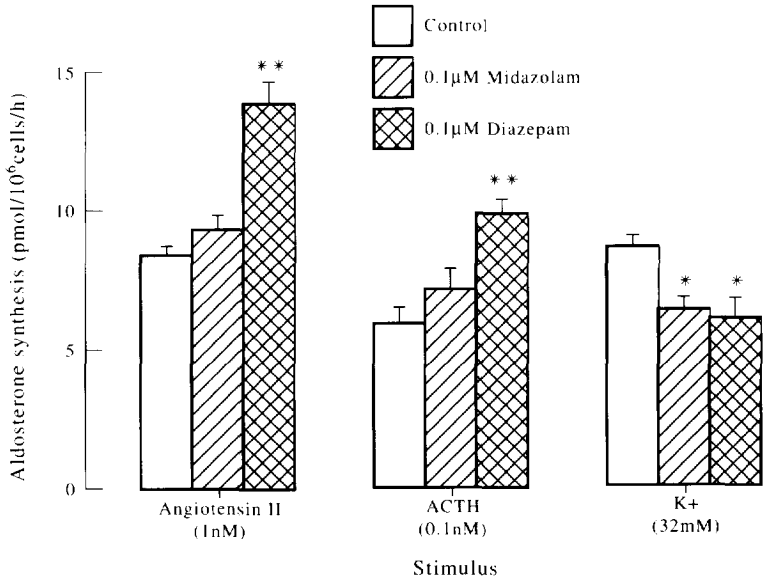

Fig. 3. Effects of midazolam and diazepam on stimulated aldosterone synthesis by cultured glomerulosa cells. Basal synthesis < 0.5 pmol/10^6 cells/h. Values shown are means ± SE, $n = 8$. $^*P < 0.05$, $^{**}P < 0.01$ compared with controls.

also increased deoxycorticosterone to aldosterone conversion. Again the effect was modest (95% increase with 0.1 μM RO5-4864). Diazepam, PK11195 and RO5-4864 at 0.1 mM all inhibited aldosterone synthesis from added deoxycorticosterone.

DISCUSSION

We have established three or four sites where benzodiazepines influence adrenocortical steroidogenesis. Microsomal hydroxylation reactions are inhibited by diazepam and midazolam [10, 12]. With K_i values in the micromolar range, it seems unlikely that this site is an important therapeutic consideration. Nevertheless we have not conducted an exhaustive survey of all benzodiazepine products. Differences between the relative potencies of diazepam and midazolam as inhibitors of 17- and 21-hydroxylase are indicative of a range of biological activity. There may be other drugs which are more potent inhibitors.

A second adrenocortical site for benzodiazepine actions appears to be linked to a plasma membrane peripheral-type receptor. As in other tissues such as the heart [15], calcium fluxes are inhibited in adrenocortical cells by diazepam and midazolam [12]. Our observations have been confirmed by more elegant electrophysiological studies [13]. T- and L-type calcium channels are blocked by a variety of benzodiazepines. From the hierarchy of these channel blocking effects, it would seem that peripheral-type receptors are involved. Receptors have been identified in the plasma membrane as well as on mitochondria of steroidogenic tissues [2, 3]. The movement of Ca^{2+} into zona glomerulosa cells is particularly important in the control of aldosterone synthesis. More than any other steroidogenic type, the zona glomerulosa cell is sensitive to small changes in potassium ion concentration which cause depolarization of the cell membrane and the opening of calcium channels which triggers steroidogenesis. Consequently, inhibitory effects of benzodiazepines reflect their calcium channel activity. Sub-micromolar concentrations of midazolam and diazepam inhibit calcium uptake and steroidogenesis in glomerulosa cells. Calcium uptake by fasciculata and reticularis cells is less sensitive. Inhibition of ACTH-stimulated cortisol synthesis requires higher concentrations of midazolam and probably reflects direct 21-hydroxylase competition rather than ion channel activity. The greatest stimulatory effects of benzodiazepines have generally been observed in isolated mitochondria [4, 6, 7] or cell lines where 21- and/or 17-hydroxylase do not feature in the steroidogenic pathway [5, 8]. In these circumstances, a clear stimulatory effect involving the facilitation of mitochondrial cholesterol transport via mitochondrial peripheral type-receptors has been demonstrated. Despite the involvement of a benzodiazepine-sensitive 21-hydroxylase step in the biosynthetic pathway, we too observed a small increase in aldosterone output with diazepam, RO5-4864 and PK11195. This effect was seen only with nanomolar concentrations of benzodiazepines (10–100-fold less than required to inhibit microsomal enzymes or calcium influx) and only in cells cultured for 6 days when basal output but not maximal steroidogenic capacity was much reduced compared with freshly isolated cells.

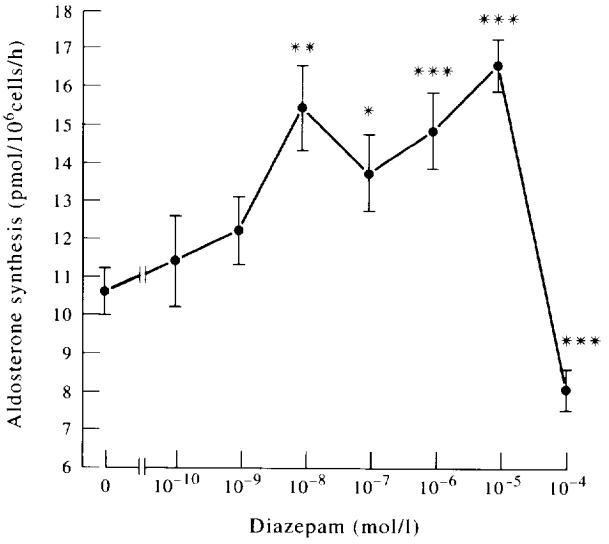

Fig. 5. Concentration dependent effects of diazepam on aldosterone synthesis from added deoxycorticosterone (5 μM) by cultured bovine zona glomerulosa cells. Values shown are means ± SE, $n = 8$. Significant differences (unpaired t-test) compared with controls are indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In a previous study, we speculated that cholesterol supply may be rate limiting for glomerulosa cell steroidogenesis [19]. Using freshly isolated cells we showed that HDL-cholesterol provides a better substrate for Ang II- or ACTH-stimulated aldosterone synthesis than LDL-cholesterol or cholesterol; the K^+ response was less affected. Given that the peripheral receptor has been linked to cholesterol transport across the mitochondrial membrane, we considered that the relatively small cholesterol stores within zona glomerulosa cells might govern responsiveness to benzodiazepines and that provision of cholesterol might enhance the effects of diazepam. HDL-cholesterol alone increased basal and Ang II-stimulated aldosterone synthesis. Diazepam in the presence of HDL-cholesterol also further increases aldosterone synthesis. Proportionally this effect was no greater than in the absence of HDL-cholesterol.

Diazepam, but not midazolam, further enhanced the stimulatory effect of Ang II and ACTH. The inhibitory effects of diazepam and midazolam were again paramount in K^+-stimulated cells. Assuming that the plasma membrane and mitochondrial peripheral-type receptors are separate entities, these data suggest firstly that the overall potency of benzodiazepines may be stimulatory or inhibitory depending on whether

mitochondrial or plasma membrane receptors are involved. Secondly, that the steroidogenic influence of benzodiazepines at each site is influenced by separate factors: the availability of cholesterol for the mitochondrial receptor; whether ion channels are active for the plasma membrane receptor. Thirdly, the activity of different benzodiazepines at each site may vary,

In common with other steroid hormones, the biosynthetic pathway of aldosterone is controlled by cholesterol side chain cleavage. Uniquely, the final mitochondrial conversion of deoxycorticosterone to aldosterone is also controlled and this may offer a further site for the actions of benzodiazepines [20]. In a previous study [10], using a high concentration of diazepam, we demonstrated inhibition of the conversion of corticosterone to aldosterone. This was confirmed in the present studies, but lower submicromolar concentrations of diazepam were stimulatory. Further studies are needed to show whether the mitochondrial receptor regulates deoxycorticosterone as well as cholesterol transport and whether, in addition, benzodiazepines are competitive inhibitors of 11β-hydroxylase enzymes which convert deoxycorticosterone to aldosterone. Again concentration dependent differences in potency between diazepam, RO5-4864 and PK11195 indicate that stimulatory and inhibitory processes are independently controlled.

In conclusion, high concentrations of benzodiazepines inhibit steroidogenesis by competing with endogenous substrates for microsomal and mitochondrial steroidogenic enzymes and by antagonizing the trophic effects of other stimuli whose actions depend on voltage sensitive calcium channels. Low concentrations of benzodiazepines acting via mitochondrial receptors are stimulatory. However, stimulatory effects on aldosterone synthesis may be difficult to demonstrate because zona glomerulosa cells are especially sensitive to the calcium blocking effects of benzodiazepines and perhaps because cholesterol supply is limited. The stimulatory effects of benzodiazepines on deoxycorticosterone to aldosterone conversion suggest that mitochondrial transport of steroids other than cholesterol may be important in regulating steroidogenesis.

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