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European Journal of Pharmacology 346 (1998) 71–76

ejp

Effects of atipamezole, detomidine and medetomidine on release of steroid hormones by porcine adrenocortical cells in vitro

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Received 5 June 1997; revised 9 January 1998; accepted 13 January 1998

Abstract

The 4-substituted imidazole type α_2-adrenoceptor ligands atipamezole, detomidine, and medetomidine were screened for actions on the release of aldosterone by a suspension of porcine adrenocortical cells with deoxycorticosterone (1 μM) as substrate. Progesterone, pregnenolone or corticosterone (all at 1 μM) were also used as substrates. With pregnenolone as substrate, drug-induced effects on the output of nine steroids (aldosterone, corticosterone, cortisol, deoxycortisol, testosterone, progesterone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone) were monitored simultaneously. The α_2-adrenoceptor antagonist atipamezole was a potent inhibitor of aldosterone release (range 10–1000 nM). The sedative α_2-adrenoceptor agonists medetomidine and detomidine also inhibited aldosterone release (range 10–1000 nM). With pregnenolone as substrate, the inhibition induced by 4-substituted imidazoles of the release of corticosterone and cortisol was more pronounced than that of aldosterone. Androstenedione and deoxycortisol release was enhanced. The 4-substituted imidazoles atipamezole, detomidine, and medetomidine inhibited mitochondrial cytochrome $P450_{11\beta/18}$ in vitro. This inhibition was unrelated to their α_2-adrenoceptor actions. The 4-substituted imidazole type α_2-adrenoceptor ligands used to control sedation/anaesthesia can alter the steroid-based defence mechanisms of the body. © 1998 Elsevier Science B.V.

Keywords: Aldosterone; Corticosterone; Cortisol; Deoxycortisol; Androstenedione; Steroid biogenesis; α-Adrenoceptor ligand; Imidazole

1. Introduction

The α_2-adrenoceptor agonists detomidine and medetomidine are recent additions to the pharmacotherapeutic arsenal as sedative-anaesthetic drugs in (veterinary) surgery (Virtainen, 1989). Atipamezole, an α_2-adrenoceptor antagonist from the same chemical group (4-substituted imidazoles), has been introduced as antidote to end α_2-adrenoceptor agonist-induced anaesthesia. Several drugs with an imidazole structure interfere with steroid biogenesis (Feldman, 1986). The 1-substituted imidazoles like the hypnotic etomidate (DeCoster et al., 1987) and the antimycotic ketoconazole especially (Nagai et al., 1986; Cough et al., 1987; Lamberts et al., 1987) are potent modulators of the biogenesis of steroid hormones in porcine adrenocortical cells in vitro (Jager et al., 1996).

This study was undertaken to screen these new drugs for effects on steroid biogenesis in vitro. Incubation of porcine adrenocortical cell suspensions with drugs for 1 h can identify acute alterations in the release of steroids. Four major intermediate products in the biosynthesis of aldosterone from cholesterol were used as substrates. This enables the localization of drug-induced interference above or below that intermediate step. In addition, measurement of eight other steroids released by the cell suspension when an early intermediate, pregnenolone, is used as substrate makes it possible to study whether the biosynthesis of other adrenocorticosteroids and adrenal androgens is affected. Compounds acting at steps in steroid biogenesis before pregnenolone, like ACTH (adrenocorticotropic hormone) or serotonin, are without effect in the substrate-driven system (Jager et al., 1994). Without substrate, however, steroid release is very low: just above or below detection levels (Jager et al., 1994). In the experimental set-up used, the release of aldosterone was used as 'reference' to select substrate and cell concentrations that enabled the detection of both increases as well as decreases in the release of corticosteroids (Jager et al., 1994, 1996);

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however, only increases can be observed in the release of testosterone and dehydroepiandrosterone (Jager et al., 1996). The observed drug-induced alterations in the release of steroids by the porcine adrenocortical cell suspension are consistent with the standard scheme for mammalian pathways of biosynthesis of adrenocorticosteroids and adrenal androgens (Bonneterre and Lefebvre, 1985; White et al., 1994; Jager et al., 1996).

A preliminary report of the present results has been published elsewhere (Jager and De Graaf, 1994).

2. Materials and methods

2.1. Preparation of adrenocortical cell suspension

Adrenals of pigs (live weight 90–100 kg) were removed at a slaughterhouse. Immediately after the abdomens were opened, the adrenals were removed and stored in ice-cold saline solution (0.9% NaCl). Within 2 h, the surrounding tissue, capsule and medulla of the adrenals were removed and the remaining tissue was minced. Minced cortical tissue suspended in Krebs solution (0.2 g/ml) was treated with collagenase: 10 mg collagenase/g tissue at 37°C for 45 min in a shaking water-bath. Depending on the size of the experiment, cells from up to 50 adrenals were pooled. After being passed through a 100-μm sieve, the cells were washed twice with Krebs solution, as follows. The cell suspension was centrifuged ($100 \times g$) for 10 min, the supernatant was decanted and the cells in the pellet were resuspended in fresh medium. Cells were kept suspended overnight in RPMI solution (about 10^6 cells/ml) at 4°C. The next morning the cells were transferred to fresh RPMI solution. The cell suspension was usually prepared in a day and the experiment was started the next morning. The release of aldosterone by the cell suspension and the number of viable cells decreased only slightly during overnight storage (about 10%). Longer storage resulted in an unacceptable loss of cells and activity.

The survival of the cells was checked by measuring the lactate dehydrogenase activity (Bergmeyer et al., 1965) of cells and supernatant before and after incubation with test compounds (Legrand et al., 1992). The survival rate was determined as the lactate dehydrogenase activity of the cells after incubation relative to that obtained before incubation; this rate was generally more than 95%. Lactate dehydrogenase activity in the medium after incubation was below the detection level, and therefore less than 0.1% of the lactate dehydrogenase activity of the cells.

2.2. Solutions and drugs used

The Krebs solution used contained (mM): NaCl (118); KCl (5.4); $NaHPO_4$ (1.6); $NaHCO_3$ (21.9); $CaCl_2$ (2.5); $MgSO_4$ (1.2); glucose (11.1). The solution was equilibrated with carbogen (5% CO_2 and 95% O_2) and the pH was 7.4 at 37 ± 0.5°C. The RPMI solution was prepared according to the instructions from the supplier (#041-02409, Gibco). The pH was 7.4 at 37 ± 0.5°C. Visible colour changes of the pH-indicator occurred at pH = 8.0 (purple) and at pH = 6.5 (yellow). The buffer capacity under these experimental circumstances was 8.3 for OH^--ions and 5.6 for H^+-ions.

The drugs used were: atipamezole (–HCl; Farmos), collagenase (type 1A; Sigma 9891), corticosterone (Sigma 2505), deoxycorticosterone (11-deoxycorticosterone; Sigma 6875), detomidine (–HCl; Farmos), DMSO (Merck), medetomidine (–HCl; Farmos), pregnenolone (Sigma 9129), progesterone (Sigma 0130). All drugs were routinely dissolved in dimethyl sulfoxide (DMSO) before they were added to the incubation medium. The DMSO in the concentration used (176 mM) did not affect the release of the steroids measured. After the drugs were added, the pH of the solution was adjusted to 7.4, when necessary.

2.3. Incubations

Before each experiment cell viability was determined by trypan blue exclusion. The final suspension of the cell pellet was based on the viability results. Throughout the experiments, cell suspensions of 1×10^6 cells/ml were used (cell count error: $\leq 10^4$ cells/ml). All incubations were done at 37 ± 0.5°C in a shaking water-bath. RPMI solution with 1 μM substrate was used to preincubate the cell suspension for 30 min. Usually incubations of 1 h were used with fresh RPMI solution and substrate. Dose-response studies were carried out with 2-ml samples of the adrenocortical cell suspension with substrate and vehicle or with substrate, vehicle and test compound. Thus, besides the controls needed for the calibration of the radioimmunoassays, every treatment vial had its own control (vehicle only) vial. Incubation was stopped by immersing the vials in boiling water for 10 min. The vials were then centrifuged, and the supernatant was stored at -20°C until analysis by radioimmunoassay. All steroids measured were stable during boiling and storage at -20°C up to 1 year.

2.4. Steroid assays and data analysis

Steroid hormones released by the cell suspension into the medium were measured directly in 25- or 50-μl samples of the thawed supernatant by specific radioimmunoassay. Exploratory measurements were made to ascertain the dilution (often eight or four times, with RPMI solution without substrate) needed to bring the final concentrations within the range of the radioimmunoassay (Jager et al., 1996). Results are expressed as nanogram steroid released by 10^6 cells/h. Aldosterone, cortisol, corticosterone, testosterone, estrone, estradiol (estradiol-17β), progesterone, 17α-hydroxyprogesterone, androstenedione and dehy-

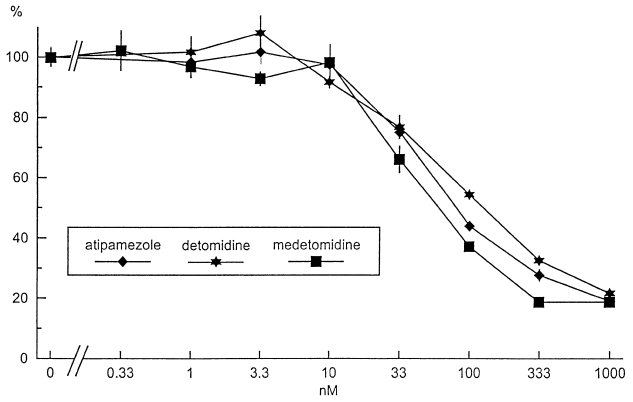

Fig. 1. Effects of the α_2-adrenoceptor ligands on aldosterone release from the adrenocortical cell suspension with 1 μM deoxycorticosterone as substrate. 100% = 2.3 ± 0.1; 2.0 ± 0.1 and 2.5 ± 0.1 ng aldosterone/10^6 cells/h for atipamezole, detomidine and medetomidine, respectively (n = 4).

droepiandrosterone were measured with radioimmunoassay kits from D.P.C., and deoxycortisol with radioimmunoassay kits from ICN Biomedicals. The cross-reaction of substrates and test compounds was compensated for by subtracting the apparent steroid production. The apparent release was constant and due to the dilution almost negligible. For example, the cross-reactivity of 1 μM pregnenolone, progesterone, deoxycorticosterone, or corticosterone in the aldosterone assay was computed as 50, 150, 100, or 50 pg/ml aldosterone before dilution, respectively.

Correction for the cross-reactivity of the medium before incubation might seem overdone as more than 2/3 of the substrate is converted during incubation. The steroids released during incubation, however, will also cross-react with the RIA used. An example can be found in the controls of Table 2. The RIA readings for aldosterone were corrected for medium with vehicle and 1 μM pregnenolone as substrate (± 50 pg/ml). Based on observations of the cross-reactivities of several steroids (1 μM) under our experimental conditions, the added, total cross-reactivity of the measured concentrations of steroids could be estimated as ± 53 pg/ml aldosterone. Calculations with the specificity data given by the supplier of the aldosterone RIA kits resulted in an estimated cross-reactivity of ± 48 pg/ml in the control situation. Thus, for the control situation our simple correction for cross-reactivity seems fairly adequate. In the medium after the control incubations, the summed cross-reactivity for the other released steroids was calculated as 2.8, 0.2, 6.6, 1.8, 10.2 and 5.0% of the reported release for the RIA of aldosterone, corticosterone, cortisol, deoxycortisol, progesterone, 17α-hydroxyprogesterone, and androstenedione, respectively. During experimental incubations, however, the release of steroids can change significantly, increasing or decreasing the contributions of the individual steroids to the total cross-reactivity. Assuming that an experimental treatment causes all pregnenolone to be converted to progesterone only, the RIA of aldosterone will yield a value that is 6% of the control value. This is a significant decrease in aldosterone release, albeit that the small amount of aldosterone detected, is a false-positive observation. Similar worst case scenarios for the other steroids measured with RIA gave comparable results. It seems that with so many 'players' even extreme changes (conversion of one steroid substrate into many steroids or comparison of many steroids with a single product steroid) are evened out.

All data are given as means ± standard error of the mean (S.E.M.). In figures, S.E.M. is presented only if it exceeds the size of the symbol. Differences between the release of steroids by control and drug-treated cell suspensions were evaluated with the *t*-test. As every treatment vial had its own concurrent control vial, all treatment vials were compared with their own controls. Differences were assumed to be real when tests gave probability levels less than 5%; n indicates the number of independent observations. In the tables and figures, the effect of drugs on steroid release by cell suspensions is expressed as a percentage of the steroid release measured after treatment to

Table 1
Effect of the α_2-adrenoceptor ligands on the aldosterone released[a] by cell suspensions incubated with different substrates

Treatment	Corticosterone (%)	Deoxycorticosterone (%)	Progesterone (%)	Pregnenolone (%)
Control (ng/10^6cells per h)[c]	0.97 ± 0.10	2.32 ± 0.24	1.72 ± 0.20	1.77 ± 0.22
Atipamezole				
0.03 μM	45 ± 4[b]	69 ± 3[b]	80 ± 3[b]	78 ± 2[b]
0.1 μM	36 ± 1[b]	41 ± 5[b]	55 ± 2[b]	54 ± 3[b]
Detomidine				
0.1 μM	46 ± 3[b]	52 ± 2[b]	57 ± 3[b]	57 ± 3[b]
0.3 μM	38 ± 3[b]	33 ± 1[b]	41 ± 2[b]	39 ± 1[b]
Medetomidine				
0.03 μM	39 ± 1[b]	46 ± 2[b]	53 ± 2[b]	52 ± 2[b]
0.1 μM	32 ± 1[b]	30 ± 1[b]	39 ± 1[b]	35 ± 2[b]

All substrates were used in a concentration of 1 μM, and also used during preincubation.
[a]Release during treatment is expressed as a percentage (mean ± S.E.M.; n = 5) of the control release (1 h incubation with 1 μM substrate and vehiculum).
[b]Significantly different from 100% ($P < 0.05$; *t*-test).
[c]To reduce the number of rows all controls were combined.

Table 2
Effect of the α_2-adrenoceptor ligands on the release of different steroids[a] by the adrenal cell suspensions[b]

Treatment	Aldosterone (%)	Corticosterone (%)	Cortisol (%)	Deoxycortisol (%)	Progesterone (%)	HO-Prog[d] (%)	Androstenedione (%)
Control (ng/10^6 cells per h)[e]	1.77 ± 0.22	116 ± 4	26 ± 1	9.4 ± 0.3	41 ± 4	17.0 ± 0.4	2.0 ± 0.1
Atipamezole (0.03 μM)	78 ± 2[c]	55 ± 2[c]	48 ± 2[c]	115 ± 13	103 ± 4	98 ± 16	133 ± 6[c]
Detomidine (0.1 μM)	57 ± 3[c]	41 ± 2[c]	32 ± 2[c]	132 ± 5[c]	108 ± 3	102 ± 18	147 ± 2[c]
Medetomidine (0.03 μM)	52 ± 2[c]	33 ± 2[c]	39 ± 3[c]	134 ± 16	104 ± 5	112 ± 25	162 ± 13[c]

[a]The release of dehydroepiandrosterone, testosterone, estrone and estradiol remained below detection limits.
[b]Release during treatment is expressed as a percentage (mean ± S.E.M.; $n = 5$) of the control release (1 h incubation with 1 μM pregnenolone and vehicle).
[c]Significantly different from 100% ($P < 0.05$; *t*-test).
[d]17α-hydroxyprogesterone.
[e]To reduce the number of rows all controls were combined.

that after concurrent vehicle treatment. To reduce the number of rows in the tables all controls were combined for presentation only.

3. Results

3.1. Effects on aldosterone release

In our protocol drugs were first tested in the concentration range 0.3–100 μM for actions on aldosterone release with deoxycorticosterone as substrate (Fig. 1). The sedative acting α_2-adrenoceptor agonists detomidine and medetomidine were effective inhibitors of aldosterone release as was their antidote atipamezole. Potency differences between these compounds were less than five-fold. The following potency order was discerned: medetomidine ≥ atipamezole ≥ detomidine. A set of adrenoceptor ligands was tested to verify whether the inhibition of aldosterone release was mediated via adrenoceptors. In the concentration range 0.3–100 μM, adrenaline, clonidine, isoprenaline, noradrenaline, phentolamine, phenylephrine, tolazoline, and yohimbine did not alter the release of aldosterone by the adrenocortical cell suspension.

3.2. Influences of the different substrates

Using different substrates, we tried to gain some insight into the site of action of the α_2-adrenoceptor ligands. From the results summarized in Table 1, it is clear that also in the presence of other substrates, aldosterone release was inhibited by the 4-substituted imidazole type α_2-adrenoceptor agonists and antagonist. Comparison of aldosterone release with the different substrates in the presence of atipamezole, detomidine, or medetomidine did not result in clear differences, although the reduction with corticosterone as substrate was often larger. The smallest reductions were observed with progesterone as substrate.

3.3. Effects on the release of nine steroids

With pregnenolone as a substrate the effects of these three compounds on the release of nine steroids released concurrently by the cell suspension were studied at concentrations that induced a half-maximal inhibition of aldosterone release (Table 2). Although aldosterone is further down the biogenesis pathway, the release of both corticosterone and cortisol was reduced to a greater extent than that of aldosterone. In contrast, the release of deoxycortisol tended to increase, the release of progesterone and 17α-hydroxyprogesterone was unchanged, and the output of androstenedione was enhanced. The release of testosterone, dehydroepiandrosterone, estrone, and estradiol remained below detection level.

4. Discussion

Atipamezole, detomidine and medetomidine, which are noted for their rather selective actions on the α_2-adrenoceptor (Hong et al., 1994), were potent inhibitors of aldosterone release. The dose-response curves shown in Fig. 1 start at concentrations 10-fold lower than those of the most potent drugs tested in this system (Jager et al., 1994, 1996). Other more-or-less specific α-adrenergic ligands were without comparable activity. Our results imply that this inhibition is not mediated via adrenoceptors.

The actions of atipamezole, medetomidine, and detomidine were identical with small differences in potency. The similarity of the inhibition by the three compounds with all four substrates (Table 1) indicated that the inhibition was due to interference with C18-hydroxylation/oxidation, the final step in the biogenesis of aldosterone via corticosterone. The observation that the inhibition of the release of corticosterone (and cortisol) was stronger than that of the release of aldosterone (Table 2) suggests that the alternate pathway, bypassing corticosterone, is less susceptible to inhibition. As the release of deoxycortisol tended to increase, inhibition of hydroxylation at C11 has to be

assumed. It is now generally (Orth et al., 1992) assumed that one cytochrome enzyme, $P450_{11\beta,18}$ (CYP11), mediates these mitochondrial transformations (Yanagibashi et al., 1986, 1988). Different enzymes seem to exist in the zona glomerulosa and in the zona fasciculata (Lauber et al., 1987; Müller et al., 1991; White et al., 1994). Inhibition of these enzymes by the 4-substituted imidazoles seems to explain our findings. The enzyme involved in the alternate pathway seems less susceptible to the imidazoles.

The hypothesis about the presence of at least two enzymes $P450_{11\beta,18}$ in porcine adrenocortical cells, based on observations with pharmacological tools (Jager et al., 1996), seems to fit the picture constructed on the basis of clinical, biochemical, and genetic data for humans (White et al., 1994). The human enzyme coded by the *CYP11B1* gene, which has mostly C11β-hydroxylation activity, producing cortisol and corticosterone, is regulated by ACTH, and has only some C18-hydroxylation and C18-oxidation activity, might be identical with the porcine enzyme most susceptible to imidazoles. The human enzyme coded by the *CYP11B2* gene, aldosterone synthase, which synthesizes aldosterone from deoxycorticosterone without the release of intermediate steroids, might form the alternate pathway in porcine adrenocortical cells. This human enzyme is regulated, however, by angiotensin only and not by ACTH, whereas in porcine cells ACTH stimulates the release of aldosterone and cortisol, but not the release of corticosterone. This difference might be due to differences in time scale: stimulation of enzyme expression versus stimulation of enzyme activity. A high-affinity and/or speedy conversion of the *CYP11B2* enzyme for corticosterone could explain the observation that it does not release corticosterone in humans, but uses corticosterone as substrate in the porcine biosynthesis of aldosterone.

It is tempting to speculate about the involvement of the imidazole structure in the inhibition of the $P450_{11\beta/18}$ enzyme of the adrenocortical mitochondrion. The 1,5 bi-substituted imidazole hypnotic etomidate only affects $P450_{11\beta/18}$ (Jager et al., 1996). The 1-substituted imidazole antimycotic ketoconazole affects the same enzyme and also 17α-hydroxylase (Jager et al., 1996). Of the 2-substituted imidazole α_1-adrenoceptor antagonists, phentolamine and tolazoline had no effect on adrenocortical steroidogenesis. If the drugs used in the present study are representative, one might expect an interference with the functioning of cytochrome $P450_{11\beta/18}$ by 1- and 4-substituted imidazoles.

Reports on interference by the 4-substituted imidazoles of *P*450 enzymes and/or steroidogenesis are sparse and concern mostly α_2-adrenoceptor agonists. In a pharmacokinetic study with human volunteers (Kivistö et al., 1994), sedation, hypotension and tachycardia were observed at plasma levels of dexmedetomidine above 10 nM, the lower end of the dose-response curves in vitro (Fig. 1). The plasma levels achieved in veterinary practice with recommended dosages are around 250 nM for detomidine (Salonen et al., 1989). This 'therapeutic' concentration is at the high end of the concentration range of the dose-response curves presented in Fig. 1. In a tolerance study with human volunteers in which the lowest dose of atipamezole was four times higher than the dose of detomidine recommended for veterinary practice, a clear reduction in the plasma cortisol levels was observed, which did not increase with increasing dosages (Karhuvaara et al., 1989). If porcine adrenocortical cells in vitro are comparable to human adrenocortical cells in vivo, it would be expected that the cortisol reduction is not dose-dependent in humans: the reduction was already maximal at the lowest dosage used with the volunteers.

Also in dogs a medetomidine-induced reduction of both basal and ACTH-stimulated plasma cortisol levels was reported with hypnotic doses, an effect which was maximal after 3 h and returned to normal after 6 h (Maze et al., 1991). The authors also reported a dose-dependant inhibition by medetomidine of ACTH-stimulated corticosterone release by rat adrenocortical cells in vitro. A comparison of the dose-response curves with those presented in Fig. 1 suggests an about hundred times higher sensitivity of the porcine system to both stereoisomers of medetomidine and etomidate. Both species differences and 'only substrate' versus ACTH-stimulated steroid release might underlie this difference in sensitivity.

The reported medetomidine-induced inhibition of testosterone metabolism by rat hepatic *P*450 enzymes (Pelkonen et al., 1991) illustrates the possibility that the adrenocortical $P450_{11\beta/18}$ is more sensitive to a general *P*450 inhibition by these 4-substituted imidazoles. As atipamezole seems a suitable drug to end medetomidine- or detomidine-induced hypnosis/sedation/anaesthesia (Virtainen, 1989, MacDonald et al., 1989), an interaction (probably an addition) of their actions regarding these *P*450 enzymes is very likely and forms a potential hazard, as they also influence the biotransformation of other xenobiotics.

Both medetomidine and atipamezole are reported to attenuate hyperalgesia induced in rats (Mansikka and Pertovaara, 1995), an observation that might be explained in part by attenuation of central and peripheral steroid release. Also, a reduction of the 'stress hormones' by these imidazoles could enhance the sedative effects mediated via α_2-adrenoceptors. In this respect, the reports about effects of imidazoles on insulin secretion are worth mentioning. These effects might be mediated via α_2-adrenoceptors (Nishimura et al., 1994) or via other receptors (Chan et al., 1997; Berdue et al., 1997). Another interesting observation is the reversal of the hardening effect of hormones on the prostate by atipamezole (Constantinou and Omata, 1996; Horváth et al., 1996).

Used to control sedation/anaesthesia in (veterinary) surgery, α_2-adrenoceptor agonists and antagonist of the 4-substituted imidazole type can compromise the steroid-based defence mechanisms of the body that are needed for recovery.

Acknowledgements

It is a pleasure to acknowledge the help of the employees and meat inspectors of the slaughterhouse in Bussum. We express our gratitude for the donations of pure and certified samples of the drugs used in this study by Farmos (Turku, SF). Joop De Bree (Dept. Mathematics) helped us with the revision of this paper.

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