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Effects of cyclosporine-A on steroid secretion of dispersed rat adrenocortical cells

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With 4 figures and 3 tables

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Summary

The acute effect of cyclosporine-A (CSA), a potent immunosuppressive agent, on the secretory activity of dispersed rat adrenocortical cells was investigated. The production of the following steroid hormones was assayed by high performance liquid chromatography: pregnenolone (PREG), progesterone (PROG), 11-deoxycorticosterone (DOC), corticosterone (B), 18-hydroxy-11-deoxycorticosterone (18OH-DOC), 18-hydroxycorticosterone (18OH-B) and aldosterone (ALDO); B and ALDO outputs were also measured by radioimmunoassay.

Low concentrations of CSA (0.1–0.2 mg/ml) enhanced basal, but not ACTH- or angiotensin-II (ANG-II) 10^{-8} M-stimulated, secretions of PREG, non-18-hydroxylated steroids (PROG, DOC and B) and 18-hydroxylated steroids (18OH-DOC, 18OH-B and ALDO) of both zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZF/ZR) cells. Middle concentrations of CSA (from 0.3 to 0.5 mg/ml) did not affect PREG yield, nor did they alter basal and ACTH-stimulated post-PREG output of both ZG and ZF/ZR cells; however, they elicited a marked decrease in ANG-II-enhanced production of 18-hydroxylated steroid by ZG cells. Concentrations of CSA higher than 0.5 mg/ml strikingly reduced either basal and agonist-stimulated overall steroidogenesis of both ZG and ZF/ZR cells.

These findings suggest that CSA at low concentrations strongly stimulates the conversion of cholesterol to PREG (i. e. the rate-limiting step of steroidogenesis), while at middle concentrations it did not affect this early step, but specifically interferes with the intracellular events which transduce the stimulatory signal of ANG-II on the late steps of mineralocorticoid production (i. e. the conversion of B to ALDO). At higher concentrations, CSA probably exerts a cytotoxic effect.

Introduction

Cyclosporine-A (CSA) is a fungal cyclic peptide with potent immunosuppressive activity, which is currently employed for the control of organ allograft rejection (COHEN et al. 1984). Unfortunately, the clinical use of CSA is limited by the occurrence of some side-effects, including hepatotoxicity (BACKMAN et al. 1986; MASON 1989), nephrotoxicity (FARNWORTH et al. 1984; NEILD et al. 1985; RACUSEN and SOLEZ 1988) and severe systemic hypertension (SCHACHTER 1988).

The mechanism underlying the development of the so-called 'CSA hypertension' is not fully understood. Experimental findings obtained in rats chronically administered therapeutical doses of CSA (10–20 mg/kg) suggest the involvement of the renin-angiotensin system: in fact, plasma renin activity (PRA) is markedly increased (LUSTIG et al. 1987; STERN et al. 1987; REBUFFAT et al. 1989) and CSA seems to directly stimulate kidney renin release (BAXTER and DUGGIN 1984; KURZ et al. 1988; LUSTIG et al. 1988). However, CSA-induced PRA rise occurs without notable changes in plasma aldosterone (ALDO) concentration (LUSTIG et al. 1987; STERN et al. 1987; REBUFFAT et al. 1989), an effect which has been ascribed to a specific blockade by CSA of the angiotensin-II (ANG-II) secretagogue action on the adrenal zona glomerulosa (ZG) (STERN et al. 1987). We confirmed this last contention, but additionally demonstrated that a 30-day treatment with CSA (20 mg/kg/day) also provokes a marked inhibition of the growth and steroidogenic *in-vitro* capacity of both ZG and zonae fasciculata/reticularis (ZF/ZR) cells of rat adrenals, probably caused by an impairment of

protein synthesis (REBUFFAT et al. 1989). The CSA-induced decrease in steroidogenic capacity of adrenocortical cells would also account for the significantly diminished glucocorticoid-response to stress observed in CSA-treated rats (HIRANO et al. 1988; KAPUR et al. 1992) that cannot be satisfactorily explained by the controversial effect of CSA on the pituitary ACTH release (WHITE 1982; RYFFEL et al. 1983; REBUFFAT et al. 1989; KAPUR et al. 1992).

In front of the relatively large mass of investigations dealing with the chronic effect of CSA on adrenals, the studies of the acute *in-vitro* effects of CSA on adrenocortical cells are very scarce. As far as we are aware, STERN et al. (1987) found that CSA 10^{-6} M slightly depresses ANG-II-stimulated ALDO response of rat ZG cells, while MOROSHITA et al. (1990) described a stimulatory action of CSA $10^{-7}/10^{-6}$ M on basal ALDO secretion of rabbit ZG cells.

It therefore seemed worthwhile to investigate the *in-vitro* effects of increasing concentrations of CSA on the basal and agonist-stimulated secretory activity of both ZG (capsular) and ZF/ZR (inner) cells of the rat adrenal cortex.

Materials and methods

Preparation of dispersed adrenocortical cells

Male Wistar rats (300 ± 30 g body weight) were decapitated, and their adrenal glands were promptly removed and freed of capsular fat. The glands were gently decapsulated to separate ZG, and then hemisected; decapsulated adrenal halves were enucleated to remove zona medullaris. Dispersed capsular and inner adrenocortical cells were obtained by collagenase/DNase I digestion and mechanical disaggregation (SZALAY 1981). The viability of isolated cells was checked by the trypan-blue exclusion test and found to be higher than 90 %. Inner-cell contamination in capsular-cell preparations, as evaluated by phase-microscopy, was always less than 6 %. Dispersed cells obtained from 6 rats were pooled to obtain a single cell suspension, and 6 cell preparations for each incubation experiment were employed. Dispersed cells were put in Medium 199 (DIFCO, Detroit, U. S. A.) and potassium-free Krebs-Ringer bicarbonate buffer with 0.2 % glucose (2:1 vol/vol) containing 5 mg/ml human serum albumin.

Aliquots of each cell suspension (3 x 10^5 cells/ml) were incubated as follows: (i) increasing concentrations (from 0.1 to 0.8 mg/ml) of CSA (Sandimmune; Sandoz, Milan, Italy), (ii) increasing concentrations of CSA in the presence of ANG-II 10^{-8} M or ACTH 10^{-8} M (Peninsula, Merseyside, U. K.), and (iii) CSA (0.1, 0.4 or 0.7 mg/ml) with or without ANG-II 10^{-8} M (capsular cells) and ACTH 10^{-8} M (inner cells), in the presence or absence of cyanoketone 10^{-5} M (WIN 24540; Sterling-Winthrop, Guilford, U. K.); according to AGUILERA et al. (1981), this concentration of cyanoketone is able to prevent further metabolism of pregnenolone (PREG). The incubation was carried out for 90 min in a shaking bath at 37°C in an atmosphere of 95 % 0_2 and 5 % CO_2.

Radioimmunoassay (RIA)

Corticosterone (B) and ALDO were extracted from incubation media and purified (SIPPELL et al. 1978), and their concentrations were measured by the following commercial kits.

CORTX-RIA kit (Eurogenetix, Milan, Italy). Sensitivity: 25 pg/ml. Cross-reactivity: B and cortisol, 100 %; 11-deoxycorticosterone (DOC) and progesterone (PROG), 2 %; other steroids, less than 0.001 %. Intra- and interassay variations: 5.1 % and 7.8 %, respectively.

ALDO-CTK2 (IRE-Sorin, Vercelli, Italy). Sensitivity: 5 pg/ml. Cross-reactivity: ALDO, 100 %; 17-iso-ALDO and other steroids, less than 0.1 %. Intra- and interassay variations: 7.6 and 9.4 %, respectively.

High performance liquid chromatography (HPLC)

The concentrations of PREG, PROG, DOC, 18-hydroxy-11-deoxycorticosterone (18OH-DOC), B, 18-hydroxycorticosterone (18OH-B) and ALDO in the incubation media were assayed.

The samples (2 ml) were added with 10 ng dexamethasone as internal standard, and then extracted with 10 ml dichloromethane. The extracts were washed twice with 2 ml 0.1 N NaOH and 1 ml distilled water and then evaporated to dryness under vacuum and redissolved in 50 µl methanol (BELLONI et al. 1990). The recovery of steroids was 85 ± 8 % (SD).

The samples were injected via Rheodyne 7105 valve (Cotati, U. S. A.) in a Perkin-Elmer HPLC (Norwalk, U. S. A.), and steroids were assayed according to the technique of O'HARE et al. (1976). Steroid hormones were measured by UV absorbance, using a variable wavelength spectrometer (LC-75; Perkin-Elmer); PREG was detected at 290 nm, and other 4-ene, 3-one steroids at 240 nm (sensitivity: 5 pmol/ml). We used a Perkin-Elmer 410 LC pump, which is able to give a very constant composition of the mobile phase; the reproducibility of the retention times was between 1 and 3 % (CV). Steroid hormones were identified by comparison of their retention times with those of the standards. This was done in normal phase, using Licrosorb Si-100 (Merck, Milan, Italy) with hexane-propanol as eluent, and in reverse phase, using the following C-18 columns: Spherisorb ODS-2 (Phase Separation, Deeside, U. K.), Hypersil ODS (Shandon, Cheshire, U. K.) and Nucleosil C-18 (Macherey-Nagel; Düren, Germany); these columns were tested with two chromatographic systems: MeOH-H_2O (40–100 %) and Acn-H_2O (25–100 %) gradients. The form of the gradients was concave-exponential, similar to that used by O'HARE et al. (1976). Moreover, for initial identification, the same sample was chromatographed alone or with the addition of the standards; this procedure was used during the routine analyses to confirm the results of some difficult sample. Quantification of steroid hormones was based on peak-area measurement. The response of the detector was satisfactorily linear over the range of 5–1000 pmol, and directly proportional to the mass of steroid hormones injected.

Statistics

Data were expressed as means ± SD or SE, and their statistical comparison was done by ANOVA, followed by the Multiple Range Test of Duncan.

Results

RIA findings

Low concentrations of CSA (0.1–0.2 mg/ml) caused a significant increase (about 60 %) of basal ALDO and B productions by dispersed capsular and inner adrenocortical cells, respectively. Middle concentrations of CSA (from 0.3 to 0.5 mg/ml) did not apparently affect basal steroidogenesis, while high concentrations (from 0.6 to 0.8 mg/ml) evoked a marked depression of both ALDO (–64/–70 %) and B (–70/–80 %) output (Figs 1 and 2).

Until a concentration of 0.5 mg/ml, CSA did not change ACTH (10^{-8} M)-stimulated ALDO and B yields by capsular and inner cells, respectively. Starting from a concentration of 0.6 mg/ml, CSA strikingly reduced ACTH-stimulated production of ALDO (–70/–80 %) and B (–85/–95 %) (Figs 1 and 2). Again, low concentrations of CSA (0.1–0.2 mg/ml) did not alter ANG-II (10^{-8} M)-stimulated ALDO secretion of capsular cells; however, higher concentrations of CSA elicited a dose-dependent (from 0.3 to 0.7 mg/ml) decrease of ALDO output, which attained a maximum of about –90 % (fig. 1).

HPLC findings

The effects of low (0.1 mg/ml), middle (0.4 mg/ml) and high (0.7 mg/ml) concentrations of CSA on basal and ANG-II (10^{-8} M)-stimulated steroidogenesis of capsular cells, and on basal and ACTH (10^{-8} M)-stimulated steroidogenesis of inner cells were examined.

Basal post-PREG output of capsular cells was represented by PROG (17 %), DOC (15 %), B (33 %), 18OH-DOC (15 %), 18OH-B (8 %) and ALDO (12 %), non-18-hydroxylated and 18-hydroxylated steroids accounting for about 65 % and 35 % of the total post-PREG yield (table 1). The low concentration of CSA raised (47 %) the total basal post-PREG output of capsular cells, by evoking similar increases in both non-18-hydroxylated and 18-hydroxylated hormone outputs. The middle concentration of CSA did no alter capsular-cell steroidogenesis, while the high concentration induced a net suppression of the total basal post-PREG secretion (about –70 %), due to comparable decreases in the production of the entire spectrum of non-18-hydroxylated and 18-hydroxylated hormones (table 1).

Basal post-PREG output of inner cells consisted of PROG (16 %), DOC (13 %), B (64 %) and small amounts of 18OH-DOC and 18OH-B; ALDO was below the limit of sensitivity of our assay. Thus, non-18-hydroxylated steroids accounted for about 93 % of the overall basal post-PREG secretion (table 1). The effects of CSA on basal steroid secretion of inner cells were similar to those exerted on ZG cells: the low concentration of CSA raised (48 %) and the high concentration decreased (-73 %) total post-PREG yield, while the middle concentration was without any apparent effect (table 1).

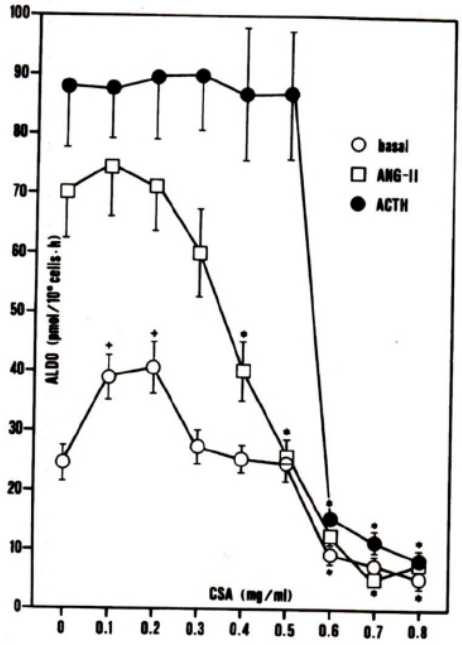

Fig. 1. Effect of increasing concentrations of CSA on basal and agonist-stimulated ALDO secretion of dispersed rat ZG cells. Data are means ± SE (n = 6). +, $P < 0.05$ and *, $P < 0.01$ from the respective control group (0 group).

ANG-II stronlgy enhanced total post-PREG output by capsular cells (2.7-fold), by raising the secretion of both non-18-hydroxylated and 18-hydroxylated steroids. The first ones were exclusively represented by B, which underwent a 5.6-fold increase, being PROG and DOC productions apparently suppressed. As to the 18-hydroxylated steroids, 18OH-DOC secretion did not evidence significant variations, whereas productions of 18OH-B and ALDO displayed 3-fold and 4-fold rises, respectively, after ANG-II exposure (compare tables 1 and 2). The low concentration of CSA did not affect ANG-II-stimulated ZG steroidogenesis. The middle concentration of CSA did not induce alterations in the overall post-PREG secretion; however, the production of non-18-hydroxylated steroids displayed an appreciable rise (33 %), which was not significant due to the high intragroup variability, while that of 18-hydroxylated steroids underwent a marked lowering (–72 %). 18OH-DOC secretion was suppressed, and the outputs of 18OH-B and ALDO showed comparable decreases (–64/–69 %). The high concentration of CSA induced striking reductions in the yields of

Table 1. Effects of CSA on basal post-PREG secretion of dispersed rat adrenocortical cells. Results (pmol/10^6 cells/h) are expressed as mean ± SD (n = 6).

	ZG				ZF/ZR			
	Control	CSA-1	CSA-2	CSA-3	Control	CSA-1	CSA-2	CSA-3
PROG	30.9 ± 10.3	44.6 ± 15.2+	24.1 ± 8.0	8.4 ± 3.1*	40.2 ± 12.6	65.6 ± 23.5+	35.6 ± 14.1	6.5 ± 3.2*
DOC	27.2 ± 8.9	40.1 ± 13.8+	21.2 ± 5.3	9.0 ± 2.7*	31.8 ± 11.5	41.3 ± 12.8	28.5 ± 8.6	7.4 ± 3.0*
B	60.5 ± 21.4	93.2 ± 30.6+	62.4 ± 23.4	18.7 ± 7.2*	163.2 ± 6.2	235.7 ± 79.2+	170.9 ± 50.6	55.7 ± 17.5*
18OH-DOC	28.3 ± 7.5	36.2 ± 13.0	25.1 ± 7.2	9.6 ± 3.0*	10.3 ± 4.6	15.9 ± 6.1*	–	–
18OH-B	15.0 ± 6.4	20.2 ± 6.3	18.3 ± 7.0	–	8.6 ± 3.7	18.1 ± 8.4*	10.7 ± 3.1	–
ALDO	20.5 ± 7.1	34.2 ± 9.2*	24.2 ± 6.8	5.2 ± 2.4*	–	–	–	–
total post-PREG steroids	182.4 ± 62.4	268.6 ± 87.8+	175.3 ± 59.3	50.9 ± 19.2*	254.3 ± 94.2	376.6 ± 129.7+	245.7 ± 76.9	69.6 ± 24.1*
non-18OH steroids	118.6 ± 41.5	177.9 ± 60.5+	107.7 ± 36.9	36.1 ± 13.5*	235.4 ± 83.1	342.6 ± 117.3+	230.0 ± 73.8	69.6 ± 24.1*
18OH steroids	63.8 ± 21.0	90.7 ± 31.4+	67.6 ± 24.1	14.8 ± 6.2*	18.9 ± 9.0	34.0 ± 15.2+	10.7 ± 3.1	–

CSA-1, 0.1 mg/ml; CSA-2, 0.4 mg/ml; CSA-3, 0.7 mg/ml; +, $P<0.05$ and *, $P<0.01$ from control group.

Table 2. Effects of CSA on agonist-stimulated post-PREG secretion of dispersed rat adrenocortical cells. Results (pmol/10^6 cells/h) are expressed as means ± SD (n = 6).

	ZG (ANG-II 10^{-8} M)				ZF/ZR (ACTH 10^{-8} M)			
	Control	CSA-1	CSA-2	CSA-3	Control	CSA-1	CSA-2	CSA-3
PROG	–	–	38.6 ± 12.2	–	–	–	15.3 ± 6.2	–
DOC	–	5.6 ± 2.5	15.4 ± 6.1	6.1 ± 3.0	60.8 ± 20.5	50.1 ± 14.8	70.2 ± 18.7	–
B	341.3 ± 130.6	338.6 ± 115.4	401.5 ± 142.8	91.2 ± 34.1*	1212.5 ± 418.1	1308.4 ± 451.2	1280.5 ± 408.5	407.1 ± 151.3*
18OH-DOC	20.1 ± 7.1	14.6 ± 5.4	–	–	–	15.3 ± 7.0	–	–
18OH-B	45.4 ± 16.2	51.3 ± 15.8	16.2 ± 6.1*	14.3 ± 5.8*	80.6 ± 23.4	70.6 ± 19.4	85.2 ± 28.5	35.2 ± 8.9*
ALDO	81.8 ± 20.9	78.4 ± 19.9	25.4 ± 9.2*	10.9 ± 3.*	–	–	–	–
total post-PREG steroids	488.6 ± 175.2	488.5 ± 159.6	497.1 ± 177.1	122.5 ± 49.5*	1353.9 ± 465.7	1444.4 ± 498.1	1451.2 ± 463.2	442.3 ± 160.8*
non-18OH steroids	341.3 ± 130.6	344.2 ± 118.0	455.5 ± 162.5	97.3 ± 38.2*	1273.3 ± 440.2	1358 ± 468.9	1366.0 ± 438.6	407.1 ± 151.3*
18OH steroids	147.3 ± 44.9	144.3 ± 41.3	41.6 ± 16.0*	25.2 ± 9.8*	80.6 ± 23.4	85.9 ± 27.2	85.2 ± 28.5	35.2 ± 8.9*

Explanation as in Table 1.

Table 3. Effects of CSA on basal and agonist-stimulated PREG production by dispersed rat adrenocortical cells. Results (pmol/10^6 cells/h) are expressed as means ± SD (n = 6).

	ZG		ZF/ZR	
	Basal	ANG-II 10^{-8} M	Basal	ACTH 10^{-8} M
Control	208.4 ± 71.4	611.9 ± 220.2	316.1 ± 110.6	1011.4 ± 358.2
CSA-1	411.8 ± 139.1*	595.3 ± 211.5	654.8 ± 251.4*	988.7 ± 318.9
CSA-2	250.1 ± 87.2	650.7 ± 308.3	363.4 ± 140.1	1182.3 ± 400.8
CSA-3	59.7 ± 15.6*	170.8 ± 54.1*	96.1 ± 35.7*	301.5 ± 128.7*

Explanations as in Table 1.

both non-18-hydroxylated (–71 %) and 18-hydroxylated (–83 %) hormones (table 2).

ACTH provoked a 5.3-fold increase in the total post-PREG secretion of inner cells, which was almost exclusively due to the rise in the production of non-18-hydroxylated steroids (B rose by about 7.5-fold) (compare tables 1 and 2). The low and middle concentrations of CSA did not cause any significant change in the ACTH-stimulated ZF/ZR-cell secretion, while the high concentration of CSA provoked a conspicuous reduction, due to comparable decreases of both non-18-hydroxylated (-68 %) and 18-hydroxylated (-56 %) steroids (table 2).

ANG-II and ACTH induced a 3-fold rise in the PREG synthesis by dispersed capsular and inner adrenocortical cells (table 3). The low concentration of CSA evoked a 2-fold increase in the basal (but not in the agonist-stimulated) PREG production, while the middle concentration did not apparently affect it. The high concentration of CSA caused comparable lowerings (about -70 %) of both basal and agonist-stimulated PREG yields of either types of cell preparations (table 3).

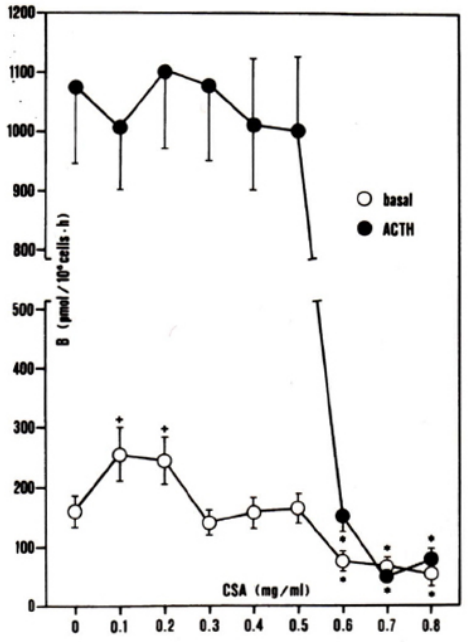

Fig. 2. Effect of increasing concentrations of CSA on basal and agonist-stimulated B secretion of dispersed rat ZF/ZR sells. Data are means ± SE (n = 6). +, $P < 0.05$ and +, $P < 0.01$ from the respective control proup (o group).

Discussion

Our present findings indicate that CSA exerts a rather complex acute effect on steroid secretion of dispersed rat adrenocortical cells. Such an effect varies according to the dose of CSA, the type of cell preparation (capsular or inner cells) and the type of agonist employed to enhance steroidogenesis (ANG-II or ACTH).

To summarize, it results that (i) low doses (0.1–0.2 mg/ml) of CSA enhance basal, but not agonist-stimulated steroidogenesis of both ZG and ZF/ZR cells, (ii) middle doses (0.3–0.5 mg/ml) are without effect on basal and ACTH-stimulated steroidogenesis of both ZG and ZF/ZR cells, but significantly decrease ANG-II-enhanced mineralocorticoid secretion of ZG cells, and (iii) high doses of CSA (0.6–0.8 mg/ml) cause a net decrease of both basal and agonist-stimulated steroid secretion of ZG and ZF/ZR cells. More insight can be obtained by expressing the effects of low, middle and high concentrations of CSA as percent changes in the production of PREG, total post-PREG steroids, non-18-hydroxylated steroid and 18-hydroxylated steroids, as evaluated by HPLC (figs 3 and 4). Under basal conditions, each of the three concentrations of CSA elicits the same effect on the four parameters both in ZG and ZF/ZR cells. The same occurs in the case of agonist-stimulated steroid output, with the notable exception that the middle concentration of CSA specifically decreases ANG-II-stimulated production of 18-hydroxylated steroids by ZG cells (fig. 4, upper panel, D).

Taken together our findings suggest that CSA exerts a dose-dependent biphasic effect on the early step of steroidogenesis, i. e. the conversion of cholesterol to PREG (for review, see HANUKOGLU 1992). At a low concentration CSA stimulates PREG synthesis, while at concentrations over 0.2 mg/ml it is ineffective. The stimulation of

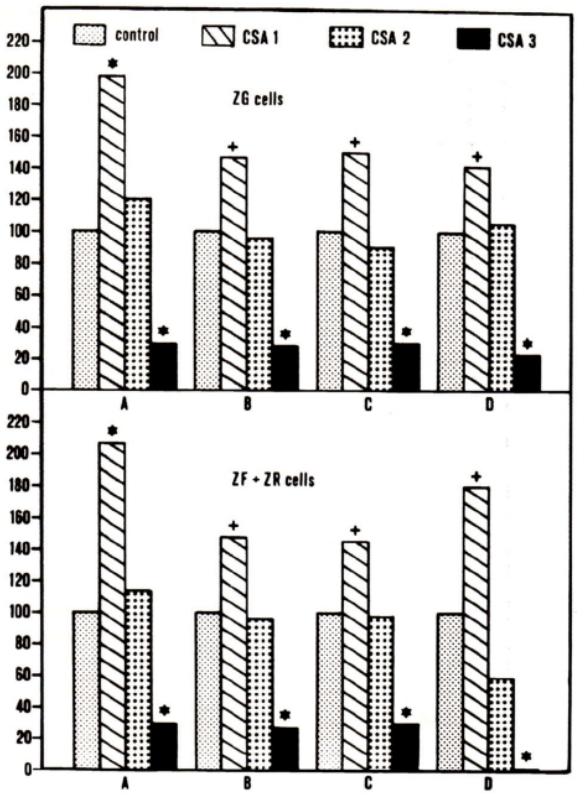

Fig. 3. Percent changes induced by low (1), middle (2) and high (3) concentrations of CSA on the basal production of PREG (A), total post-PREG steroids (B), non-18-hydroxylated steroids (C) and 18-hydroxylated steroids (D) by ZG and ZF/ZR cells. +, $P < 0.05$ and *, $P < 0.01$ from control group.

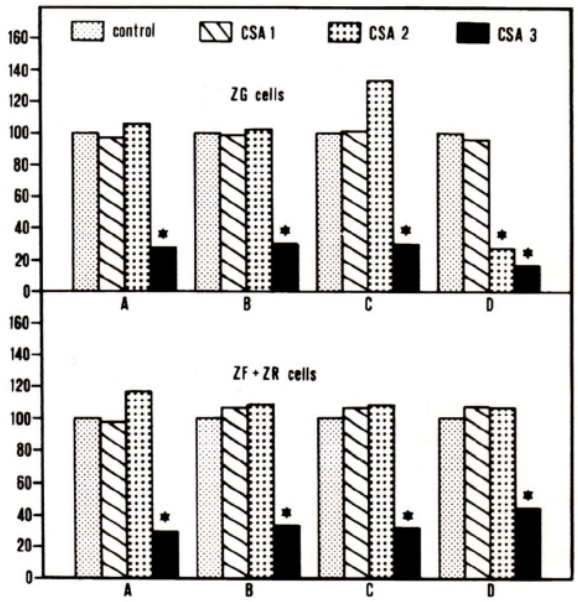

Fig. 4. Percent changes induced by low (1), middle (2) and high (3) concentrations of CSA on the agonist-stimulated production of PREG (A), total post-PREG steroids (B), non-18-hydroxylated steroids (C) and 18-hydroxylated steroids (D) by ZG (ANG-II 10^{-8} M) and ZF/ZR (ACTH 10^{-8} M) cells. *, $P < 0.01$ from control group.

PREG production, which is the rate-limiting step of steroidogenesis (for review, see MILLER 1988), obviously is a reflection on the secretion of the entire spectrum of post-PREG steroids. The positive effect of the low concentration of CSA on the conversion of cholesterol to PREG cannot conceivably manifest itself in the presence of maximal effective (10^{-8}M) concentrations of ACTH and ANG-II (VINSON et al. 1985; HAUSDORFF et al. 1989), since both these agonists strongly and specifically enhance this early step of steroid synthesis (for review, see VINSON et al. 1992).

Only tentative hypotheses can be advanced about the mechanism underlying this effect of CSA, which parenthetically appears to be in keeping with the results obtained by MORISHITA et al. (1990) in rabbits. CSA could theoretically bind to the membrane receptors for ACTH and ANG-II, thus activating the post-receptorial cascade transducing the secretagogue signal of these agonists. However, this contention does not seem to be tenable, inasmuch as the overall effects of ACTH and ANG-II on steroidogenesis of ZF/ZR and ZG cells are markedly different from those elicited by low concentrations of CSA, since they involve stimulation not only of the early, but also of the late steps of steroidogenesis (for review, see VINSON et al. 1992). CSA is an extremely hydrophobic compound that readily penetrates cell membranes (KAHAN 1985). Hence, it may be reasonable to conceive that CSA acts intracytoplasmatically by enhancing the transport of intracellular stored cholesterol to mitochondria and/or the activity of the mitochondrial cytochrome-$P450_{scc}$, the enzyme involved in the cleavage of the cholesterol side-chain (for review, see HANUKOGLU 1992). The early step of steroid synthesis is a very complicated process, involving the SCP_2 protein (that binds free-cholesterol and carries it to the mitochondria) and the translocation of cholesterol from the outer to the inner mitochondrial membrane, where cytochrome-$P450_{scc}$ is located (for review, see JEFCOATE et al. 1992); this last process, in turn, requires the presence of steroidogenic labile proteins, among which an endogenous ligand (called diazepam-binding inhibitor) that binds to the mitochondrial benzodiazepine receptors (for review, see WHITEHOUSE 1992). Further studies are needed to ascertain the exact locus at which CSA is able to activate the early step of steroidogenesis.

Middle concentrations of CSA (from 0.3 to 0.5 mg/ml) do not affect the conversion of cholesterol to PREG, but are able to exert a specific anti-ANG-II effect that can manifest itself only in ZG cells, which in rodents are the only adrenocortical cells provided with ANG-II receptors (for review, see VINSON et al. 1992). These data appear to

agree with those of STERN et al. (1987), who found that CSA 10^{-6} M significantly attenuates ALDO response of rat ZG cells to ANG-II, but not to ACTH and potassium. As pointed out above, the possibility that CSA may interfere with ANG-II binding to its membrane receptors can be excluded, since in this case a middle concentration of CSA should also depress the early step of ALDO synthesis in ZG cells. A more convincing possibility is that CSA may specifically interfere with one of the specific intracellular events mediating the acute secretory response of ZG cells to ANG-II, i. e. the conversion of B to 18OH-B and ALDO by intramitochondrial cytochrome-$P450_{c18}$ (ALDO synthase) (HANUKOGLU 1992). It is well known that different mechanisms are involved in the intracellular transduction of ALDO secretagogue signals of ACTH and ANG-II (for review, see SPÄT 1988; QUINN and WILLIAMS 1992; VINSON et al. 1992). ACTH mainly acts by activating adenylate-cyclase and cyclic-AMP-dependent protein kinase A, a process which is relatively Ca^{2+}-independent (TAIT et al. 1987; HAUSDORFF et al. 1989). Conversely, ANG-II mainly acts by activating phospholipase C that catalyzes the hydrolysis of phosphoinositides to inositol triphosphate and diacylglycerol. The major action of inositol triphosphate is to rise cytosolic free Ca^{2+} concentration by causing the release of Ca^{2+} from intracellular stores, thus activating calmodulin; diacylglycerol is thought to activate the Ca^{2+}-dependent protein kinase C. According to RASMUSSEN et al. (1987), the calmodulin-mediated branch of the acute adrenoglomerulotropic effect of ANG-II plays a pivotal role in initiating ALDO response being the protein kinase C branch mainly involved in the sustained response that requires the activation not only of cytochrome-$P450_{c18}$, but also of the early steps of steroidogenesis. As previously pointed out CSA readily penetrates plasma membrane, and in T-lymphocytes (its main target), binding of CSA to a cytoplasmic binding protein (cyclophilin) inhibits some calmodulin-mediated events (HAIT et al. 1986; COLOMBANI and HESS 1987), including the expression of interleukin-2 gene (MANGER et al. 1986). Thus, it appears reasonable to advance the hypothesis that middle doses of CSA may interfere with the calmodulin-mediated transduction of ANG-II acute secretagogue effect on ZG cells. The possibility that CSA may directly interfere with cytochrome-$P450_{c18}$ can be ruled out by our RIA data showing that middle doses of CSA do not inhibit the ACTH-induced stimulation of B to ALDO conversion.

Independently of the type of adrenocortical-cell preparation and of the agonist employed to enhance steroid secretion, high doses of CSA (higher than 0.5 mg/ml) cause a profound depression of the production of PREG, non-18-hydroxylated steroids and 18-hydroxylated steroids. We propose that this finding may be interpreted as an unspecific toxic effect of CSA. Compelling evidence is available that high doses of CSA exert a toxic effect *in vivo* (see Introduction) and *in vitro* (BÄCKMAN et al. 1988; BENNETT et al. 1991), and CSA over 10^{-6} M has been found to completely block ALDO secretion by rabbit ZG cells (MORISHITA et al. 1990). CSA was found to exert a strong inhibitory effect on protein synthesis in rat hepatocytes (BÄCKMAN et al. 1988), and it is well known that steroidogenesis is dependent on the continuous synthesis of labile (rapid turnover) proteins, that are required in the early rate-limiting step of this process (for review, see JEFCOATE et al. 1992; VINSON et al. 1992).

Conclusions

Our findings indicate that CSA exerts dose-dependent pleiotropic effects on rat adrenal steroidogenesis *in vitro:* low concentrations (0.1–0.2 mg/ml) and high concentrations (over 0.5 mg/ml) stimulate and depress steroid synthesis, respectively, while middle concentrations (between 0.3 and 0.5 mg/ml) specifically inhibit ANG-II-stimulated mineralocorticoid secretion by ZG cells. CSA doses usually employed in *in vivo* experiments with rats vary from 5 to 100 mg/kg (i. p.), those higher than 30 mg/kg being strongly cytotoxic (for review, see RACUSEN and SOLEZ 1988). By considering that the i. p. absorption rate of peptides in rats is near to 90 % and by assuming that blood volume in rats averages 35–40 mg/kg (REAVEN et al. 1988; ANDREIS et al. 1991), we can roughly calculate that the plasma concentration reached by CSA is about 25 µg/ml per mg/kg of i. p. *bolus* administered CSA. Thus, the low, middle and high concentrations of CSA, as defined in the present study, would be obtained by i. p. administration of about 4–8, 12–20 and 24–32 mg/kg of CSA. On the ground of these considerations, our present results appear to be in complete agreement with those of previous *in-vivo* studies (LUSTIG et al. 1987; STERN et al. 1987; REBUFFAT et al. 1989). In fact, the daily administration of 10–20 mg/kg of CSA provokes 'middle' blood concentrations of this peptide, which obviously, despite stimulating kidney renin release and ANG-II production, strongly blunt the ALDO secretagogue effect of ANG-II, thus producing in rats a state of hypertension-associated hyperreninemic hypoaldosteronism.

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