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Pituitary Adenylate-Cyclase Activating Peptide Enhances Aldosterone Secretion of Human Adrenal Gland: Evidence for an Indirect Mechanism, Probably Involving the Local Release of Catecholamines

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ABSTRACT

Evidence is accumulating that the adrenal medulla exerts a paracrine control on the secretory activity of the cortex by releasing catecholamines and several regulatory peptides. Pituitary adenylate-cyclase activating peptide (PACAP) is contained in the adrenal medulla of several mammalian species and in human pheochromocytomas. Thus, we investigated whether PACAP exerts a modulatory action on steroid secretion by the human adrenal cortex *in vitro*. Adrenal slices (including both capsule and medulla) and dispersed adrenocortical cells (obtained from the gland tail deprived of medulla) were employed. Both adrenal preparations secreted aldosterone (ALDO) and cortisol in response to 10 nmol/L ACTH. PACAP (10 nmol/L) was found to enhance basal ALDO production by adrenal slices, but not by dispersed cells. PACAP was ineffective on cortisol secretion of both preparations. Adrenal slices displayed a marked ALDO, but not cortisol, secretory response to 100 nmol/L isoprenaline or noradrenaline. *l*-Alprenolol (1 μmol/L), a specific β-adrenoceptor antagonist, completely suppressed the ALDO response to both β-adrenoceptor agonists and 10 nmol/L PACAP, without *per se* altering basal ALDO output by adrenal slices. PACAP (10 nmol/L) induced a net rise in catecholamine release by adrenal slices. Taken together, our present findings suggest that PACAP indirectly stimulates ALDO secretion by the human adrenal cortex, probably by eliciting the local release of catecholamines by medullary chromaffin cells that are also scattered in the cortical tissue, especially the zona glomerulosa. (*J Clin Endocrinol Metab* **81:** 169–173, 1996)

NUMEROUS lines of evidence indicate that the adrenal medulla exerts a paracrine control on the secretory activity of the cortex in many mammalian species by releasing catecholamines and several regulatory peptides that may act either directly on adrenocortical cells or on the gland vasculature (for review, see Refs. 1 and 2). The morphological background of this paracrine mechanism may be, in addition to the abundant adrenergic and peptidergic nerve fibers of medullary origin reaching the cortex (2), the presence of abundant interdigitations between medullary and cortical tissues (3, 4). No data are available on this topic as far as the human adrenal gland is concerned; however, the recent demonstration of islets of chromaffin cells intermingled with adrenocortical cells, especially in the subcapsular zona glomerulosa, suggests that important cortico-medullary interactions may be operative in human adrenals (5).

PACAP-38 is a 38-amino acid peptide first isolated from ovine hypothalamus based on its ability to stimulate adenylate cyclase in rat corticotropes (6, 7). PACAP is contained in the adrenal medulla of several mammalian species (mouse, hamster, rat, cow, and pig) (8–11) and has recently been reported to enhance *in vitro* steroid secretion of frog interrenals (12, 13) and rat adrenal slices (14) as well as to induce a moderate increase in cortisol release by adrenals of conscious calves (15).

The presence of PACAP-like immunoreactivity has not yet been demonstrated in normal human adrenals, but PACAP is particularly abundant in some human pheochromocytomas (16). It, therefore, seemed worthwhile to examine whether PACAP affects the secretory activity of the human adrenal cortex *in vitro*. Our findings suggest that PACAP exerts a mineralocorticoid, but not glucocorticoid, secretagogue effect via an indirect mechanism, probably involving a local release of catecholamines.

Materials and Methods

Fragments of adrenal glands were obtained from consenting adult patients (35–45 yr old) undergoing unilateral nephrectomy for kidney cancer. Starting from 2 weeks before surgery, patients were kept on a normal diet, without any medication able to alter adrenal function; adrenal glands appeared histologically normal. Portions of the adrenal head and tail that, respectively, contain and do not contain medulla (17) were removed, placed in potassium-free Krebs-Ringer bicarbonate buffer with 0.2% glucose at 4 C, and immediately carried to our laboratory. Head fragments were cut into slices, always including the gland capsule and medulla; tail fragments were employed to obtain dispersed adrenocortical cell preparation by collagenase digestion and mechanical disaggregation (18).

Adrenal slices and dispersed cells obtained from each adrenal gland were placed in medium 199 (Difco, Detroit, MI) and Krebs-Ringer bicarbonate buffer with 0.2% glucose containing 5 mg/mL human serum albumin and incubated (8–10 mg/mL or 3×10^5 cells/mL, in replicates of five each) as follows: 10 nmol/L ACTH, 10 nmol/L PACAP-38 (Pen-

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insula, Merseyside, UK), or without any peptide; and 10 nmol/L PACAP-38, 100 nmol/L isoprenaline, or 100 nmol/L noradrenaline (Sigma Chemical Co., St. Louis, MO) in the presence or absence of 1 μmol/L *l*-alprenolol (Sigma; only adrenal slices). The concentrations of ACTH and PACAP were those determined to be maximally effective *in vitro* (14, 19). The concentrations of isoprenaline and noradrenaline, β-adrenoceptor agonists, were also those determined to be maximally effective (20), and the concentration of *l*-alprenolol, a β-adrenoceptor antagonist (21), was previously found to completely suppress the secretagogue effect of 10^{-7} mol/L isoprenaline in rat adrenal slices (14). The incubation was carried out for 90 min in a shaking bath at 37 C in an atmosphere of 95% O_2 and 5% CO_2. The medium was collected and kept frozen at −80 C until hormonal assays.

The concentrations of progesterone, 11-deoxycorticosterone (DOC), corticosterone (B), 18-hydroxycorticosterone (18OH-B), aldosterone (ALDO), 11-deoxycortisol, cortisol, and cortisone were measured by high pressure liquid chromatography (HPLC). The samples (1 mL) were added with 100 mg dexamethasone as internal standard, then extracted with 10 mL dichloromethane. The extracts were washed twice with 2 mL 0.1 N NaOH and 1 mL distilled water, then evaporated to dryness under vacuum and redissolved in 50 μL acetonitrile-water (1:1). The samples were injected via a Rheodyne 7125–075 valve (Rheodyne, Cotati, CT) in a Perkin-Elmer high pressure liquid chromatograph (series 410 LC pump, Perkin-Elmer Corp., Norwalk, CT) equipped with a LC-75 Spectrophotometric Detector and CC12 Computing Integrator (Perkin-Elmer) and assayed according to the technique of O'Hare *et al.* (22), as modified by Neri *et al.* (23). Briefly, steroids were detected by UV absorbance at 240 nm wavelength and identified by comparison of their retention times with those of the standards. This was performed in reverse phase, using a Hypersil RP 3 μm (4.6 × 100 mm) column (Alltech, Deerfield, IL) and 95% acetonitrile-5% methanol as eluent. The 18-min exponential concave gradient (organic phase-water, 27–100%), generated by the computer of the pump corresponded to curve 4 of the series 410 LC high pressure liquid chromatograph. A good separation between steroid hormones assayed was obtained (Fig. 1), and the final recovery of steroids was 80–85%. Quantification of steroid hormones was based on peak area measurement; the sensitivity of our assay system was 1 pmol/mL, and the response of the detector was satisfactorily linear over the range of 1–1000 pmol and directly proportional to the mass of steroid hormone injected. Intra- and interassay variations were 5.9% and 7.6%, respectively.

ALDO and cortisol concentrations were also measured by RIA (without previous extraction), using commercial kits purchased by IRE-Sorin [Vercelli, Italy; ALDO-CTK2 kit: sensitivity, 5 pg/mL; cross-reactivity: ALDO, 100%; 17-iso-ALDO and other steroids (including 18OH-B), <0.1%; intra- and interassay variations, 7.5% and 8.9%; cortisol RIA kit: sensitivity, 30 pg/mL; cross-reactivity: cortisol, 100%; 11-deoxycortisol, 4.8%; B, 3%; progesterone, 0.5%; DOC, 0.02%; other steroids, <0.01%; intra- and interassay variations, 6.2% and 7.8%].

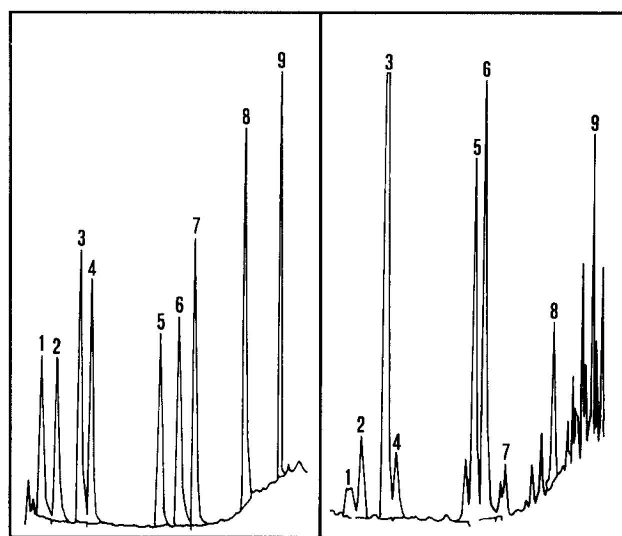

FIG. 1. HPLC separation of steroid standards (*left panel*) and an example of a chromatogram of steroid hormones released by a human adrenal slice under basal conditions (*right panel*). 1, 18OH-B; 2, ALDO; 3, cortisol; 4, cortisone; 5, dexamethasone (internal standard); 6, B; 7, 11-deoxycortisol; 8, DOC; 9, progesterone.

The catecholamine concentration in the incubation medium was measured, without previous alumina purification and concentration, by HPLC, using a reverse phase column (150 × 4 mm; BioSil ODS-5S, Bio-Rad Laboratories, Hercules, CA) and a glassy carbon electrochemical detector (TL-5, Bioanalytical Systems, Lafayette, IN), as described by Saiani and Guidotti (24). Catecholamines were about 50% adrenaline and 50% noradrenaline, and the sensitivity of the assay was approximately 3 pmol/mL. The intraassay variation was 7%.

Data obtained from each adrenal gland were averaged and expressed as the mean ± SD of three separate experiments (three adrenals from three patients). In the case of catecholamine assay, data were the mean ± SD of six measurements performed on slices from one adrenal head. The statistical comparison of results was performed using ANOVA, followed by the multiple range test of Duncan, or by Student's *t* test in the case of catecholamine assay.

Results

HPLC assay (Table 1) showed that our adrenal slice preparations produced significant amounts of both glucocorticoid and mineralocorticoid hormones. ACTH (10 nmol/L) increased both cortisol (2.7-fold) and ALDO yields (4.4-fold) as well as those of 18OH-B (3.8-fold) and B (2.0-fold); conversely, it decreased progesterone release (−76%). PACAP (10 nmol/L) did not affect cortisol secretion, but markedly enhanced ALDO and 18OH-B production (3.0- and 2.8-fold, respectively) without changing that of B and DOC; progesterone release was decreased, but not significantly. ACTH (10 nmol/L) increased both cortisol (6.9-fold) and ALDO (3.8-fold) secretion by dispersed adrenocortical cells (as measured by RIA). On the contrary, PACAP had no apparent effect (Table 2).

RIA demonstrated that 100 nmol/L isoprenaline or noradrenaline, like 10 nmol/L PACAP, evoked a significant rise in ALDO secretion by adrenal slices (from 3.2- to 2.6-fold) without significantly affecting cortisol production. *l*-Alpropanol (1 μmol/L) did not alter the basal secretion of adrenal slices, but it completely suppressed the ALDO response to both β-adrenoceptor agonists and PACAP (Table 3).

TABLE 1. Effects of 10^{-8} mol/L ACTH and 10^{-8} mol/L PACAP on steroid production by human adrenal slices

	pmol/mg · h		
	Basal	ACTH	PACAP
Progesterone	12.7 ± 6.8	3.0 ± 0.6[a]	7.3 ± 1.3
11-Deoxycortisol	14.5 ± 3.4	17.6 ± 3.9	14.1 ± 3.6
Cortisol	235.6 ± 67.5	638.8 ± 121.5[b]	219.1 ± 50.1
Cortisone	29.9 ± 5.4	25.7 ± 4.9	20.3 ± 4.5
DOC	10.1 ± 2.9	10.6 ± 7.7	12.5 ± 4.6
B	121.5 ± 15.3	244.6 ± 37.7[b]	108.4 ± 14.9
18OH-B	12.8 ± 1.7	48.8 ± 9.7[b]	35.9 ± 7.1[b]
ALDO	10.2 ± 2.0	45.1 ± 10.7[b]	31.8 ± 6.4[b]

Data are the mean ± SD of three separate experiments.
[a] $P < 0.05$ *vs.* respective basal value.
[b] $P < 0.01$ *vs.* respective basal value.

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TABLE 2. Effects of 10^{-8} mol/L ACTH and 10^{-8} mol/L PACAP on steroid production by dispersed human adrenocortical cells

	pmol/10^6 cells · h	
	Cortisol	ALDO
Basal	301.5 ± 101.6	15.8 ± 1.9
ACTH	2081.4 ± 460.2[a]	59.5 ± 16.1[a]
PACAP	361.7 ± 125.4	14.5 ± 6.2

Data are the mean ± SD of three separate experiments.
[a] $P < 0.01$ *vs.* respective basal value.

TABLE 3. Effects of 10^{-7} mol/L β-adrenoceptor agonists, 10^{-8} mol/L PACAP and 10^{-6} mol/L *l*-alprenolol on steroid production by human adrenal slices

	pmol/mg · h			
	Cortisol		ALDO	
	Control	*l*-Alprenolol	Control	*l*-Alprenolol
Basal	225.8 ± 75.1	295.2 ± 85.9	22.3 ± 8.4	20.1 ± 6.9
Isoprenaline	285.7 ± 68.9	306.7 ± 96.2	71.8 ± 17.5[a]	19.5 ± 6.0[b]
Nor-adrenaline	258.1 ± 81.4	230.4 ± 68.5	58.6 ± 14.8[a]	22.6 ± 5.9[b]
PACAP	281.9 ± 69.2	245.8 ± 71.5	64.4 ± 14.2[a]	24.7 ± 7.1[b]

Data are the means ± SD of three separate experiments.
[a] $P < 0.01$ *vs.* respective basal value.
[b] $P < 0.01$ from the respective control value.

The basal secretion of catecholamines by adrenal slices was very low, and 10 nmol/L ACTH did not affect it. PACAP (10 nmol/L) induced about an 8-fold increase in catecholamine release (Fig. 2).

Discussion

Our human adrenal preparations under basal conditions secrete much more cortisol than ALDO; this expected finding can be reasonably ascribed to the minor mass of zona glomerulosa tissue compared to that of zona fasciculata contained in the adrenal fragments. Our preparations display clear-cut glucocorticoid and mineralocorticoid responses to ACTH, demonstrating a well preserved steroidogenic enzymatic machinery. ACTH is known to acutely enhance the activity of 11β-hydroxylase, 17α-hydroxylase, and ALDO synthase (for review, see Refs. 25 and 26); accordingly, not only is the production of ALDO and cortisol increased by ACTH, but so is that of B and 18OH-B, the main precursors of ALDO in humans (for review, see Refs. 25 and 26), whereas the release of progesterone is markedly decreased, probably due to its increased utilization in steroidogenesis.

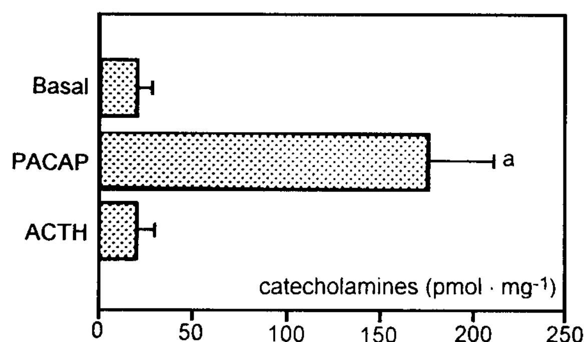

FIG. 2. Effects of 10 nmol/L PACAP and 10 nmol/L ACTH on catecholamine release by human adrenal slices including medullary chromaffin tissue. *Bars* are the mean ± SD (n = 6). a, $P < 0.01$.

In keeping with the results of earlier studies (14), PACAP was found to stimulate ALDO and 18OH-B release by human adrenal slices. As PACAP, in contrast to ACTH, does not significantly change B release, it seems reasonable to suggest that this peptide specifically enhances the activity of ALDO synthase (*i.e.* the conversion of B to ALDO via 18OH-B). This could be possible in humans, whose adrenals, in contrast to those in the bovine, possess distinct genes coding for 11β-hydroxylase and ALDO synthase (for review, see Ref. 27). Preliminary results confirm that PACAP as well as noradrenaline (see below) specifically stimulate the late step of ALDO synthesis in humans.

However, a direct action of PACAP on adrenocortical cells can be ruled out, inasmuch as this peptide does not exert any ALDO secretagogue action on dispersed cell preparations. Thus, an indirect effect mediated by an intraadrenal factor(s) is postulated. Compelling evidence indicates that catecholamines are able to stimulate steroidogenesis, as zona glomerulosa ALDO secretion is their main target in rodents and bovines (21, 28–30). Our present findings with β-adrenoceptor agonists indicate that this also occurs in humans. Previously, we demonstrated that in rats, the adrenocortical secretagogue effect of PACAP is not direct, but probably involves medullary chromaffin cells (14). As shown for vasoactive intestinal polypeptide (31–33) and neuropeptide Y (33), it has been suggested that PACAP, which parenthetically possesses a high degree of similarity with VIP (6, 7), enhances the release of catecholamines that, in turn, elicit the secretion of ALDO by zona glomerulosa cells.

The hypothesis that in humans, as in rats, the mechanism underlying the ALDO secretagogue action of PACAP involves an increased release of catecholamines is supported by the following lines of evidence: 1) *l*-alprenolol, a specific β_1-receptor antagonist, completely suppresses the ALDO response of human adrenal slices not only to isoprenaline and noradrenaline, but also to PACAP; and 2) PACAP, but not ACTH, elicits a clear-cut catecholamine release by adrenal head slices including medulla, a preliminary finding in keeping with those reported in the rat, pig, and bovine (9, 34, 35). It must be noted that taken together, our results are consistent with the demonstration that PACAP receptors are present in medullary chromaffin, but not in adrenocortical cells (36, 37).

Before concluding, it remains to be discussed why PACAP does not increase glucocorticoid release by human adrenals, as it does in frogs (12, 13), rats (14), and calves (15). As our preparations display normal basal and ACTH-stimulated glucocorticoid secretion, a lesion of the 17α-hydroxylase pathway of steroidogenesis can be excluded. The lack of specific PACAP receptors in adrenocortical cells indicates that the glucocorticoid response to this peptide (like the mineralocorticoid one) is indirect. In fact, in calves, it seems

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to depend on an increased blood flow in adrenals (15), and in the rat, it appears to be mediated by the intraadrenal CRH/ACTH system (14), which is located in chromaffin cells (38–41). Parenthetically, PACAP has been reported to evoke a sizable release of CRH by calf adrenals (15). Our findings may indicate that either 1) the CRH-ACTH system is not operative in the human adrenal medulla or in chromaffin cell islets scattered in the cortical tissue (5); or 2) human medullary chromaffin cells possessing CRH/ACTH-releasing activity are not sensitive to PACAP.

The importance of the indirect mineralocorticoid effect of PACAP in the regulation of human adrenal function under normal and pathological conditions remains to be ascertained. However, we wish to propose a few considerations that could provide the basis for future investigations. As mentioned in the introduction, PACAP is present in high concentrations in some human pheochromocytomas, where it probably enhances catecholamine release (16). Hence, by taking into account the possibility of paracrine control of the human adrenal cortex by chromaffin tissue, our findings could acquire relevance in explaining the pathophysiological background of some clinical cases of Conn adenomas (42, 43) or primary aldosteronism (44) associated with catecholamine-secreting pheochromocytomas.

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