

## ACTH-Induced Cortisol Secretion Is Mediated by cAMP and PKC in Various Adrenocortical Adenomas

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**Abstract.** We examined the role of PKC in cortisol secretion from adrenocortical adenomas. Isolated cells were prepared from aldosterone producing adenoma (APA, n=5), APA complicated with pre-clinical Cushing's syndrome (APA+PC, n=1), PC (n=2), and cortisol producing adenoma (CPA, n=5). They were stimulated with 100 nM ACTH, 1  $\mu$ M forskolin (FS), 1  $\mu$ M tetradecanoyl phorbol 13-acetate (TPA), and 100 nM angiotensin II (AngII). ACTH was most potent to secrete cortisol. FS also stimulated cortisol secretion, but did less-potently. TPA and AngII also stimulated cortisol secretion significantly in cells from CPA. Furthermore, ACTH- and TPA-induced PKC $\alpha$  and  $\beta$  translocations from cytosol to membrane were observed in adenoma cells from APA+PC, PC, and CPA. In conclusion, it was suggested that ACTH-induced cortisol secretion may be mediated by both PKC and protein kinase A in adrenocortical adenomas, and that PKC-mediated signal transduction may be involved in ACTH-induced cortisol secretion in CPA.

**Key words:** Cortisol secretion, ACTH, Phorbol ester, PKC, Adrenocortical adenoma

(Endocrine Journal 44: 661–670, 1997)

IT IS known that 12-O-tetradecanoyl phorbol-13-acetate (TPA) alone, an activator of protein kinase C (PKC) [1], affects the enzymes required for steroid synthesis in adrenal cortical cells [2–4]. It has been shown that angiotensin II (AngII) stimulates adrenocortical glomerulosa cells that secrete aldosterone [5]. In fact, the AngII-induced aldosterone secretion has been demonstrated to accompany hydrolysis of phosphoinositides [6] and translocation of PKC from cytosol to membrane in glomerulosa cells [7].

On the other hand, ACTH causes secretion of cortisol from adrenocortical fasciculata cells and aldosterone from glomerulosa cells [8]. In general, ACTH action has been considered to be mediated

via cyclic AMP [9], but some other mechanisms are thought to be involved in the promotion by ACTH of steroidogenesis. One possibility is that ACTH induces hydrolysis of polyphosphoinositides and *de novo* synthesis of diacylglycerol (DG) production [10] which is activating PKC. Another possibility is that ACTH induces Ca<sup>2+</sup> influx via an increase in intracellular cyclic AMP [11]. These results suggest that PKC may act as a second messenger of ACTH.

In this study, we examined *in vitro* cortisol secretion stimulated with ACTH, TPA and forskolin (FS) in resected adrenocortical adenomas and the adjacent adrenal tissues from patients with various adrenocortical adenomas to clarify the mechanism by which ACTH induces cortisol secretion. In some cases, we also examined ACTH- or TPA-induced PKC $\alpha$  and  $\beta$  translocation from cytosol to membrane in isolated cortisol producing adenomas.

Received: March 13, 1997

Accepted: July 8, 1997

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## Materials and Methods

### Subjects

Five patients with aldosterone producing adenoma (APA), one APA complicated with pre-clinical Cushing's syndrome (PC) (APA+PC), 2 PC due to adrenocortical adenoma, and 5 cases of Cushing's syndrome due to cortisol producing adenoma (CPA) were diagnosed clinically as previously described [12–15]. Diagnostic criteria for PC were as follows: no Cushingoid appearance, absence of cortisol suppression after 1 mg overnight dexamethasone suppression test, no circadian rhythm of plasma cortisol, scintigraphic ( $^{131}\text{I}$ -iodocholesterol) appearance of the unilateral uptake with contralateral suppression. Especially, in the case of APA complicated with PC, plasma cortisol levels were not suppressed by dexamethasone and there was no circadian rhythm of plasma cortisol, although laboratory data, clinical manifestations and pathological findings corresponded to typical APA. In 4 of 5 patients with APA, 1 mg dexamethasone suppression test was performed as shown in Table 1. The main clinical and hormonal characteristics of these patients are shown in Table 1.

### Materials

Collagenase (Type V), 12-O-tetradecanoyl phorbol-13-acetate (TPA), forskolin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). AngII was donated by Ciba Geigy Pharmaceutical Co. (Takarazuka, Japan). ACTH (Cortrosyn) was from Daiichi Seiyaku Co. (Tokyo, Japan).

### Methods

Cell preparation: Resected tumors were characterized histologically as adenomas. Resected adenoma and adjacent adrenal tissue were immersed in ice-cold Krebs-Ringer phosphate (KRP) buffer equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and then cut into small pieces. The pieces were treated with 5 mg/ml collagenase and 0.01% soybean trypsin inhibitor at 37 °C for 20 min in KRP buffer and passed through silk mesh, then washed three times with glucose-free KRP buffer containing 1% BSA as described previously [16, 17]. Isolated cells were resuspended ( $4\text{--}8 \times 10^6/\text{ml}$ ) in glucose-free KRP buffer, and divided into plastic tubes, in order to estimate the time course

**Table 1.** Clinical and hormonal characteristics of the patients

Case	Age	Sex	F	F'	UF	Ald	ACTH	Disease	Tumor size (cm)
1	52	F	23.7	1.3	120	446.1	32.7	APA	$1.6 \times 1.3 \times 1.2$
2	70	F	13.6	2.4	41	184.4	18.5	APA	$0.8 \times 1.0 \times 1.2$
3	44	F	13.7	1.7	83	955.8	16.8	APA	$1.8 \times 2.0 \times 2.0$
4	58	F	9.7	1.4	74	207.7	15.5	APA	$1.6 \times 1.6 \times 1.2$
5	59	F	9.8	/	70	440.0	29.0	APA	$1.6 \times 1.5 \times 0.8$
6	46	F	12.6	11.7	134	982.0	11.2	APA+PC	$2.6 \times 1.8 \times 2.0$
7	54	F	8.6	7.2	112	65.7	19.3	PC	$1.7 \times 1.7 \times 1.7$
8	36	F	8.3	8.3	229	45.0	12.5	PC	$2.2 \times 1.6 \times 1.5$
9	55	M	21.7	ns	231	25.0	4.7	CPA	$2.8 \times 2.8 \times 2.5$
10	52	F	20.3	ns	651	25.0	4.0	CPA	$2.3 \times 2.3 \times 1.5$
11	27	F	25.0	ns	463	29.2	4.0	CPA	$3.0 \times 2.0 \times 2.0$
12	28	F	26.8	ns	703	26.9	4.0	CPA	$3.0 \times 2.1 \times 3.2$
13	47	F	22.0	ns	410	25.0	5.0	CPA	$7.0 \times 4.0 \times 3.0$

F, plasma cortisol (6–16  $\mu\text{g}/\text{dl}$ ); F', plasma cortisol after 1 mg dexamethasone suppression test. ns, non-suppressible after 1 and 8 mg dexamethasone suppression test. UF, urine cortisol (<120  $\mu\text{g}/\text{day}$ ); ACTH (10–50  $\text{pg}/\text{ml}$ ); Ald, plasma aldosterone (40–140  $\text{pg}/\text{ml}$ ). Values in parentheses show normal range. CPA, Cortisol producing adenoma; APA, Aldosterone producing adenoma; PC, Pre-clinical Cushing's syndrome; APA+PC, APA complicated with PC.

of cortisol secretion and PKC immunoreactivity. The viability of the cells was approximately 80–85% as assessed by trypan blue exclusion.

Cortisol secretion from isolated adenoma cells: Following incubation at 37 °C for 30 min, cells were stimulated with 100 nM ACTH, 1  $\mu$ M TPA, or 1  $\mu$ M FS and taken at different times as described in the figure legend. The reaction was terminated by the addition of ice-cold KRP buffer. To determine the cortisol secretion, each tube was centrifuged at 2000 rpm for 10 min and the cortisol concentration in the supernatant was measured by RIA (Gamma Coat Cortisol Kit, Incstar Corporation, Stillwater, MN).

Immunoblot analysis of PKC: Other cells were washed with ice-cold homogenizing buffer (20 mM Tris/HCl, pH 7.5, 2 mM EGTA, 0.25 M sucrose, 20 mM 2-mercaptoethanol, 0.1 mM PMSF, 20  $\mu$ g/ml leupeptin) and homogenized. The homogenate was centrifuged at  $105,000 \times g$  for 60 min and the supernatant was used as the cytosolic fraction. The pellet was incubated and sonicated in ice-cold homogenizing buffer containing 1% Triton X-100, then centrifuged at  $105,000 \times g$  for 60 min to obtain the supernatant (solubilized membrane fraction). The protein concentration in each fraction was determined by the method of Bradford [18] with bovine serum albumin (BSA) as the standard. Immunoreactive PKC was measured as follows. Cytosolic (40  $\mu$ g) and membrane protein (30  $\mu$ g) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated first with anti-PKC $\alpha$  or PKC $\beta$  antiserum (GIBCO Co., NY) at 4 °C for 3–7 days. Subsequently, the proteins were incubated with anti-rabbit  $\gamma$ -globulin complexed to alkaline phosphatase (Sigma Co., St Louis, MO) at room temperature for 60 min. Finally, development was performed with 25 mg o-tetrazotized dianisidine, and 25 mg  $\beta$ -naphthyl acid phosphate per 100 ml sodium borate buffer (120 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 9.7 mM MgSO<sub>4</sub>) [19]. In some cases immunoblot analysis was performed with the ECL system (Amersham, Little Chalfont, UK) after incubation with the first antibody. Immunoreactive PKC $\beta$  recognized at 80 kDa was quantitated by densitometric scanning.

## Results

### *Cortisol secretion in aldosterone producing adenomas (APA) and adjacent adrenal tissues*

ACTH increased cortisol secretion to 600% of the basal level at 30 min in adenoma cells of APA (Fig. 1, upper panel). Forskolin (FS) also increased cortisol secretion to 500%, and AngII and TPA

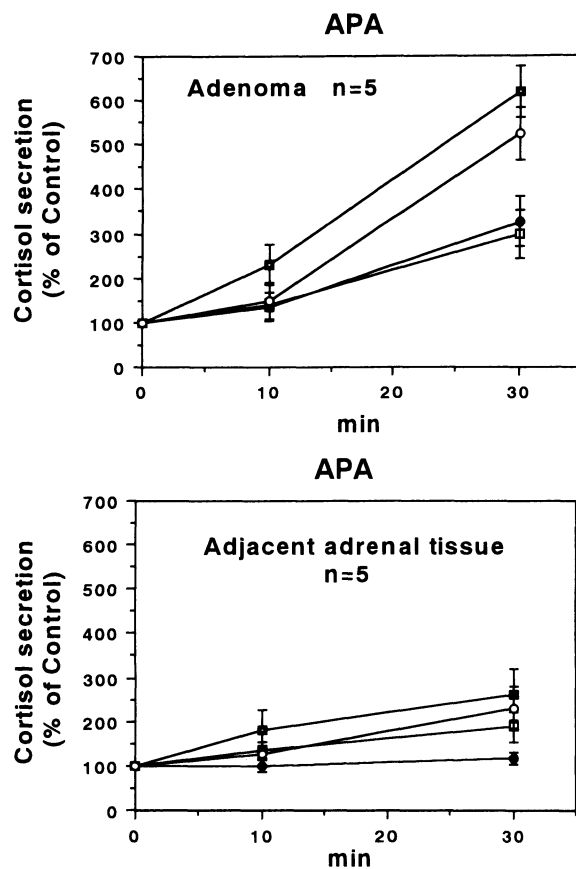


Fig. 1. Cortisol secretion stimulated by ACTH, forskolin (FS), angiotensin II (AngII) or TPA in five aldosterone producing adenomas and adjacent adrenal tissues. Isolated adrenal cells from adenoma tissue (upper panel) and adjacent adrenal tissue (lower panel) were stimulated with 100 nM ACTH (■), 1  $\mu$ M FS (○), 100 nM AngII (●), or 1  $\mu$ M TPA (□) for 0, 10 and 30 min after preincubation for 30 min. Cortisol secretion was expressed as the mean  $\pm$  SEM of percentage increases from the basal level (100%) in five cases. The basal cortisol values in adenoma and adjacent adrenal cells were  $2.0 \pm 1.3$  and  $4.2 \pm 2.0$   $\mu$ g/ $10^6$  cells, respectively.

(1  $\mu$ M) raised secretion to approximately 290% (Fig. 1, upper panel). These results clearly show that cortisol secretion was observed in APA. On the other hand, ACTH, FS or TPA increased cortisol secretion to approximately 200% from the basal level in adjacent adrenal tissues, but AngII did not stimulate cortisol secretion in adjacent adrenal tissues (Fig. 1, lower panel).

*Cortisol secretion in APA complicated with pre-clinical Cushing's syndrome (APA+PC)*

In adenoma cells, ACTH-induced cortisol secretion was maximal among agonists studied (Fig. 2, upper panel). TPA induced minimal cortisol secretion to only 125% of the basal level (Fig. 2, lower panel). In adjacent adrenal cells, agonist-induced cortisol secretion was noticeably lower than in adenoma cells. ACTH and AngII stimulated cortisol secretion, but TPA did not (Fig. 2, lower panel).

*Cortisol secretion in pre-clinical Cushing's syndrome (PC)*

ACTH-induced cortisol secretion increased to 425% of the basal level at 30 min and 790% at 120 min in adenoma cells taken from cases 1 and 2, respectively (Fig. 3, upper and middle panels). FS-induced cortisol secretion increased to 280% in case 1 and to 725% in case 2. FS-induced cortisol secretion was higher than TPA-induced cortisol secretion (increases to 250% at 30 min in case 1 and 510% at 120 min in case 2). AngII-induced secretion in adenoma cells was compatible to TPA-induced production (Fig. 3, upper). Cortisol production in adjacent adrenal tissue was studied only in case 2. ACTH, FS and TPA stimulated cortisol secretion to 540%, 420% and 210% at 60 min from the basal level, respectively (Fig. 3, lower panel).

*Cortisol secretion in cortisol producing adenomas (CPA)*

ACTH, TPA, and AngII increased cortisol secretion to 700%, 550% and 500% from the basal level at 60 min, respectively. FS-induced increase in cortisol secretion was lower than the others, at a 300% increase from the basal level. These results

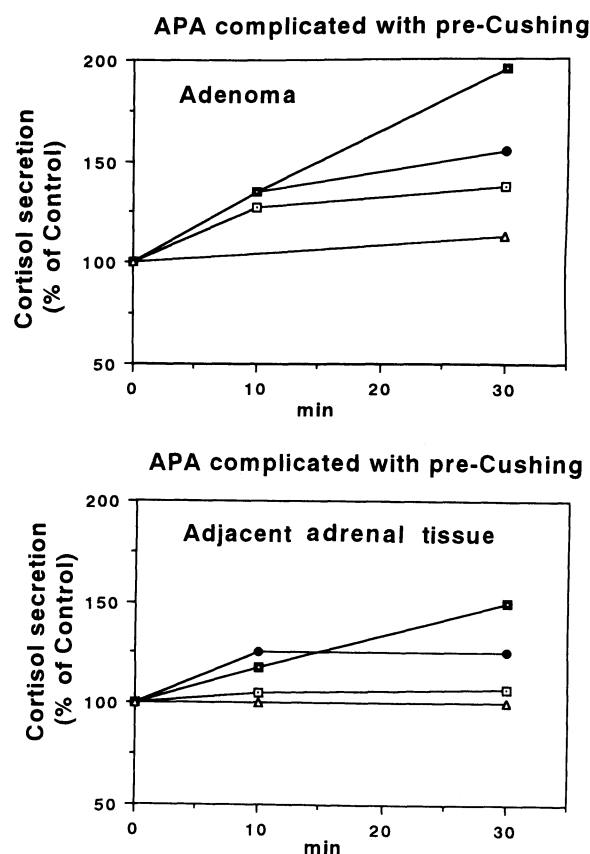


Fig. 2. ACTH-, Angiotensin II (AngII)-, or TPA-induced cortisol secretion in adenoma and adjacent adrenal tissue from a patient with aldosterone producing adenoma complicated with pre-clinical Cushing's syndrome. Isolated adrenal cells from adenoma tissue (upper panel) and adjacent adrenal tissue (lower-panel) were stimulated with 100 nM ACTH (■), 100 nM AngII (●), 1  $\mu$ M TPA (□) or vehicle (△) for 0, 10 and 30 min after preincubation with for 30 min. The basal cortisol values in adenoma and adjacent adrenal cells were 8 and 0.8  $\mu$ g/ $10^6$  cells, respectively.

show that ACTH was thus the most potent to stimulate secretion of cortisol in all of adrenocortical adenomas examined. TPA also significantly stimulated cortisol secretion in CPA, but less in APA, APA+PC and PC (Fig. 4).

*Agonist-induced PKC $\alpha$  translocation from cytosol to membrane in adenoma cells from the patients with APA + PC and PC*

Translocation of PKC $\alpha$  was studied in adenoma cells from APA+PC by using 1  $\mu$ M TPA, 100 nM

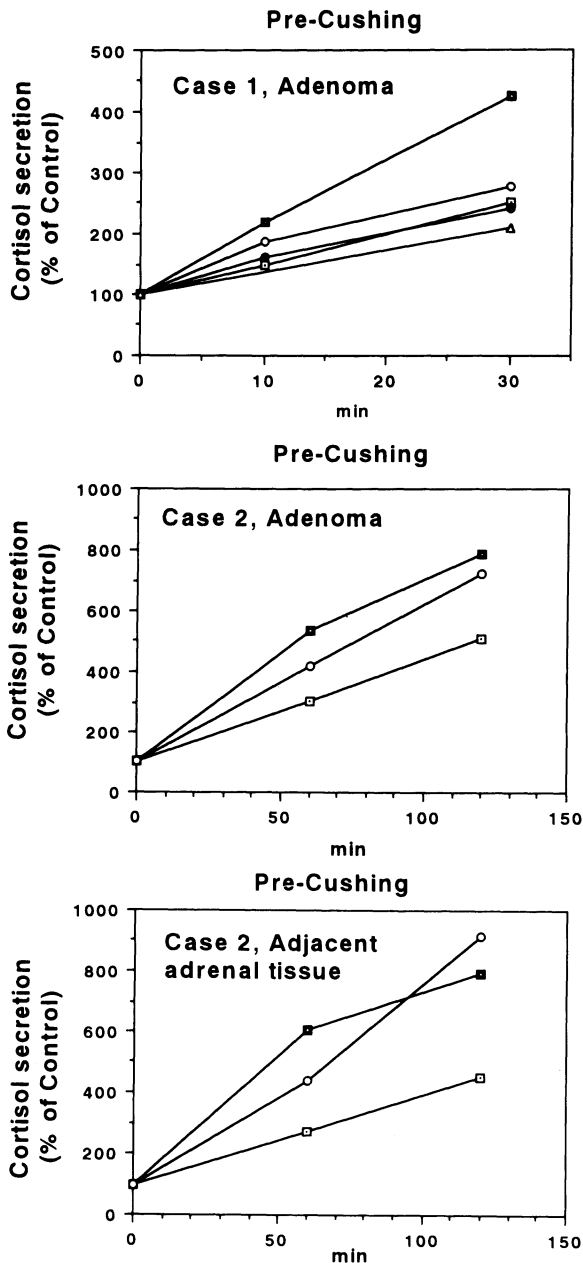


Fig. 3. Cortisol secretion in adenoma and adjacent adrenal tissue from 2 patients with pre-clinical Cushing's syndrome. Isolated cells from 2 adenoma tissues (upper and middle panels) and adjacent adrenal tissue (lower panel; adjacent adrenal tissue in case 1 could not be used because the tissue was atrophic) were stimulated with 100 nM ACTH (■), 1  $\mu$ M TPA (□) and 1  $\mu$ M forskolin (FS) (○), 100 nM AngII (●) or vehicle (△) for 0, 10, 30, 60 and 120 min after preincubation for 30 min. The basal cortisol values in adenoma and adjacent adrenal cells were 3.4 and 2  $\mu$ g/10<sup>6</sup> cells, respectively.

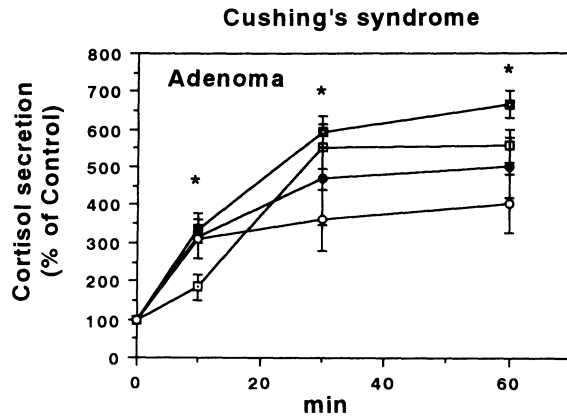
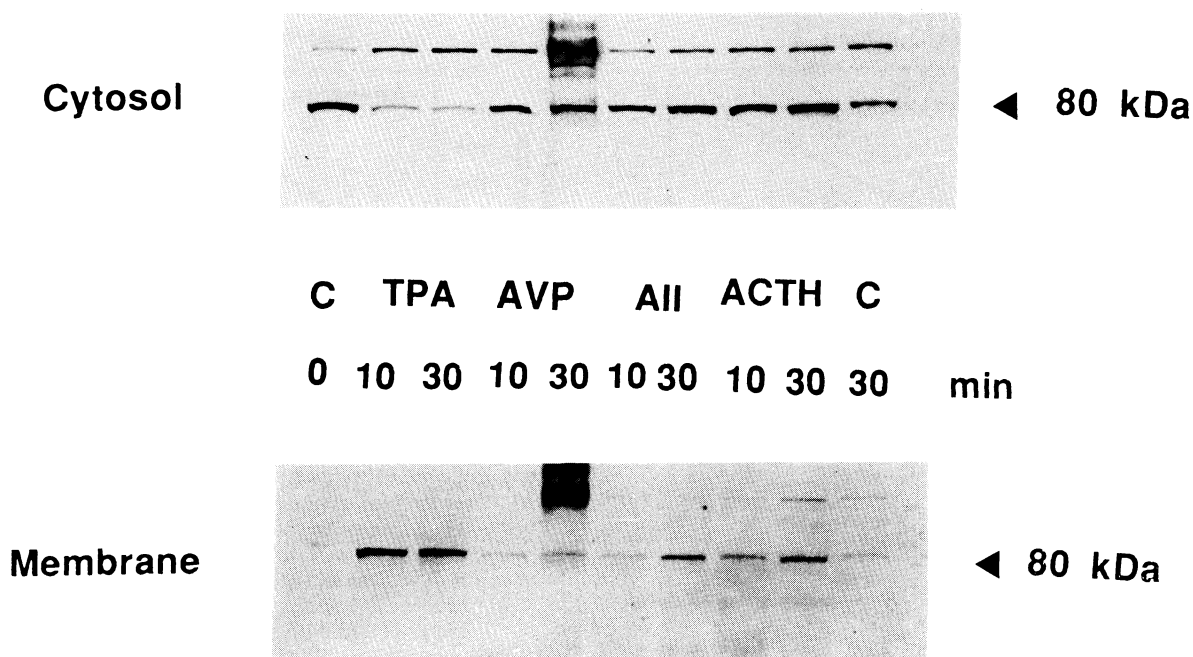


Fig. 4. Cortisol secretion in cortisol producing adenomas from 5 patients with Cushing's syndrome. Isolated adenoma cells were stimulated with 100 nM ACTH (■), 1  $\mu$ M TPA (□), angiotensin II (AngII) (●) or 1  $\mu$ M forskolin (FS) (○) for 0, 10, 30 and 60 min after pretreatment with for 30 min. Cortisol secretion was expressed as the mean  $\pm$  SEM of percentage increases from the basal level (100%) in five cases. The basal cortisol value in adenoma cells was  $15.8 \pm 6.0$   $\mu$ g/10<sup>6</sup> cells. \* $P < 0.05$  determined by standard *t*-test. ACTH-induced cortisol secretion for 10, 30 and 60 min vs. TPA-induced cortisol secretion for 10 min, FS-induced cortisol secretion for 30 and 60 min, respectively.

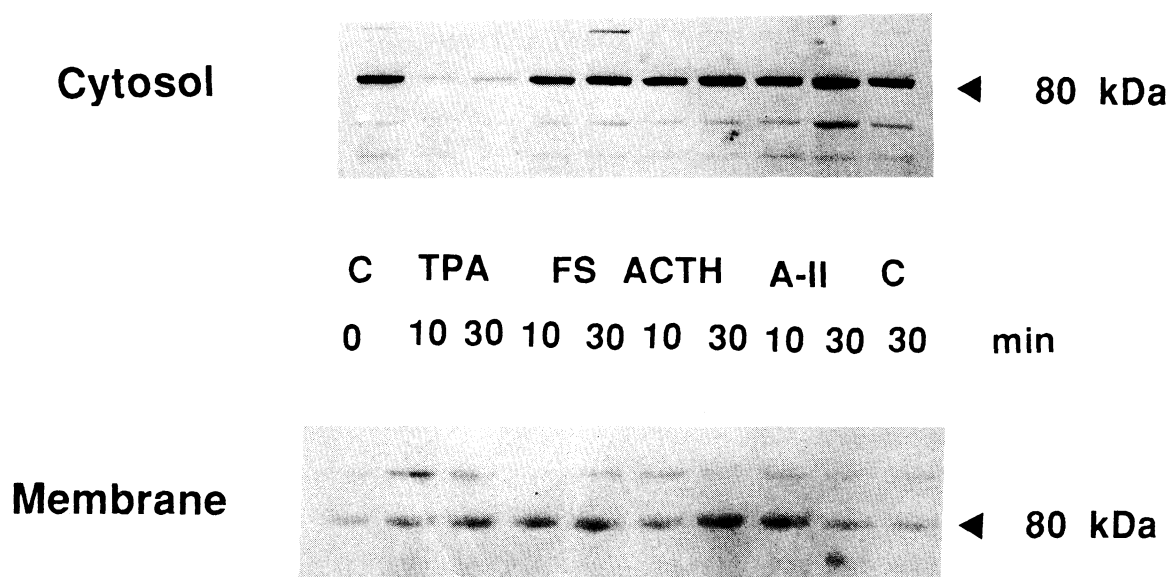
arginine-vasopressin (AVP), 100 nM AngII, and 100 nM ACTH (Fig. 5). Marked TPA-induced PKC $\alpha$  translocation was observed. AVP, AngII and ACTH also increased membrane-associated PKC $\alpha$  (Fig. 5). In adenoma cells from PC, TPA-induced translocation from cytosol to membrane was clearly observed (Fig. 6). Although the decrease in cytosolic PKC $\alpha$  under the stimulation with FS, ACTH or AngII was weak, an increase in membrane-associated PKC $\alpha$  by these ligands was observed (Fig. 6).

#### *Redistribution of PKC $\alpha$ and PKC $\beta$ by ACTH and TPA in adenoma cells from CPA*

Redistribution of PKC $\alpha$  and PKC $\beta$  was studied in adenoma cells from the patients with CPA (Fig. 7). In case 1 to 3 we observed TPA- and ACTH-induced of PKC $\alpha$  translocation as shown in Fig. 7-a. Translocation of PKC $\beta$  was clearly induced by TPA and ACTH in cases 2 and 3 (Fig. 7-b). In cases 4 and 5, membrane-associated PKC $\beta$



**Fig. 5.** Immunoblot analysis of PKC $\alpha$  translocation from cytosol to membrane by 1  $\mu$ M TPA, 100 nM ACTH, 100 nM AVP or 100 nM AngII in adenoma cells from a patient with aldosterone producing adenoma complicated with pre-clinical Cushing's syndrome. Isolated adenoma cells were stimulated with TPA, ACTH, AVP, or AngII for 0, 10 and 30 min. Each cytosolic (40  $\mu$ g) and membrane associated-protein (30  $\mu$ g) was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, subjected first to immunologic detection with PKC $\alpha$  antibody and second to the ECL system.



**Fig. 6.** Immunoblot analysis of PKC $\alpha$  translocation from cytosol to membrane by 1  $\mu$ M TPA, 100 nM ACTH, 100 nM AngII or 1  $\mu$ M forskolin (FS) for 0, 10 and 30 min in adenoma cells from a patient with pre-clinical Cushing's syndrome. Each cytosolic (40  $\mu$ g) and membrane-associated protein (30  $\mu$ g) was subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and subjected first to PKC $\alpha$  antibody and second to the ECL system.

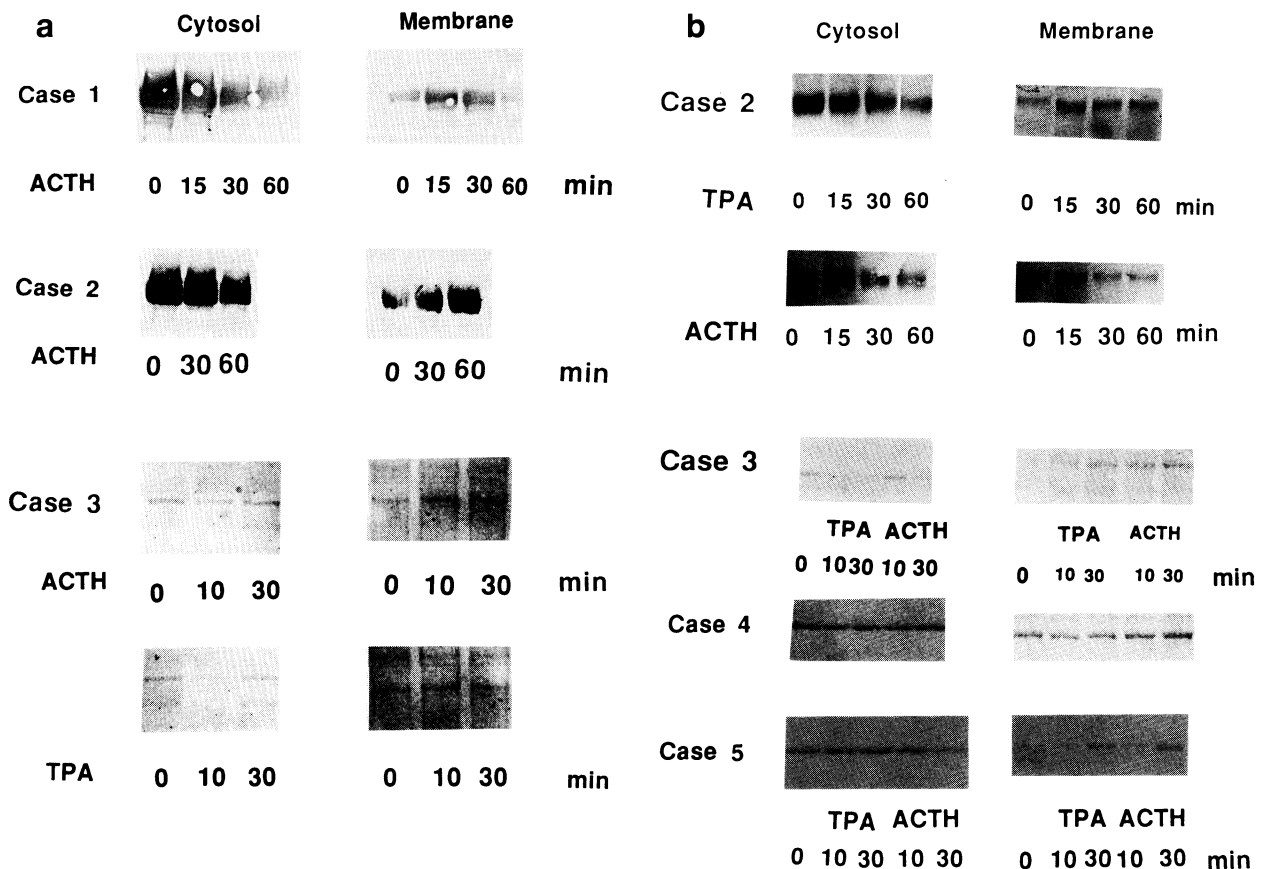


Fig. 7. Redistribution of PKC $\alpha$  (a) and  $\beta$  (b) from cytosol to membrane stimulated by 100 nM ACTH or 1  $\mu$ M TPA in cortisol producing adenoma cells from 5 patients with Cushing's syndrome. Isolated adenoma cells were stimulated with ACTH or TPA. The cytosolic and membrane-associated proteins were obtained and subjected to immunoblot analysis as indicated in Fig. 5 and 6 legends.

immunoreactivity increased in the presence of TPA and ACTH, although the changes in the cytosolic compartment were minimal. In summary, PKC $\alpha$  and  $\beta$  immunoreactivities were present in CPA, and ACTH and TPA induced redistribution of both isoforms.

### Discussion

ACTH stimulates cortisol production by fasciculata/reticularis cells through a stimulatory guanine nucleotide-binding protein (Gs)-coupled receptor which stimulates the cAMP-dependent pathway [20, 21]. AngII acts through an inhibitory guanine nucleotide-binding protein (Gi)-coupled receptor which inhibits adenyl cyclase, and also acts via stimulatory G protein regulator of

phospholipase C (Gq)-coupled AT $_1$  receptor which stimulates phospholipase C and PKC [22–24]. It has been reported that PKC activation and cortisol secretion are closely associated [3, 4]. TPA enhanced cortisol secretion in guinea pigs [4]. In addition, synthesis of dihydroepiandrosterone, androstenedione, and cortisol was stimulated by TPA, but ACTH-stimulated steroid synthesis was attenuated by TPA, with this attributable to a reduction in PKC [3]. We also reported previously that ACTH, TPA and AngII stimulated aldosterone secretion concomitant with translocation of PKC from cytosol to membrane in human aldosterone producing adenoma, and that in the adjacent fasciculata/reticularis cells of these aldosteronomas, TPA weakly stimulated redistribution of PKC and cortisol secretion [16]. Recently, cross-talk between phosphoinositides, cAMP, and

tyrosine kinase pathway was found in bovine adrenocortical fasciculata/reticularis cells [25], but it remains unclear to what extent cortisol secretion is correlated with phorbol ester-induced cortisol secretion, and whether ACTH-induced cortisol secretion is actually mediated through PKC activation and cAMP production.

Against this background, we examined ACTH, AngII, forskolin, and TPA-stimulated cortisol secretion in various kinds of cortisol producing adenomas in APA, APA+PC, PC and CPA. In these adenomas we also examined PKC $\alpha$  and  $\beta$  redistribution by various agonists. As indicated in Figs. 1, 2, 3 and 4, phorbol ester increased cortisol secretion to 290%, 125%, 250% and 550% from the basal level at 30 min, in the cases of APA, APA+PC, PC and CPA, respectively. As indicated in Figs. 5, 6 and 7, ACTH caused a decrease in cytosolic PKC $\alpha$  and  $\beta$  and an increase in membrane-associated PKC $\alpha$  and  $\beta$  in APA+PC, PC and CPA. These results suggest that TPA-induced cortisol secretion in CPA is highest among adenomas from APA, APA+PC and CPA. Furthermore, participation of PKC activation was proportionally enhanced concomitant with the increases in urinary and plasma cortisol levels in ACTH-induced cortisol secretion (data not shown). These findings indicate that PKC translocation is a prerequisite step for ACTH-induced signal transduction in cortisol producing adenoma. As indicated in Fig. 6, FS also stimulated redistribution of PKC $\alpha$  isoform. Probably, this is due to the Ca<sup>++</sup> influx caused by cAMP as previously indicated [11], but the true mechanism is still controversial.

On the other hand, our data also showed that the ACTH-stimulated cortisol secretion was not different from FS-stimulated cortisol secretion in APA and adrenal tissue adjacent to APA. Moreover, the cortisol secretion profile caused by ACTH and forskolin in the adenoma was similar to that in adrenal tissue adjacent to PC as indicated in Fig. 3, middle and lower panels. Previously, dexamethasone-nonsuppressible cortisol secretion in two cases with APA has been reported to be like a case of APA + PC as indicated in Fig. 2 [19]. In those cases, P450c17 m-RNA expression was

increased in tumor tissue, but no *in vitro* secretion study has been performed. These results also indicate that ACTH-induced cortisol secretion is mediated by a cAMP-dependent system in the case of normal cortisol secreting adrenocortical tissue like adjacent adrenal fasciculata/reticularis cells.

As indicated in Figs. 2, 3 and 4, AngII-induced cortisol secretion from adenomas in CPA and PC was observed to be like TPA. Recently, it was reported that AngII-induced cortisol secretion was mediated via AT<sub>1</sub> receptor which was coupled to phospholipase C [24]. Interestingly, AngII did not increase cortisol secretion from the adjacent adrenal tissue in APA, as indicated in Fig. 1. This may be due to the lack of AT<sub>1</sub> receptor expression in the adjacent adrenal tissue to APA.

In summary, first, phorbol ester-induced cortisol secretion in CPA was highest among the various grades of cortisol and aldosterone producing adenomas and adjacent adrenal tissues. Second, ACTH-induced PKC $\alpha$  and  $\beta$  translocation from cytosol to membrane in APA+PC, PC and CPA adenomas was observed. Third, ACTH-induced cortisol secretion may be mediated by PKC activator/translocation and a cAMP-dependent system in the case of various adrenocortical adenomas. Finally, PKC-mediated signal transduction may play an important role in ACTH-induced cortisol secretion from adrenocortical adenoma in CPA.

### Acknowledgments

The authors thank Dr. N. Yamakita, Matsunami General Hospital, Dr. H. Murase, Daiyukai General Hospital, Dr. T. Shimada, Hajima City Hospital, Dr. M. Ishimori, Gifu Red Cross Hospital for providing hormone producing adenomas and Miss M. Shinoda, Gifu University School of Medicine for technical assistance.

This work was partly supported by the grant for "Disorders of Adrenal Hormones" (Chairman: Professor H. Nawata) from the Ministry of Health and Welfare of Japan.



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