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# Involvement of the Cytoskeleton in the Mechanism of Action of Endothelin on Frog Adrenocortical Cells

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**In a previous report, we have shown that endothelin-1 (ET-1) is a potent stimulator of corticosterone and aldosterone secretion by frog adrenocortical cells. In the present study, we examine the possible involvement of cytoskeletal elements in the mechanism of action of ET-1 on corticosteroid secretion from frog adrenal gland. The microfilament disrupting agent cytochalasin B ($5 \times 10^{-5}$ M) induced a reversible inhibition of the spontaneous secretion of corticosteroid and blocked the response of adrenocortical cells to ET-1 ($5 \times 10^{-9}$ M). In contrast, the antimicrotubular agent vinblastine ($10^{-5}$ M) and the intermediate filament inhibitor β-β′ iminodipropionitrile ($10^{-3}$ M) had virtually no effect on both spontaneous and endothelin-induced steroidogenesis. Taken together, these results indicate that, in the frog adrenal gland, the integrity of the microfilament network is required for the corticotropic activity of ET-1 whereas microtubules and intermediate filaments are apparently not involved in the mechanism of action of ET-1.**

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## INTRODUCTION

The involvement of microtubules and microfilaments in the process of peptide hormone release is clearly established [1]. In contrast, proof of the involvement of the cytoskeleton in the secretory activity of steroid hormone-producing cells has proved more elusive. However, there is now increasing evidence that cytoskeletal elements play specific roles in the control of steroidogenesis.

Microfilaments appear to be involved in the transport of cholesterol to the mitochondria in adrenocortical cells [2] and/or in the mechanism of translocation of desoxycorticosterone from the endoplasmic reticulum to the mitochondria [3, 4]. In addition, the integrity of the microfilament network is required for the response of adrenocortical cells to ACTH [5, 6]. In fact, microfilaments appear to be involved in the mechanism of action of most corticotropic factors including angiotensin II, serotonin and vasoactive intestinal peptide [6, 7].

The antimicrotubular agent vinblastine does not impair the spontaneous secretion of corticosteroids by adrenocortical cells in human [5], rat [8], duck [9] and frog [10]. Concurrently, vinblastine has no effect on the steroidogenic response of frog adrenal gland to angiotensin II and acetylcholine [7]. In contrast, vinblastine inhibits ACTH-induced steroidogenesis in rat [3] and frog [10].

The intermediate filament disrupting agent β-β′iminodipropionitrile (IDPN) has no effect on the secretion of corticosteroids under basal conditions [5, 11, 12]. Studies on the involvement of intermediate filaments in the mechanism of action of ACTH have produced controversial results. In the Y-1 cell line, IDPN was found to enhance ACTH-induced steroidogenesis [13]. Conversely, it has been reported that IDPN inhibits the response of rat adrenocortical cells to ACTH [11], whereas in human and frog adrenals, IDPN does not affect the corticotropic action of ACTH [5, 12].

Recent studies indicate that the vasoconstrictor peptide endothelin is a potent stimulator of corticosteroid secretion [14–16]. In particular, endothelin-1 (ET-1) stimulates corticosterone and aldosterone secretion by the frog adrenal gland in a dose-dependent manner [17]. The aim of the present study was to investigate the possible involvement of cytoskeletal elements in the steroidogenic response of frog adrenal gland to ET-1.

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## EXPERIMENTAL

### *Animals*

Adult male frogs (*Rana ridibunda*) originating from Albania were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). The animals were maintained for at least 8 days in glass tanks with running water, in controlled conditions of temperature (8°C) and illumination (lights on from 06.00 to 18.00 h). Animal treatment was performed according to the recommendations of the French Ethics Committee and under the supervision of authorized investigators.

### *Secretagogues and reagents*

Synthetic ET-1 (human and porcine sequence), cytochalasin B and HEPES were purchased from Sigma (St Louis, MO, U.S.A.). Vinblastine was obtained from Eli-Lilly (St Cloud, France). IDPN was supplied by Eastman Kodak (Rochester, NY, U.S.A.). [1,2,6,7-$^3$H]Corticosterone and [1,2,6,7-$^3$H]aldosterone were purchased from Amersham Int. (Bucks., England).

### *Perifusion technique*

For each perifusion experiment, six animals were killed by decapitation, and the kidneys were quickly removed. The adrenal (interrenal) tissue was carefully dissected, freed of renal tissue, sliced with scissors, and preincubated in 5 ml Ringer's solution (100 mM NaCl, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 15 mM HEPES, 2 mg/ml glucose, and 0.3 mg/ml bovine serum albumin). The Ringer's solution was gassed with a 95% $O_2$–5% $CO_2$ mixture before use. The perifusion technique has been described previously [18]. Briefly, adrenal fragments were rinsed twice with 5 ml Ringer's solution, mixed with Bio-Gel P2 and transferred into siliconized glass columns delimited by Teflon roundels. The adrenal tissue was perifused with Ringer's solution at a constant flow rate (200 $\mu$l/min), with pH (7.35) and temperature (24°C) constant throughout the experiment. The effluent perifusate was collected as 5-min fractions, and the samples immediately frozen until assay. After an equilibration period of 2 h, the experimental procedure began. Secretagogues were dissolved in gassed Ringer's solution and infused into the columns at the same flow rate as Ringer's alone, by means of a multichannel peristaltic pump. Cytochalasin B was dissolved in ethanol, and the final dilutions made up in Ringer's solution so that ethanol concentrations were always lower than 0.25%. Previous studies have shown that ethanol concentrations up to 1% have no effect on corticosteroid production [19].

### *Corticosteroid radioimmunoassay*

Corticosterone and aldosterone concentrations were directly determined by RIA, without prior extraction, as previously described [20]. The assay methods were sensitive enough to detect 20 pg of corticosterone and 5 pg of aldosterone. The specificity of the antibodies has been previously evaluated by determining their cross-reactivities with 34 different steroids or related compounds [20]. None of the secretagogues interfered in the assay. For both assays, the intra- and interassay coefficients of variation were lower than 4 and 10%, respectively.

### *Calculations*

Each perifusion pattern represents the mean profile of corticosteroid output ($\pm$SEM) established over at least three independent experiments. The levels of corticosterone and aldosterone released were expressed as percentages of the basal values calculated as the mean of eight samples (40 min), collected at the beginning of the perifusion experiment.

## RESULTS

It has previously been shown that repeated pulses of ET-1 cause attenuation of the response of frog adrenal tissue to the peptide [17], in the light of this desensitization phenomenon a single pulse of ET-1 ($5 \times 10^{-9}$ M) was administered to perifused adrenal slices. In the absence of cytoskeleton disrupting agents, a 20-min infusion of ET-1 induced a significant increase in corticosterone and aldosterone secretion [47 and 45%, respectively; Fig. 1(A)]. Prolonged infusion of cytochalasin B ($5 \times 10^{-5}$ M) caused a marked inhibition of corticosterone and aldosterone output [$-84$ and $-86$%, respectively; Fig. 1(B)]. However, a rapid recovery of the initial corticosteroid secretion rate was observed after withdrawal of cytochalasin B. During prolonged exposure of adrenal tissue to the microfilament disrupting agent, the stimulatory effect of ET-1 ($5 \times 10^{-9}$ M) on corticosterone and aldosterone production was virtually abolished.

Figure 2 shows the effects of vinblastine, a microtubular disrupting agent and IDPN, an intermediate filament inhibitor on the response of frog adrenal gland to ET-1. In control conditions, infusion of ET-1 ($5 \times 10^{-9}$ M; 20 min) caused an increase in corticosterone (+59%) and aldosterone (+62%) secretion [Fig. 2(A)]. Addition of vinblastine ($10^{-5}$ M) to the perifusion medium during 6 h did not affect the response of interrenal slices to ET-1 [Fig. 2(B)]. As shown in Fig. 2(C), a 6 h administration of IDPN ($10^{-3}$ M) had no effect on both spontaneous and ET-1-evoked secretion of corticosterone and aldosterone.

## DISCUSSION

Previous studies have shown that the activity of the amphibian adrenal gland is regulated by multiple factors including peptide hormones and neurotransmitters. In particular, frog adrenocortical cells are stimulated by a number of vasoactive agents such as angiotensin II [21], arginine vasotocin [22] and serotonin [23]. We have recently shown that ET-1, a very

powerful vasoconstrictor peptide [24, 25], exerts a stimulatory effect on steroid release from frog adrenal tissue *in vitro* [17]. ET-1 was also found to enhance corticosteroid secretion in rabbit [14], rat, human [16] and bovine adrenocortical cells [15].

The present report describes the first investigation on the role of cytoskeletal elements in the mechanism of action of endothelin on adrenocortical cells. As previously observed [6, 26], the microfilament disrupting agent cytochalasin B induced a marked reduction of the spontaneous secretion of corticosterone and aldosterone. During the administration of cytochalasin B, the stimulatory effect of ET-1 was suppressed, indicating that microfilaments play a key role in the mechanism of action of ET-1. In fact, it has already been shown that cytochalasin B inhibits the action of various corticotropic factors including ACTH [6], angiotensin II, serotonin and acetylcholine [7]. Since the actions of these regulatory factors are mediated by different second messenger systems, it appears that microfilaments must play a crucial role in a late and common step of the steroid biosynthetic pathway. We have previously shown that the action of ET-1 on adrenocortical cells is mediated by generation of eicosanoids through the cyclooxygenase pathway [17]. We have also shown that the stimulatory action of prostaglandins on corticosteroid secretion is totally blocked by cytochalasin B [6]. Taken together, these data support the concept that microfilaments are involved in a late step of endothelin-induced stimulation of corticosteroidogenesis, beyond the activation of the arachidonic acid cascade.

The antimicrotubular agent vinblastine has been widely used to investigate the role of microtubules in steroid secretion by adrenocortical cells. The data reported so far indicates that microtubules are involved in the mechanism of action of corticotropic factors coupled to adenylate cyclase, such as ACTH and serotonin [7, 10]. In contrast, microtubules do not play

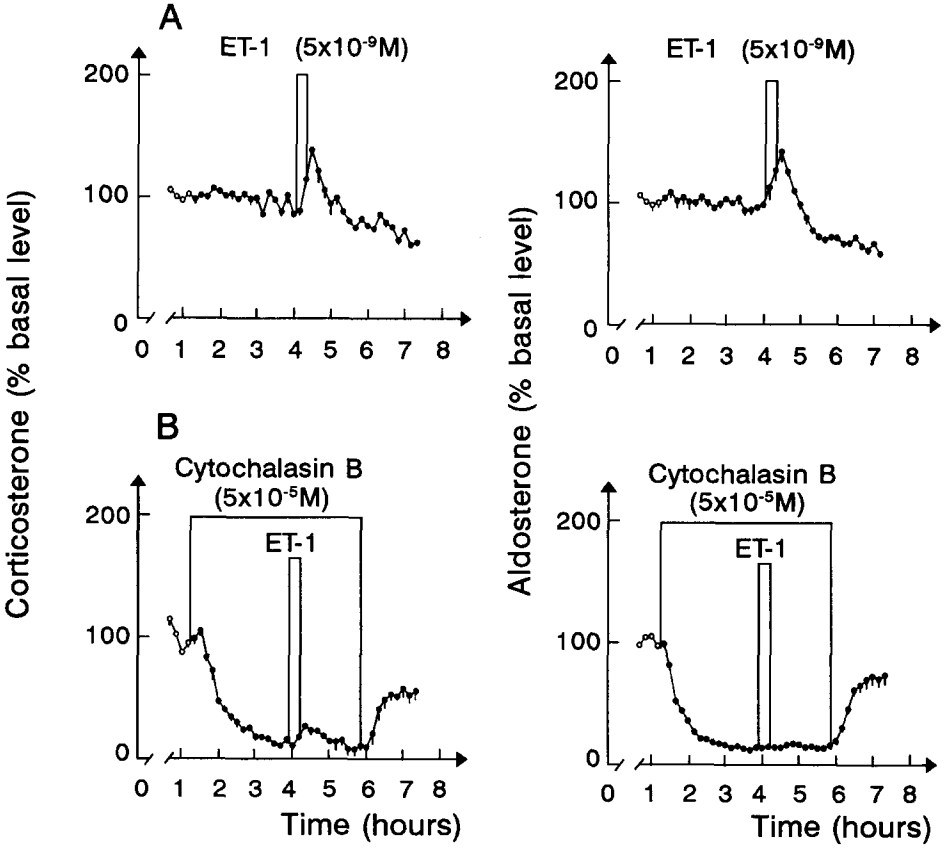


**Fig. 1. Effect of ET-1 alone or during prolonged infusion of cytochalasin B on corticosterone and aldosterone secretion by perifused frog adrenal slices. (A) Control experiment showing the effect of ET-1 ($5 \times 10^{-9}$ M; 20 min) on corticosteroid production. (B) Effect of ET-1 during prolonged administration of cytochalasin B on corticosteroid secretion. A single pulse of ET-1 ($5 \times 10^{-9}$ M; 20 min) was given 150 min after the beginning of cytochalasin B administration ($5 \times 10^{-5}$ M). The profiles represent the mean secretion pattern of three independent perifusion experiments. Each point is the mean corticosteroid production (expressed as a percentage of spontaneous steroid output) of two consecutive fractions collected during 5 min. The spontaneous level of steroid release (100% basal level) was calculated as the mean of eight consecutive fractions (40 min) collected at the beginning of the perifusion experiment (○—○). The mean secretion rates of corticosterone and aldosterone in these experiments were (A) $14.6 \pm 0.8$ and $5.8 \pm 1.0$ and (B) $21.5 \pm 2.1$ and $8.4 \pm 0.5$ pg/interrenal gland per min, respectively.**

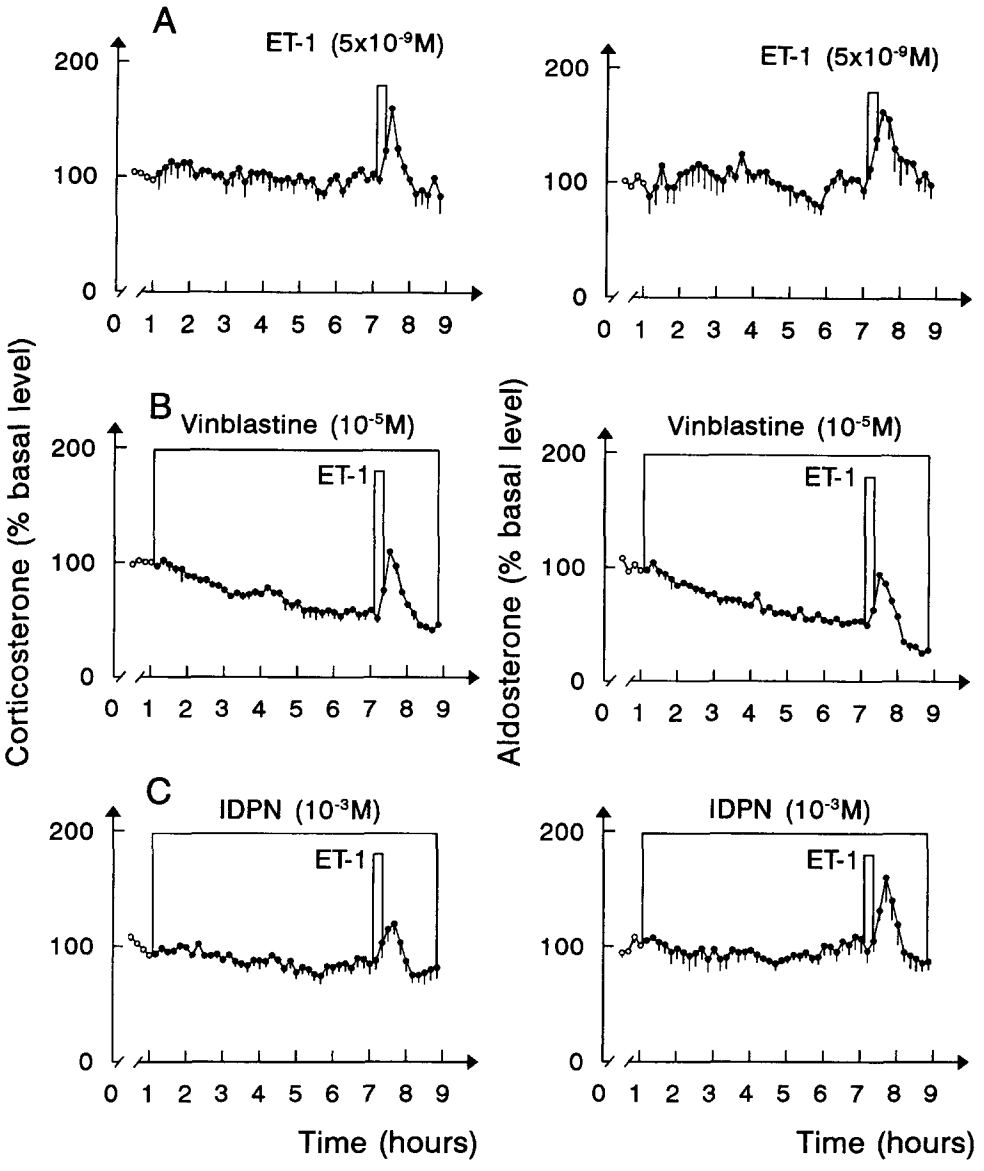


**Fig. 2. Effect of ET-1 alone or during prolonged infusion of vinblastine or IDPN on corticosterone and aldosterone secretion by perifused frog interrenal gland. (A) Control experiment showing the effect of ET-1 ($5 \times 10^{-9}$ M; 20 min) on corticosterone and aldosterone secretion. (B) Effect of ET-1 during prolonged infusion of vinblastine on corticosteroid secretion. Six hours after the beginning of vinblastine administration ($10^{-5}$ M), a 20-min pulse of ET-1 ($5 \times 10^{-9}$ M) was infused. (C) Effect of ET-1 during prolonged administration of IDPN on corticosteroid secretion. Six hours after the beginning of IDPN infusion ($10^{-3}$ M), a pulse of ET-1 ($5 \times 10^{-9}$ M; 20 min) was added. The mean secretion rates of corticosterone and aldosterone in these experiments were (A) $9.1 \pm 1.0$ and $2.0 \pm 0.2$, (B) $31.0 \pm 2.7$ and $24.5 \pm 3.3$ and (C) $21.3 \pm 3.3$ and $14.0 \pm 3.6$ pg/interrenal gland per min, respectively.**

a significant role in the spontaneous secretion of corticosteroids [7–10, 27] and do not influence the activity of corticotropic factors coupled to phospholipid metabolism [7]. Consistent with this notion, the present study shows that vinblastine does not impair the response of frog adrenal tissue to ET-1. The absence of effect of vinblastine on corticosteroidogenesis cannot be ascribed to the lack of activity of the drug since it has previously been shown that a 6-h perifusion with vinblastine causes total disruption of the microtubular network [7]. The fact that prostaglandin-induced steroidogenesis is not affected by vinblastine [28] supports the concept that microtubules are not involved in the stimulatory action of ET-1 on adrenocortical cells.

There is general agreement that intermediate filaments do not play a significant role in the basal secretion of corticosteroids in vertebrates [5, 11, 12]. However, it has been shown that the intermediate filament inhibitor IDPN causes a substantial reduction of angiotensin II-induced corticosteroid release [12]. Since the steroidogenic actions of angiotensin-II and ET-1 are both mediated through activation of

phospholipid metabolism, we have investigated the effect of IDPN on ET-1-stimulated corticosteroid secretion. The present results show that IDPN does not affect the response of adrenal tissue to ET-1, and thus indicate that intermediate filaments do not significantly contribute to the activation of adrenocortical cells by ET-1.

In conclusion, the present data show that, in the frog adrenal gland, the integrity of microfilaments is required for the response of adrenocortical cells to ET-1 stimulation. The results also indicate that microtubules and intermediate filaments are not involved in the corticotropic effect of ET-1.

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