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In Vitro Study of the Effect of Urotensin II on Corticosteroid Secretion in the Frog *Rana ridibunda*

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Urotensin II is a cyclic dodecapeptide that was originally isolated from the fish urophysis, the terminus of a neurosecretory system located in the caudal area of the spinal cord. We have recently isolated and characterized urotensin II in the brain of a tetrapod, the frog *Rana ridibunda*. Recent reports, suggesting that urotensin II may stimulate cortisol secretion in fish, prompted us to investigate the possible effects of fish and frog urotensin II on corticosteroid secretion in amphibians. Exposure of perifused frog adrenal slices to goby (*Gillichthys mirabilis*) urophysis extracts induced a marked stimulation of corticosterone and aldosterone secretion. In contrast, at concentrations ranging from 10^{-10} to 10^{-6} M, synthetic goby urotensin II had no effect on corticosteroid production. Similarly, infusion of synthetic frog urotensin II (10^{-10} to 10^{-6} M) did not modify the spontaneous release of corticosterone and aldosterone. In addition, frog urotensin II had no effect on ACTH- and angiotensin II-induced secretion of corticosteroids. These results show that in frog, urotensin II does not modulate spontaneous and ACTH- or angiotensin II-evoked adrenal steroidogenesis.

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INTRODUCTION

Teleosts and elasmobranchs possess, in the caudal portion of their spinal cord, a neurosecretory system analogous to the hypothalamo–neurohypophysial complex [1]. The neurohemal organ of this system, called urophysis, contains factors that, among other effects, activate the motility of the urinary bladder [2] and exert osmoregulatory actions [3]. Two families of biologically active peptides have been isolated from the fish urophysis: urotensins I, which are homologous to mammalian CRF [4] and urotensins II which exhibit structural similarity with somatostatin [5].

Urotensin II is a cyclic dodecapeptide that was first identified in the urophysis of the goby *Gillichthys mirabilis* [5]. Subsequently, the structure of urotensin II was established in several species of fish (reviewed in Conlon *et al.* [6]). Recently, we have characterized urotensin II in the brain of the frog *Rana ridibunda* [7], demonstrating for the first time that the peptide is present in tetrapods (Fig. 1).

Pharmacological studies indicate that fish urotensin II exerts a large spectrum of biological activities. In the goldfish, high affinity urotensin II-binding sites have been found in different organs including kidney, intestine and brain [8]. Concurrently, an effect of urotensin II on blood pressure [9, 10] and osmoregulation [3] has been reported in mammals. Several lines of evidence suggest that urotensin II could also be involved in the regulation of adrenocortical activity [8]. For instance, in teleost fish, the decrease in plasma cortisol observed during transfer from sea water to fresh water is associated with a reduction of urotensin II concentration in the urophysis [11]. In addition, it has recently been shown that urotensin II stimulates the production of 1α-hydroxycorticosterone in the dogfish [6].

In the present study, we have examined the effect of fish urophysial extracts, as well as synthetic urotensin II, on steroid release from the frog adrenal gland *in vitro*. We have also investigated the possible action of synthetic frog urotensin II on ACTH- and angiotensin II-induced corticosteroid secretion.

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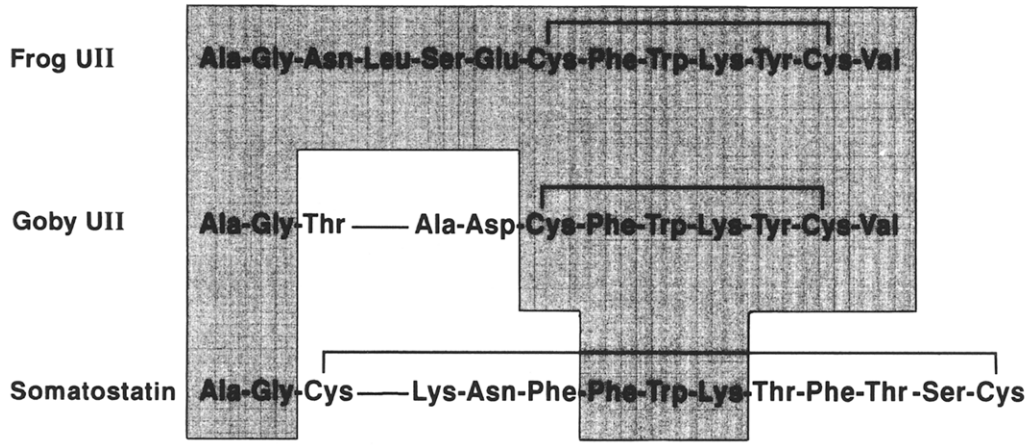

Fig. 1. A comparison of the primary structures of frog urotensin II, goby urotensin II and somatostatin. Shaded areas donate sequence identity.

EXPERIMENTAL

Animals

Adult male frogs (*Rana ridibunda*) weighing 30–40 g, were obtained from a commercial supplier (Couétard, Saint-Hilaire de Riez, France). To reduce stress from transport, the animals were kept for at least 1 week before use in glass tanks at constant temperature (8°C) and under artificial illumination (lights on: 06:00–20:00 h).

Secretagogs and reagents

Preparation of goby (*Gillichthys mirabilis*) urophysial extracts and determination of the urotensin II bioactivity of the samples were performed according to Pearson *et al.* [5]. Synthetic goby urotensin II was obtained from Sigma (St Louis, MO). Frog urotensin II was synthesized by Dr D. D. Smith as described previously [7]. Synthetic ACTH was kindly provided by Drs R. H. Andreatta and V. Rasetti (Ciba-Geigy, Basel, Switzerland). The angiotensin II analog [Sar^1-Val^5]-angiotensin II was a gift from Dr E. Escher (University of Sherbrooke, Québec, Canada). HEPES [*N*-(2-hydroxy-ethyl)piperazine-*N'*-(2 ethanesulfonic acid)] was purchased from Merck (Darmstadt, Germany).

Perifusion technique

Frogs were killed by decapitation between 08.00 and 09.00 h and the kidneys were quickly removed. The interrenal (adrenal) tissue was dissected free of renal parenchyma, sliced and preincubated at 24°C for 15 min in 5 ml Ringer's solution. The Ringer consisted of 15 mM HEPES buffer, 112 mM NaCl, 15 mM $NaHCO_3$, 2 mM $CaCl_2$ and 2 mM KCl and was supplemented with 2 mg glucose/ml and 0.3 mg bovine serum albumin (BSA)/ml. The incubation medium was continuously gassed with a 95% O_2–5% CO_2 mixture. The perifusion column was constructed according to Leboulenger *et al.* [12]. The tissue slices were layered between several beds of Bio-Gel P2 (Bio-Rad, Richmond, CA) into a perifusion chamber (0.8 cm^3) limited by two Teflon plungers (equivalent of 12 adrenal glands per chamber). The adrenal slices were perifused with Ringer at constant flow rate (200 μl/min), temperature (24°C) and pH (7.4). The column effluent was collected as 5-min fractions which were kept frozen

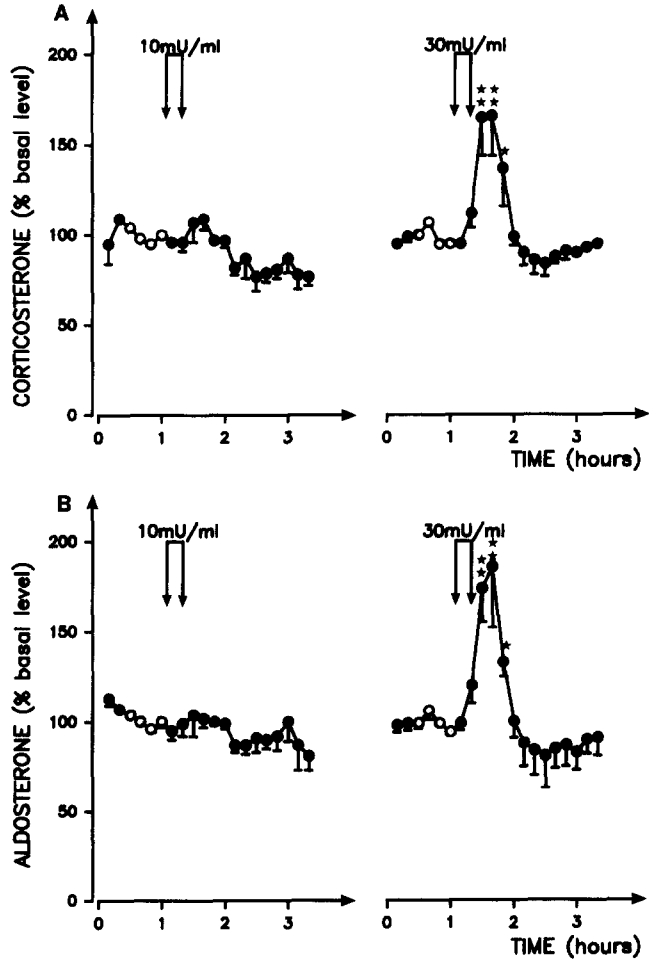

Fig. 2. Effect of crude goby urophysial extracts on corticosterone (A) and aldosterone (B) secretion from perifused frog adrenal slices. The goby urophysial extracts containing 10 or 30 mU/ml of bioactive urotensin II were infused for 20 min. The profiles represent the mean secretion pattern of 3 independent perifusion experiments. The spontaneous level of steroid production (100% basal level) was calculated as the mean of 8 samples just preceding the infusion of the secretagogue (○—○) $\star P < 0.05$; $\star\star P < 0.01$.

Table 1. Effect of goby or frog urotensin II on corticosterone and aldosterone secretion by perifused frog adrenal slices

	Corticosterone (% basal level ± SEM)	Aldosterone (% basal level ± SEM)
Control	100	100
Goby UII 10^{-10} M	91.2 ± 7.7	96.9 ± 4.7
Goby UII 10^{-9} M	92.8 ± 4.2	108.0 ± 17.6
Goby UII 10^{-8} M	100.1 ± 3.8	99.5 ± 13.5
Goby UII 10^{-7} M	94.8 ± 3.1	101.5 ± 4.2
Goby UII 10^{-6} M	105.0 ± 6.4	109.0 ± 4.2
Frog UII 10^{-10} M	92.2 ± 2.3	92.6 ± 2.4
Frog UII 10^{-9} M	94.9 ± 3.2	98.7 ± 6.8
Frog UII 10^{-8} M	96.2 ± 2.0	97.2 ± 1.3
Frog UII 10^{-7} M	97.3 ± 2.6	104.1 ± 5.4
Frog UII 10^{-6} M	93.2 ± 3.5	100.5 ± 8.4

The data were obtained from experiments similar to those presented in Fig. 2. Each dose of urotensin II was administered during 20 min. The control values (100%) represent the mean secretion rate during the 40-min period just preceding the administration of urotensin II. The values represent the mean secretion rates ± SEM calculated from 3 independent experiments.

until assay. The experimental procedure started after a 2-h stabilization period.

Corticosteroid radioimmunoassays

Corticosterone and aldosterone concentrations were determined by radioimmunoassays (RIA), without prior extraction, in 100–200 μl of effluent perifusate, as described previously [13, 14]. Direct measurement of corticosterone and aldosterone has been validated by RIA quantification of corticosteroids after high performance liquid chromatography analysis of the effluent perifusate [15]. The working ranges of the assays were 20–5000 pg for corticosterone and 5–640 pg for aldosterone. For both assays, the intra- and interassay coefficients of variation were <3 and 6%, respectively.

Calculations

Each perifusion pattern was calculated as the mean (± SEM) profile of corticosteroid release established in at least three independent experiments. The levels of corticosterone and aldosterone secretion were expressed as percentages of the basal values calculated as the mean of 8 samples (40 min), taken just before the administration of the first secretagogue.

For statistical analysis, stimulated values were calculated as the net area under the peaks (mean ± SEM) corresponding to 12 consecutive samples (1 h) starting 10 min after the addition of the secretagogue. Statistical significance between the basal and experimental values was examined using the Student's paired *t*-test.

RESULTS

Figure 2 shows that infusion of a goby urophysis extract corresponding to 10 mU/ml bioactive urotensin II did not modify the secretion of corticosterone and aldosterone by frog adrenal slices. Administration of a urophysis extract containing 30 mU/ml bioactive urotensin II induced a significant stimulation of corticosterone and aldosterone secretion (+66 and +86%, respectively). The lag period between the infusion of the extract and the beginning of the adrenal response was about 10 min. The maximum effect was observed 40 min after the onset of administration of the tissue extract. In contrast, exposure of perifused frog adrenal slices to graded concentrations of synthetic goby urotensin II (ranging from 10^{-10} to 10^{-6} M) did not affect the secretion of corticosterone and aldosterone (Table 1). Similarly, administration of increasing concentrations of synthetic frog urotensin II (10^{-10} to 10^{-6} M) had no effect on corticosteroid release.

The possible action of urotensin II on ACTH- and angiotensin II-evoked secretion of corticosteroids was investigated during long-term infusion (3.5 h) of frog urotensin II (10^{-7} M). During the experiments, the urotensin II solution was renewed every 30 min to overcome possible degradation. Figure 3 shows that frog urotensin II did not modify the kinetics and the response of interrenal slices to a brief administration of ACTH (10^{-9} M). Similary, frog urotensin II had no effect on angiotensin II-induced stimulation of corticosterone and aldosterone secretion (Fig. 4).

DISCUSSION

Various neuropeptides are capable of directly modulating the activity of the adrenal cortex [16, 17]. Especially, in amphibians, it has been demonstrated that pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal peptide, arginine vasotocin (AVT) and several tachykinins can stimulate corticosteroid secretion *in vitro* [18–21]. We have recently characterized urotensin II as a novel peptide present in the frog brain [7]. In fish, urotensin II is thought to play a role in the control of osmoregulation and cardiovascular activity [1]. In addition, recent data indicate that urophysial peptides stimulate cortisol secretion in the flounder *Platichthys flesus* [11] and in the goldfish

Carassius auratus [8]. These observations led us to investigate the possible effect of urotensin II on adrenal steroidogenesis in the frog *Rana ridibunda*.

In a pilot experiment, we found that extracts of goby urophysis induce a significant stimulation of corticosterone and aldosterone secretion from perifused frog adrenal tissue. This observation was regarded as a clue to the possible action of urotensin peptides on corticosteroid release. However, subsequent studies revealed that neither synthetic goby urotensin II nor synthetic frog urotensin II could mimic the stimulatory effect of goby urophysis extracts. Thus, further studies are required to characterize the active compound, contained in the fish urophysis, which is responsible for stimulation of steroidogenesis in frogs. Previous data indicate that various neuropeptides, including AVT,

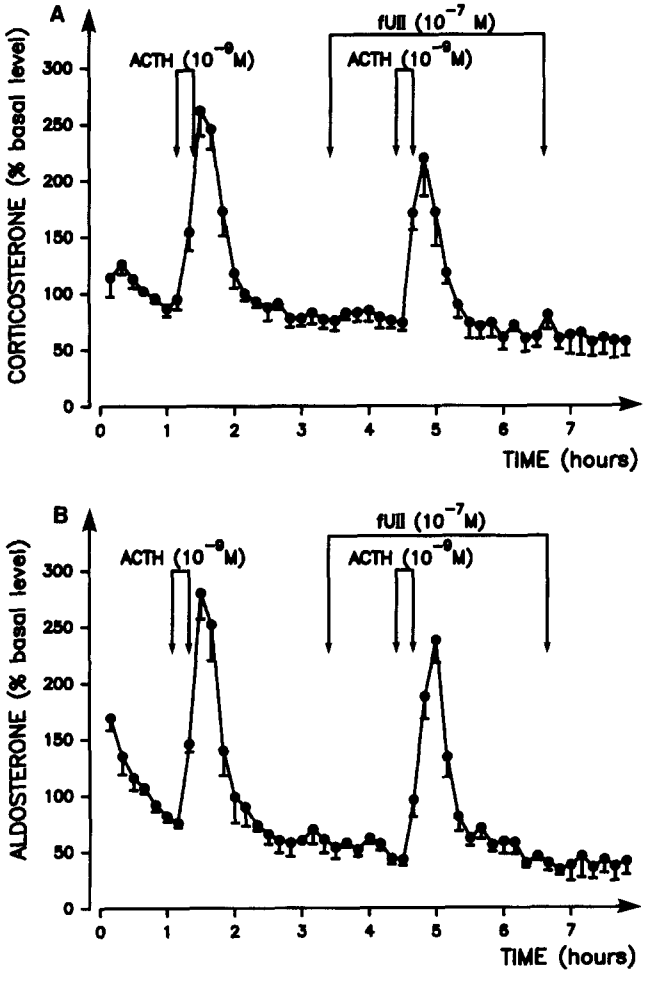

Fig. 3. Effect of ACTH alone or during prolonged administration of frog urotensin II on corticosterone (A) and aldosterone (B) secretion by perifused frog adrenal slices. After a 120-min equilibration period, a first pulse of ACTH (10^{-9} M) was administered for 20 min. The glands were allowed to stabilize for 90 min; frog urotensin II (10^{-7} M) was then infused for 3.5 h. One hour after the onset of urotensin II infusion, a second 20-min pulse of ACTH was administered. The profiles represent the mean secretion pattern of 4 independent perifusion experiments. The spontaneous level of steroid secretion (100% basal level) was calculated as the mean of 8 samples just preceding the infusion of the first dose of ACTH.

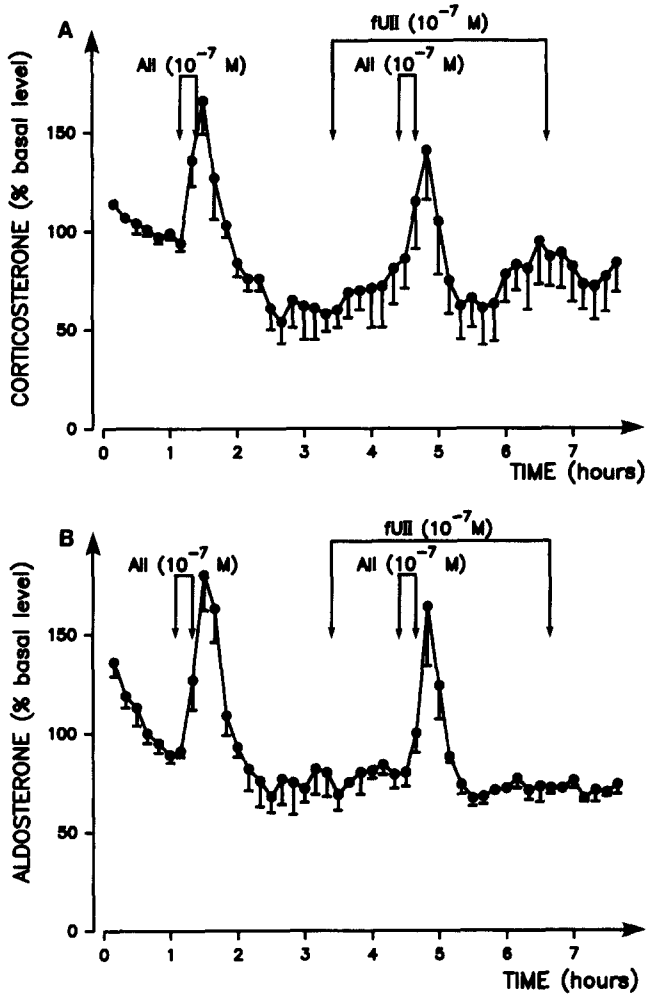

Fig. 4. Effect of the angiotensin II analog [Sar¹-Val⁵]-angiotensin II (10^{-7} M) alone or during prolonged administration of frog urotensin II (10^{-7} M) on corticosterone (A) and aldosterone (B) secretion by perifused frog interrenal slices. See legend to Fig. 2 for other designations.

are present in the urophysis of teleosts [22, 23]. It has also been shown that the caudal neurosecretory system of fish contains a high concentration of acetylcholine [24]. Since both AVT and acetylcholine are potent activators of adrenocortical cells in amphibians [25], the corticotropic activity of goby urophysis extracts might be attributed to one of these compounds. The urophysis also contains large amounts of urotensin I, a CRF-related peptide [26]. A direct effect of urotensin I on adrenocortical cells cannot be excluded, although we have previously observed that CRF does not affect corticosteroid secretion from the frog adrenal gland *in vitro* (unpublished data). Alternatively, the fish urophysis may contain novel neuropeptide(s) with corticotropic potency.

Urotensin II exhibits structural similarities with somatostatin (Fig. 1). Both urotensin II and somatostatin inhibit the release of prolactin from the rostral pars distalis of fish [27], suggesting that urotensin II may interact with somatostatin receptors. In this respect, the lack of effect of urotensin II on steroid secretion is consistent with previous studies which

showed that somatostatin does not modulate the release of corticosteroids in the frog [28].

We have previously shown that the atrial natriuretic peptide ANF, although having no direct effect on frog corticosteroidogenesis, can attenuate the response of the adrenal gland to other corticotropic factors [29]. In order to investigate whether urotensin II could exert a similar modulatory action, we have studied the effect of frog urotensin II on the response of frog adrenal slices to ACTH and angiotensin II. These two corticotropic peptides were chosen because they act through two distinct pathways: ACTH mainly stimulates the adenylate cyclase system [30], whereas the action of angiotensin II is essentially mediated through polyphosphoinositide hydrolysis [31] and prostaglandin formation [32]. The present data demonstrate that frog urotensin II does not affect the response of adrenal slices to ACTH and angiotensin II. Studies on structure–activity relationships of urotensin II-related peptides have shown that these molecules are sensitive to oxidation and that the integrity of the disulfide bridge is required for their biological activity [33, 34]. We have thus taken precautions to avoid chemical alteration of the peptide. During prolonged infusion of urotensin II, the peptide solution was renewed every 30 min. Also, in several experiments, the Ringer was supplemented with 0.02% ascorbic acid to avoid oxidation of the peptide. Therefore, the lack of effect of urotensin II cannot be ascribed to degradation of the peptide during the perifusion experiments.

Taken together, the present data indicate that, whereas urotensin II may play a role in adrenal steroidogenesis in fish, it has no effect on corticosteroid secretion in frog. Although the cyclic portion of urotensin II, which is thought to correspond to the biologically active region of the molecule, has been fully conserved during evolution [7, 33], the physiological role of urotensin II appears to have diverged during the emergence of terrestrial life.

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