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STRUCTURE–ACTIVITY RELATIONSHIPS OF MONOMERIC AND DIMERIC SYNTHETIC ACTH FRAGMENTS IN PERIFUSED FROG ADRENAL SLICES

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Summary—The effect of synthetic monomeric and dimeric ACTH fragments on spontaneous and ACTH(1–39)-evoked steroidogenesis in frog interrenal tissue was studied *in vitro*. Infusion of ACTH fragment 11–24 (10^{-6} M) or its dimeric conjugates, attached either by their *N*-terminal, Glu(11–24)$_2$, or their *C*-terminal amino acid, (11–24)$_2$Lys, had no effect on the spontaneous release of corticosteroids. The monomer ACTH(11–24) and the dimer Glu(11–24)$_2$ were also totally devoid of effect on the steroidogenic response to ACTH(1–39) (10^{-9} M). In contrast, the (11–24)$_2$Lys conjugate (10^{-6} M) significantly decreased ACTH-induced stimulation of corticosterone and aldosterone (−63 and −62%, respectively). The dimeric conjugate of the fragment ACTH(7–24), linked through the *C*-terminal ends, (7–24)$_2$Lys (10^{-6} M), was also completely devoid of effect on basal steroidogenesis but caused a marked decrease of ACTH-evoked corticosterone and aldosterone release (−72 and −80%, respectively). Conversely, infusion of the dimer (1–24)$_2$Lys gave rise to a dose-related stimulation of corticosterone and aldosterone release. The time-course of the steroidogenic response to the dimer was similar to that of ACTH(1–24). The 1–24 conjugate was 70 times less potent than the monomers ACTH(1–24) and ACTH(1–39).

These results suggest that amphibian adrenocortical cells contain only one class of ACTH receptor which recognizes the 11–24 domain of ACTH with an affinity which depends on the presence of a strong potentiator segment, located at the *N*-terminus end of ACTH(1–39). Since the ACTH-dimers are thought to induce cross-linking of the receptors, our results suggest that aggregation of ACTH receptors causes a down-regulation of the receptors.

INTRODUCTION

Although the secretion of adrenocortical hormones is regulated by peptide hormones [1–4], neurotransmitters [5–8] and electrolytes [1, 4, 9], there is clear evidence that ACTH plays a pivotal role in the control of corticosteroid secretion. Studies have been conducted to determine the structure–activity relationships of various ACTH fragments in mammalian adrenocortical cells [10–12]. Using various animal models, the sequence (1–24) of the ACTH molecule exhibited full biological activity, whereas the *C*-terminal (25–39) segment only exerted protective functions [13]. Within the sequence (1–24), the fragment (4–10) bears the corticotropic message, while the sequence (11–24) provides the affinity of the ACTH molecule for membrane receptors [13, 14].

Among non-mammalian vertebrates the sequence of ACTH was determined in birds [15, 16] and fish [17, 18]. In the toad *Xenopus laevis*, the primary structure of ACTH was deduced from the cDNA sequence of the precursor molecule pro-opiomelanocortin. The structure of α-MSH, corresponding to the *N*-terminal sequence of ACTH was remarkably well preserved during vertebrate evolution [19–21]. However, there is little information available concerning the structural requirement for ACTH fragments in lower vertebrates.

Covalent dimers of ACTH short-chain analogues have been used to explore the position of the binding segment of ACTH and to develop potent competitive antagonists of ACTH on mammalian adrenocortical cells [22, 23]. In the present study we have investigated the structure–activity relationships of ACTH analogues in amphibian adrenocortical cells, using several synthetic ACTH fragments, including four covalent dimeric conjugates.

EXPERIMENTAL

Secretagogues and reagents

Synthetic human ACTH(1–39) and ACTH(1–24) were kindly provided by Drs Scheibli and Andreatta

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Table 1. Chemical structure and abbreviation of the ACTH analogs

ACTH (1-39)	1——7——11——24——39 SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF
ACTH (1-24)	SYSMEHFRWGKPVGKKRRPVKVYP
ACTH $(1-24)_2$Lys	SYSMEHFRWGKPVGKKRRPVKVYP K-NH_2 SYSMEHFRWGKPVGKKRRPVKVYP
ACTH (7-24)	FRWGKPVGKKRRPVKVYP
ACTH $(7-24)_2$Lys	FRWGKPVGKKRRPVKVYP K-NH_2 FRWGKPVGKKRRPVKVYP
ACTH (11-24)	KPVGKKRRPVKVYP
ACTH $(11-24)_2$Lys	KPVGKKRRPVKVYP K-NH_2 KPVGKKRRPVKVYP
ACTH Glu$(11-24)_2$	KPVGKKRRPVKVYP Ac-E KPVGKKRRPVKVYP

(Ciba–Geigy, Basel, Switzerland). Dimeric ACTH(11–24), (7–24) and ACTH(1–24) fragments were synthesised using a conventional method of peptide synthesis [22, 23]. The structure of the different ACTH-related molecules used in the present study is shown in Table 1. HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was purchased from Merck (Darmstadt, F.R.G.). Bovine serum albumin (BSA; fraction V) was obtained from Boehringer–Mannheim (Meylan, France).

Animals

Adult male frogs (*Rana ridibunda*) of 40–50 g body weight, originating from Egypt, were purchased from a commercial supplier (Couétard, Saint-Hilaire de Riez, France). The animals were kept at least one week before use at constant temperature (8°C) under artificial illumination (light on: 06.00–20.00 h) in glass tanks continuously supplied with tapwater.

Perifusion technique

Frogs were killed by decapitation between 08.00 and 09.00 h and the interrenal (adrenal) tissue was dissected free of renal parenchyma. For each perifusion experiment 12 adrenal glands were sliced and preincubated at 24°C for 15 min in 5 ml Ringer's solution consisting of 15 mM HEPES buffer, 112 mM NaCl, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, supplemented with 2 mg glucose/ml and 0.3 mg 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.*[24]. Briefly, the interrenal slices were layered into a siliconized glass column between several beds of Bio-Gel P2 (Bio-Rad, Richmond, Calif., U.S.A.). The tissue was perifused at a rate of 0.20 ml/min with Ringer's solution. The pH (7.35) and the temperature (24°C) of the perifusion medium were kept constant throughout the experiments. The tissue was perifused for 2 h before the experimental manipulation commenced. The column effluent was collected as 5-min fractions during a 8–10-h period and the fractions were immediately frozen until assay.

Steroid radioimmunoassay

Corticosterone and aldosterone concentrations were directly determined by radioimmunoassay (RIA) without prior extraction in 30–50 μl of effluent perifusate [25, 26]. Direct measurement of corticosterone and aldosterone was validated by RIA quantification of corticosteroids after high-performance liquid chromatography analysis of the effluent perifusate [27]. The assays were sensitive enough to detect 20 pg corticosterone and 5 pg aldosterone. For both assays the intra- and inter-assay coefficients of variation were lower than 4 and 10%, respectively.

Calculations

Each perifusion pattern was calculated as the mean (±SEM) profile of corticosteroid output, established over at least three independent experiments. Corticosterone and aldosterone productions were expressed as percentages of the basal values calculated as the mean of eight 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) taken 10 min after the onset of the secretagogue. Student's t-test was employed to determine statistical significance between experimental values.

RESULTS

Effect of graded doses of ACTH(1–39) on corticosteroid secretion

Figure 1 shows that infusion of increasing concentrations of ACTH(1–39) (3.16×10^{-11}–10^{-8} M) induced a dose-related stimulation of corticosterone and aldosterone release. The lower dose which caused stimulation of steroidogenesis was 3.16×10^{-11} M. Half-maximum effect was observed after administration of 3.16×10^{-10} M ACTH(1–39). The maximal response was obtained 30 min after the beginning of the infusion of ACTH(1–39).

Effect of ACTH(1–24) and dimeric ACTH(1–24)$_2$Lys on corticosteroid secretion

Administration of increasing concentrations of ACTH(1–24) (3.16×10^{-11}–3.16×10^{-8} M) to perifused interrenal slices caused a dose-dependent increase of corticosteroid release (Fig. 2a). For concentrations ranging from 10^{-9} to 10^{-6} M, infusion of ACTH(1–24)$_2$Lys, the ACTH(1–24) dimer attached by its N-terminal amino acid, also gave rise to a dose-related stimulation of corticosterone and aldosterone release (Fig. 2b). The time-course of the steroidogenic response to the dimer was similar to that of monomeric ACTH(1–39) and ACTH(1–24), with respect to the lag-period (15 min) and the

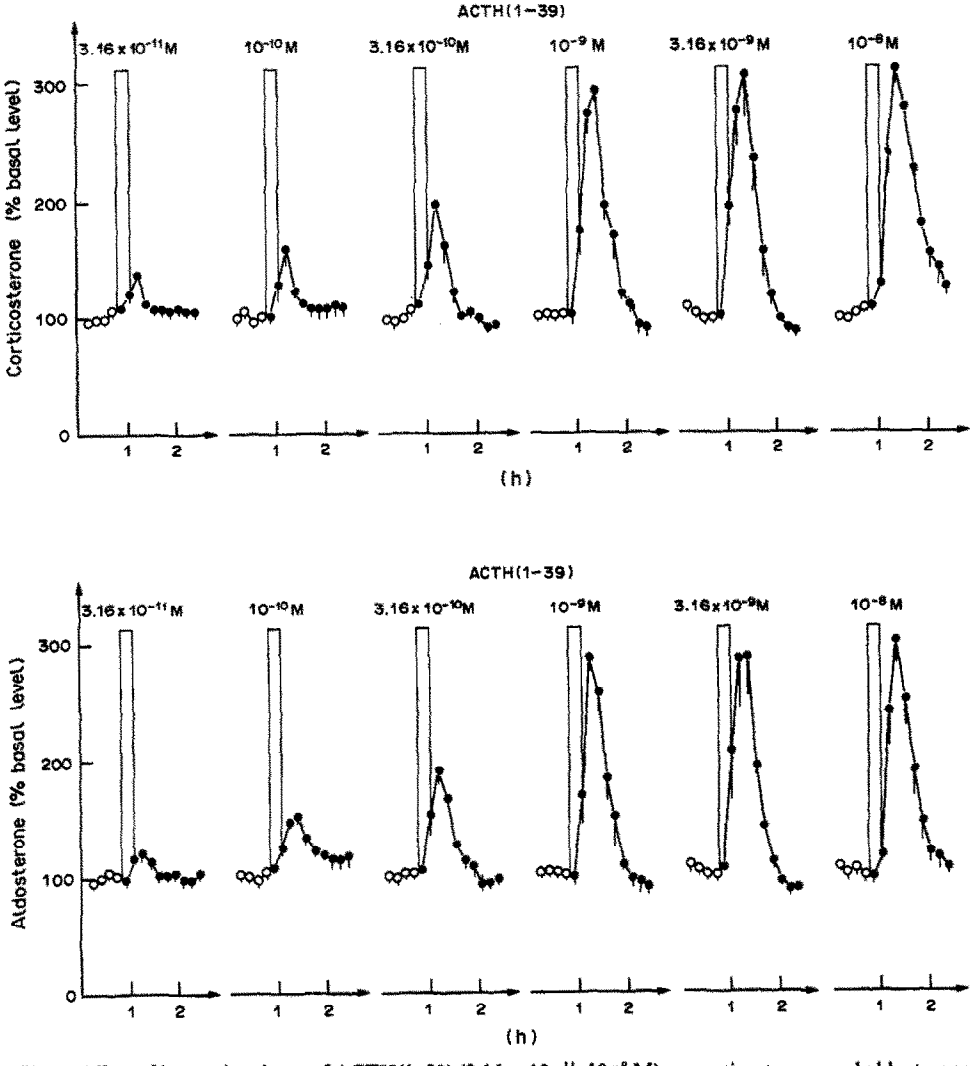

Fig. 1. Effect of increasing doses of ACTH(1–39) (3.16×10^{-11}–10^{-8} M) on corticosterone and aldosterone release. After a 120-min stabilization period, the interrenal slices were perifused for 20 min with increasing concentrations of ACTH(1–39). The profiles represent the mean secretion pattern of 3 independent experiments. The spontaneous level of steroid production (100% basal level) was calculated as the mean of the 8 samples just preceding the infusion of ACTH(1–39). Mean basal levels of corticosterone and aldosterone secretion in these experiments were 94 ± 17 and 52 ± 7 pg/ml per interrenal gland, respectively.

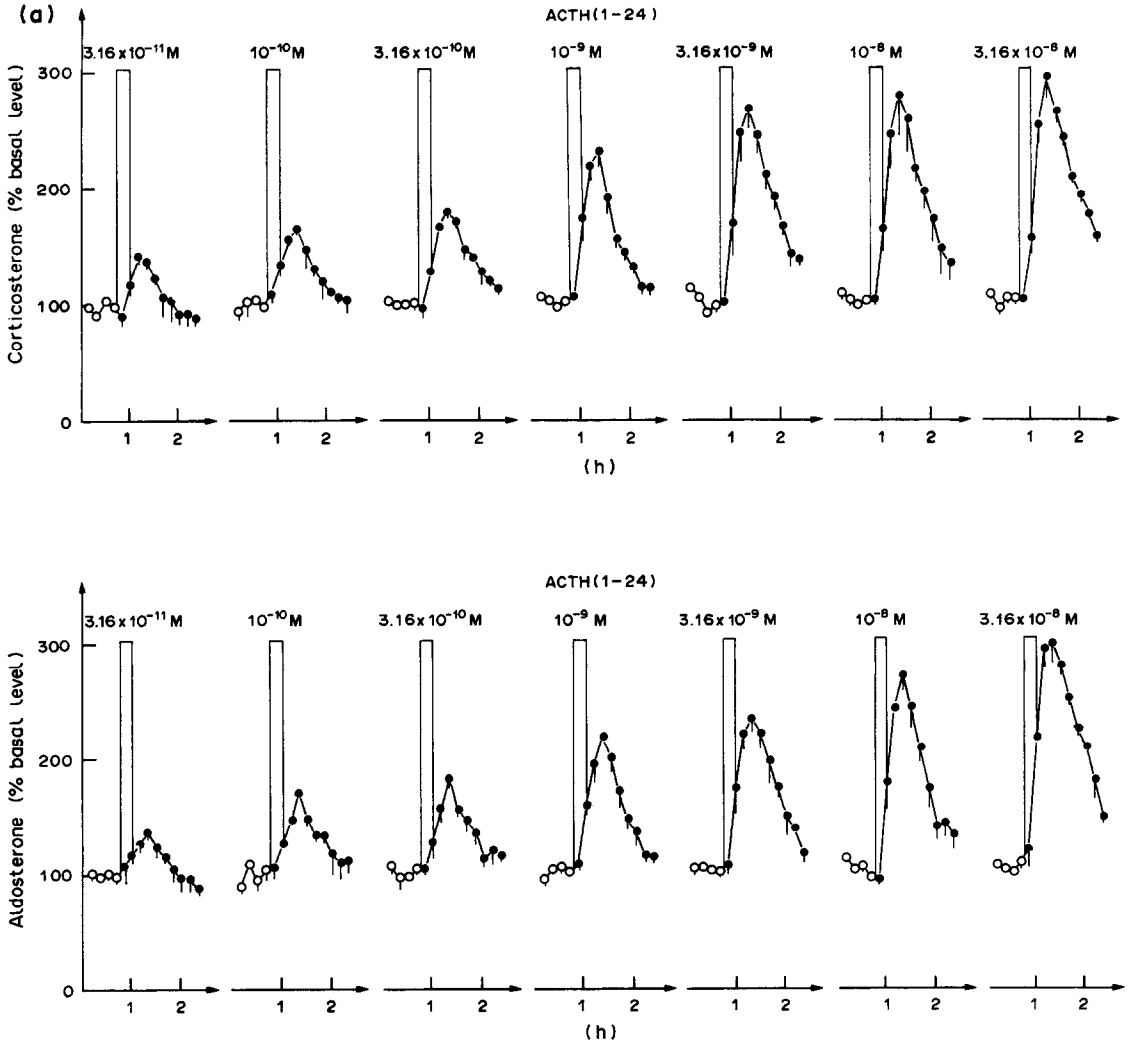

Fig. 2(a). *Continued opposite.*

delay between the onset of the stimulation and the maximum response (30 min). However, the ACTH(1–24)$_2$Lys conjugate was at least 70 times less potent than ACTH(1–24). The lowest dose which induced a steroidogenic response of interrenal glands was 10^{-9} M for the ACTH(1–24)$_2$Lys dimer and 3×10^{-11} M for the ACTH(1–24) monomer. Half-maximum stimulation was observed with 3.16×10^{-10} M ACTH(1–24) and 10^{-8} M ACTH(1–24)$_2$Lys. Nevertheless the maximum responses obtained with the two peptides were not significantly different.

Effect of ACTH(7–24) and dimeric ACTH(7–24)$_2$Lys on corticosteroid secretion

Administration of the ACTH fragment (7–24) (10^{-6} M) to perifused interrenal slices did not affect the spontaneous release of corticosteroids and had no effect on ACTH(1–39)-evoked steroidogenesis (Fig. 3a). The dimer (7–24)$_2$Lys, obtained through dimerization of two ACTH(7–24) fragments by means of a lysine amide spacer on the carboxylic group [23], did not modify basal production of corticosterone and aldosterone but significantly depressed the steroidogenic response to ACTH(1–39) (−72% for corticosterone and −80% for aldosterone output, respectively; $P < 0.02$) (Fig. 3b).

Effect of ACTH(11–24), dimeric ACTH Glu(11–24)$_2$ and dimeric ACTH(11–24)$_2$Lys on corticosteroid secretion

Figure 4a shows that a 100-min perifusion with the ACTH(11–24) fragment (10^{-6} M) did not modify spontaneous or ACTH(1–39)-evoked release of corticosterone and aldosterone. In addition, ACTH Glu(11–24)$_2$, the ACTH(11–24) dimer in which the *N*-terminal amino acid of the monomer is attached through a *N*-acetylglutamic acid spacer, was also totally devoid of effect on spontaneous and ACTH(1–39)-induced corticosteroid secretion (Fig. 4b). Infusion of the dimeric ACTH fragment, (11–24)$_2$Lys, in which the monomers are attached through a 100-Å long lysine amide spacer by their *C*-terminal end, did not affect basal production of

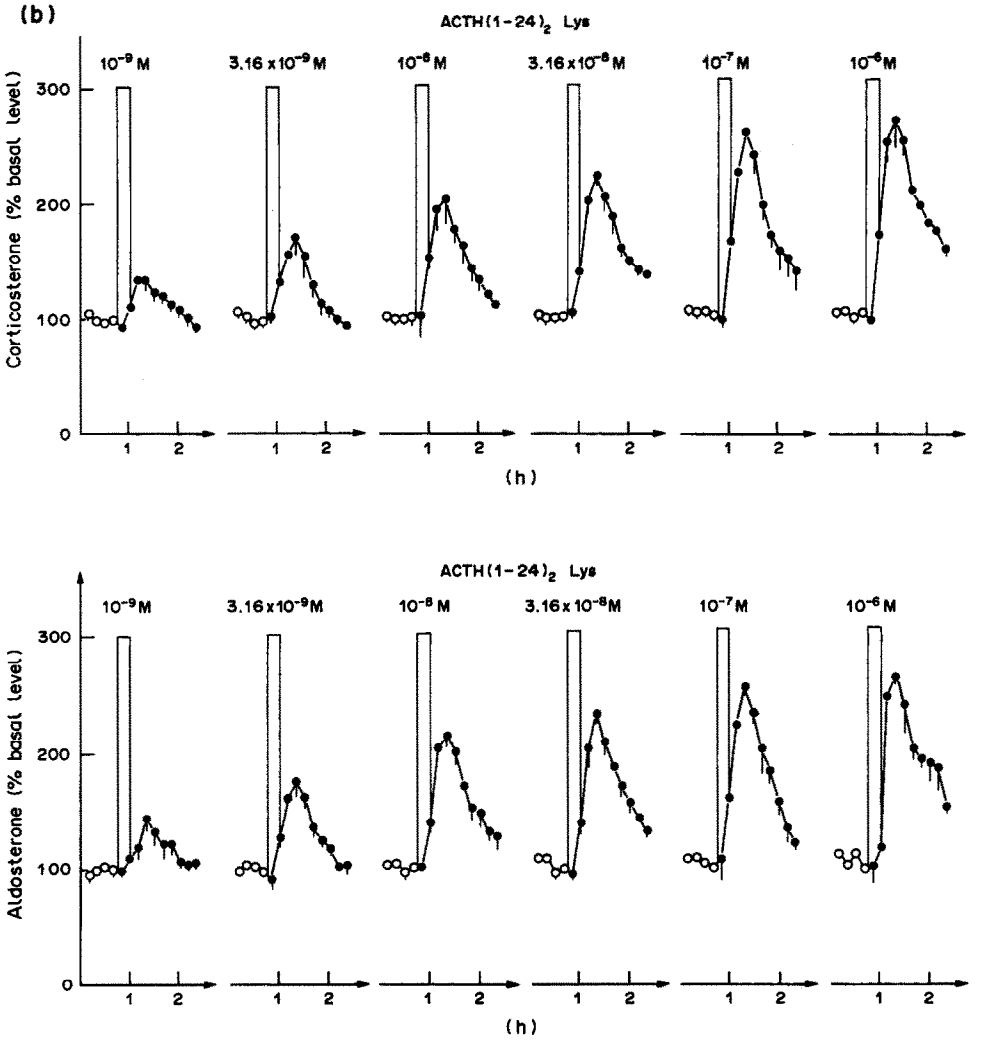

Fig. 2(b).

Fig. 2. Effect of increasing concentrations of (a) the ACTH(1–24) monomer (3.16×10^{-11}–3.16×10^{-8} M), or (b) the ACTH(1–24)$_2$Lys dimer (10^{-9}–10^{-6} M), on corticosterone and aldosterone secretion. Mean basal levels of corticosterone and aldosterone output in these experiments were (a) 140 ± 33 and 46 ± 13, and (b) 98 ± 23 and 54 ± 29 pg/min per interrenal gland, respectively. See legend to Fig. 1 for other designations.

corticosteroids from perifused adrenal slices (Fig. 4c). In contrast, the (11–24)$_2$Lys dimer markedly reduced ACTH(1–39)-evoked stimulation of corticosterone and aldosterone secretion (−63 and −62%, respectively; $P < 0.02$).

DISCUSSION

In adult mammals, the major circulating form of ACTH is the peptide (1–39) [28, 29]. Vinson *et al.*[30] suggested that the ACTH molecule comprises two distinct determinants, corresponding to the sequences (1–13) and (18–24), which respectively contain the corticotropic signal for zona fasciculata and zona glomerulosa cells. In amphibians, previous studies have shown that the adrenal gland contains only one category of steroidogenic cells [31], suggesting that the organization of the corticotropic message in lower vertebrates might be different from that existing in mammals. Infusion of ACTH(1–39) to perifused frog adrenocortical cells induced a dose-related stimulation of corticosterone and aldosterone secretion. In addition, the kinetics of the steroidogenic effect of the ACTH(1–24) fragment was similar to that obtained with ACTH(1–39). Therefore, in amphibians, the *C*-terminal part of ACTH(1–39) plays only a minor role in the regulation of corticosteroid production [32]. The (25–39) region of the ACTH molecule may have only protective functions, as previously suggested in mammals [13, 14]. The dimeric conjugate of ACTH(1–24), ACTH(1–24)$_2$Lys, also exerted agonistic activity on frog interrenal cells. However, while the kinetics of the steroidogenic response of the cells to the dimer was similar to that of the monomer ACTH(1–24), the (1–24) conjugate was at least 70 times less potent than the monomer. Thus dimeriza-

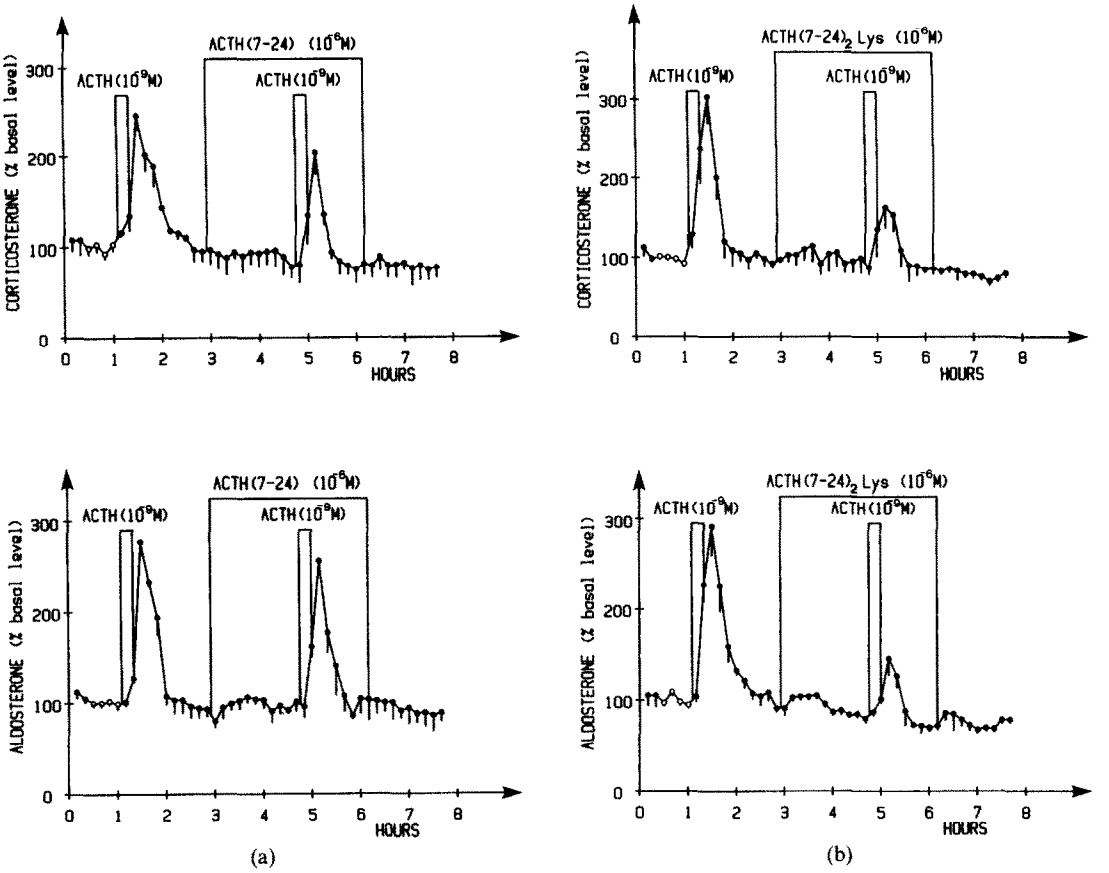

Fig. 3. Effect of ACTH(1–39) alone or during prolonged infusion of (a) the ACTH(7–24) monomer, or (b) the ACTH(7–24)$_2$Lys dimer, on corticosterone and aldosterone secretion. After a 120-min equilibration period, a first pulse of ACTH(1–39) (10^{-9} M) was administered for 20 min. The glands were allowed to stabilize for 90 min, and ACTH(7–24) or ACTH(7–24)$_2$Lys (10^{-6} M each) were then infused for 200 min. 110 min after the beginning of ACTH(7–24) or ACTH(7–24)$_2$Lys infusion, a second 20-min pulse of ACTH(1–39) was administered. The profiles represent the mean secretion pattern of three independent perifusion experiments. The spontaneous level of steroid secretion (100% basal level) was calculated as the mean of 8 samples taken immediately before the infusion of the first pulse of ACTH(1–39). Mean basal levels of corticosterone and aldosterone release in these experiments were (a) 37 ± 10 and 22 ± 14, and (b) 56 ± 28 and 14 ± 3 pg/min per interrenal gland, respectively.

tion of ACTH fragments through their *C*-terminal end markedly affect the corticotropic potency of the peptide. Administration of synthetic ACTH fragment (7–24) to perifused interrenal slices revealed that deletion of the *N*-terminal hexapeptide totally suppressed the corticotropic effect of ACTH. In rat, the corticotropin-inhibiting peptide (CIP), a naturally occurring peptide which corresponds to the sequence ACTH(7–38), also appeared to be devoid of intrinsic steroidogenic activity [33, 34]. Since the (25–39) fragment of ACTH has apparently no corticotropic function, ACTH(7–24) can be regarded as a short-chain agonist of CIP. However, in our model, ACTH(7–24) had no effect on ACTH(1–39)-evoked steroidogenesis, while in rat ACTH(7–38) inhibits ACTH(1–39)-stimulated release of corticosteroids [34]. These results suggest that the organization of the hormonal message in the *N*-terminal region of the ACTH molecule is slightly different in mammals compared to amphibians. In addition, previous studies have shown that the ACTH fragments (1–10) and (4–10), which have a marked corticotropic effect in mammals [12, 13], are very weak stimulators of corticosteroid secretion from frog adrenocortical tissue [32]. Dimerization of the ACTH fragment (7–24) by its *C*-terminal end markedly affected the activity of the peptide, since formation of the conjugate transformed the inactive fragment (7–24) into a potent antagonist of ACTH(1–39). This difference in potency between the monomer and the dimer suggests that the loss of activity of the ACTH(1–24)$_2$Lys dimer was not due to a decrease in the affinity of the dimeric conjugate to the receptor.

To determine more precisely the structure–activity relationships of the ACTH molecule in frog interrenal cells, we tested the effect of shorter ACTH fragments. Infusion of ACTH(11–24) had no effect on spontaneous or ACTH(1–39)-induced steroidogenesis. This result is in agreement with previous studies which showed that ACTH(11–24) was almost

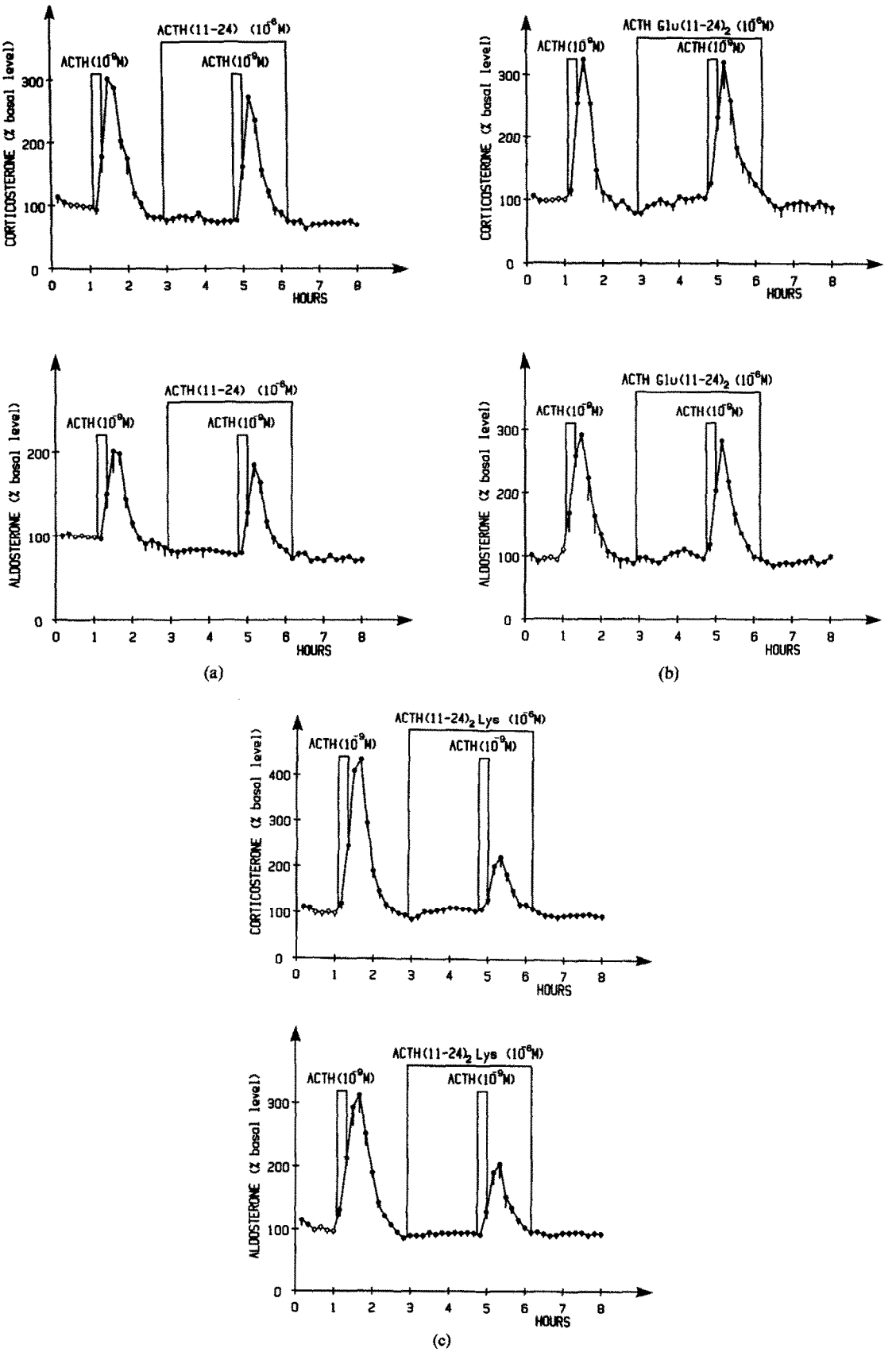

Fig. 4. Effect on corticosterone and aldosterone secretion of ACTH(1–39) (10^{-9} M) alone or during prolonged infusion of (a) the ACTH(11–24) monomer (10^{-6} M), or (b) the ACTH Glu(11–24)$_2$ dimer (10^{-6} M), or (c) the ACTH(11–24)$_2$Lys dimer (10^{-6} M). Mean basal levels of corticosterone and aldosterone release in these experiments were (a) 103 ± 37 and 32 ± 5, (b) 79 ± 18 and 29 ± 13, and (c) 65 ± 21 and 14 ± 3 pg/min per interrenal gland, respectively. See also legend of Fig. 3.

totally devoid of activity on bovine fasciculata and reticularis cells [35]. However, while ACTH(11–24) was previously considered as an antagonist of ACTH(1–39) [14], recent data obtained in rat adrenocortical cells have shown that the ACTH fragment (11–24) potentiates the steroidogenic effect of ACTH(1–39) and, at high concentrations ($>10^{-6}$ M), acts as an ACTH agonist [36]. In frog adrenocortical cells, the ACTH $Glu(11–24)_2$ dimer was also totally inactive on basal and ACTH(1–39)-evoked steroidogenesis. Conversely, the $ACTH(11–24)_2Lys$ dimer exhibited a marked antagonistic activity on the steroidogenic response to ACTH(1–39). However, this $ACTH(11–24)_2Lys$ dimer appeared to be less potent than the dimer $ACTH(7–24)_2$. The fact that the ACTH $Glu(11–24)_2$ dimer, which is linked by its *N*-terminal amino-acid, was totally inactive, while the $ACTH(11–24)_2Lys$ dimer had a marked antagonistic action, points out the importance of the *N*-terminal region of the 11–24 sequence for the binding of the ACTH molecule to its receptor. However, the major effect of a dimer is probably to induce cross-linking of the receptors. This mechanism is considered as one of the first steps of the response of the cells, even in the presence of a monomeric peptide [37, 38]. Nevertheless, in our experiments, dimerization of the ACTH fragments by their *C*-terminal end decreased the corticotropic activity of the molecule or transformed an inactive peptide into an antagonist. Similar observations have been reported in bovine adrenal cells [35], while other studies indicate that dimerization of an antagonist can lead to the formation of an agonist [38, 39]. Thus, in frog adrenal gland, our results suggest that the dimeric ACTH fragments $(7–24)_2Lys$ and $(11–24)_2Lys$ likely interfere with the binding of ACTH and (or) inhibit the internalization of the receptors. However, since dimerization of the ACTH fragment (1–24) decreased its corticotropic activity, but apparently did not modify the binding capacity of the molecule, we suggest that the dimers may affect the movement of the receptors. Such a down-regulation of the cross-linked ACTH receptors may be caused by alteration of the relations of the receptors with the microtubular network which is involved in the capping mechanism of the receptors [40] and in the response of frog adrenocortical cells to ACTH [41].

In summary, this structure–activity study indicates that, in frog adrenocortical tissue, the 11–24 domain of the ACTH molecule does not possess any intrinsic activity. Addition of the sequence 7–10 is not sufficient to restore the corticotropic activity of the molecule. Since the ACTH(7–24) fragment is also totally devoid of antagonistic activity we suggest that the affinity of the sequence 11–24 for ACTH receptors is much lower in amphibians than in mammals. Moreover, recent data suggest the presence, in bovine adrenocortical cells, of two classes of ACTH receptors specific of the sequences 1–10 and 11–24 [42]. Thus, since the ACTH fragments (4–10) and (1–10) do not exhibit any steroidogenic activity in frog [32], our results support the existence of only one class of ACTH receptors, which recognize the domain 11–24, the affinity of which is modulated by a strong potentiator site, located at the *N*-terminal end of the ACTH molecule. Our data also suggest that aggregation of ACTH receptors by dimeric ACTH fragments may be responsible for a down-regulation of the receptors as previously shown for various peptide receptors [43].

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