Potentiation of the antagonistic effect of $ACTH_{11-24}$ on steroidogenesis by synthesis of covalent dimeric conjugates

Jean-Luc Fauchère, Michel Rossier, Alessandro Capponi and Michel B. Vallotton

Institute of Biotechnology, Swiss Federal Institute of Technology, CH 8093 Zurich, Switzerland and *Division of Endocrinology, University Hospital, CH 1211 Geneva 4, Switzerland*

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Covalent dimerization of the adrenocorticotropin fragment $ACTH_{11-24}$ increases its antagonistic potency on the ACTH-induced steroidogenesis in isolated bovine fasciculata/reticularis cells by 3 orders of magnitude when the C-termini are linked via a 10 Å long spacer. This strong potentiation, probably mediated by cross-linking of the receptors, was shown to be dependent on the point of attachment of the monomeric fragment to the spacer, thus providing information about the position of the binding site in the hormonal segment and about the distance of the receptors on the cell surface.

Adrenocorticotropin inhibitor *Covalent dimerization* *Receptor aggregation* *Hormone action potentiation*

1. INTRODUCTION

$ACTH_{11-24}$ has been demonstrated to be a competitive antagonist of $ACTH_{1-39}$ and of $ACTH_{1-10}$ for the stimulation of steroidogenesis in isolated adrenal cells [1]. This observation fits the current theory of the organization of the information in the ACTH molecule according to which the sequence 11–24 is not involved in activation but provides affinity to the receptor [2]. Covalent dimeric conjugates of peptide hormones [3,4] and of non-peptide drugs [5,6] have been suggested to act as bivalent ligands and to be able to cross-link receptors. No such conjugates have been reported in the ACTH field so far. In addition, there is a need for potent ACTH antagonists, the potency of the natural [7] and synthetic [8,9,10] inhibitors known to date being generally low.

The purpose of our work was therefore to prepare bivalent conjugates of ACTH fragments, starting with antagonistic moieties, and paying special attention to the point of attachment of the peptide and to the structure of the spacer. We have synthesized the two compounds shown in fig.1, in which two $ACTH_{11-24}$ fragments are covalently attached through either their terminal carboxy or terminal amino groups. The two functional groups of

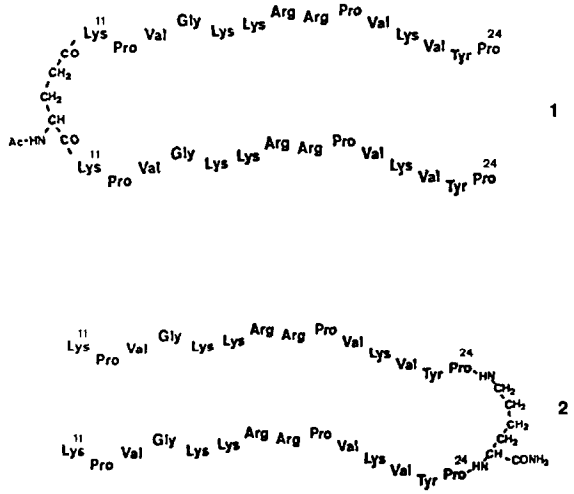

Fig.1. Chemical structure of the dimeric conjugates of $ACTH_{11-24}$ obtained with the spacers *N*-acetylglutamic acid (**1**) and lysine amide (**2**), respectively.

Abbreviations: according to the IUPAC-IUB Commission on Biochemical Nomenclature, (1967) Eur. J. Biochem. 1, 375; ACTH, adrenocorticotropic hormone

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acetylglutamic acid and of lysine amide, respectively, were chosen for the covalent binding of the hormonal fragments. In this way, the spacer also was an amino acid attached and protected throughout via amide bonds. Measuring steroidogenesis in the isolated adrenal cell system, we were able to show that compounds **1** and **2** exhibited the potentiating effect expected for such bivalent conjugates, and that the effect was very sensitive to the structure of the dimer.

2. MATERIALS AND METHODS

Compounds **1** and **2** (fig.1) were synthesized from the protected fragment H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys-(Boc)-Val-Tyr-Pro-OH,2HCl industrially prepared and kindly provided by CIBA-Geigy, Basel, and from the derivatized spacers Ac-Glu-OBzl and H-Lys(Fmoc)-NH_2 (prepared in our laboratory), using the methods of classical peptide synthesis in solution [11]. The products were isolated as pure hydrochlorides after cleavage of the protecting groups under acidic conditions and gel filtration on Sephadex L60 in methanol. Details of the synthesis will be published elsewhere [12]. The ratio of glutamic acid in conjugate **1** and of lysine in **2**, to the other amino acids after total hydrolysis and amino-acid analysis, was a convenient means to prove covalent dimerization.

Isolated bovine fasciculata/reticularis cells were prepared by collagenase dispersion by a modification of the method of Haning et al. [13] as described elsewhere [14]. One ml aliquots of the cell suspension (300000 cells) were incubated for 60 min at 37°C under 95% O_2/5% CO_2, in the presence of various concentrations of $ACTH_{1-24}$ and/or the synthetic $ACTH_{11-24}$ monomer and dimers. At the end of the incubation period, the cells were sedimented by centrifugation, and the corticosterone (B) content determined by radioimmunoassay, either directly or after CCl_4 extraction, using a rabbit antiserum raised against corticosterone-21–hemisuccinate–BSA conjugate and dextran charcoal separation. No difference in B levels was observed, whether the media had been assayed directly or extracted with CCl_4. Cross-reaction with cortisol was less than 1% and this steroid was found to be produced in equivalent amounts to corticosterone.

3. RESULTS

The dimeric conjugates **1** and **2** failed to activate B production at any dose up to 10^{-5} M. The monomer $ACTH_{11-24}$ was inactive at doses lower than 10^{-6} M, showing however a light enhancement of B over the basal value 13.0 ± 2.5 to 15.6 ± 2.5 ng/ml per h (not shown).

In the presence of a fixed amount (10^{-11} M) of the agonist $ACTH_{1-24}$, the response was not affected by $ACTH_{11-24}$ up to 2×10^{-5} M, while the two dimers **1** and **2** markedly depressed or completely suppressed B production, respectively (fig.2).

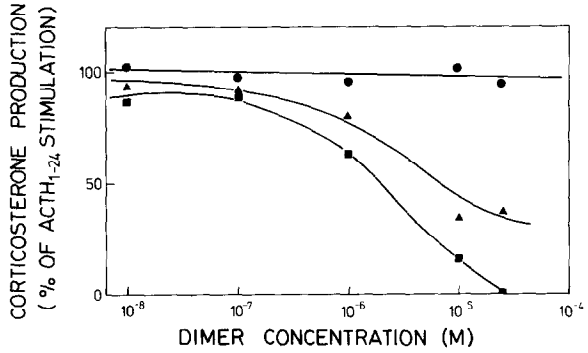

Fig.2. Effects of $ACTH_{11-24}$ (●), of dimer **1** (▲) and **2** (■) on corticosterone production induced by $ACTH_{1-24}$ at 10^{-11} M. Each point is the mean of triplicate determinations from two separate experiments.

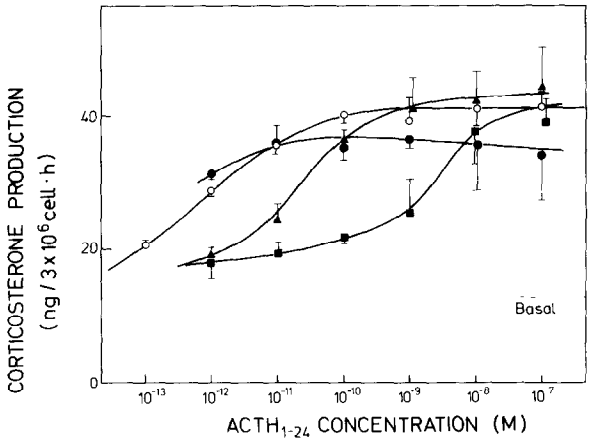

Fig.3. Log dose response curves of $ACTH_{1-24}$ induced B production in the absence of antagonist (○) or in the presence of 10^{-5} M $ACTH_{11-24}$ (●) or of dimer **1** (▲) or **2** (■). Each point is the mean (± SE) of triplicate determinations from two separate experiments.

The log dose response curve of $ACTH_{1-24}$ for B production at constant inhibitor concentration (10^{-5} M) was shifted to the right by the two dimers while the monomer $ACTH_{11-24}$ had little effect. From computer analysis the dose to induce 1/2 B_{max} was found to be 5.3 ($\pm$ 3.1) $\times$ 10^{-13} M for the agonist alone or 3.92 ($\pm$ 1.08) $\times$ 10^{-11} M and 2.14 ($\pm$ 0.92) $\times$ 10^{-9} M in the presence of conjugate **1** or **2**, respectively.

4. DISCUSSION

Due to the bivalent structure of the new derivatives, an evaluation of the inhibitory constant according to the classical kinetic model for competitive inhibition is no longer possible. However, the 75- and 4050-fold increase of the ED_{50} for $ACTH_{1-24}$ at 10^{-5} M of conjugate **1** and **2**, respectively, compared to the hardly measurable effect of the monomer $ACTH_{11-24}$ under the same conditions, represents a most significant effect of covalent dimerization. A direct visualization of the molecular processes involved at the cellular surface is impossible without the effector molecules being radioactively or fluorescently labelled. However, it is reasonable to admit that potentiation is primarily due to cross-linking of the receptor sites. The ensuing potency increase considerably greater than the statistical factor of two can tentatively be explained by a more favorable entropy change for the bridging by a single bivalent ligand than for the univalent binding of two bivalent ligands [6]. Cross-linking generally induces a microaggregation of the receptors and the latter process is thought to be necessary for the generation of an immediate biological response, also when monovalent agonists are applied [15]. When using antagonists, microaggregation can be promoted by complexes of the antagonist with bivalent antibodies, leading in some instances to the conversion of the antagonist to an agonist [4,16]. However, with our inhibitors no such conversion took place and it was the antagonistic effect which was amplified. Our dimeric conjugates, in contrast to the monomer, might indeed cause an aggregation of the receptor sites. However, the resulting clusters would not be functional. Moreover, they would prevent further receptor stimulation either through alteration of the binding sites or by blocking the access to them. Support for the postulated mechanism might be gained from the synthesis (in progress) and testing of bivalent conjugates of the agonist $ACTH_{1-24}$.

The difference in potency between the two conjugates **1** and **2** is also very informative. The stronger effect observed for conjugate **2** is indicative of a better interaction with the receptor sites and therefore of stronger binding elements near the N-terminal region of the monomer. Taking in account an approximate length of 7–10 Å for the spacer and of 22 Å [17] for the monomer, a mean distance of 25–35 Å between two receptors can be estimated. Variation of the spacer length (not performed here) may help to settle these values more precisely and to optimize the antagonistic potency. Finally, it seems reasonable to expect that starting with N-terminal elongated monomers of $ACTH_{11-24}$ [18], even stronger ACTH inhibitors should be obtained by the same method of covalent dimerization.

REFERENCES

[1] Seelig, S., Sayers, G., Schwyzer, R. and Schiller, P.W. (1971) FEBS Lett. 19, 232–234.
[2] Schwyzer, R. (1977) Ann. NY Acad. Sci. 297, 3–26.
[3] Shimohigashi, Y., Costa, T., Matsuura, S., Chen, H.C. and Rodbard, D. (1982) Mol. Pharmacol. 21, 558–563.
[4] Conn, P.M., Rogers, D.C., Stewart, J.M., Niedel, J. and Sheffield, T. (1982) Nature 296, 653–655.
[5] Paton, W.D. and Zaimis, E.J. (1949) Br. J. Pharmacol. 4, 381–400.
[6] Porthogese, P.S., Ronsisvalle, G., Larson, D.L., Yim, C.B., Sayre, L.M. and Takemori, A.E. (1982) Life Sci. 31, 1283–1286.
[7] Lee, C.Y., McPherson, M., Licko, V. and Ramachandran, J. (1980) Arch. Biochem. Biophys. 201, 411–419.
[8] Hofmann, K., Montibeller, J.A. and Finn, F.M. (1974) Proc. Natl. Acad. Sci. USA 71, 80–83.
[9] Ways, D.K., Mahaffee, D.D. and Ontjes, D.A. (1979) Endocrinology 104, 1028–1035.
[10] Bristow, A., Gleed, C., Fauchère, J.L., Schwyzer, R. and Schulster, D. (1980) Biochem. J. 186, 599–603.
[11] Fauchère, J.L. and Schwyzer, R. (1981) in: The Peptides (Gross, E. and Meienhofer, J. eds) vol.3, pp.203–252, Academic Press, New York.
[12] Fauchère, J.L. (1985) Helv. Chim. Acta 68, in press.

[13] Haning, R., Tait, S.A. and Tait, J.F. (1970) Endocrinology 87, 1147–1167.
[14] Capponi, A.M., Lew, P.D., Jornot, L. and Vallotton, M.B. (1984) J. Biol. Chem. 259, 8863–8869.
[15] King, A.C. and Cuatrecasas, P. (1981) N. Engl. J. Med. 305, 77–88.
[16] Schechter, Y., Hernaez, L., Schlessinger, J. and Cuatrecasas, P. (1979) Nature 278, 835–838.
[17] Schiller, P.W. (1972) Proc. Natl. Acad. Sci. USA 69, 975–979.
[18] Fauchère, J.L. and Petermann, C. (1978) Helv. Chim. Acta 61, 1186–1192.