[9-ISOLEUCINE]$ACTH_{1-24}$, A COMPETITIVE ANTAGONIST OF $ACTH_{1-24}$ INDUCED CYCLIC AMP AND CORTICOSTERONE PRODUCTION

Satyendra Kumar

Division of Molecular and Cellular Endocrinology
School of Medicine
Case Western Reserve University, Cleveland, Ohio 44106

Received August 13,1975

SUMMARY

Substitution of tryptophan^9 in $ACTH_{1-24}$ by isoleucine results in complete loss of biological activity. A dose of 3.4×10^{-5} M per ml fails to stimulate corticosterone and cyclic AMP production. This analogue inhibits cyclic AMP production and corticosterone production induced by $ACTH_{1-24}$ in isolated adrenal cortex cells. The I_{50} values for corticosterone and cyclic AMP inhibition are 2.3×10^{-6} M and 3.4×10^{-6} M respectively.

INTRODUCTION

Since the demonstration that $ACTH_{11-24}$ is a competitive inhibitor of $ACTH_{1-39}$ (1) an increasing number of reports describing antagonists have appeared in the literature (2,3,4,5,6). The capacity of $ACTH_{11-24}$ to act as

---

Abbreviations used are those recommended by the IUPAC - IUB commission on Biochemical Nomenclature Symbols for Amino Acid Derivatives and Peptides, (1972), Arch. Biochem. Biophys. 150, 1; DCC, dicyclohexylcarbodiimide; Metrp, methyltryptophan; Nal, naphthylalanine; Pmp, pentamethylphenylalanine. I_{50} is the concentration of [Ile^9]$ACTH_{1-24}$ required to shift the log concentration curve of $ACTH_{1-24}$ by a factor of 2 to the right.

Melting points were determined on Fisher-John's apparatus and are uncorrected. Microanalyses were performed by Baron Consulting Co., Orange, Conn. Thin layer chromatography was done on silica gel plates and spots were detected by ninhydrin, Pauly's and chlorax-KI-starch reagents. The following solvent systems were used: I, $CHCl_3$: MeOH (9:1); II, BuOH: AcOH: H_2O (4:1:1); III, $CHCl_3$: MeOH (85:15); IV, BuOH: AcOH: H_2O: Pyridine (30:6:24:20); V, Sec. BuOH satd with 3% NH_3; VI, MeOH; VII, $CHCl_3$: MeOH (1:1). For amino acid analysis samples were hydrolyzed with constant boiling HCl at 110° for 24 hr and analyzed on Durrum D-500 analyzer. Electrophoresis was carried out on Whatman 3M chromatography paper on Savant instrument. The electrophoretic behavior of [Ile^9]$ACTH_{1-24}$ is reported here by the ratio of its migration to that of simultaneously run lysine standard.

Copyright © 1975 by Academic Press, Inc.
All rights of reproduction in any form reserved.

an antagonist is presumed to be due to loss of the active core (amino acids 4-10) and retention of a region (11-24) of the ACTH molecule which contributes importantly to the affinity of the hormone for its receptor. Recently, modifications of amino acids in the active center, especially tryptophan at position 9 (the sole tryptophan in the ACTH molecule) have uncovered competitive antagonists of the ACTH molecule. For example, [Trp(Nps)^9]$ACTH_{1-24}$ (2) and [Trp(Dnps)^9]$ACTH_{5-24}$ (3) antagonize the stimulatory action of $ACTH_{1-24}$ when added to suspensions of isolated adrenal cortex cells. Substitution of phenylalanine or Nα-methyltryptophan for tryptophan results in the conversion of the molecule to an antagonist of $ACTH_{1-24}$ measured by activation of adenylate cyclase in plasma membrane fragment of bovine adrenal cortex (6). We now report the results of substitution of tryptophan with isoleucine.

MATERIALS AND METHODS

$ACTH_{1-24}$ was provided by Dr. H. Strade, Organon Ltd., West Orange, New Jersey. Protected ACTH fragments: Boc-Ser-Tyr-Ser-Met-$NHNH_2$ and H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t-di-hydrochloride, were generous gifts from Dr. W. Rittel, Ciba-Geigy AG, Basel, Switzerland. Protected amino acids were purchased from Bachem, 4077 Glencoe Avenue, Marina Del Rey, California 90291.

A suspension of isolated rat adrenal cortex cells was prepared by the method of Sayers et al., (7). The cells were dispersed in Krebs-Ringer bicarbonate buffer containing glucose (0.2%) (KRBG) and [8-^{14}C] adenine, 30 μCi. After dispersion, the cells were centrifuged at 300 g for 10 min at room temperature. The pellet was resuspended in 80 ml of KRBG buffer containing calcium (7.65 mM), bovine serum albumin (0.5%) and lima bean trypsin inhibitor (0.1%). Aliquots of the suspension, 0.9 ml in volume together with 0.1 ml vehicle or with 0.1 ml of vehicle to which ACTH has been added were incubated in an atmosphere of 95% O_2:5% CO_2. After 60 minutes incubation, methylene chloride was added to each beaker. An aliquot from the methylene chloride phase was analyzed for corticosterone (8) and an aliquot from the aqueous phase was analyzed for cyclic [8-^{14}C]AMP by the method of Kuo and DeRenzo (9) as adapted for use in isolated cortex cells suspension by Beall and Sayers (10).

SYNTHESIS

[Ile^9]$ACTH_{1-24}$ was prepared using basically the same plan that was used by Schwyzer et al., for the snythesis of $ACTH_{1-24}$ (11). The final acylation of the protected C-terminal tetradecapeptide by protected N-terminal decapeptide was done by dicyclohexylcarbodiimide and N-hydroxysuccinimide (13).

Z-Ile-Gly-OMe (I). Was prepared by DCC condensation of Z-Ile-OH and Gly-OMe in CH_2Cl_2 and crystallized from EtOAc (yield 89%); mp 131-132°; $[\alpha]_D^{24}$ -25° (c.2.0, AcOH); R_f^I 0.86.

Boc-Arg(NO_2)-Ile-Gly-OMe (II). Z-group was removed from I by HBr-AcOH. The hydrobromide was neutralized and condensed with Boc-Arg(NO_2)-OH in DMF:CH_2Cl_2 (50:50) using DCC. Crystallized from isopropanol: petroleum either (yield 60.41%); mp 152-154°; $[\alpha]_D^{24}$ -34.48° (c, 1.3, MeOH). R_f^{III} 0.56; Anal. calcd. for $C_{20}H_{38}O_8N_7$: C, 47.61; H, 7.53; N, 19.44. Found C, 47.37; H, 7.29; N, 19.19.

Z-Phe-Arg(NO_2)-Ile-OMe (III). Boc-group was removed from II by TFA. The TFA salt was neutralized and reacted with Z-Phe-ONSu, to obtain III. Crystallized from EtOH, (yield 80.4%); mp 220-222°; $[\alpha]_D^{24}$ -17.31° (c, 1.15, DMF). R_f^{I} 0.52; R_f^{II} 0.86; Anal. calcd. for $C_{22}H_{44}N_8O_9.H_2O$: C, 54.70; H, 6.55; N, 15.95. Found C, 54.92; H, 6.25; N, 15.91.

Z-Glu(OBu^t)-His-Phe-Arg(NO_2)-Ile-Gly-OMe (IV). Z-group was removed from III by HBr-AcOH. For liberating the free amine from hydrobromide salt and for acylation with Z-Glu-His-$NHNH_2$, method of Schwyzer et al., (12) was followed. Crystallized from MeOH-Ether (yield 66%); mp 215-216°; $[\alpha]_D^{24}$ -27° (c, 1.0. DMF); R_f^{II} 0.71; R_f^{IV} 0.83; R_f^{V} 0.87. Anal. calcd. for $C_{47}H_{67}O_{13}N_{12}$: C, 56.00; H, 6.65; N, 16.68. Found C, 55.86; H, 6.45; N, 16.31.

H-Glu(OBu^t)-His-Phe-Arg-Ile-Gly-OH (V). The protected hexapeptide methyl ester (IV) was hydrolyzed in dioxane by 1N NaOH and hydrogenated over 10% palladium charcoal to give V; R_f^{IV} 0.54; R_f^{VI} 0.18.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Ile-Gly-OH (VII). This protected decapeptide was prepared by coupling Boc-Ser-Tyr-Ser-$NHNH_2$ with V by azide method. The crude product was purified by reprecipitation from DMF solution with H_2O, (yield 76%); mp 222-225° (decomp. sinters at 215°); $[\alpha]_D^{24}$ -12.58° (c, 1.03, DMF); R_f^{VI} 0.75; R_f^{VII} 0.51.

[Ile^9]$ACTH_{1-24}$ (VIII). The protected decapeptide (VII, 188mg) was condensed with H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t-di-hydrochloride (366mg) by DCC-HOSu method following the procedure of Riniker and Rittel (13). The crude product was subjected to counter current distribution in the system $CHCl_3$-CCl_4-MeOH-buffer (5:2:8:4); (buffer: AcOH, (28.5ml) NH_4OAc (19.25g) made up to 1000 ml with H_2O). After 360 transfers (60 tube manual machine, 10ml each phase), by single withdrawal technique, the desired compound was located in fundamental tubes 30-60. Thin layer chromatography of lower phase (in system VII) showed that it was still contaminated. The fractions were pooled together and lyophilized. The protected peptide was treated with 90% TFA (5ml), in the dark under N_2 with occasional shaking. After 40 min it was concentrated below 30° in vacuo and anhydrous, peroxide-free, cold ether was added. The precipitate filtered and dried in vacuo. Trifluoroacetate ions were exchanged for acetate ions. The residue dissolved in 0.01M NH_4OAc buffer pH 4.6 (2ml).

and applied to a CMC-column (2x30 cms). It was eluted with the same buffer at a flow rate of 1·5 - 1·8 ml/min and fractions were collected every 10 mins. After 27 fractions were collected a gradient was produced by adding 0.2M NH_4OAc buffer (pH 6.5) through a mixing flask containing 1000 ml of starting buffer. After 58 fractions, the flask containing 0.2M buffer was replaced by 0.4M NH_4OAc buffer (pH 6.5). Fractions 108 to 120, which contained the desired compound were pooled togehter and lyophilized until all the NH_4OAc disappeared. The residue was treated by thioglycolic acid to reduce any methionine sulfoxide (14); 25mg; $[\alpha]_D^{26}$ -71.61° (c, 0.37, 90% AcOH); R_f^{IV} 0.40; R_f^{IV} (cellulose) 0.55; Amino acid composition of acid hydrolysate: $Arg_{3.06}$ $Lys_{4.13}$ $His_{0.91}$ $Phe_{0.92}$ $Tyr_{1.82}$ $Ile_{0.98}$ $Met_{0.91}$ $Val_{2.96}$ $Gly_{1.92}$ $Pro_{2.89}$ $Glu_{1.00}$ $Ser_{1.75}$. After electrophoresis at 1000V for 60 min in 2N AcOH only one component could be detected: E(Lys) 0.8.

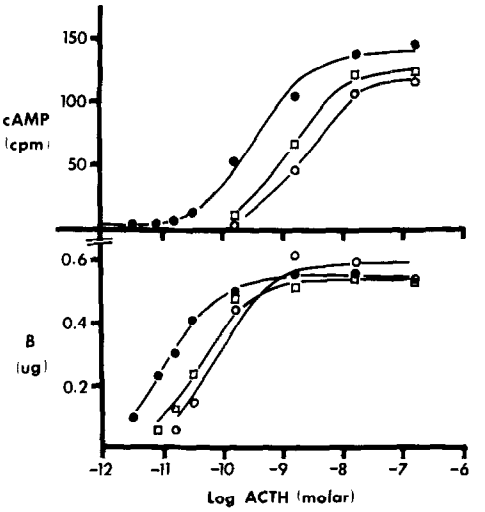

Fig. 1. Log concentration response curve for $ACTH_{1-24}$ acting alone (●) and for $ACTH_{1-24}$ acting in combination with 6.8×10^{-6} M $[Ile^9]ACTH_{1-24}$ (□) or with 1.36×10^{-5} M $[Ile^9]ACTH_{1-24}$ (○). The abscissa is log of the molar concentration of $ACTH_{1-24}$. The top ordinate is cyclic [8-^{14}C] AMP in cpm; the bottom ordinate is corticosterone in micrograms. The values for cyclic [8-^{14}C] AMP and for corticosterone are net, cyclic [8-^{14}C] AMP or corticosterone produced in samples with $ACTH_{1-24}$ minus blanks (cyclic [8-^{14}C] or corticosterone in samples without $ACTH_{1-24}$). Both concentrations of $[Ile^9]ACTH_{1-24}$ when acting alone did not increase cyclic [8-^{14}C] AMP or corticosterone above blanks. Blanks for cyclic [8-^{14}C] AMP equaled 4.0 cmp; for corticosterone, 0.02μg. $ACTH_{1-24}$ and $[Ile^9]ACTH_{1-24}$ were added to the cell suspensions at the same time and the cell suspensions were incubated for 60 minutes.

RESULTS AND DISCUSSION

In the design of antagonists of ACTH, considerable attention has been focused on the tryptophan at position nine. A dominant characteristic of the amino acid substituted for Trp^9 in ACTH is aromaticity. Substitution of phenylalanine (6), pentamethylphenylalanine (15) and naphthylalanine (16) for Trp^9 have resulted in analogues with low steroidogenic activity. In plasma membrane preparations $[Gln^5,Phe^9]ACTH_{1-20}$ amide and $[N\alpha\text{-Metrp}^9]ACTH_{1-24}$ have been reported as antagonists of ACTH induced activation of adenylate cyclase (6). $[Lys^8,Phe^9]ACTH_{1-19}$ was tested for antagonist properties and was not found to inhibit the steroidogenic action of $ACTH_{1-19}$ (16). An interesting feature of all these analogues is that they have an aromatic substituent at position nine and posses low but definite steroidogenic acti-

vity. Possibly the biological activity of these analogues is related to the aromaticity of the substituents. With this in mind an aliphatic amino acid isoleucine was substituted for tryptophan. [Ile^9]$ACTH_{1-24}$ at a concentration of 3.4×10^{-5} molar is inactive for corticosterone and cyclic AMP production in suspensions of isolated adrenal cortex cells. Its potency as an antagonist was evaluated by constructing concentration response curves for $ACTH_{1-24}$ in the absence and in the presence of [Ile^9]$ACTH_{1-24}$ (Fig. 1). Log-concentration response curves obtained in the presence of [Ile^9]$ACTH_{1-24}$ were essentially parallel to and shifted to the right of the log concentration response curves for $ACTH_{1-24}$ alone. Apparent dissociation constants for [Ile^9]$ACTH_{1-24}$ determined by corticosterone production and by cyclic AMP production equalled 2.3×10^{-6} M and 3.5×10^{-6} M respectively.

The apparent dissociation constants of [Ile^9]$ACTH_{1-24}$ are lower than those of $ACTH_{11-24}$ which means it is more potent antagonist. However, these dissocation constants of [Ile^9]$ACTH_{1-24}$ are higher than those of $ACTH_{7-23}$ amide. This was unexpected, since $ACTH_{7-23}$ amide does not even have the first six amino acids. One possible interpretation is that the isoleucine moiety, because of its different sterochemistry might be causing steric hindrance for the amino acids in 1 through 8 region in such a fashion as to markedly impair the ability of the hormone to attach to the receptor.

[Ile^9]$ACTH_{1-24}$ is the only ACTH analogue reported so far with full complement of first 24 amino acids which is totally devoid of agonist activity. But with the loss of agonistic activity this analogue appears also to have lost affinity. Our efforts are continuing to synthesize more potent antagonists of ACTH.

ACKNOWLEDGMENT

The author is very grateful to Dr. G. Sayers for his interest and valuable suggestions and Mary Vegh, Mary Rupnow and Edith Hanzmann for technical assistance.

This work was supported by NSF Grant No. BMS75-02378.

REFERENCES

1. Seelig, S., Sayers, G., Schwyzer, R., and Schiller, P. (1971) Fed. Eur. Biochem. Soc. Lett. 19, 232-234.
2. Seelig, S., Kumar, S., and Sayers, G. (1972) Proc. Soc. Exp. Biol. Med. 139, 1217-1219.
3. Kumar, S., Seelig, S., and Sayers, G. (1972) Biochem. Biophys. Res. Cummun. 49, 1316-1322.
4. Moyle, W. R., Kong, Y. C., and Ramachandran, J. (1973) J. Biol. Chem. 248, 2409-2417.
5. Sayers, G., Seelig, S., Kumar, S., Karlanganis, G., Schwyzer, R., and Fujino, M. (1974) Proc. Soc. Exp. Biol. Med. 145, 176-181.
6. Hofmann, K., Judith, M. A., and Francis, F. M. (1974) Proc. Nat. Acad. Sci. 71, 80-83.
7. Sayers, G., Swallow, R. L., and Giordano, N. D. (1971) Endocrinology 88, 1063-1068.
8. Silber, R. H., Busch, R. D., and Oslapas, R. (1958) Clin. Chem. 4, 278-285.
9. Kuo, J. F., and DeRenzo, E. C. (1969) J. Biol. Chem. 244, 2252-2260.
10. Beall, R. J., and Sayers, G. (1972) Arch. Biochem. Biophys. 148, 70-76.
11. Schwyzer, R., and Kappeler, H. (1963) Helv. Chim. Acta 46, 1550-1572.
12. Schwyzer, R., and Kappeler, H. (1961) Helv. Chim. Acta 44, 1991-2002.
13. Riniker, B., and Rittel, W. (1970) Helv. Chim. Acta 53, 513-519.
14. Hofmann, D., Finn, F. M., Limmeti, M., Montibeller, J., and Zanetti, G. (1966) J. Am. Chem. Soc. 88, 3633-3639.
15. VanNipsen, J. W., and Tesseer, C. I. (1975) Int. J. Pept. Prot. Res, 7, 57-67.
16. Blake, J. and Li, C. H. (1975) J. Med. Chem. 18, 423-426.