STRUCTURE-ACTIVITY STUDIES OF MSH-RELEASE-INHIBITING HORMONE*

Maria E. CELIS, Satoe HASE† and Roderich WALTER†
Instituto de Investigacion Medica Mercedes y Martin Ferreyra, Cordoba, Argentina and
†Departments of Physiology and Psychiatry, Mount Sinai Medical and Graduate Schools of the City University of New York, New York, N.Y. 10029, USA

Received 25 August 1972

1. Introduction

The release and inhibition of release of melanocyte-stimulating hormone (MSH) from rat pituitary are under the control of two hormonal factors present in the hypothalamus [1]. Both are fragments of oxytocin resulting from an enzymic degradation of this neurohypophyseal hormone [2–5]. It was established that the chemical structure of the MSH-release-inhibiting factor (MSH–R–IF) is H-Pro–Leu–Gly-NH_2 [3, 4, 6], and more recently we reported that the pentapeptide H-Cys–Tyr–Ile–Gln–Asn-OH (hereafter referred to as releasing peptide, R-p), possesses the capacity to release pituitary MSH and increase MSH plasma levels [7].

In this communication we show that not only MSH–R–IF (the C-terminal tripeptide of oxytocin) but also H-Pro–Lys–Gly-NH_2 and H-Pro–Arg–Gly-NH_2 (the C-terminal tripeptides of lysine- and arginine-vasopressin, respectively), inhibit *in vivo* the release of MSH from rat pituitary. These latter peptides are less potent than MSH–R–IF; other analogs of MSH–R–IF are inactive. R-p is inhibited by MSH–R–IF and several of its analogs. Tocinoic acid, the ring component of oxytocin (H-Cys–Tyr–Ile–Gln–Asn–Cys-OH), is inactive both in the release of MSH and in MSH-release inhibition in the intact rat.

* Abbreviations of amino acid derivatives and peptides are in accordance with the IUPAC–IUB Commission on Biochemical Nomenclature, J. Biol. Chem. 247 (1971) 977. The amino acids (except glycine) had the L-configuration unless otherwise stated. All new peptides were analyzed for C, H and N, and the values found differed maximally by 0.3% from the theoretical values. The structure of the tripeptides was also confirmed by amino acid analysis. Pyroglu stands for pyroglutamic acid (2-pyrrolidone-5-carboxylic acid).

2. Materials and methods

H-Cys–Tyr–Ile–Gln–Asn-OH (R-p) and crystalline H-Pro–Leu–Gly-$NH_2 \cdot \frac{1}{2} H_2O$ [8] (MSH–R–IF, I) were the same preparations as those used in earlier studies [3, 4, 7]. Tocinoic acid was supplied by Dr. V.J. Hruby [9], and H-Pro–Lys–Gly-$NH_2 \cdot 2HCl \cdot \frac{1}{2} H_2O$ (II) was a gift from Dr. S. Sakakibara [10]. Z-Pro–Arg-(Tos)–Gly-NH_2 [11] was deprotected with HF [12]; the HF salt was converted to H-Pro–Arg–Gly-$NH_2 \cdot 2AcOH$ (III), which was purified by column chromatography on silica gel using MeOH containing 10% AcOH ($[\alpha]_D^{20}$ −16.1° (*c* 1, H_2O)). H-D-Leu–Gly-$NH_2 \cdot AcOH$ (mp 233–235° dec., $[\alpha]_D^{20}$ −29.5° (*c* 1, MeOH) was obtained by catalytic hydrogenolysis (10% Pd-C, R.T., MeOH containing dil. AcOH) of Z–D-Leu–Gly-NH_2 (mp 86–88°, $[\alpha]_D^{20}$ + 9.8° (*c* 1.2, $CHCl_3$)), which was obtained by acylation of H-Gly-$NH_2 \cdot HCl$ with Z–D-Leu–ONp in the presence of one equivalent Et_3N. H-D-Leu–Gly-NH_2 was allowed to react with Z–Pro–ONp and the resulting Z–Pro–D-Leu–Gly-NH_2 exhibited the physical data reported by Schneider et al. [13]; the tripeptide was subjected to catalytic hydrogenolysis and purification by preparative thin-layer chromatography on silica gel G (90% MeOH) yielding H-Pro–D-Leu–Gly-$NH_2 \cdot$ Crystalline H-Leu–Gly–$NH_2 \cdot$ AcOH (mp 241–242° dec., $[\alpha]_D^{20}$ + 28.0° (*c* 1, MeOH)) was obtained from Z-Leu–Gly-NH_2 prepared according to Boissonnas et al. [14]. The dipeptide was elongated with Z–Pyroglu–OPcp (mp 148–149°, $[\alpha]_D^{20}$ −38.9°

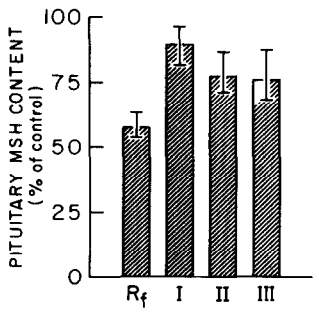

Fig. 1. Inhibition of pituitary MSH release *in vivo* by synthetic C-terminal tripeptides of mammalian neurohypophyseal hormones. The left bar indicates the residual pituitary MSH content after i.v. injection of an homogenate of stalk median eminence. The other 3 bars indicate the MSH content after injection of H-Pro–Lys–Gly-NH_2 (II), H-Pro–Arg–Gly-NH_2 (III), and H-Pro–Leu–Gly-NH_2 (I) (5 ng of each peptide per rat).

(*c* 1, DMF)) in the presence of one equivalent of *N*-methylmorpholine to give crystalline Z–Pyroglu–Leu–Gly-NH_2 (mp 217–218°, $[\alpha]_D^{20}$ –28.2° (*c* 0.8, DMF)), which upon catalytic hydrogenation yielded crystalline H-Pyroglu–Leu–Gly-NH_2 (mp 174–175°, $[\alpha]_D^{20}$ –17.7° (*c* 0.9, MeOH)).

The effect of tri- and dipeptides on *in vivo* pituitary MSH levels was determined in male rats (180–200 g) according to a procedure reported earlier [2–4]. In experiments in which tocinoic acid was tested for its capacity to *inhibit* the release of MSH from the pituitary, the incubated homogenate of stalk median eminence [2–4] was replaced by the acid. Tocinoic acid was injected directly to test its effect on *release* of MSH from the pituitary.

The effect of the various peptides on MSH plasma levels was studied using male rats (180–200 g) prepared the day before the experiment as described in [7]. All agents were injected in a volume of 0.2 ml. The amount of MSH released into the blood plasma was compared with an α-MSH standard (a generous gift from Dr. S. Lande), and is expressed in ng MSH per ml of plasma. All these studies were carried out in Argentina during the winter months, when toad skin (*Bufo arenarum*) is at its highest sensitivity to MSH.

Table 1
Inhibition of MSH-release by tripeptide fragments of the neurohypophyseal hormones, oxytocin, lysine-vasopressin and arginine-vasopressin*.

Tripeptide fragment	Plasma MSH (ng/ml)			
	Exp. 1	Exp. 2	Exp. 3	Average
H-Pro–Leu–Gly–NH_2 (I)	1.0	0.9	0.8	0.9
H-Pro–Lys–Gly–NH_2 (II)	1.3	1.0	0.9	1.1
H-Pro–Arg–Gly–NH_2 (III)	1.9	3.1	1.0	2.6
Controls (R-p only)	3.4	3.5	4.1	3.7

* In triplicate experiments, 20 ng of I, II, or III was injected into the cannulated jungular veins of untreated male rats; three min later 80 ng of H-Cys–Tyr–Ile–Gln–Asn-OH (R-p) was also injected into these animals. Control animals received R-p only. Ten min after the administration of R-p rats were decapitated and the plasma MSH concentration (ng/ml) was measured using α-MSH as standard.

3. Results and discussion

In an effort to assess the structural requirements necessary for the specific action of the MSH–R–IF (I) molecule, we have compared the activities of the synthetic C-terminal tripeptides of lysine- and arginine-vasopressin with that of oxytocin in inhibiting MSH release from male rat pituitary *in vivo*. As can be seen in fig. 1, H-Pro–Lys–Gly-NH_2 (II) and H-Pro–Arg–Gly-NH_2 (III) inhibit the release of MSH, but are less active than equivalent doses of I. These results agree with our earlier finding that the enzymic degradation of any of the three mammalian neurohypophyseal hormones by a hypothalamic microsomal preparation from male rats yields MSH-release-inhibiting activity [2–4].

In the present study we also tested di- and tripeptide analogs of MSH–R–IF, including H-Leu–Gly–NH_2, H-D-Leu–Gly-NH_2, H-Pro–D-Leu–Gly-NH_2, and H-Pyroglu–Leu–Gly-NH_2, but found them to be inactive. These data are in accord with our contention [4] that there are highly specific structural requirements for the N- and C-terminal residues of the MSH–R–IF molecule that are critical to its activity, but

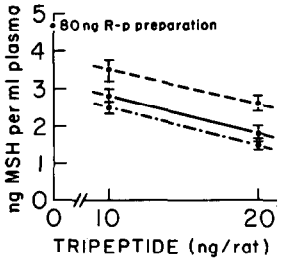

Fig. 2. Inhibition of pituitary MSH release by 10 and 20 ng of H-Pro–Leu–Gly-NH_2 (- : -), H-Pro–Lys–Gly-NH_2 (——), and H-Pro–Arg–Gly-NH_2 (- - -) on the MSH plasma level produced by 80 ng of H-Cys–Tyr–Ile–Gln–Asn-OH preparation. The experiments were performed as described in the legend of table 1. Each point is the average of two experiments; vertical lines indicate standard error.

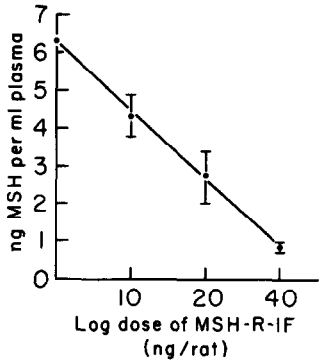

Fig. 3. Dose-response of H-Pro–Leu–Gly-NH_2 as an inhibitor of MSH release in the presence of a constant amount of H-Cys–Tyr–Ile–Gln–Asn-OH, the releasing peptide R-p. The injection of 10, 20, 40 or 80 ng of H-Pro–Leu–Gly-NH_2 was followed 3 min later by an injection of 80 ng of (R-p) into the cannulated jungular vein of the male rat. Ten min thereafter the animal was decapitated and the plasma was analyzed for MSH activity. Results of control animals, injected with 80 ng of (R-p), are shown on the ordinate. Each point represents the average of three experiments; vertical lines indicate standard error.

that modifications in the side chain of the penultimate residue are tolerable, provided the L-configuration is retained.

Although Bower et al. [15] recently reported that synthetic tocinoic acid (the ring component of oxytocin) inhibits MSH release from the rat pituitary *in vitro*, we have not been able to demonstrate that *in vivo* administration of tocinoic acid (10–100 ng) results in either depletion of MSH from the pituitary, or inhibition of the MSH-release stimulated by R-p in the intact rat. Likewise, tocinoic acid showed no inhibitory activity in lesioned frogs [16]. In this context, it should also be noted that H-Pro–Leu–Gly-NH_2 (MSH–R–IF) inhibits the release of pituitary MSH in both *in vitro* and *in vivo* experiments [4], as might be expected of a natural principle.

We next evaluated the relative MSH-release inhibitory activity of MSH–R–IF, of H-Pro–Lys–Gly-NH_2 in a second *in vivo* system. Constant amounts of the R-p preparation were injected into rats, with and without the addition of one of these tripeptides; 10 min later the animals were decapitated, and the MSH in the plasma was measured. The R-p preparation was given to stimulate the release of pituitary MSH, and animals injected only with R-p served as controls. The relative reduction in MSH of animals injected with both R-p and a tripeptide served as an index of the degree of inhibition of MSH release exerted by the tripeptide.

The data in table 1 and fig. 2 reveal that MSH–R–IF is the most potent inhibitor of the pentapeptide R-p, but that the other two tripeptides are also inhibitory. The inhibitory effect of MSH–R–IF was demonstrated to be dose-dependent; introduction of increasing amounts of the inhibitor progressively reduced the plasma concentration of MSH relative to that of controls. Under the present experimental conditions, approx. 60 ng of MSH–R–IF reduced plasma MSH levels below detection limits (fig. 3), suggesting that no MSH, or very little, was released from the rat pituitary.

When constant amounts of MSH–R–IF are injected, the capacity of this peptide to inhibit the release of MSH from the pituitary diminishes with time; these data are summarized in table 2. In control groups, rats injected with 20 ng of R-p only showed a strong elevation of MSH plasma levels, reaching an average of 6.6 ± 0.3 ng MSH per ml plasma. In rats first injected with 40 ng of the MSH–R–IF and then with R-p either 3 or 5 min later, MSH could not be detected in the plasma. However, in rats receiving R-p 7 or 9 min after the administration of MSH–R–IF, plasma concentrations reached 3.5 ± 0.5 and 5.3 ± 0.3, respectively. On the basis of these data the most

Table 2
Reduction with time of MSH–R–IF activity *in vivo**.

Time of R-p administration (min)	Plasma MSH (ng/ml)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Average
3	Neg.	Neg.	Neg.	Neg.	Neg.
5	Neg.	Neg.	Neg.	Neg.	Neg.
7	4.6	2.5	3.3	3.6	3.5 ± 0.5
9	5.4	5.4	4.9	5.7	5.3 ± 0.3
Controls	6.5	6.5	6.8	6.6	6.6 ± 0.3

* Sixteen male rats were divided in four groups and were injected with 40 ng each of MSH–R–IF at time zero. Each animal was then given 20 ng of R-p 3, 5, 7 or 9 min later, respectively. Four additional rats served as controls, receiving 20 ng of R-p only. Ten minutes after administration of R-p, the rats were decapitated and the plasma MSH (ng/ml) was measured.

probable explanation for this drop in activity is a combination of enzymic degradation of MSH–R–IF in blood and various tissues, together with renal clearance. It has been found that MSH–R–IF can be inactivated by rat serum [17].

Acknowledgements

The very personal interest of Drs. M. Sapirstein and S. Taleinsnik in this work is greatly valued. The authors are also grateful to Dr. J. Chakravarty for his help in preliminary synthetic studies, and to Mrs. I. Mintz for performing amino acid analyses. This work was supported in part by the U.S. Public Health Service Grant AM-13567, the Organization Psychiatry Fund (G8-4280), and by the Consejo Nacional de Investigaciones Cientificas y Technicas de la Republica Argentina.

References

[1] S. Taleinsnik and M.E. Tomatis, Endocrinology 81 (1967) 819.
[2] M.E. Celis and S. Taleinsnik, Int. J. Neurosci. 1 (1971) 223.
[3] M.E. Celis, S. Taleinsnik, I.L. Schwartz and R. Walter, Biophys. J. 11 (1971) 98a.
[4] M.E. Celis, S. Taleinsnik and R. Walter, Proc. Natl. Acad. Sci. U.S. 68 (1971) 1428.
[5] M.E. Celis and S. Taleinsnik, Experientia 27 (1971) 1481.
[6] R.M.G. Nair, A.J. Kastin and A.V. Schally, Biochem. Biophys. Res. Commun. 43 (1971) 1376.
[7] M.E. Celis, S. Taleinsnik and R. Walter, Biochem. Biophys. Res. Commun. 45 (1971) 564.
[8] M. Zaoral and J. Rudinger, Coll. Czech. Chem. Commun. 20 (1955) 1183.
[9] V.J. Hruby, C.W. Smith, D.K. Linn, M.F. Ferger and V. du Vigneaud, (as cited in [15]).
[10] S. Sakakibara, T. Fukuda, Y. Kishida and I. Honda, Bull. Chem. Soc. Japan 43 (1970) 3322.
[11] R.L. Huguenin and R.A. Boissonnas, Helv. Chim. Acta 45 (1962) 1629.
[12] S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, Bull. Chem. Soc. Japan 40 (1970) 2164; R.H. Mazur and G. Plume, Experientia 24 (1968) 661.
[13] C.H. Schneider and V. du Vigneaud, J. Am. Chem. Soc. 84 (1962) 3005.
[14] R.A. Boissonnas, St. Guttman, P.-A. Jaquenoud and J.-P. Waller, Helv. Chim. Acta 38 (1955) 1491.
[15] A. Bower, M.E. Hadley and V.J. Hruby, Biochem. Biophys. Res. Commun. 45 (1971) 1185.
[16] R.M.G. Nair, A.J. Kastin and A.V. Schally, Biochem. Biophys. Res. Commun. 47 (1972) 1420.
[17] A.J. Kastin, A.V. Schally and S. Viosca, Proc. Exptl. Biol. Med. 137 (1971) 1437.