EVIDENCE THAT PRO-LEU-GLY-NH_2, TOCINOIC ACID, AND DES-CYS-TOCINOIC ACID DO NOT AFFECT SECRETION OF MELANOCYTE STIMULATING HORMONE

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Received December 11,1972 , Revised January 22,1973

Summary. Pro-Leu-Gly-NH_2, tocinoic acid (Cys-Tyr-Ile-Gln-Asn-Cys-OH), and des-Cys-tocinoic acid (Cys-Tyr-Ile-Gln-Asn-OH) were studied for their effects on release of melanocyte stimulating hormone (MSH) from rat pituitaries. These peptides neither blocked nor stimulated secretion in cell culture systems. Pro-Leu-Gly-NH_2 also failed to alter MSH release when added to whole- and hemi-pituitary incubation systems.

The hypothalamus appears to control secretion of melanocyte stimulating hormone (MSH) through one or more MSH-release inhibiting factors ("MRIF" or "MIF"). Two groups of investigators have proposed L-Pro-L-Leu-Gly-NH_2 as one of the inhibiting factors (1-3); a third asserts that this tripeptide lacks the inhibiting activity while tocinoic acid* and its amide possess it (4,5), but this claim has, in turn, been questioned (6). Thus, MRIF cannot presently be viewed as unequivocally identified, as are the hypothalamic hormones responsible for release of luteinizing hormone and thyrotropin. Until several laboratories can agree on these identifications, including that of the releasing factor (7), pharmacological and clinical investigations with these agents (such as those under way in Parkinson's disease and depression) are apt to produce misleading inferences regarding the role of MSH secretion.

MATERIALS AND METHODS

Two types of *in vitro* releasing experiments were performed. The first employed incubation of whole pituitaries (or, in one experiment, hemi-

*L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-OH

pituitaries). Glands were removed from the brains of Sprague-Dawley male rats, weighing about 200 g, following death in a CO_2 chamber. After

a two-hour preincubation at 37° in Ca-free Krebs-Ringer bicarbonate buffer (KRB, 1 ml per gland) under 95% O_2, 5% CO_2, glands were incubated for 20 minutes in normal KRB. The test agent was usually present during both incubations, but the results did not differ when it was present only during the shorter incubation.

The second system employed cultures of enzymatically dispersed cells from whole pituitaries (8), plating about 1.2 x 10^6 cells per dish, and utilizing them when cell confluency was reached (3-4 days). Test agents were dissolved in Eagle's medium from which the amino acids were deleted, and each treatment was accorded to 5 culture dishes. Incubations were carried out at 37° for 3 hours, following which the supernatant solutions were pooled for assay.

MSH was assayed by its promotion of melanin pigment dispersion in segments of frog skin (male Rana pipiens) according to the experimental design of Shizume, et al (9). Darkening was determined on a Zeiss PMQ II spectrophotometer (chromatogram attachment used with expanded scale in the reflectance mode). The method was standardized by samples of synthetic α-MSH obtained from Dr. S. Lande (Yale) and Dr. J. Gelzer (CIBA-Geigy), and as little as 0.3 ng of MSH could be assayed. Frogs from different suppliers varied in their sensitivity to MSH and could not be mixed. Moreover, owing to variations sometimes contributed by different frogs of the same supplier and different skin sites of the same frog, effects of various treatments were evaluated by analysis of variance adapted specifically to latin square designs (10). Within a given releasing experiment, treated and control systems were compared for their MSH content only at the same dilution prior to frog skin assay (1/300 to 1/1000 for the whole- and hemi-pituitary incubations; 1/20 to 1/100 for the cell culture incubations). Results were recorded and analyzed as reduction in reflectance.

Two different batches of L-Pro-L-Leu-Gly-NH_2 were synthesized and tested; both were assessed by TLC and amino acid analysis as essentially pure. Tocinoic acid and des-Cys-tocinoic acid were kindly supplied by Dr. V. Hruby and were similarly evaluated.

Table I

EFFECT OF PRO-LEU-GLY-NH_2 ON MSH RELEASE IN SHORT-TERM GLAND INCUBATIONS

Exp. No.	Glands/Flask	Tripeptide Concn. (μg/ml)	MSH (Reduction in Reflectance) Control	Experimental	P
1	3	10	2.0 ± 0.2*	4.5 ± 0.9	<0.05
2	3	100	4.0 ± 1.3	2.9 ± 0.7	NS**
2	3	10	4.0 ± 1.3	7.1 ± 1.6	NS
2	3	1	4.0 ± 1.3	5.5 ± 1.1	NS
3	4	500	5.6 ± 0.4	7.5 ± 1.6	NS
3	4	50	5.6 ± 0.4	10.7 ± 1.1	<0.01
3	4	5	5.6 ± 0.4	7.3 ± 0.5	NS
4	4	50	19.5 ± 3.4	26.2 ± 4.2	<0.05
4	4	10	19.5 ± 3.4	23.0 ± 4.1	NS
5	8	50	9.4 ± 1.7	7.4 ± 1.9	NS
	16 halves	50	8.4 ± 2.3	5.8 ± 4.3	NS

* Mean ± S.E.M.
** Not significant.

RESULTS AND DISCUSSION

Table I summarizes the effects of Pro-Leu-Gly-NH_2 in short-term whole- and hemi-pituitary incubations. At levels of the tripeptide nearly 2 x 10^3 times greater than Celis, et al (3) employed in similar experiments and 50 times greater than the highest level of Bower, et al (4), no significant inhibition of release appeared. Indeed, the only three statistically significant changes were all in the direction of enhanced release (experiments 1, 3, 4).

Table II shows the effects of Pro-Leu-Gly-NH_2 in ten cell culture releasing experiments. Two (experiments 11 and 12) indicated MRIF activity and eight indicated no MRIF activity by this peptide.

Tocinoic acid and des-Cys-tocinoic acid were both studied in cell

Table II

EFFECT OF PRO-LEU-GLY-NH_2 ON MSH RELEASE IN CELL CULTURE

Exp. No.	Tripeptide Concn. (μg/ml)	MSH (Reduction in Reflectance) Control	Experimental	P
6	10	2.6 ± 0.3	2.9 ± 0.9	NS
7	10	9.0 ± 2.7	11.3 ± 3.5	NS
8	100	5.6 ± 1.5	4.1 ± 0.6	NS
9	100	7.0 ± 1.8	9.3 ± 1.4	NS
10	100	4.1 ± 0.3	3.4 ± 1.6	NS
11	100*	6.9 ± 2.4	-0.4 ± 0.8	<0.05
11	100**	2.0 ± 0.6	-0.5 ± 0.9	<0.05
12	100	12.4 ± 0.9	6.6 ± 1.3	<0.01
13	100	3.6 ± 0.7	4.2 ± 1.0	NS
14	100	3.1 ± 1.2	2.4 ± 0.3	NS
15	100*	5.1 ± 1.7	6.0 ± 0.8	NS
15	100**	4.0 ± 0.9	2.9 ± 0.9	NS

* Culture supernatant diluted 1/20 before bioassay.
** Culture supernatant diluted 1/40 before bioassay.

cultures at 20 μg/ml and proved to be completely ineffective (Table III). This concentration exceeds or is in the range of the tocinoic acid levels reported as inhibiting release from pituitaries of rats (4) or hamsters (5); it exceeds by a factor of about 10^3 the levels of TRH and LRH found effective in rat pituitary cell cultures (8).

There is obviously no harmony between the various investigations into the identity of MRIF and MRF. The trouble would seem to lie predominantly with the two aspects of the assay: secretion and MSH determination. For example, application of test material directly to the surface of the frog

Table III

EFFECTS OF TOCINOIC ACID AND DES-CYS-TOCINOIC ACID ON MSH RELEASE IN PITUITARY CELL CULTURES

Exp. No.	Additive*	MSH (Reduction in Reflectance) Control	Experimental	P
13	Tocinoic Acid (T.A.)	3.6 ± 0.7	5.3 ± 2.3	NS
	Des-Cys-T.A.	3.6 ± 0.7	3.7 ± 0.9	NS
14	Tocinoic Acid	3.1 ± 1.2	4.9 ± 1.6	NS
	Des-Cys-T.A.	3.1 ± 1.2	3.9 ± 0.5	NS
15	Tocinoic Acid	4.3 ± 0.8	4.1 ± 0.6	NS
	Des-Cys-T.A.	4.3 ± 0.8	2.9 ± 1.1	NS

*Concentrations 20 μg/ml for each additive in each experiment.

pituitary *in situ* gives an equivocal model for MSH release: similar doses of melatonin and Pro-Leu-Gly-NH_2 produced skin lightening by this procedure despite evidence that melatonin and MIF produce opposite effects on the pituitary MSH content (1).

The depletion assay itself raises problems. Celis, *et al* (3), in describing their *in vitro* assays, observed that the concentration of MSH in homogenates of pituitaries was the same regardless of the presence or absence of Pro-Leu-Gly-NH_2. Nonetheless, for their *in vivo* assays, they injected the peptide and measured release by pituitary depletion. The massive quantities in the gland relative to the amount released and variations in this quantity from rat to rat (11) make such residual measurements highly questionable.

Releasing experiments with isolated pituitaries (undissected, anterior, or halved) are carried out widely, but their validity rests on the assumptions

that the contents of a given hormone and the distribution of cell types are fairly uniform within and between glands and that the glands are not damaged. Shapiro, et al found that the total MSH content could vary by 300 percent among rat pituitaries (11). Guillemin and Vale reported that quarters of the same pituitary responded with large variations to identical doses of a releasing hormone (12). Moreover, there is evidence (12) that a 4-hour incubation (4) can lead to necrosis of pituitary tissue.

Variations within glands or between glands cannot easily be overcome by pooling large numbers. When we set up six control flasks (no test agent), each containing four whole pituitaries, and followed the incubation with a 6 x 6 latin square analysis of frog skin darkening, the variance ratio, F, for columns ("treatments") was highly significant, 34.4, far exceeding F for rows (frogs) and diagonals (skin sites). With means ranging between 9.3 and 21.7 the presence of test agents in one or more flasks could easily have been misinterpreted.

The use of cell cultures diminishes these problems. In preparing cultures, the pituitary cells are dispersed and randomized before being distributed to dishes (in the present study at a seed level of more than a million cells per dish). Essentially, this converts a batch of pituitary glands into a single population of cells capable of highly consistent responses to releasing factors within a given experiment (8).

In the bioassay for MSH it seems reasonable to replace visual scoring of hundreds of melanophores (1, 3) with instrumental detection of light reflectance. Moreover, with a randomized design coupled to the t test for significance (4), additions by frogs and skin site to the variation will be inadequately controlled, heightening the chance that an active agent will be overlooked. Hopefully, a discriminating radioimmunoassay will obviate this aspect of the problem.

ACKNOWLEDGMENT

The authors are indebted to Dr. W.H. McGregor and Mr. W. McKinley for their valuable contributions in peptide synthesis and analysis.

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