168. Hormone-Receptor Interactions. Demonstration of Two Message Sequences (Active Sites) in α-Melanotropin

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In memoriam *Josef Rudinger*

Summary. The purpose of this investigation was to elucidate the biological significance of the tripeptide sequence 11–13,-Lys-Pro-Val · NH_2 contained in α-melanocyte-stimulating hormone. To this end the *in vitro* melanotropic activities of 21 synthetic peptides related to the hormone were determined and compared with one-another and with results reported in the literature. The tripeptide amide, H · Lys-Pro-Val · NH_2, its N^{α}-acetylderivative, and -weakly, but distinctly- N^{α}-acetyl-L-lysine-amide were found to be hormonally active. The following conclusions were drawn: (1) α-melanophore-stimulating hormone possesses two message sequences (active sites), -Met-Glu-His-Phe-Arg-Trp-Gly-, and -Lys-Pro-Val · NH_2 which are capable of independently triggering the hormone receptor responsible for melanin dispersion. (2) In correct covalent combination, the two message sequences act in a 'cooperative' manner to potentiate their activities on the receptors. (3) -Lys-Pro-Val- is an address sequence in adrenocorticotropic hormone and a message sequence in α-melanocyte-stimulating hormone. This implies that the two receptors (adrenal and melanocyte), albeit recognizing -Met-Glu-His-Phe-Arg-Trp-Gly- as stimulus, differ fundamentally in their response to -Lys-Pro-Val-.

1. Introduction. – This report deals with the one-dimensional organization of information in polypeptide hormones [1].

α-Melanocyte-stimulating hormone (α-MSH, α-melanotropin), β-melanocyte-stimulating hormone (β-MSH, β-melanotropin), and adrenocorticotrophic hormone (ACTH, adrenocorticotropin) are structurally related, Table 1. They belong to the class of polypeptide hormones with sychnological organization [2]. This means that discrete sequences of adjacent amino-acids ('continuate words') are responsible for different components of the biological activity [3a, b].

Table 1. *Primary structures of melanotropic and corticotropic hormones- α-MSH, β-MSH (bovine), and ACTH. Common sequences framed*

	1 10 20 24 39
ACTH	SYSMEHFRWGKPVGKKRRPVKVYP-----F
α-MSH	Ac-SYSMEHFRWGKPV-NH_2
β-MSH	DSGPYKMEHFRWGSPPKD

1) We thank the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* for subsidy. This work is part of the doctoral thesis of *A. E.* Abbreviations and nomenclature according to *E. Wünsch*: «Synthese von Peptiden», Bd. 15, Teil 1 of «*Houben-Weyl*, Methoden der organischen Chemie», *E. Müller*, ed., Georg Thieme Verlag, Stuttgart, 1974 (three-letter symbolism), and *Margaret O. Dayhoff*: «Atlas of Protein Sequence and Structure 1972», Vol. 5, National Biomedical Research Foundation, Silver Spring, Maryland (one-letter symbolism).

Harris [4] had already suggested that the common biological activity of the three hormones on melanocytes might be due to the common heptapeptide sequence, -Met-Glu-His-Phe-Arg-Trp-Gly-. Synthetic experiments have shown this to be true, Table 2. The same sequence is also capable of eliciting the adrenal steroidogenic response typical of ACTH [5]. It has therefore been called the hormonal active site [6] or *message sequence* [2]. It is supposed to contain the structural elements necessary for triggering the biological responses, or – in other words – for providing the stimulus for both the MSH and ACTH receptors of melanocytes and adrenal cells.

Table 2. *Melanotropic activity of synthetic peptides related to the first message sequence of α-MSH, β-MSH, and ACTH*

No.	Sequence	U/mmol	Compound No. or reference ()
1	Ac·SYSMEHFRWGKPV·NH_2	$4 \cdot 10^{10}$	1 (10)
2	MEHFR	0	(16)
3	SYSMEHFR	0	(16)
3a	FR	$6 \cdot 10^{2}$	3a (10)
4	FRWG	$6 \cdot 10^{2}$	4 (10)
5	Ac·FRWG	$6 \cdot 10^{3}$	5 (10)
6	FRLG	$3 \cdot 10^{3}$	6 (10)
7	HFRW	$6 \cdot 10^{3}$	(17)
8	HFRWG	$2 \cdot 10^{4}$	(18a,b, 19,20)
9	EHFRWG	$2 \cdot 10^{5}$	(21)
"	"	$1 \cdot 10^{5}$	9 [1]
10	Ac·EHFRWG	$4 \cdot 10^{5}$	10 (10)
11	QHFRWG	$2 \cdot 10^{5}$	(22b)
12	GHFRWG	$2 \cdot 10^{5}$	(23)
13	MEHFRWG	$1 \cdot 10^{6}$	(24)
14	MQHFRWG	$3 \cdot 10^{5}$	(22a,25)
15	SMQHFRWG	$7 \cdot 10^{5}$	(16,26)
16	Ac·SMEHFRWG	$4 \cdot 10^{6}$	16 (10)
17	SYSMEHFRWG	$3 \cdot 10^{6}$	17 (10)
18	SYSMQHFRWG	$4 \cdot 10^{6}$	(27a,b,28,22c)
19	Ac·SYSMEHFRWG	$1 \cdot 10^{7}$	(3a)

[1] Prepared from Z·E(OtBu)HFRWG [22a,b,c] by Dr. *Georg Karlaganis*.

In the case of ACTH, the peptides 1–3 and 11–24 are inactive *per se*, but potentiate the biological activity of the message sequence to maximal values if combined by the correct covalent bonds [5]. As a free tetradecapeptide, sequence 11–24 reversibly associates with lipocytes and adrenal cells (unpublished experiments in this laboratory) and specifically inhibits the action of ACTH and its message sequence [7]. Binding of the decapeptide amide 11–20 to bovine adrenal cortical membrane fractions has also been demonstrated [8a, b]. Consequently, stretches 11–20 and 11–24 have been referred to as binding sites [8a, b], or *address sequences* [2][2]).

[2] We find the name 'binding site' somewhat misleading, because stretches 1–3 and 4–10 also contribute to receptor affinity (unpublished experiments). The term 'address sequence' is to be preferred, because it is this stretch (11–20 or 11–24) that contains the structural information necessary for 'addressing' the attached message sequence to the correct target cells of the adrenal cortex. This is achieved by providing *additional, cell-specific* (i.e. *receptor-specific*) *affinity*.

In the case of α-MSH, the sequences 1–3 and 11–13, that are also contained in ACTH, potentiate the melanotropic activity of the central heptapeptide. It has been tacitly assumed that – because it is part of the corresponding sequence in ACTH – the tripeptide amide 11–13 might qualify as a melanocyte-specific address.

We have studied this assumption by preparing amino-acid and peptide derivatives related to -Lys-Pro-Val · NH_2 and testing them for melanotropic activity *in vitro* (assay essentially that of *Shizume, Lerner & Fitzpatrick* [9], but modified to be statistically more pleasing). Instead of being inactive and inhibitory, the tripeptide and its acetyl derivative exhibited agonistic potency in almost the same order of magnitude as the central heptapeptide (Table 3, Fig. 1).

This means that the melanocyte receptors recognize -Lys-Pro-Val- as message, whereas the adrenal receptors recognize it as part of the (non-stimulating) adress.

Table 3. *Melanotropic activity of synthetic peptides related to the C-terminal second message sequence of α-MSH and extending into the N-terminal first message sequence*

No.	Sequence	U/mmol	Compound No. or reference ()
20	PV·NH_2	0	20 (10)
21	GK·NH_2	$8 \cdot 10^2$	21 [1]
22	Ac·K·NH_2	$4 \cdot 10^3$	22 (10)
23	KPV·NH_2	$3 \cdot 10^4$	23 "
24	Ac·KPV·NH_2	$8 \cdot 10^4$	24 "
25	Ac·WGKPV·NH_2	$6 \cdot 10^5$	25 "
26	FRWGKPV·NH_2	$1 \cdot 10^6$	Liddle in (29)
"	"	$2 \cdot 10^4$	Schally in (29)
"	"	$4 \cdot 10^5$	(28)
27	Ac·FRWGKPV·NH_2	$5 \cdot 10^6$	27 (10)
28	Ac·EHFRWGKPV·NH_2	$5 \cdot 10^8$	28 "
29	Ac·MEHFRWGKPV·NH_2	$3 \cdot 10^9$	29 "
30	Ac·SMEHFRWGKPV·NH_2	$7 \cdot 10^9$	(30)
31	Ac·YSMEHFRWGKPV·NH_2	$2 \cdot 10^{10}$	31 (10)
1	Ac·SYSMEHFRWGKPV·NH_2	$4 \cdot 10^{10}$	(31, 32, 33, 12)

[1]) Prepared by Dr. *Ernst Fischer* in this laboratory.

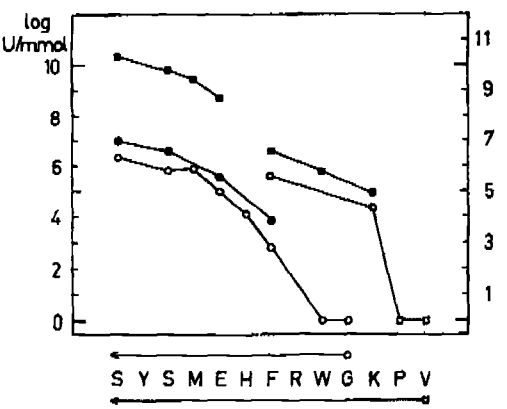

Fig. 1. *Melanotropic activity in vitro of peptides and peptide derivatives obtained by sequence extension 10 → 1* (○, ●) *and 13 → 1* (□, ■). Open symbols indicate unsubstituted, dark symbols acetylated N-terminal amino groups

Our unexpected finding introduces the necessity of explaining not only the potentiating interaction between message-recognizing and address-recognizing sites of hormone receptors, but also those between two otherwise independent message-recognizing sites. It also raises the question of possible evolutionary interconversions of the two types of site in hormone receptors.

2. Material and Methods. – *Synthetic peptides* were prepared by a classical approach and were analytically pure [10].

Bioassays. The melanotropic activity of peptides was determined *in vitro* using essentially the reflectometric assay of *Shizume, Lerner & Fitzpatrick* [9] as modified for microquantities by *Geschwind & Huseby* [11]. We encountered disturbing fluctuations in our first results. A separate investigation revealed that this was due to the fact that the variations in reflectance are relatively large in the first 2 h during which the calibration is usually carried out. Typical observations are shown in Fig. 2: 20 'competent' skins [11] were alternately washed for 1 h with *Ringer* solution and incubated for 1 h with *Ringer* solution containing 1 U/ml of α-MSH (prepared according to [10], *cf.* [12]). The decrease of reflectivity is displayed on the ordinate as a relative effect, *i.e.* the difference of reflectivity of every single skin between treatment with 1 U at 240 min and the base-value determined after washing at 30 min was arbitrarily taken as the 100%-value. Five skins were grouped together and the mean value displayed in Fig. 2. The variations are especially small (5–10%) between the 3rd and 8th hour, and are greater before and after.

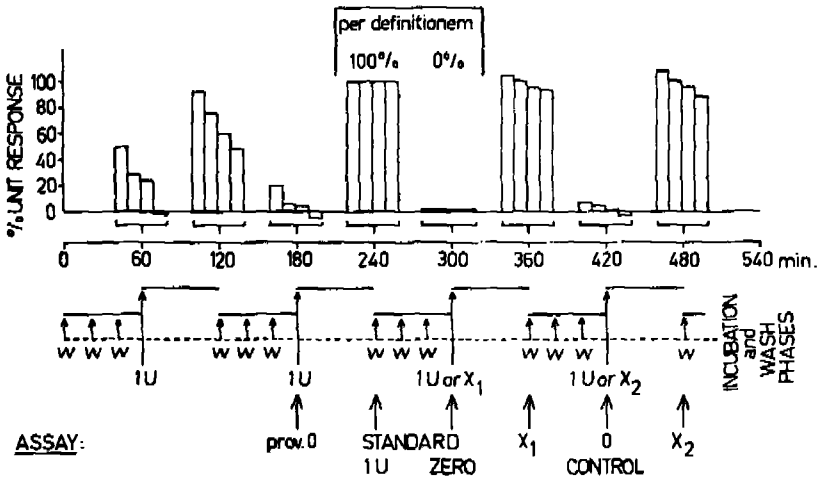

Fig. 2. *Effect of 1 α-MSH-U/ml on 20 skins of Rana pipiens in a typical experiment.* Modified assay procedure. Explanation see text

Therefore, our test procedure was carried out as follows: After a 60 minutes' wash with *Ringer* solution, the skins were pretreated for 1 h with 1 U of α-MSH, and then again washed for 1 h. At the end of this cycle (180 min) an approximate zero-value was determined. After a second cycle of incubation and washing, the zero-value was definitely determined (300 min). Only those skins were used that displayed a reflectance-difference of at least 8 reflectance units for 1 U of α-MSH and were otherwise suitable according to the criteria of *Kastin, Miller & Schally* [13]. Incubation with the unknown sample and washing provided the x_1-value ('zero' at 300 min *vs.* incubation value at 360 min); x_2 was determined after the fourth cycle ('zero' *vs.* 480 min). The x_2-values were used as approximate values for unknown samples, the exact activities of which were determined as x_1 in a next experiment. The reported values are the means of 3 assays with 3 different concentrations of the same compound (in the relative range of about 1:1.5:2) giving an approximately linear relationship of the observed responses. Trivial pH effects were excluded by careful control after addition of the compound to be tested.

All assays were performed with specimens of *Rana pipiens*, 7.5–9 cm long, that had been kept in the light on a white surface for 48 h preceding the experiment.

3. Results. – The *in vitro* melanotropic potencies are expressed in Units per millimole because of the large differences between the molecular weights of the peptides investigated. Tables 2 and 3 list the agonistic potencies of peptides related to the 'common' heptapeptide sequence, -Met-Glu-His-Phe-Arg-Trp-Gly- (–MEHFRWG–), and to the C-terminal tripeptide sequence of α-MSH, -Lys-Pro-Val · NH_2 (–KPV · NH_2), respectively. Examples from the literature were included and repeated to test the consistency of our methods. Fig. 1 displays the effects of chain elongations from C-terminal Gly^{10} and Val · $NH_2{}^{13}$ towards the N-terminus: the values were selected from Tables 2 and 3.

The highest activities measured required about 10^{-7} mg of compound per ml (~10^{10} U/g), the lowest ones (~10^{3} U/g) up to 1 mg, intermediate activities typical of the message sequences (~10^{5}–10^{6} U/g) about 1–10 μg. Zero potency was assigned if more than 1 mg/ml of the compound elicited no reflectance change. The time dependance of reflectance changes during the incubation phase was the same for α-MSH and two representatives of the 'second message sequence', Ac · Lys-Pro-Val · NH_2 and Ac · Lys · NH_2, with a half-time of approximately 15 min. The half-time for the wash phase was somewhat longer for the amino-acid and tripeptide derivatives, than for α-MSH, but was also complete after 1 hour (Fig. 3).

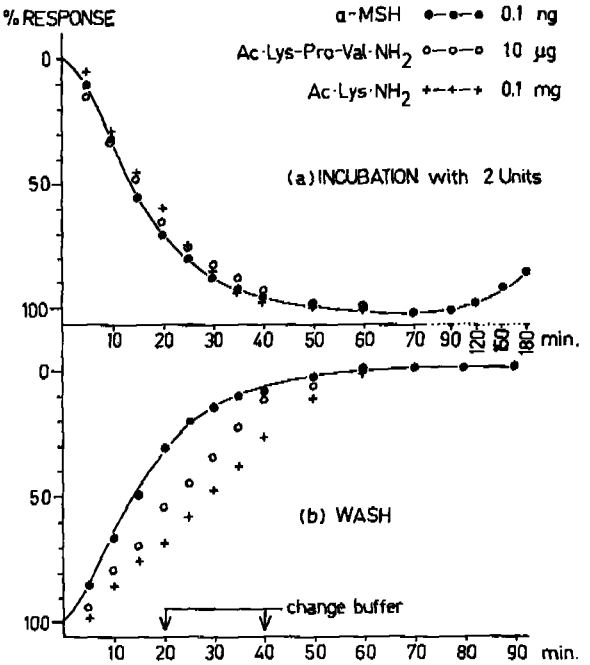

Fig. 3. *Time dependence of the melanotropic response of 20 Rana pipiens skins each for three peptides during prolonged incubation* (a) *and wash* (b) *phases*

4. Discussion. – The usual *in vitro* assay [9, 11] was modified to reduce statistical fluctuations. The obtained activity values in Units [9] are derived from the linear part of the dose-response curves. They are not identical with the potencies usually expressed as hormone concentrations necessary to elicit half-maximal response. Their ratios

(for different compounds) are assumed to reflect the ratios of these potencies. They are, under these premisses, valid expressions for the relative melanotropic activities of the peptides investigated.

We found the activity of synthetic α-MSH [10] and of the synthetic hexapeptide, EHFRWG, to be – within the limits of experimental error – identical with the values reported in the literature, Table 2. Furthermore, the activities of compounds from this investigation and from the literature, which constitute a structural series, were found to follow an uninterrupted, smooth curve. This indicates that our results are quantitatively compatible with those of earlier workers (Tables 2 and 3, Fig. 1).

In the case of low ($\sim 10^5$ U/mmol) and very low ($\sim 10^2$ U/mmol) activities, we feel quite sure that they result from specific hormone-receptor interactions for 3 reasons: (1) Acetylation at the N-terminus produces an approximately 2- to 10-fold rise in activity, much the same as with peptides exhibiting high activity (Fig. 1). (2) The time course of the response during the incubation and wash phases is approximately the same for α-MSH, Ac · KPV · NH_2, and Ac · K · NH_2; slight differences during the wash phase are probably due to the larger amounts of amino-acid and tripeptide derivatives present and to solubility differences (Fig. 3). (3) Lysine and arginine are inactive even in high doses.

The 7 peptides related to the common heptapeptide N-terminal message sequence –MEHFRWG– prepared for this study (**4**, **5**, **6**, **9**, **10**, **16** and **17**) fit well into the series compiled from the literature (Table 2). The search for the smallest melanotropically active unit within this message sequence is not yet complete: **4** indicates that histidine, **7** that glycine is not essential. In the remaining, presumably active tripeptide Phe-Arg-Trp, tryptophane probably does not play a key role. It can be replaced by phenylalanine and pentamethylphenylalanine in the complete α-MSH sequence with retention of activity [14]. The latter amino-acid has charge-transfer donor properties similar to those of tryptophan [15]. Aromaticity and charge-transfer donor properties, however, are not essential, because tryptophan can be replaced by leucine as in the tetrapeptide **6**. Trp cannot be completely eliminated (**2**, **3**). In this study, the simple dipeptide FR (**3a**) was found to be about as active as FRWG; whether it triggers the first or the second message site of the receptor is still unknown (it is possible that FR acts by virtue of its structural analogy to Ac · K · NH_2 (**22**). However, a mixture of 10 mg/ml each of phenylalanine and arginine was inactive).

An approximate activity plateau is reached in the heptapeptides **13** and **14** with a mean activity of about $6 \cdot 10^5$ U/mmol. The N-terminal tripeptide sequence enhances activity about 3- to 5-fold (**17**). N-terminal acetylation at various stages (tetrapeptides **4/5**, hexapeptides **9/10**, octapeptides **15/16**, and decapeptides **18/19**) activates between 2- and 10-fold (Fig. 1). The methionine sulfur appears to have no specific effect: Ac · Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val · NH_2 (**29a**) [10] (replacement of Met by Nle, L-norleucine, not included in the Tables) displays an activity of $2 \cdot 10^9$ U/mmol, about 60–70% of that of **29**.

Within the N-terminal tripeptide sequence of α-MSH, lysine and Pro-Val · NH_2 (**20**) are inactive (Table 3). Gly-Lys · NH_2 (**21**) and Ac · Lys · NH_2 (**22**) elicit low, reproducible melanotropic activity. This is strongly enhanced in the complete sequence Lys-Pro-Val · NH_2 (**23**) and in the acetylated tripeptide (**24**). The activity attained is greater than that of tetrapeptides (**4**–**7**) contained in the N-terminal mes-

sage sequence, and comparable to that of pentapeptide **8**, hexapeptides **9–12**, and even heptapeptides **13** and **14**.

It could be argued that this second message sequence, by virtue of the basic lysine side-chain, triggers a receptor site responsive to the (basic) arginine residue in a rather unspecific manner and is therefore not a message sequence in its own right. This argument appears to be disproved by the data of Fig. 1. Covalent combination of active peptides from the first and second message sequences leads to peptides with activities corresponding more closely to the products than to the sums of component activities, e.g. Ac·EHFRWGKPV·NH_2 (**28**), from Ac·EHFRWG (**10**) and KPV·NH_2 (**23**) (or **1** from **19** and **23**; **27** from **5** and **23** is about intermediate between sum and product). Mixtures of 0.1 μg/ml of Ac·EHFRWG (**10**) plus 10 μg/ml Ac·KPV·NH_2 (**24**) showed no multiplicative effect.

The α-MSH-specific dipeptide sequence Trp-Gly enhances the activity of **24** between 5- and 10-fold (**25**). We suggest that this is due to enhancement of specific receptor affinity, much the same as by Ser-Tyr-Ser in the pairs **29/1** or **13/17**.

It appears that the melanocyte α-MSH receptor contains two message-recognizing sites, one for MEHFRWG, the N-terminal or first message sequence of α-MSH, and one for KPV·NH_2, the C-terminal or second message sequence. The two sites can either operate alone or in combination to trigger melanin dispersion. In combination, they have a multiplicative, 'cooperative' effect. Whether they produce melanin dispersion by the same, or by different mechanisms remains to be elucidated (cAMP?, cGMP?, others?).

The adrenal response to ACTH is not triggered by KPV, only by MEHFRWG. This indicates a major difference between the two receptors. Whether or not they are descended from a common ancestral receptor with a change from an address-recognizing site for KPV to a message-recognizing site for the same tripeptide sequence, or *vice versa*, is another question, outside the scope of this investigation.

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169. Preparation of Neutral Ionophores for Alkali and Alkaline Earth Metal Cations and their Application in Ion Selective Membrane Electrodes

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(9. IV. 75)

Summary. The preparation of a series of non-cyclic, uncharged ligands able to selectively complex alkali and alkaline earth metal cations is described. These molecules are designed to be used as carriers for cations through membranes. Some of the compounds show high Ca^{2+} and Na^{+} selectivity, respectively, in liquid membrane electrodes.

1. Introduction. – Certain uncharged, lipophilic complexing agents for cations behave as carriers for these ions through membranes [1] [2] and are therefore attractive components for ion selective liquid membrane electrodes [2] [3]. Although quite a number of such cyclic compounds has been described [2–6] only few are potentially useful components in liquid membrane electrodes [3] [7]. A series of non cyclic synthetic ligands showing high ion selectivity as well as carrier properties for