THE MELANOCYTE STIMULATING ACTIVITY OF N-NITROSO-2-CHLOROETHYL-CARBAMOYL DERIVATIVES OF α-MELANOTROPIN FRAGMENTS

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Received February 15th, 1988
Accepted May 3rd, 1988

The authors feel to be honoured by the opportunity to dedicate this paper to the memory of Karel Bláha. He was over decades one of the greatest friends of all members of the Hungarian team, founder and life and soul of the cooperation between peptide chemists in Prague and Budapest. His amiable personality will be never forgotten.

A number of N-nitroso-2-chloroethyl-carbamoyl (Q(NO)) derivatives of α-melanotropin fragments have been synthesized and their effect on the frog skin melanocytes studied. Peptides substituted in this way possess the biological activity of the parent compounds, indicating that they preserved their receptor recognizing ability. These compounds can therefore serve as affinity labels. Some of these derivatives, related to the C-terminal sequence of α-melanotropin show prolonged darkening reaction, which does not influence the subsequent reaction of melanocytes with α-melanotropin. The Q(NO)-derivative of a fragment derived from the classical active site of the hormone shows, however, inhibition of the effect of α-melanotropin. It can be concluded that the latter peptide acts through the melanotropin receptor, while others, related to the C-terminal sequence of the hormone through another mechanism.

The N-nitroso-2-chloroethylcarbamoyl (Cl–CH_2–CH_2–N(NO)–CO, Q(NO)) derivatives of polypeptide hormones are chemically reactive compounds, in principle capable of covalently modifying the hormone receptor. A basic condition of this behavior is that the hormone molecule, or its still biologically active smaller fragments should not lose their receptor recognizing ability by substitution with the Q(NO)-group, and after binding these compounds should find a suitable reactive amino acid side chain in a favorable position on the receptor. With this working hypothesis in mind, we started the synthesis of the Q(NO)-derivatives of some C-terminal fragments of α-melanotropin[1]. The remarkable antitumor activity of these compounds has been reported earlier[2]. In the present paper the melanocyte stimulating activity

of these compounds, as well as our first observations on their interaction with the melanotropin receptor are described.

It is known that small fragments of α-melanotropin (tri- and tetrapeptides) possess melanocyte stimulating (melanin dispersing) activity characteristic of the intact hormone, though this effect is by six orders of magnitude lower than that of the whole hormone[3].

Moreover, a common property of the melanotropin fragments and the melanotropin sequence itself is that acetylation of the terminal amino group enhances their melanocyte stimulating activity (for a review, see ref.[4]). Therefore, it was to be expected that biologically active C-terminal fragments (Lys-Pro-Val-NH_2, Gly-Lys-Pro-Val-NH_2, Trp-Gly-Lys-Pro-Val-NH_2) would preserve their activity when substituted with the Q(NO)-group. For a better comparison, the corresponding N-acetyl derivatives have also been prepared and tested.

For certain reasons[2] we synthesized and tested a tetrapeptide derivative (Q(NO)-Pro-Lys-Pro-Val-NH_2) slightly differing in structure from the C-terminal sequence of α-melanotropin. Finally, in order to investigate the behavior of the Q(NO)-derivatives of compounds more resembling in activity to the natural hormone, a hexapeptide derivative related to the so-called classical active site of the α-melanotropin, Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe, was also prepared.

EXPERIMENTAL

The peptides investigated are compiled in Table I. Some of these (Lys-Pro-Val-NH_2, Gly-Lys-Pro-Val-NH_2 and Trp-Gly-Lys-Pro-Val-NH_2 in the free and N^{ε}-acetylated form) were synthesized by us and other authors earlier, as reviewed in ref.[4]. Synthesis of Q(NO)-Lys-Pro-Val--NH_2, Q(NO)-Gly-Lys-Pro-Val-NH_2 and Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2 is described in ref.[1]. A brief report of the synthesis of H-Pro-Lys-Pro-Val-NH_2 appeared in ref.[2]. Synthesis of the other compounds is detailed in the following. Abbreviations common in the peptide synthesis are used. The solvent systems applied for thin layer chromatography are the following: A: ethyl acetate–pyridine–acetic acid–water (60:20:6:11); B: ethyl acetate–pyridine–acetic acid–water (60:40:12:22); C: butanol–pyridine–acetic acid–water (4:1:1:1); D: butanol–acetic acid–water (4:1:1); adsorbent Merck Kieselgel 60.

Ac-Pro-Lys(Boc)-Pro-Val-NH_2 (*I*)

H-Pro-Lys(Boc)-Pro-Val-NH_2 (ref.[2]) (200 mg, 0.36 mmol) was dissolved in 4 ml of DMF, and 4-nitrophenyl acetate (90 mg, 0.5 mmol) was added to the solution. After standing overnight at room temperature the solvent was removed in vacuo, and the residue was purified on a silica gel column in the solvent system A, to give 170 mg (82%) of the protected acetyl tetrapeptide *I*. R_F: 0.53 (A), 0.83 (C); $[\alpha]_{589}^{23}$ −105.7 (*c* 1, methanol). For $C_{28}H_{48}N_6O_7$ (580.7) calculated: 14.47% N; found: 14.09% N.

Ac-Pro-Lys-Pro-Val-NH_2.HCl (*II*)

Tetrapeptide *I* (130 mg) was suspended in 3 ml of 3 M HCl–ethyl acetate. After stirring for 15 min ether was added, the precipitate filtered and washed on the filter with ether. The product was dissolved in water and freeze-dried to give 110 mg (95%) of *II*. R_F: 0.49 (C), 0.09 (D); $[\alpha]_{589}^{23}$ −100.7 (*c* 0.88, methanol). For $C_{23}H_{41}ClN_6O_5$ (517.1) calculated: 16.24% N; found: 15.90% N.

Q(NO)-Pro-Lys-Pro-Val-NH_2.HCl (*III*)

Q(NO)-Pro-Lys(Boc)-Pro-Val-NH_2 (ref.[2]) (673 mg, 1mmol) was kept in 10 ml 0.12 M HCl–98% formic acid at 0 C for 10 min. After concentration in vacuo the residue was dissolved in cold water and freeze-dried. The crude product was purified on a silica gel column in a cold room (+5 C) in solvent system B, to give 90 mg (15%) of *III*. R_F: 0.66 (B), 0.65 (C). UV spectrum, λ_{max} (ε): 395 (83). For $C_{24}H_{42}Cl_2N_8O_6$ (609.6) calculated: 11.63% Cl; found: 12.00% Cl.

Z-His-D-Phe-OMe (*IV*)

Z-His-N_2H_3 (10.9 g, 36 mmol) was suspended in 100 ml of DMF and chilled to −5 C. To this suspension 28.1 ml 6 M HCl (168 mmol) was added under stirring in 5 min. Azide was prepared by the addition of 2.9 g (42 mmol) $NaNO_2$ in 6.3 ml water, and added to the solution of D-Phe-OMe prepared from 9 g (42 mmol) hydrochloride and 29.3 ml (210 mmol) triethylamine in 100 ml DMF at −5 C. The reaction mixture was stirred at the same temperature for 30 min, and for 2 − 3 h at 0 C. After standing overnight at room temperature the precipitated triethylammonium chloride was filtered and the solution evaporated in vacuo. For purification the residue was dissolved in ethyl acetate–water (200 − 50 ml), the organic layer washed with bicarbonate and water, dried and evaporated. Crystallization from methanol–ether yielded 9.78 g (60.3%) of *IV*, m.p. 158 − 159 C. R_F: 0.33 (A), 0.74 (C); $[\alpha]_{589}^{23}$ −6.0 (*c* 1, methanol). For $C_{24}H_{26}N_4O_5$ (450.5) calculated: 63.99% C, 5.82% H, 12.44% N; found: 63.55% C, 5.87% H, 12.48% N.

H-His-D-Phe-OMe.HCl (*V*)

The protected dipeptide *IV* (8.3 g, 18.4 mmol) was dissolved in 60 ml methanol containing one equivalent of HCl, and hydrogenated in the presence of 1.2 g 10% Pd C catalyst. After filtration the solvent was removed in vacuo and the residue crystallized from ethanol–ether, to yield 6.12 g (94%) of *V*, m.p. 152 − 153 C. R_F: 0.19 (A), 0.52 (B), 0.55 (C); $[\alpha]_{589}^{23}$ +16.7 (*c* 1, methanol). For $C_{16}H_{21}ClN_4O_3$ (352.8) calculated: 54.47% C, 6.00% H, 15.88% N; found: 53.73% C, 5.78% H, 15.54% N.

Z-Gly-His-D-Phe-OMe (*VI*)

Dipeptide *V* (2 g, 5.7 mmol) was dissolved in 20 ml DMF, and at 0 C triethylamine (0.79 ml, 5.7 mmol) was added. The precipitated hydrochloride was filtered off, and Z-Gly-OPCP (2.59 g, 5.7 mmol) was added to the solution. After 2 h of stirring at 0 C the reaction mixture was allowed to stand overnight at room temperature, the solvent evaporated in vacuo, the residue dissolved in ethyl acetate, washed with water and bicarbonate, dried and evaporated. The residue was powdered under ethyl acetate, collected and crystallized from methanol–ether. Yield 2.04 g (70.9%), m.p. 182 − 185 C. R_F: 0.46 (A), 0.76 (B); $[\alpha]_{589}^{23}$ −8.8 (*c* 1, methanol). For $C_{26}H_{29}N_5O_6$ (507.5) calculated: 61.52% C, 5.77% H, 13.79% N; found: 61.44% C, 5.68% H, 13.37% N.

Z-Gly-His-D-Phe-N_2H_3.H_2O (*VII*)

Protected tripeptide ester *VI* (1.7 g, 3.5 mmol) was dissolved in 18 ml methanol and hydrazine (0.60 ml, 12.3 mmol) was added. After standing for 1 h at room temperature, the mixture was allowed to stand overnight in refrigerator. The precipitated hydrazide was collected and crystallized from methanol–ether. Yield 1.6 g (88.8%), m.p. 174 – 176 C. R_F: 0.32 (A); $[\alpha]_{589}^{23}$ +10.1 (*c* 1, DMF). For $C_{25}H_{31}N_7O_6$ (525.5) calculated: 57.13% C, 5.95% H, 18.64% N; found: 57.04% C, 6.13% H, 18.60% N.

Z-Gly-His-D-Phe-Arg-Trp-Gly-OMe.2 CH_3COOH (*VIII*)

Hydrazide *VII* (1.65 g, 3.15 mmol) was dissolved in a mixture of DMF–tetrahydrofuran containing 0.033 g (9.46 mmol) HCl, the solution chilled to −15 C, and isoamyl nitrite (0.47 ml, 3.45 mmol) was added dropwise, stirring was continued for 15 min, and the resulting azide solution was added to 1.74 g (3.15 mmol) H-Arg-Trp-Gly-OMe.2 CH_3COOH and 1.76 ml (12.6 mmol) triethylamine in 2 ml DMF. The reaction mixture was stirred for 2 h at −10 C, and allowed to stand in refrigerator overnight. The solvents were removed in vacuo, the residue powdered under ether, collected, and purified by silica gel column chromatography (solvent A). Yield 1.21 g (39.7%); R_F: 0.22 (A), 0.70 (C); $[\alpha]_{589}^{23}$ −17 (*c* 1, methanol). For $C_{49}H_{62}N_{12}O_{13}$ (1 027.1) calculated: 57.30% C, 6.10% H, 16.36% N; found: 57.62% C, 6.28% H, 16.00% N.

H-Gly-His-D-Phe-Arg-Trp-Gly-OMe.3 HCl (*IX*)

Protected hexapeptide *VIII* (1.0 g, 0.97 mmol) was hydrogenated in methanol in the presence of 0.2 g Pd C catalyst and 0.11 g (3 mmol) HCl. The filtered solution was concentrated in vacuo, the residue dissolved in ethanol and precipitated with ether, to yield 0.81 g (95%) hexapeptide ester trihydrochloride. R_F: 0.30 (B), 0.12 (C); $[\alpha]_{589}^{23}$ −50.2 (*c* 1, methanol). For $C_{37}H_{51}Cl_3N_{12}O_7$ (882.3) calculated: 12.06% Cl; found: 11.87% Cl. Amino acid analysis: Gly 1.96, His 0.96, Phe 1.00, Arg 1.09.

Ac-Gly-His-D-Phe-Arg-Trp-Gly-OMe.2 HCl (*X*)

Hexapeptide *IX* (100 mg, 0.11 mmol) was dissolved in 1.0 ml DMF, neutralized with triethylamine, and 4-nitrophenyl acetate (20 mg, 0.11 mmol) was added. The pH was kept between 7 and 8, and the mixture stirred for 4 h. The acetylated peptide was precipitated with ether and purified on silica gel column using a solvent mixture of ethyl acetate–pyridine–acetic acid–water (45:20:6:11) as eluent. The pure product was obtained by a final precipitation from methanol–ether, to yield 40 mg (40%). R_F: 0.48 (C), 0.36 (B). For $C_{39}H_{52}Cl_2N_{12}O_8$ (887.8) calculated: 18.92% N; found: 18.63% N.

Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe.2 CH_3COOH (*XI*)

Hexapeptide *IX* (0.46 g, 0.52 mmol) was dissolved in 5 ml DMF and N-nitroso-2-chloroethyl-carbamic acid N-hydroxysuccinimide ester (0.15 g, 60 mmol) was added. The pH was kept between 7 and 8 by the addition of diisopropylethylamine for 15 min, the product precipitated with ether and purified on silica gel column in a cold room (solvent B), to yield 230 mg (43%) of *XI*. R_F: 0.56 (B), 0.64 (C); UV spectrum, λ_{max} (ε): 400 (92). For $C_{44}H_{59}ClN_{14}O_{13}$ (1 027.5) calculated: 3.45% Cl; found: 3.50% Cl.

Biological Evaluation

For the estimation of the melanocyte stimulating activity, the frog skin reflectometric method of Shizume et al.[5] was used with slight modification. The pH of the Ringer solution was maintained between 7.0 – 7.2. Synthetic α-melanotropin served as reference substance. Before the experiments animals (*Rana ridibunda*) were kept at constant illumination for 24 h at +15 C. Light reflectance was measured with a Zeiss SPEKOL spectrophotometer equipped with reflectance accessories. An usual run of measurement consisted of 60 min preincubation of the skin in the Ringer, 60 min with the standard α-MSH, 60 min rinse and the actual measurement with the substance in question. The Q(NO)-derivatives were dissolved immediately before the measurement to minimize the danger of decomposition (see later).

RESULTS AND DISCUSSION

The melanocyte stimulating activity of the free peptides, their N-acetyl and Q(NO)-derivatives are shown in Table I. From the data it is clearly seen that acetylation of the free peptides enhances the biological activity without exception, even in cases, where the sequence does not exactly correspond to that of the natural hormone (*II* and *X*). There is no similar change if the Q(NO)-group is introduced, the activity of these derivatives equals to that of the free peptide or decreases to an insignificant extent. This means that the Q(NO)-derivatives preserve the receptor recognizing ability of the parent peptides, thus a basic condition of the affinity labelling is fulfilled.

TABLE I
The melanocyte stimulating activity of peptides related to the C-terminal sequence of α-melanotropin

No.	Peptide	Activity, units mmol
	H-Lys-Pro-Val-NH_2	$0.8 \cdot 10^4$
	Ac-Lys-Pro-Val-NH_2	$3 \cdot 10^4$
	Q(NO)-Lys-Pro-Val-NH_2	$0.6 \cdot 10^4$
	H-Gly-Lys-Pro-Val-NH_2	$1 \cdot 10^4$
	Ac-Gly-Lys-Pro-Val-NH_2	$5 \cdot 10^4$
	Q(NO)-Gly-Lys-Pro-Val-NH_2	$0.5 \cdot 10^4$
	H-Trp-Gly-Lys-Pro-Val-NH_2	$0.7 \cdot 10^5$
	Ac-Trp-Gly-Lys-Pro-Val-NH_2	$6 \cdot 10^5$
	Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2	$0.4 \cdot 10^5$
	H-Pro-Lys-Pro-Val-NH_2	$0.7 \cdot 10^4$
II	Ac-Pro-Lys-Pro-Val-NH_2	$4 \cdot 10^4$
III	Q(NO)-Pro-Lys-Pro-Val-NH_2	$1 \cdot 10^4$
IX	H-Gly-His-D-Phe-Arg-Trp-Gly-OMe	$0.7 \cdot 10^9$
X	Ac-Gly-His-D-Phe-Arg-Trp-Gly-OMe	$1.4 \cdot 10^9$
XI	Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe	$0.4 \cdot 10^9$

The skins treated with α-melanotropin always regained their original reflectance on washing with Ringer solution. This is not the case when the small fragments are measured. Table II shows that the darkening (melanin dispersing effect) of quite a number of the peptides investigated remains after 60 min rinse. It should be noted that this prolonged effect is not well reproducible, it probably depends on circumstances which are not sufficiently controllable.

According to the original working hypothesis the Q(NO)-peptides should be useful as affinity labels, in other words their irreversible binding to the receptor could be expected. As the data of the Table II show, the prolonged darkening does not necessarily indicate irreversible binding, as this phenomenon can be observed even in the case of peptides lacking the Q(NO)-substituent. Whether the prolongation, that is, the situation when melanin granules remain in the dispersed state, is connected with the irreversible blocking of the melanotropin receptor, can be in principle proved by measuring the activity of the melanocytes against α-melanotropin after incubation with the peptide in question. Figure 1 shows the results of such an experiment, where Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2 was used for preincubation. These data clearly show that in spite of the observed remaining darkening no inhibition of the MSH action can be achieved either on subsequent or on simultaneous application of these peptides.

This is not the case if this experiment is performed with *XI*, a D-phenylalanine containing Q(NO)-hexapeptide related to the classical active site of α-melanotropin. We selected this compound on the basis of the findings

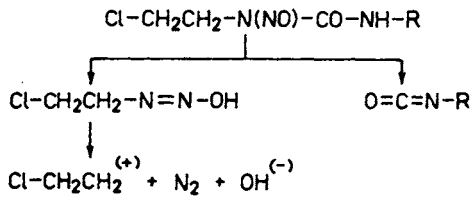
TABLE II
Remaining darkening of the skins after 60 min washing expressed as percentages of the total effect

No.	Peptide	Concentration mol l^{-1}	Remaining darkening, % (average values)
	α-Melanotropin	10^{-10}	0
	Q(NO)-Lys-Pro-Val-NH_2	10^{-4}	0
	Q(NO)-Gly-Lys-Pro-Val-NH_2	10^{-4}	0
	H-Trp-Gly-Lys-Pro-Val-NH_2	10^{-5}	50
	Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2	10^{-5}	50[a]
	H-Pro-Lys-Pro-Val-NH_2	10^{-4}	50
II	Ac-Pro-Lys-Pro-Val-NH_2	10^{-4}	50
III	Q(NO)-Pro-Lys-Pro-Val-NH_2	10^{-4}	40
IV	H-Gly-His-D-Phe-Arg-Trp-Gly-OMe	10^{-9}	30
X	Ac-Gly-His-D-Phe-Arg-Trp-Gly-OMe	10^{-9}	10
XI	Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe	10^{-9}	30

[a] In some cases no lightening but even further darkening occurred on washing.

of Schnabel and Li[6], and later of Sawyer et al.[7] according to which melanotropin fragments and analogs containing D-phenylalanine instead of the amino acid of the natural configuration show an extraordinarily high biological potency. Fig. 2 shows an approximately 30% prolonged effect and a significant and concentration-dependent inhibition of the α-melanotropin action, if the skins are preincubated with *XI*.

Investigations of the decomposition of N-nitroso-N,N'-bis(2-chloroethyl)-urea (BCNU), the classical representative of Q(NO)-compounds, by Colvin et al.[8] indicated the intermediary formation of two reactive products, a chloroethyl carbonium ion and an isocyanate (Scheme 1).



SCHEME 1

This scheme of decomposition is also valid, if R is not a simple amine but an amino acid[9] and very likely also in cases when this amino acid is the N-terminal constituent of a peptide. Our Q(NO)-peptides can therefore behave as affinity labels through the reaction of an unspecific chloroethyl carbonium ion or a specific peptide isocyanate formed during their decomposition. However, under the incubation conditions (0.1 M phosphate buffer, pH 7.4, 25 C) this decomposition is not too rapid, the half life time of Q(NO)-Lys-Pro-Val-NH_2 is 204 min, that of Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2 108 min, the formation of the reactive intermediates during the 60 min incubation time does not exceed 30% even in the case of the more labile compound.

It is not likely that the chloroethyl carbonium cation plays any role in the reaction with the melanotropin receptor, for on this basis more or less similar behavior would be expected for every Q(NO)-compound. The observations do not support this possibility, since a number of derivatives (Q(NO)-Trp-Gly-Lys--Pro-Val-NH_2, *III*, *XI*) permanently darken the skin, others (Q(NO)-Lys-Pro--Val-NH_2, Q(NO)-Gly-Lys-Pro-Val-NH_2) do not show any prolonged action. On the other hand, covalent binding of the peptide isocyanate to the receptor cannot be proved either, as prolonged effect is shown also by the Q(NO)-Pro-Lys-Pro-Val-NH_2 peptide, which, because of the secondary amino group of the N-terminal proline residue, cannot produce isocyanate on decomposition.

The data of Table II give further evidences for the non-covalent character of binding in cases of prolonged melanin dispersion, since this is a property of a number of peptides possessing only acetyl-substituent, or a free amino terminus. Keeping in mind the observation, according to which the extent of the prolonged action of one and the same compound may show a wide – up to 60% – dispersion of values, it is clear, that it would be too early to draw conclusions for the explanation of this phenomenon.

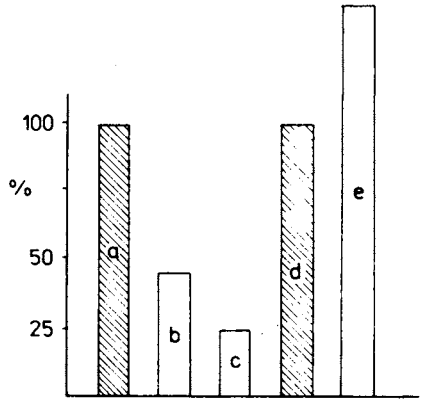


FIG. 1

Darkening of the frog skin under different conditions expressed in relation to the α-melanotropin effect. a α-MSH, $0.5 \cdot 10^{-10}$ mol l^{-1}; b Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2, $1.8 \cdot 10^{-5}$ mol l^{-1}; c remaining darkening; d α-MSH, $0.5 \cdot 10^{-10}$ mol l^{-1}; e α-MSH, $0.5 \cdot 10^{-10}$ mol l^{-1} + Q(NO)-Trp-Gly-Lys-Pro-Val-NH_2, $1.8 \cdot 10^{-5}$ mol l^{-1}

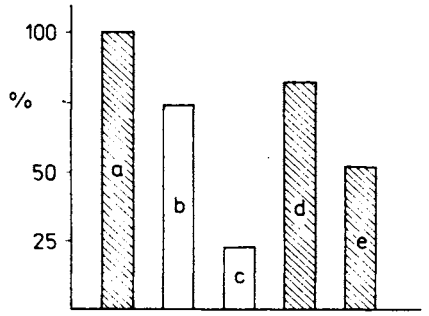


FIG. 2

Inhibition of the α-melanotropin effect after incubation with Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe. a α-MSH, $0.5 \cdot 10^{-10}$ mol l^{-1}; b Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe, $1.9 \cdot 10^{-9}$ mol l^{-1}; c remaining darkening; d α-MSH after washing, $0.5 \cdot 10^{-10}$ mol l^{-1}; e the same as d, but concentration of Q(NO)-peptide $4.5 \cdot 10^{-9}$ mol l^{-1}

The data of Fig. 2 indicate a different situation in the case of the Q(NO)-Gly-His-D-Phe-Arg-Trp-Gly-OMe hexapeptide; beside its partly irreversible darkening effect, the reactivity against α-melanotropin of the melanocytes pretreated with this Q(NO)-peptide decreases significantly; inhibition of the melanotropin receptors in a concentration dependent manner can be observed. Since the effective concentration of this Q(NO)-peptide is not too far from that of the α-melanotropin (10^{-9} mol l^{-1} vs 10^{-10} mol l^{-1}), it is very likely that this compound, containing the classical active site of α-melanotropin blocks in fact the melanotropin binding site of the receptor. Comparing this effect to the behavior of the peptide derivatives related to the C-terminal part of the α-melanotropin discussed above, we come to the conclusion that these latter peptides do not exert their melanin dispersing activity through the same melanotropin receptor, or their effect follows another, still unknown mechanism.

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