Liposome-mediated labeling of adrenocorticotropin fragments parallels their biological activity

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To test our hypothesis that specific interactions of ACTH peptides with model lipid membranes reflect the biological importance of similar interactions on target cells, we investigated the liposome-mediated labeling of ACTH fragments with the extremely hydrophobic photolabel, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine. Correlations were found between the labeling rates and the agonistic and antagonistic potencies of the peptides for in vitro steroidogenesis and inhibition of a synaptosomal protein kinase. A model for the cross-reactivity between ACTH and opioid peptides is discussed.

Peptide hormone–lipid membrane interaction *Hydrophobic photolabeling* *Adrenocorticotropin* *Dynorphin* *Enkephalin* *Endorphin* *Liposome*

1. INTRODUCTION

Adrenocorticotropin-(1–24)-tetracosapeptide ($ACTH_{1-24}$) is a very potent, synthetic fragment of the native hormone, $ACTH_{1-39}$. It induces steroidogenesis in adrenal cells (review [1]) and inhibits the phosphorylation of synaptosomal proteins [2]. ACTH also produces pharmacologic effects previously thought to be specific for opioid peptides and alkaloids [3–5]. Structure–activity relationships of ACTH are established in some detail [1,2]. At least two functionally different sequences of adjacent amino acids can be distinguished: the N-terminal *message* comprising amino acids 1–10 contains the information for triggering the responses, and the C-terminal *address* (11–24) adds specific receptor affinity and potentiates the message. Shortening of the message segment leads to weak agonists ($ACTH_{5-24}$), partial agonists ($ACTH_{6-24}$), and finally to competitive antagonists ($ACTH_{7-24}$, $ACTH_{11-24}$).

The lipid phase of the target cell membranes is thought to regulate the intrinsic affinity of receptors for the peptides [6]. However, it may also serve to facilitate receptor binding by specific, direct reaction with the agonists and antagonists [7]. Thus, we found that $ACTH_{1-24}$ is adsorbed to pure lipid membranes [8,9] and that the address remains on the surface in an extended conformation, whereas the message enters the membrane perpendicularly as a helix, establishing contacts with the hydrophobic layer [7,10,11]. For determining the structure and head-group specificity of such interactions, the extremely hydrophobic photolabel, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) of Brunner and Semenza [12] was used to label those parts of a peptide that penetrate into the hydrophobic layer of model membranes. In this work, we estimated the liposome-mediated labeling rate of ACTH peptides (which is a measure for their amphiphilicity or ease of mem-

Abbreviations: ACTH, adrenocorticotropic hormone; fragments of peptides, designated by subscript numerals, e.g. $ACTH_{1-10}$, adrenocorticotropin-(1–10)-decapeptide; $dynorphin_{1-13}$, dynorphin-(1–13)-tridecapeptide; amino acids, according to the IUPAC–IUP recommendations; PA, dipalmitoylphosphatidic acid; PC, egg yolk lecithin, phosphatidylcholine; [^{125}I]TID, 3-trifluoromethyl-(*m*-[^{125}I]iodophenyl)diazirine

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brane penetration) and compared it with their biological potency. Furthermore, the molecular anatomy and the amphiphilicity of ACTH peptides, dynorphin, β-endorphin, and leuenkephalin are discussed with regard to their biological cross-reactivity.

We concluded that model membranes are sensitive probes for the amphiphilic and conformational requirements necessary for biological activity and that, indeed, the specific interactions of peptides with model membranes may reflect the biological importance of analogous interactions with the lipid phase of the target cell membranes. Such interactions may serve to facilitate the proper interaction between peptides and their receptors.

2. MATERIALS AND METHODS

Dynorphin$_{1-13}$, α-melanotropin (α-MSH), and leuenkephalin were purchased from Bachem (Bubendorf). ACTH$_{1-10}$, ACTH$_{1-24}$, ACTH$_{1-13}$ amide, ACTH$_{5-24}$, ACTH$_{7-24}$, and ACTH$_{11-24}$ were prepared by known procedures [1]. [^{125}I]TID was synthesized as in [12] and had spec. act. 3.7×10^{11} Bq/mmol. Egg yolk lecithin (phosphatidylcholine, PC) was isolated according to [13]. Dipalmitoylphosphatidic acid (PA) was purchased from Lipid Products Ltd. (Nutfield).

Liposomes or lipid vesicles were prepared by sonication and checked by electron microscopy and oxidation index as in [14]. The preparations contained 1.5 mg lipid mixture (PC/PA, 9:1, w/w)/ml buffer A (10 mM phosphate (pH 7.3), 100 mM NaCl, 0.1 mM EDTA) in the form of liposomes with diameters around 25 nm.

Labeling was performed as follows: to 1 ml vesicle suspension as above, 10 μl of a 1 mM peptide solution and $3-10 \times 10^5$ Bq of [^{125}I]TID were added. After 15 min incubation at 23°C, the samples were screened with a $CuSO_4$ filter (cut off at 315 nm) and illuminated for 1 min with a 150 W mercury lamp that produced 1.5 W at the target, thus ensuring complete photolysis of the TID [12]. Control experiments lacked either the peptide or the liposomes.

Isolation of labeled peptides was effected by chromatography over a column (0.5 × 5 cm) of carboxymethyl cellulose (Whatman CM-52) after disruption of the liposomes with 0.05 ml Triton X-100. Initial washing with 300 ml 1 mM ammonium acetate (pH 5.5), removed practically all the by-products of photolabeling. The peptides were eluted with 1 M ammonium acetate at pH 7. Labeled enkephalin was esterified prior to chromatography to produce a net charge of 1+; identification of labeled peptides and chymotryptic hydrolysis or Edman degradation were performed as detailed in [14].

The mean radioactivity in cpm of the peptide peak fractions of 2–5 independent labeling experiments, after deduction of the blank values from controls without peptide, was regarded as the labeling rate.

3. RESULTS AND DISCUSSION

[^{125}I]TID partitions strongly in favor of the hydrophobic layers of lipid membranes [12] and into hydrophobic pockets of peptide aggregates [7,11,15]. The photolabeling of peptides with [^{125}I]TID in the presence of liposomes can be used to estimate the contribution of hydrophobic peptide–membrane interactions in the adsorption of peptides to liposomes and to distinguish them from electrostatic interactions. This has been demonstrated for ACTH peptides, dynorphin, and enkephalins by using a combination of liposome-mediated labeling with [^{125}I]TID in the presence of liposomes with different head groups, infrared attenuated total reflection spectroscopy, and equilibrium dialysis [7,10,11,14,15].

Table 1 lists the primary structures of ACTH and opioid peptides, and their liposome-mediated labeling rates. The latter are a measure of hydrophobic peptide–membrane interactions. ACTH$_{1-24}$ is strongly labeled through its message sequence, as has been demonstrated by spectroscopy [7,10] and by chymotryptic and Edman degradation [7,11,15]. ACTH$_{7-24}$ and dynorphin$_{1-13}$ are also labeled in their message segments [7,11,14]. ACTH$_{1-10}$ is not adsorbed to lipid membranes, and ACTH$_{11-24}$ is electrostatically adsorbed, but only to the membrane surface [7,10,11,15], resulting in a low liposome-mediated labeling rate in both cases. Leuenkephalin shows a low rate of labeling with PC/PA (9:1, w/w) vesicles [7,11,14] and β-endorphin was not yet investigated.

A comparison of the liposome-mediated labeling

Table 1

Primary structure and liposome-mediated labeling of selected peptides with [^{125}I]TID

Peptide	Amino acid sequence		PC/PA (9:1, w/w) liposome-mediated labeling rates
	Message	Address	
1 $ACTH_{1-24}$	+SYSMEHFRWG	– KPVGKKRRPVKVYP –	10000
2 $ACTH_{5-24}$	+EHFRWG	– KPVGKKRRPVKYYP –	7000
3 $ACTH_{7-24}$	+FRWG	– KPVGKKRRPVKVYP –	9300
4 $ACTH_{11-24}$		+KPVGKKRRPVKVYP –	2700
5 $ACTH_{1-13}$amide	+SYSMEHFRWG	– KPVnh_2[a]	5200
6 α-MSH	acSYSMEHFRWG	– KPVnh_2[a,b]	4000
7 $ACTH_{1-10}$	+SYSMEHFRWG		3000
8 $Dynorphin_{1-13}$	+YGGF	– LRRIRPKLK –	10000[c]
9 Leuenkephalin	+YGGF	– L –[d]	3000[c]
10 Dynorphin message	+YGGF		1500[c]
11 β-Endorphin	+YGGF	– MTSEKSQTPLVTLFKNAII-KNAYKKGE –	n.d.

[a] Lower case letters are used for symbols other than those for amino acids (ac, acetyl; nh_2, amide)
[b] In α-MSH, KPV·nh_2 is an additional message, not an address [1]
[c] Recognized as liposome-mediated labeling rate by comparison of head-group specificities [14]
[d] Message-address subdivision in analogy to 8
n.d., not determined

rates of ACTH peptides with two of their biological activities, steroidogenesis and inhibition of a synaptosomal protein kinase (values taken from [1,2]) revealed remarkable correlations (fig.1,2). It thus appeared likely that hydrophobic interactions of these peptides with the lipid phase of target cells, and the observed specific conformational transitions and topological arrangements induced in the peptides by the anisotropy of the membrane surroundings [7,10] may indeed be biologically significant.

The strong antagonism of $ACTH_{7-24}$ compared

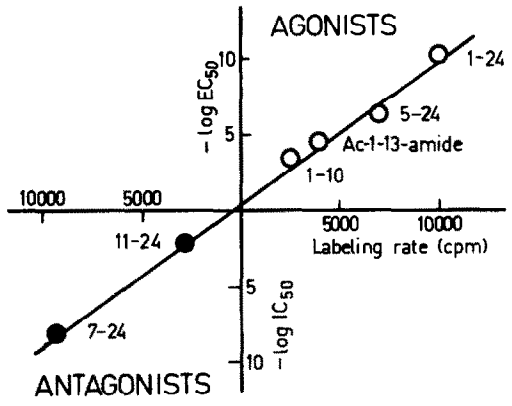

Fig.1. Correlation between in vitro steroidogenic and antagonistic potency and vesicle-mediated labeling rate of corticotropin-related peptides.

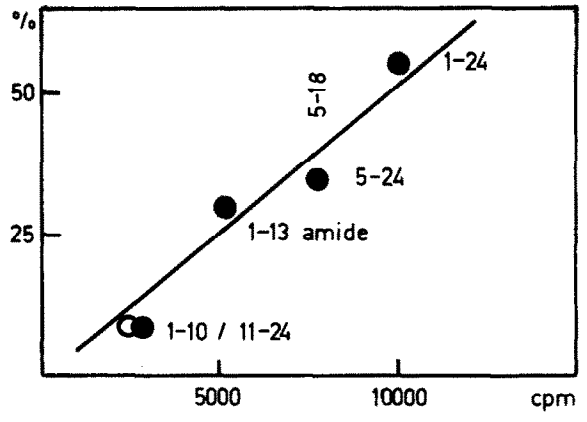

Fig.2. Correlation between vesicle-mediated labeling rate and % inhibition of a synaptosomal protein kinase [2]. Labeling of $ACTH_{5-24}$ is compared with the activity of $ACTH_{5-18}$, assuming similar potency of the two peptides.

to $ACTH_{11-24}$ (fig.1) was consistent with the observation that the message attached to the address contributes considerable hydrophobic binding force [15] but, in $ACTH_{7-24}$, lacks elements necessary for triggering receptors [1]. The very low biological activity of $ACTH_{1-10}$ (the message lacking the address) correlates well with its inability to establish hydrophobic contacts with the membrane. Thus, artificial membranes appeared to be sensitive probes for the amphiphilic and conformational requirements necessary for biologic activity.

Amphiphilicity is a characteristic of the strong agonists and antagonist of table 1: $ACTH_{1-24}$, $ACTH_{5-24}$, $ACTH_{7-24}$, $dynorphin_{1-13}$ [16], and β-endorphin [17]. It is caused by the separation into a hydrophobic message segment and a hydrophilic address segment, a feature common to all of these peptides. $ACTH_{1-24}$ and $dynorphin_{1-13}$ insert their messages into the membrane hydrophobic layers [7,11,14,15], their addresses remain on the surface of the membranes. The behaviour of β-endorphin is not known, although it is adsorbed to lipid micelles [18].

The adsorption of $ACTH_{1-24}$ to lipid membranes results in conformational transitions caused by the anisotropic surroundings: the message assumes a helical structure entering the membrane perpendicularly, and the address assumes an irregular, extended conformation on the membrane surface with the peptide bond planes oriented perpendicularly to the membrane plane [10]. We have not yet investigated the secondary structure of $dynorphin_{1-13}$, but it was reported that the message assumes no regular conformation and that the address probably is a helix with its axis parallel to the membrane surface [19]. A detailed study of β-endorphin revealed that an amphiphilic helix in the C-terminal region (residues 13–29) is an important structural determinant for the activity, probably serving to anchor the peptide to membrane surfaces, and that residues 6–12 constitute a flexible hinge between the membrane anchor 13–29 and the message 1–5 [20]. Such hinge regions had been postulated earlier for ACTH peptides on purely pharmacological grounds; e.g., Gly^{10}, Pro^{12} [1].

Dynorphin and β-endorphin are known to be much more potent than the enkephalins in opiate assays, and enkephalin is more potent than the dynorphin message segment, YGGF [16]. Thus, as shown in table 1, addition of the enkephalin 'address', L, to this message, or the addition of the dynorphin or β-endorphin addresses increases both the opiate potency and the hydrophobic interaction with lipid membranes.

It has been demonstrated that ACTH peptides exhibit opiate peptide cross reactivity since, for example, α-MSH causes similar effects as β-endorphin when injected into the periaqueductal gray matter of rats [4], and ACTH peptides inhibit electrically evoked contractions of the mouse vas deferens in a naloxone-reversible manner [3]. $ACTH_{1-39}$ was about 45-times less active than β-endorphin in the displacement of labeled dihydromorphine or naloxone from rat brain membrane preparations [5], but about 30–200-times more active than peptides derived from the message segment, residues 1–10, of ACTH.

Like $ACTH_{1-24}$, $ACTH_{1-39}$ consists of the hydrophobic message and a hydrophilic address [1]. Thus, as in the enkephalin–β-endorphin–dynorphin series, amphiphilic structure and hydrophobic interactions enhance the opiate-like binding characteristics of ACTH peptides.

In 1976, we proposed a helical model of the message segment of ACTH to account for its action on different types of receptor: steroidogenic, melanotropic, and behavioural (central nervous system) [1]. This model has been applied to explain the ACTH–β-endorphin cross reactivity [5]. It now can be refined to include the influence of the address: The address is necessary to allow the message to penetrate into the membrane and to assume a helical structure [7,10,14].

Our findings also lend support to a recently developed model of the β-endorphin receptor (review [21]). According to this hypothesis, the β-endorphin receptor is a lipoprotein complex composed of protein, with δ and $\varkappa$ opiate receptor specificity, and lipids (cerebroside sulfate), with μ specificity. In our model, the binding of the agonists to the lipid phase is strengthened by their amphiphilicity. In this process, the messages assume specific secondary structures and topological arrangements within the membrane, perhaps facilitating favorable contacts with the protein parts of the receptor. Furthermore, we have found that, in contrast to enkephalins with a free carboxy group and, hence, a preference for δ

receptors, enkephalin amides with a preference for μ receptors interact much more strongly with cerebroside sulfate liposomes than with liposomes composed of PC, PA, or phosphatidylserine [7,14]. This behaviour is similar to that of morphine [22,23].

Thus, our results indicate that the lipid phase of target cell membranes may exert significant influences on biological activity by being parts of receptors and by capturing agonists from the surrounding fluid and inducing conformations and topological arrangements of the agonist that are favorable for receptor interaction.

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