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Physicochemical interaction of opioid peptides with phospholipids and membranes

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Summary

The surface activity and opioid potency of peptide analogues of enkephalins are described. The molecules under study have as a general formula: Tyr-D · Met-Gly-Phe-Pro-NH-$(CH_2)_n$-CH_3, *n* being 5, 9 or 13. The interaction of these peptide-alkylamides with PC, PS, PI and GM_1, monolayers and their opioid activity measured by the GPI (guinea pig ileum) test are highly dependent on the hydrophobicity of the molecule. Moreover, the GPI muscle contraction records show a strong hydrophobic interaction between the peptides and the bilayer components, that holds the molecules anchored to the membrane and increases the duration of the effect.

Introduction

Activity and potency in the field of opioid molecules have been frequently related to their hydrophobicity (Fauchère and Petermann, 1980; Filippi et al., 1983). Moreover, the ability of opioid molecules to cross the blood brain barrier has been claimed to be a crucial factor in the in vivo activity (Banks et al., 1987). As far as affinity and binding assays are concerned, the existence of lipids as structural components of the opioid receptor would favour interactions with hydrophobic molecules (Cho et al., 1986). It has been suggested that the peptide sequence of enkephalins and endorphins carries the message in the N-terminal tetrapeptide, and the address in the C-terminal sequence (Sargent, 1986). According to this hypothesis, we prepared a series of enkephalin analogues having as parent compound the following peptide: Tyr-D · Met-Gly-Phe-Pro-NH_2.

The synthesis and analgesic activity of these compounds have been published elsewhere (Reig et al., 1984; Solé et al., 1985). The chemical modification introduced was the attachment of alkylamides of different length to the C terminal

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Abbreviations: PS, phosphatidylserine; PI, plant phosphatidylinositol potassium salt; GM_1, ganglioside; Chol, cholesterol; PC, phosphatidylcholine; GPI, guinea pig ileum; DMSO, dimethyl sulphoxide.

aminoacid in order to obtain peptides of different hydrophobicity. The length of alkylamides varied from six carbon atoms to fourteen, according to the general formula: Tyr-D · Met-Gly-Phe-Pro-CO-NH-$(CH_2)_n$-CH_3. These series of derivatives have the same 'message' but present differences in the 'address', that could influence their interaction with the membrane lipids and with the opioid receptor complex.

The interaction of these peptides with biological membranes has been determined in guinea pig ileum tissue. In this preparation a direct interaction between the drug and the opioid receptors can be assumed.

The surface activity of these synthetic peptides and its interaction with phospholipids and gangliosides have been studied by the technique of monomolecular layers.

Lipid monolayers, although a very simple membrane model, are very useful to study interactions between lipids and pharmacologically active molecules that act at the outer membrane level.

Materials and Methods

Chemicals

Bovine phosphatidylserine (PS), plant phosphatidylinositol, potassium salt (PI) and ganglioside (GM_1) were purchased from Supelco (Pennsylvania).

Egg yolk lecithin was obtained from Merck and was purified by column chromatography on alumina using $CHCl_3$/MeOH (9 : 1 v/v) as eluent (Singleton et al., 1965). The purity of the phospholipids was checked by TLC using coated silica gel 60 plates (Merck) and chloroform/methanol/ammonia (4 N) (9 : 7 : 2) (v/v) as eluent. Spots were visualized by spraying with perchloric acid and charring. Cholesterol was purchased from Sigma. The composition of PS/PC mixtures was 0.8 : 0.2. The three-component monolayers were composed of PS/PC/Chol 0.64 : 0.16 : 0.2.

Peptides of sequence Tyr-D · Met-Gly-Phe-Pro-CO-NH-$(CH_2)_n$-CH_3 ($n = 5$ or 9 or 13) were synthesized and characterized as described by Solé et al. (1985). Purity was checked by FAB-mass spectrometry, amino acid analysis and HPLC. Water for the Langmuir balance was prepared by distillation over potassium permanganate of single-distilled water in an all-glass apparatus. Its resistivity was always greater than 16 mΩ/cm, the pH was 5.5–6 and it was distilled daily. Chloroform (Merck pro analysi) was used as spreading solvent. DMSO was purchased from Merck.

Surface studies

Compression isotherms The compression isotherms were measured on a Langmuir film balance equipped with a Wilhelmy platinum plate, as described by Verger and De Haas (1973). The output of the pressure pick-up (Beckman LM 600 microbalance) was calibrated by recording the well known isotherm of stearic acid. This isotherm is characterised by a sharp phase transition at 25 mN m^{-1} for pure water at 20 °C. The teflon trough for measuring compression isotherms (surface area 495 cm^2, volume 309.73 ml) and penetration kinetics (124 cm^2, 237 ml) was regularly cleaned with hot chromic acid; moreover, before each experiment it was washed with ethanol and rinsed with double-distilled water. Before each run, the platinum plate was cleaned with chromic acid and rinsed with double-distilled water. Films were spread on double-distilled water using a microsyringe, and at least 10 min allowed for solvent evaporation. Films were compressed at a rate of 4.2 cm/min; changes in the compression rate did not alter the shape of the isotherms. All the isotherms were run at least three times in the direction of increasing pressure with freshly prepared films. The accuracy of the system under the conditions in which the bulk of the reported measurements were made was ± 0.5 mN m^{-1} for surface pressure.

Measurements at constant surface area Interfacial measurements were made by spreading a monomolecular lipid layer at the air/water interface in a Teflon trough. The subphase was stirred with a magnetic bar. The superficial pressure of the monolayer was initially 5 mN m^{-1}.

Peptides were injected into the subphase, the final concentration being: 1.52×10^{-5}, 1.08×10^{-5} and 5.47×10^{-6} M for P_6, P_{10} and P_{14}, respectively.

Due to their high insolubility in water the

peptides were injected into the subphase being dissolved in 60 μl of DMSO. This volume of DMSO produces, by itself, a variation of 1.2 mN m^{-1} in the superficial tension of water. This value was taken into account to correct the final superficial pressures.

Pharmacological assays

Male guinea pigs weighing 250–300 g were used. The ileum isolation procedure was carried out as described (Patton, 1955). The strip was mounted in a 13 ml organ bath containing modified Krebs-Ringer solution (mM concentrations: NaCl, 141; $NaHCO_3$, 11.93; KH_2PO_4, 1.39; $MgSO_4 \cdot 6H_2O$, 1.43; $CaCl_2$ anh., 2.97; KCl, 5.563 and glucose, 29.16), bubbled with 95% oxygen and 5% carbon dioxide, and kept at 37 °C during the experiments.

The contractions of the longitudinal muscle were recorded isometrically by means of an electronic transducer and registered on an Omniscribe recorder (Houston Instruments). The strip was stimulated by a Harvard Stimulator. Stimulation conditions were: 60 V; frequency, 0.1 Hz; delay, 0.1 ms; width, 7 ms. The volume of drug solution added ranged between 5 and 100 μl. Due to the insolubility in water of the peptides, it was necessary to use DMSO as solvent. Previously we determined that these volumes do not produce any change in the ileum contractions.

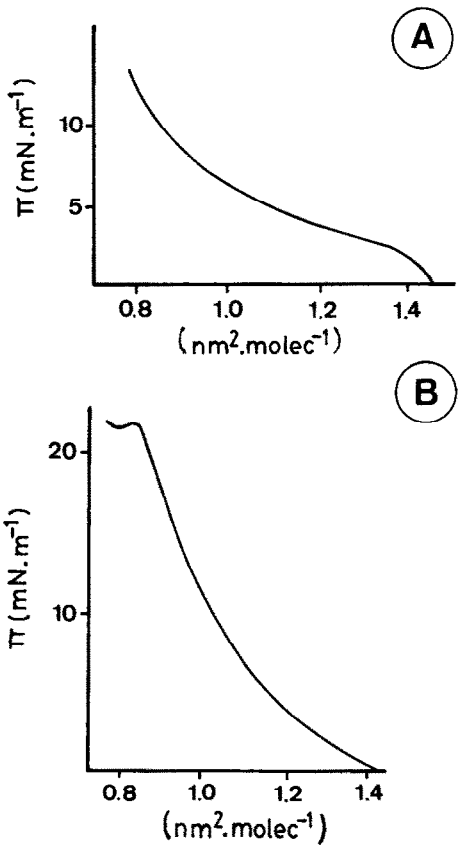


Fig. 1. Compression isotherms of (A) P_{10} and (B) P_{14} enkephalin analogue monolayers, spread on water subphases. The curves represent the mean of three determinations.

Results and Discussion

Surface activity of the peptides

Due to the highly hydrophobic character of peptides containing alkylamides, their ability to form stable monolayers was checked first. Varying amounts of the peptides were dissolved in chloroform and spread on distilled water. Compression isotherms of P_{10} and P_{14} derivatives are given in Fig. 1a and b.

Since the decrease in molecular area was negligible after two successive cycles of compression and expansion, the peptides appear to form insoluble monolayers. The compressibility of the molecules under study is highly dependent on the alkylamide chain length. Compression isotherms characteristic of gaseous state can be observed for P_6 and P_{10} derivatives and in both cases isotherms do not reach the collapse point.

In contrast, the P_{14} derivative gives liquid-expanded isotherms and its collapse pressure is 22 mN m^{-1}. The area/molecule values obtained show a strong dependence on the alkyl chain length, (Fig. 2), suggesting that the packing of the peptide molecules is different. In theory, if molecules were to be stacked perpendicular to the monolayer plane, the areas for all of them would be the same. Nevertheless, as monolayers do not exhibit a solid-state transition, one must admit that the most favourable orientation of polar groups might lead to a non-vertical chain orientation.

The high area/molecule values here described fall in the same range as those reported for short

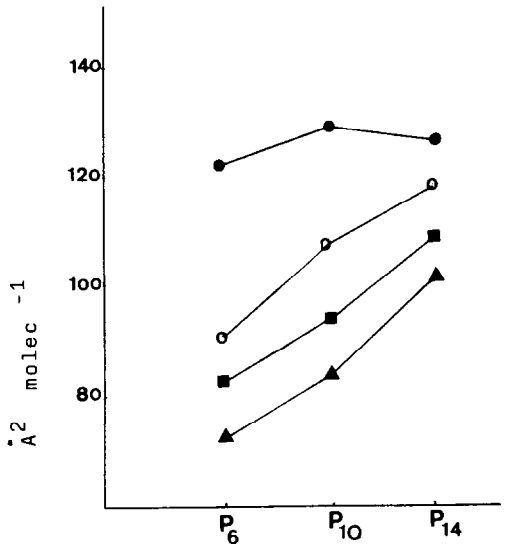


Fig. 2. Area per molecule of peptides measured at different compression pressures (mN m^{-1}): (●) 2.5, (○) 5, (■) 7.5, (▲) 10.

peptides (Fidelio et al., 1986). Moreover, the large molecular areas at surface pressures that are relevant to biological membranes (20 mN m^{-1}) are consistent with helical structure perpendicular to the interface.

Penetration kinetics of peptides in lipid monolayers

Lipids were chosen with reference to previous results obtained in our laboratory. Phosphatidylcholine as the major component of biological membranes was used to determine hydrophobic interactions, and phosphatidylserine, phosphatidylinositol and gangliosides were selected as lipids that can be involved in opioid receptor-opioid interaction. Mixtures of PS/PC and PS/PC/Chol were also included because membrane microviscosity has been demonstrated to affect the binding affinity of opioid molecules to their receptors (Heron et al., 1981). The molar composition of the lipid mixtures was chosen after a previous study of the miscibility of these components in mixed monolayers (Alsina et al., 1988/89; Reig et al., 1989).

The concentration of peptides in the subphase was 10^{-5} M, and the initial pressure of the lipid monolayers was 5 mN m^{-1}. For comparative purposes, the surface activity of the peptides in the absence of monolayers was determined and is represented in Fig. 3. The time course for surface pressure changes, following injection into the subphase, of the peptides, P_6 and P_{10} was similar, the final pressures (as expected) being higher for P_{10}.

However, in the P_{14} peptide, a delay in the onset of the rise in surface pressure is observed. This behaviour is also general for all the monolayers used in the present study. These differences in the kinetics of the penetration process are probably due to the high hydrophobicity of the P_{14} derivative.

This delay is especially clear when there is no monolayer in the surface. For this reason, in Fig. 4, the values corresponding to this experiment are those measured at 90 min. The possibility of formation of micelles has been discarded due to the fact that in a previous paper dealing with the same type of derivatives of Leu-enkephalin, these structures were not found (Reig et al., 1982). Moreover, the most usual values found in the literature for the critical micellar concentration are around 10^{-2}–10^{-4} M, and the present work has been carried out with 10^{-5} M solutions in every case.

In addition, it has been shown that the presence of micelles in the subphase, in the case of bile salts, reduces the surface pressure due to a partial solution of the monolayer molecules (Erill, 1989).

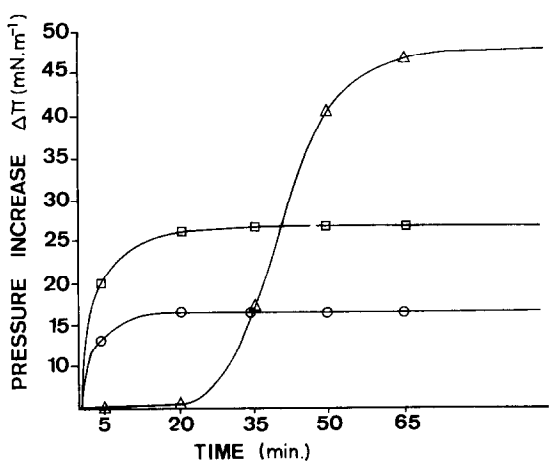


Fig. 3. Time course for surface pressure changes following injection into the subphase of the P_6, P_{10} and P_{14} enkephalin analogues. (○———○) P_6, (□———□) P_{10}, (△———△) P_{14}. Curves represent the mean of three determinations.

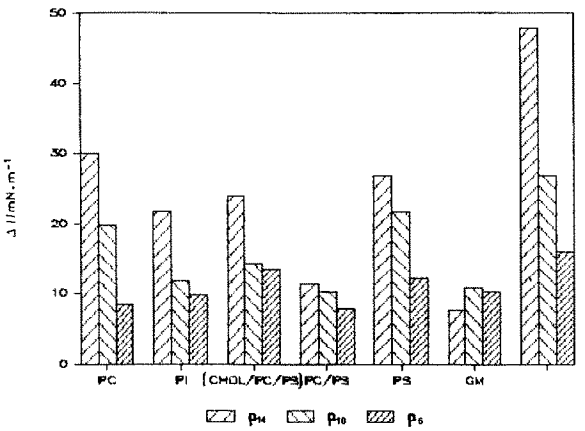


Fig. 4. Penetration pattern of enkephalin analogues in lipid monolayers at 5 mN m^{-1}. Molar composition of mixed monolayers is given in the text. The last three columns correspond to the surface activity of the peptides in absence of monolayer.

The most feasible explanation for this behaviour should be that these molecules, when in contact with the aqueous subphase, undergo some conformational change allowing the alkyl moieties to avoid the contact with water. It will take longer for these structures to reach the air/water surface and to order themselves in monolayers or to fuse with the pre-existing lipid monolayers. The penetration level is of the same order for all the lipid monolayers with two exceptions, gangliosides and PC/PS mixtures. Moreover, there is a gradation in the pressure increases produced by the peptides, that are related to their hydrophobic characteristics. This behaviour appears to exclude the existence of specific interactions between acidic phospholipids (PS, PI) and the peptides.

The low degree of interaction (measured as pressure increases) between gangliosides and the peptides under study is difficult to explain. Others (Fidelio et al., 1981) have studied interactions between gangliosides and membrane proteins, and the values obtained are of the same order as those we obtained.

One can suggest that the hydroxyl groups of gangliosides hinder the penetration of the alkyl chains of the peptide derivatives independently of their length.

Mixtures of PS/PC give expanded monolayers as has already been described (Alsina et al., 1988/89), but the presence of cholesterol in these mixtures produces a condensing effect (Reig et al., 1989), the area/molecule values of mixed monolayers being lower than for pure components. Related to this, in a previous paper, we have described the negative effect of cholesterol as far as the penetration of opiate molecules in this system is concerned. In the present case, the results obtained are not in agreement with the previous data; this could be attributed to the greater hydrophobicity and different structure and rigidity of the molecules under study.

Considering that the ΔG_M^E for PS/PC (0.8 : 0.2) = 298.04 cal mol^{-1} (at 8 mN m^{-1}) (Reig et al., 1982) and ΔG_M^E for PS/PC/Chol (0.64 : 0.56 : 0.2) = −627.8 cal mol^{-1} (at 8 mN m^{-1}) (Alsina et al., 1990), one can appreciate that the three-component monolayer is more stable. This fact would suggest that the penetration of subphase molecules would be higher in PS/PC monolayers than in PS/PC/Chol as in the case of heterocyclic opiate molecules (Alsina et al., 1988/89, Reig et al., 1989). Nevertheless, differences in surface pressure increases were very small in the above-cited experiments and the maximum pressure variation does not reach 10 mN m^{-1}.

One can thus suggest that the apparently anomalous values here reported must be attributed to the highly hydrophobic character of the peptide alkyl amides. Moreover, the alkyl chains will probably be incorporated into the monolayer as a new lipid, the monolayer in this case being composed of three or four components, respectively, and having its own miscibility characteristics.

Pharmacological activity of peptides

The in vitro assays in guinea pig ileum preparations were carried out according to the description given in Materials and Methods.

As far as the interaction of these peptides with the receptors exposed in the outer part of the membrane is concerned, this test is very simple and can provide information about the influence of hydrophobic factors.

Considering the IC_{50} values given in Table 1, one can see that for short alkyl chains, some type of interference occurs that influences negatively

TABLE 1

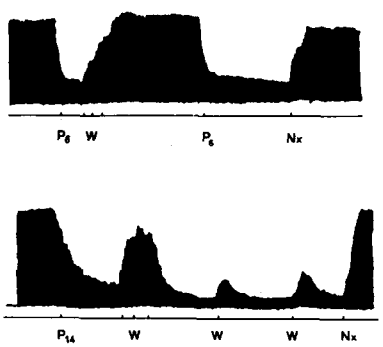
Activity values of the peptides in the GPI assay

Peptide	IC_{50} (M)
Parent compound	1.09×10^{-8}
P_6	4.341×10^{-7}
P_{10}	7.9×10^{-10}
P_{14}	5.300×10^{-11}

General formula: Tyr-D·Met-Gly-Phe-Pro-NH-$(CH_2)_n$-CH_3 for P-6, $n = 5$; P-10, $n = 9$; P-14, $n = 13$.

the opioid molecule-receptor interaction but for longer alkyl chains a hydrophobic effect must predominate that favours the attachment of the peptide to the receptor, contributing in a favorable way to the potency of the whole molecule. It is a matter for debate whether the interaction is direct on the hydrophobic domain of the receptor or on the lipid matrix that surrounds it.

This second possibility has been suggested by Schwyzer (1986) in the sense that lipid bilayer membranes can serve not only as matrices for receptors but also interact with many neuropeptides. In this model the lipid bilayer could act as an antenna to capture the peptides and facilitate their encounter with the receptors. Looking at the GPI registers for P_6 and P_{14} (Fig. 5), one can observe that the recuperation of contractions by



P_6 : Tyr - D - Met - Gly - Phe - Pro - CONH - $(CH_2)_5$ - CH_3

P_{14} : Tyr - D - Met - Gly - Phe - Pro - CONH - $(CH_2)_{13}$ - CH_3

Nx : Naloxone

W : Washings

Fig. 5. Graphic registers corresponding to the GPI test for the (P_6) and (P_{14}) derivatives.

repetitive washings is lower for the more hydrophobic derivative. Moreover, after an initial recuperation of the contraction level, it decays without a new addition of the peptide. This effect suggests the existence of a strong hydrophobic linkage between the alkyl chain and the phospholipid components of the membrane that holds the whole molecule attached to the bilayer, allowing the peptide moiety to fluctuate in equilibrium between the associated and free form.

The fact that the analogues are active in this assay shows that the chemical modification introduced has not changed the selectivity for the μ-receptor, that is the most abundant receptor type in this tissue. Nevertheless, it would be necessary to submit these peptides to a binding assay in order to be sure of this fact.

As a conclusion, one can say that by modifying drastically the hydrophobic-hydrophilic balance of an opioid molecule without changing the message fragment, one can modulate the residence time and affinity of these molecules by their receptor. Moreover, concerning the hypothesis about the pertinence of acidic phospholipids to the opioid receptor structure, the lack of specific interactions between our peptides and these phospholipids does not support it. Nevertheless, this does not exclude the possibility that these phospholipids could act in the opioid receptor as structural elements more than as active parts.

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