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ORIGINAL ARTICLE

Interaction of methionine–enkephalins with raft-forming lipids: monolayers and BAM experiments

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Abstract Enkephalins (Tyr-Gly-Gly-Phe-Met/Leu) are opioid peptides with proven antinociceptive action in organism. They interact with opioid receptors belonging to G-protein coupled receptor superfamily. It is known that these receptors are located preferably in membrane rafts composed mainly of sphingomyelin (Sm), cholesterol (Cho), and phosphatidylcholine. In the present work, using Langmuir's monolayer technique in combination with Wilhelmy's method for measuring the surface pressure, the interaction of synthetic methionine–enkephalin and its amidated derivative with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), Sm, and Cho, as well as with their double and triple mixtures, was studied. From the pressure/area isotherms measured, the compressional moduli of the lipids and lipid–peptide monolayers were determined. Our results showed that the addition of the synthetic enkephalins to the monolayers studied led to change in the lipid monolayers characteristics, which was more evident in enkephalinamide case. In addition, using Brewster angle microscopy (BAM), the surface morphology of the lipid monolayers, before and after the injection of both enkephalins, was determined. The BAM images showed an increase in surface density of the mixed surface lipids/enkephalins films, especially with double and triple component lipid mixtures. This effect was more pronounced for the enkephalinamide as well. These observations showed that there was an interaction between the peptides and the raft-forming lipids, which was stronger for the amidated peptide, suggesting a difference in folding of both enkephalins. Our research demonstrates the potential of lipid monolayers for elegant and simple membrane models to study lipid–peptide interactions at the plane of biomembranes.

Keywords Methionin-enkephalins · Raft-forming lipids · Langmuir monolayers · Compressional modulus · Brewster angle microscopy

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Introduction

More than 10 years are necessary to develop and introduce a new drug into clinical practice. A crucial step in the development of peptide based opioid ligands for therapeutic purposes is the design of ligands with desirable biological properties coupled with the proper biophysical properties to permit access from the blood to receptor sites in the brain (Romanowski et al. 2002). Moreover, it is of great importance for the preclinical studies that the model systems and the methods which will be chosen to be simultaneously easy to operate with and very informative. The Langmuir monolayer technique, especially in combination with Brewster angle microscopy for visualization of the surface morphology of the monolayers, is one such method and can be used as the first step in preclinical research for drugs that exert their biological activity by interaction with the cell membrane.

During the past decades, after the discovery of enkephalins in 1975, endogenous opioid peptides have attracted much interest due to their proven antinociceptic action (Fuxe et al. 1988). The mechanism of their action has been considered and they have been structurally modified to increase their resistance during their pharmaceutical administration and to strengthen their effect on target cells.

Enkephalins (Tyr-Gly-Gly-Phe-Met/Leu) are neurotransmitters found in the human central nervous system, especially in the regions of the brain and spine associated with diffuse pain pathways (Hucho 1986; Kruk and Pycock 1991). They are involved in a wide variety of physiological processes: the inflammatory and immune response (Shipp et al. 1991); gastrointestinal physiology, especially in ion transport (Cheng et al. 1996); learning and memory (Gallagher 1982; Gallagher et al. 1983; Messing et al. 1979); emotional behaviors (Nieto et al. 2005); respiration and in inhibition of pain signals (Fuxe et al. 1988), etc.

So far it is known that enkephalins act as agonists of the opioid receptors which belong to the superfamily of G-proteins coupled receptors (GPCRs). Methionine–enkephalin (Met-enk) acts via the main subtypes of receptors referred to as μ (mu)-, δ (delta)-, and κ (cappa)-, as well as ζ (zeta)-receptors (Childers 1991), whereas the first three receptor subtypes mediate the classic opioid effects of Met-enk, ζ-receptors are reported to be involved in the non-opioid actions of the peptide, i.e. the inhibitory effect on cell growth (Malendowicz et al. 2005). In addition, it is known that GPCRs are localized mainly in membrane "rafts" (Huang et al. 2007, 2008), which consist mostly of sphingomyelin (Sm), cholesterol (Cho), and phosphatidylcholine (De Almeida et al. 2003; Frazier et al. 2007). It is believed that to achieve their biological function, enkephalins must be transported from an aqueous phase to the lipid-rich environment of their membrane bound receptor proteins (Liu et al. 2006). In a complementary model called "membrane catalysis", Sargent and Schwyzer (1986) have proposed that, more specifically, the opiate peptides would interact with the membrane lipids prior to receptor binding. This interaction would allow the neuropeptides to adopt an appropriate conformation suitable for their binding. The membrane would thus catalyze the peptide–receptor interaction.

In order to better understand the interaction of enkephalins with neuronal membranes, various membrane mimetic systems have been studied over the years: giant unilamelar vesicules (GUV) (Boyanov et al. 2005; Mutafchieva et al. 2005), bicelles (Marcotte et al. 2003), multilamellar vesicles (Marcotte et al. 2004), lipid monolayers (Bourhim et al. 1993; Ege and Lee 2004), etc. It is generally accepted that electrostatic interactions with negatively charged lipids are important for the association of enkephalins with membranes (Deber and Behnam 1984) and that these neuropeptides interact hydrophobically with zwitterionic lipids (Deber and Behnam 1984; Marcotte et al. 2003). In addition, a recent study of Met-enk at phosphatidylcholine and phosphatidylglycerol monolayers has suggested that the insertion of enkephalins in the membranes depends mainly on hydrophobic interactions (Tsanova et al. 2012).

The aim of the present work was to study the interactions of synthetic Met-enk and its amidated derivative, Met-enk-NH_2, with the raft-forming lipids, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), Sm, and Cho, as well as with their mixtures and to compare the penetrating ability of both enkephalins into model membranes of different lipid composition. In this regard the Langmuir monolayer technique, in combination with Wilhelmy's method for measuring the surface pressure, was used. Moreover, by Brewster angle microscopy (BAM) the difference in surface morphology of lipids and lipid–peptide films, as a result of the interactions were observed.

Materials and methods

Manual stepwise solid-phase techniques were applied to obtain Methionine–enkephalins. The peptides were synthesized on a Rink-amide resin using a Fmoc-strategy with DIC/HOBt activation. The crude peptides were purified by preparative TLC and their purity was checked by analytical HPLC. The results of electrospray ionization mass spectrometry (ESI–MS) were in agreement with the expected results. Met-enk and Met-enk-NH_2 stock solutions (10 mM) were prepared by dissolving the peptides in a physiological solution (0.15 M NaCl). POPC, Sm from bovine brain, and Cho were purchased from Sigma-Aldrich. Lipids stock solutions (1 mg/ml) were prepared by dissolving them in chloroform.

All measurements were made in Langmuir trough MicroTrough X (Kibron Inc., Finland). The apparatus uses the Wilhelmy method with a platinum wire probe attached to the microbalance sensor head. Fixed amounts of POPC, Sm, and Cho solutions, as well as their preliminary formed equimolar double and triple mixtures were spread at the surface with a Hamilton microsyringe to a surface concentration of 150 $Å^2$ per lipid molecule over a subphase of 0.15 M NaCl (pH 5.77) at 30 °C. Fifteen minutes were awaited for evaporation of the solvent, and afterwards the enkephalins were injected in the subphase under the formed monolayers to a final volume concentration of 0.1 mM. After reaching an equilibrium surface pressure (π, mN/m) of the lipid–peptides films, monolayer area compression was performed at slow rate (3.75 $Å^2$/molecule/min) up to 20 % of their initial area and the

π/area (A, Å^2/molecule) isotherms were recorded. The surface pressure was measured with accuracy of ±0.01 mN/m. The trough temperature was controlled within ±0.5 °C. Each measurement was repeated at least three times with each separate sample.

From the π/A-isotherms measured for each of the monolayers studied the compressional moduli (Cs^{-1}, mN/m) were calculated according to the following formula:

$$Cs^{-1} = -A_\pi \left(\frac{d\pi}{dA}\right)_T,$$

where A_π is the area per molecule at the indicated surface pressure π.

For BAM experiments in a Langmuir trough, insoluble lipid monolayers were formed at the air–water interface by spreading the lipids solutions over the subphase of 0.15 M NaCl. The amount of the lipids was calculated by the pressure/area isotherms to correspond to $\pi \approx 10$ and 30 mN/m. After the formation of the monolayers, the peptides were injected into the subphase to a final volume concentration of 0.1 mM. The changes in the morphology of the monolayers after the addition of the enkephalins was monitored, and BAM images were recorded with dimensions of 696 × 520 pixels using Micro BAM2 (Nima Technology Ltd, Coventry, UK). The polarizer and analyzer were set to *p*-polarization and incoming laser light was limited to an angle of incidence of 53 ± 2° (Brewster angle for water or aqueous solutions). All measurements were made at 30 °C. The images presented in this work were further processed by reducing their original size up to 296 × 220 pixels.

Results

The interaction of synthetic Methionin-enkephalin with the raft-forming lipids, POPC, Sm, and Cho alone and with their double and triple mixtures at monolayers was studied. In order to observe the effect of the C-terminal amidation of the neuropeptide, the penetrating ability of the modified enkephalin (Met-enk-NH_2) into the model membranes was studied. Compressional π/A-isotherms of the lipid films before and after the addition of the peptides were recorded, using the Langmuir monolayer technique in combination with Wilhelmy's method for measuring the surface pressure and then analyzed. The compressional moduli, Cs^{-1}, were then calculated to observe the lateral elasticity of the corresponding monolayers, from the isotherms, and the results were presented as a function of surface pressure. Our results showed that both peptides penetrated into the monolayers composed of POPC, Sm, and Cho alone at low surface pressure values, corresponding to liquid-expanded state of the films, which was more pronounced for phosphatidylcholine and sphingomyelin monolayers (Fig. 1, panels with index 1). This effect was more obvious for the amidated Met-enk in the studied three single lipids. At

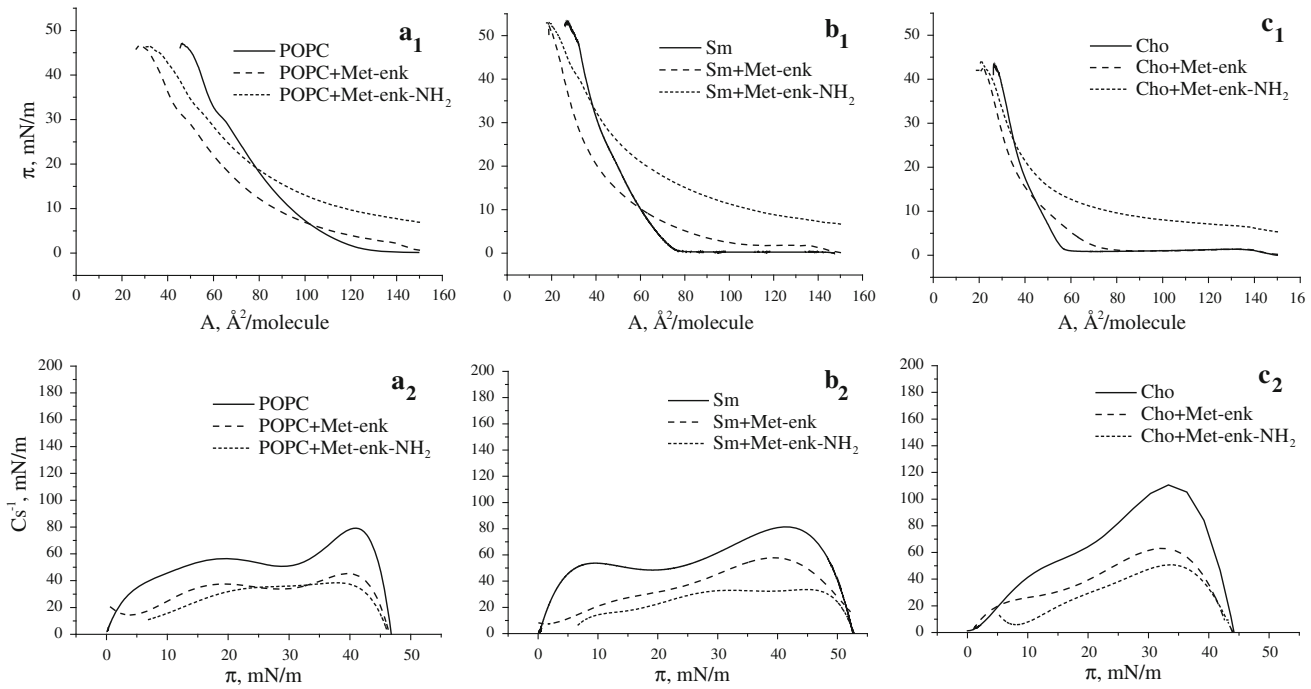

Fig. 1 Compressional π/A-isotherms (*panels* with index 1) and compressional moduli (*panels* with index 2) of lipid monolayers composed of POPC (**a**), Sm (**b**), and Cho (**c**) alone and after the addition of Met-enk, and Met-enk-NH_2

more compact monolayers (high surface pressure values, resp.) the addition of both peptides led to lower values of the area per lipid molecule at given π, especially in case of Met-enk, suggesting structure reorganization of the films. The most significant changes at the air–liquid interfaces were observed at POPC (Fig. 1, panel a_1), followed by Sm (Fig. 1, panel b_1) and Cho (Fig. 1, panel c_1) monolayers.

After the analysis of all the isotherms, it was visible that the lift-off of π/A-isotherms (relevant to gas to liquid-disordered phase transition) could not be noticed after the insertion of both neuropeptides. The compression of lipid–peptides monolayers, which started at higher π values, compared with pure lipid films, was more pronounced for the Met-enk-NH_2.

The effects observed were confirmed by Cs^{-1}/π dependencies for the monolayers obtained (Fig. 1, panels with index 2). The results showed that the curves for the mixed lipids–Met-enk films followed the shape of the pure lipids Cs^{-1}/π curves; Cs^{-1} minimums (i.e. liquid-expanded to liquid-condensed phase transitions) appeared at approximately same surface pressure values for the pure and mixed monolayers. In contrast, the amidation of the neuropeptides resulted in a significant change in the elasticity behavior of the mixed lipids–Met-enk-NH_2 films. Regarding the maximum values of Cs^{-1}, it was obvious that they were much lower after the addition of both enkephalins (which corresponds to higher lateral film elasticity), especially in case of the amidated derivative. The difference in the elasticity of pure and mixed monolayers was most significant at Cho films (Cs^{-1} for Cho was ca. 115 mN/m, while this value for Cho-Met-enk was ca. 60 mN/m, and 50 mN/m for Cho-Met-enk-NH_2, Fig. 1, panel c_2). For a comparison, the compressional modulus of POPC monolayers reached maximum value at $\pi = 40$ mN/m of ca. 80 mN/m, whereas the addition of the peptides led to its lowering to about 40 and 30 mN/m for Met-enk and Met-enk-NH_2, resp. (Fig. 1, panel a_2); a similar trend followed the respective Cs^{-1} values for the Sm films (Fig. 1, panel b_2).

Since the compressional moduli reflect the surface reorganization and elasticity change of the film, due to interactions of the different lipids with both enkephalins, we thus could estimate most significant effect of increasing elasticity for Cho (Fig. 1, panel c_2), followed by Sm (Fig. 1, panel b_2) and POPC (Fig. 1, panel a_2) films. In addition, in case of the three lipids the effects of amidated Met-enk-NH_2 were more pronounced than that of Met-enk.

More detailed analyses of the interaction of the enkephalins and the studied monolayers were provided by Brewster angle microscopy. BAM images showed a difference in the morphology of the mixed single lipid–enkephalin monolayers only at $\pi \approx 10$ mN/m, i.e. at more "diluted" state of the lipid films, especially in case of Sm and Cho monolayers (Fig. 2). The addition of Met-enk to the model membranes led to the formation of random domains in POPC monolayer, visible as white dots in the loose black film, while the effect of the peptide to Sm morphology led to bigger domains as compared with the pure monolayer. A negligible increase in surface density of the film was visible in the Cho-Met-enk as well. However, a more significant effect in the morphology of the monolayers was determined after the injection of the amidated neuropeptide into the subphase, which was more pronounced in case of Cho, where the vacant lipid molecules area, seen as black holes in the pure cholesterol film was filled, and the monolayer was gray with decreased contrast of the picture. In POPC films bigger domains were visible as a result of the insertion of Met-enk-NH_2, whereas in case of Sm no visible change in the domains type was noticed, except that they looked a little denser as compared with the pure lipid monolayer.

At a surface pressure of ca. 30 mN/m the effect of the addition of both enkephalins was visible only in POPC monolayers, where Met-enk led to a formation of smaller but denser domains in the films, while Met-enk-NH_2 resulted in bigger domains (Fig. 2).

More interesting effects were observed in the double- and triple-component lipid model membranes either in π/A-isotherms and compressional moduli, or in BAM experiments.

As with the single lipids, the addition of the peptides to the Cho-containing lipid mixtures led to insertion of Met-enkephalins into the monolayers at lower values of π (Fig. 3, panels with index 1), and this effect was more significant in the case of the amidated derivative. At more compact monolayer, both peptides led to reorientation of the lipid molecules, manifested by a lowering of the area per lipid molecule in case of the double lipid mixtures, especially at Sm/Cho monolayers (Fig. 3, panel b_1). In the triple raft mixture, however, Met-enk had an opposite effect, suggesting a different type of interaction with the lipid monolayer (Fig. 3, panel c_1).

Regarding the compressional moduli of the above mentioned lipid mixtures, the effect of the injection of the neuropeptides into the subphase under the double- and triple-component monolayers was more pronounced as compared with π/A-isotherms (Fig. 3, panels with index 2). Cs^{-1} minimums, i.e. phase transitions, were negligible for both added enkephalins in all curves. The maximum values of the compressional moduli were lower in double and triple lipid–peptide films as with the single lipid–enkephalin films (Fig. 1, panels with index 1). The difference in Cs^{-1} maximums, however, between pure lipid and lipids–Met-enk-NH_2 model membranes was much bigger as compared with ΔCs^{-1} between lipids and lipids–Met-enk monolayers: in case of POPC/Cho mixture the

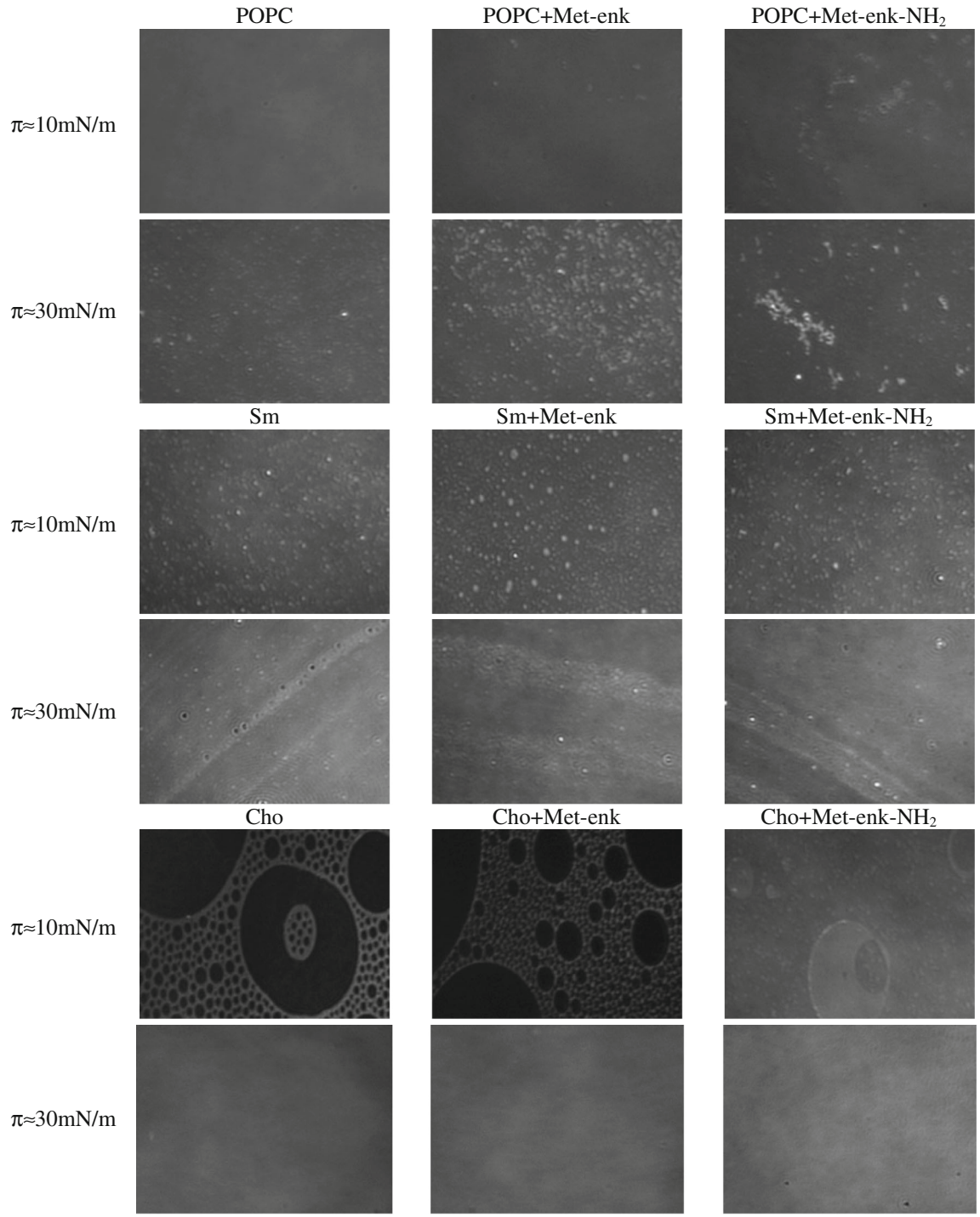

Fig. 2 BAM images of lipid monolayers composed of POPC, Sm, and Cho alone and after the injection of Met-enk, and Met-enk-NH_2 at surface pressure values of ca. 10 and 30 mN/m

compressional modulus reached value of ca. 110 mN/m, whereas the addition of Met-enk lowered this parameter to ca. 90 mN/m, and the C-terminal amidation of the peptide led to a significant decrease of up to 50 mN/m (Fig. 3, panel a_2). At the monolayers composed of Sm/Cho and the raft mixture with added POPC, an almost identical tendency in reducing maximum Cs^{-1} was observed: in both cases lipid films without peptides showed values of about 160 mN/m; the monolayers with Met-enk reached Cs^{-1} of ca. 120 mN/m, while the addition of Met-enk-NH_2

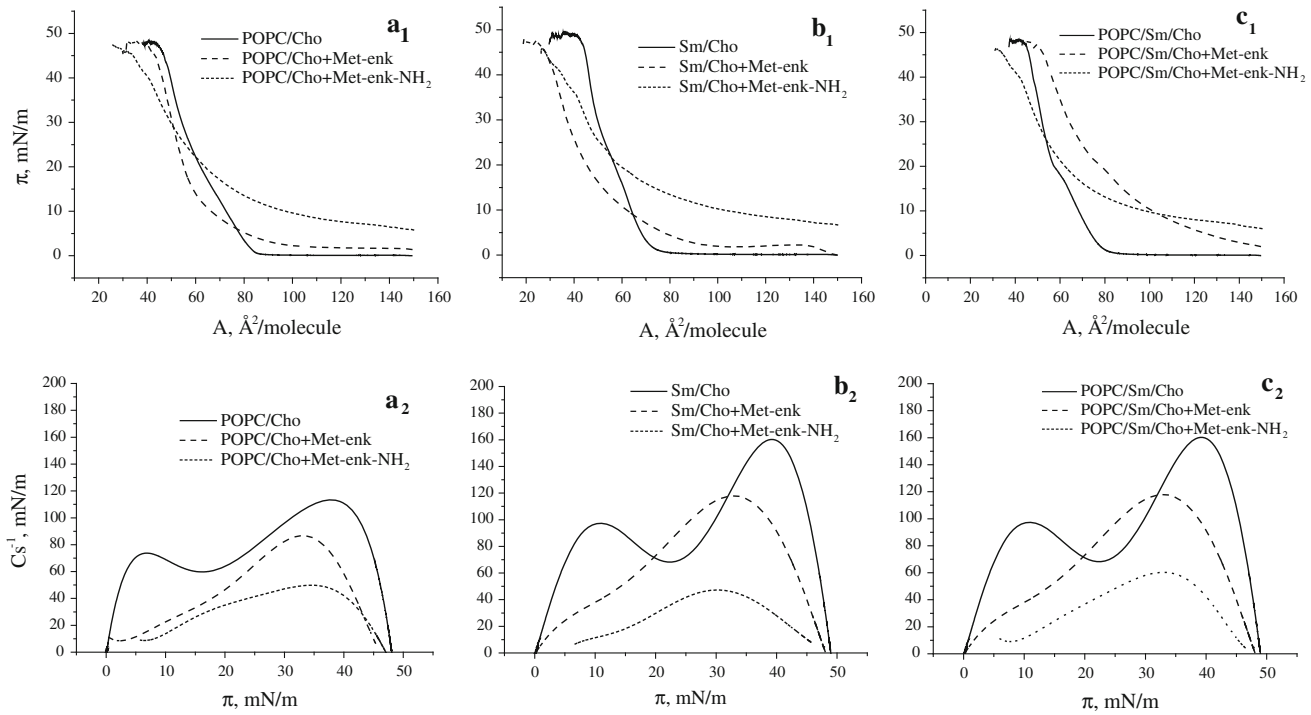

Fig. 3 Compressional π/A-isotherms (*panels* with index1) and compressional moduli (*panels* with index 2) of lipid monolayers composed of equimolar mixtures of POPC/Cho (**a**), Sm/Cho (**b**), and POPC/Sm/Cho (**c**) alone and after the addition of Met-enk, and Met-enk-NH_2

dramatically changed this parameter to ca. 50 and 60 mN/m for the double- and the triple-component lipids mixtures, resp. (Fig. 3, panels b_2 and c_2).

Mixtures containing Sm and Cho had the highest Cs^{-1} maximum (about 160 mN/m at $\pi = 40$ mN/m), i.e. the lowest surface elasticity and again the effects of increasing elasticity of the mixtures with amidated Met-enk-NH_2 were more pronounced than that with Met-enk.

BAM experiments for the visualization of the interaction of the enkephalins with the double- and triple-component lipid mixtures showed a significant change in the monolayer morphology only at a surface pressure of ca. 10 mN/m (Fig. 4). It was seen that the insertion of Met-enk into the POPC/Cho film resulted in obvious increase in surface density of the monolayer, i.e. more homogenous surface with much less vacant from molecules spaces, seen as black holes. In case of Sm/Cho the effect was less noticeable; however, the tendency for reducing the free area remained, and the grid-like domains in the pure lipid film lowered their sizes at $\pi \approx 10$ mN/m. The smallest change in the surface morphology was found at the raft mixture, which was observed as reducing the gaps number in the monolayer. The addition of the amidated derivative of Met-enk in all three mixtures resulted in a drastic and similar effect: significant homogenization of the films, seen as changing their contrast to more gray scale and reducing the contrast of the domains formed. At a surface pressure of ca. 30 mN/m, i.e. dense monolayer, there was no visible change in the surface morphology, respectively, in the packing of the molecules before and after the addition of both peptides (Fig. 4).

Discussion

The interaction of synthetic enkephalins with lipid model membranes is pivotal for understanding the mechanism of their biological activities. It is commonly accepted that this interaction primarily arises from both hydrophobic and electrostatic effects (Deber and Behnam 1984; Marcotte et al. 2003; Tsanova et al. 2012). Thus, to perform their biological functions, peptides usually are attracted by either hydrophobic or electrostatic force or both to incorporate into the cell membrane and reach the target receptor sites (Liu et al. 2006). Our interest on the study of the penetrating ability of Methionin-enkephalin and its C-terminal amidated derivative with monolayers composed of raft-forming lipids arose from the fact that most probably GPCRs, including opioid receptors, prefer membrane "rafts" (Huang et al. 2007, 2008); it is believed that these neuropeptides interact with the nerve cell membrane to adopt a bioactive conformation that will then fit onto the receptors (Behnam and Deber 1984; Deber and Behnam 1985; Gysin and Schwyzer 1983; Sargent and Schwyzer

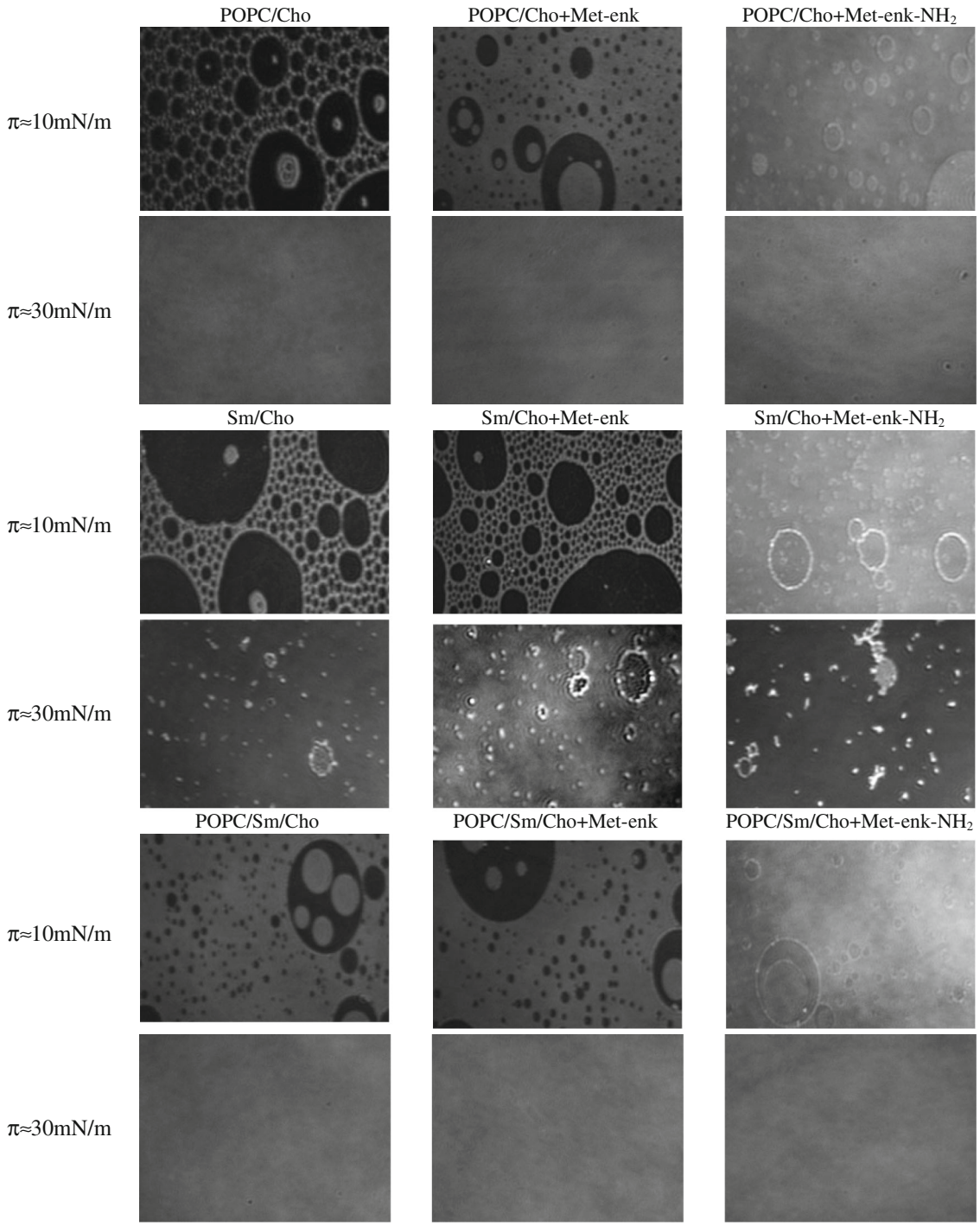

Fig. 4 BAM images of lipid monolayers composed of equimolar mixtures of POPC/Cho, Sm/Cho, and POPC/Sm/Cho alone and after the injection of Met-enk, and Met-enk-NH_2 at surface pressure values of ca. 10 and 30 mN/m

1986). Moreover, it is of a great importance that the preclinical studies of a novel drug to be conducted by simple, but informative methods, such as Langmuir monolayer technique and BAM.

Our results on the pressure/area isotherms and the compressional moduli showed that the addition of both Met-enk and Met-enk-NH_2 affected the surface properties and elasticity of the monolayers, composed of POPC, Sm, and Cho alone. The π/A-isotherms of the pure lipid monolayers measured were in agreement with those obtained by other authors (Prenner et al. 2007; Wydro 2012). The addition of Met-enk to the lipid films led to penetration of

the enkephalin into all of the studied monolayers at low surface pressure values, i.e. at loose films, and this effect was less pronounced in case of cholesterol (Fig. 1, panels with index 1). Moreover, the penetrating ability of the amidated derivative was more obvious in all isotherms. It was also noticed that the phase transition from gas to liquid-expanded state after the injection of both enkephalins was insignificant and the compression started at higher π. The tendency observed is logical, since there is a free area for incorporation of the peptide molecules between the lipids. However, at high surface pressure, i.e. more dense films the curve changes showed less area per molecule in the mixed lipids–peptide films suggesting reorientation and rearrangement of the molecules, forming the monolayers. Cholesterol is known to change the molecular packing at monolayers (Nelson and Cox 2008); therefore, we can speculate that the amphiphilic enkephalins dislocate Cho-molecules from the films. Most probably this effect was due to the fact that cholesterol has no charge at the pH studied, as compared with the zwitterionic POPC and Sm, leading to a specific folding of the peptides.

These hypotheses were confirmed by the compressional moduli of the films calculated by the respective isotherms (Fig. 1, panels with index 2). According to the literature, compressional modulus values under 100 mN/m correspond to a low degree of ordering of the lipid tails, composing the monolayers, while the higher values of this parameter demonstrate lowering the elasticity of the monolayer, i.e. dense packing of the hydrophobic parts of the molecules (Kodama et al. 2004). Our data revealed that while in case of Met-enk the phase transition from liquid-expanded to liquid-condensed phase state of the monolayer remained at almost same values of surface pressure, C-terminal amidation of the peptide led to unnoticeable minimum of the compressional modulus. Most probably, this was as a result of different type of interaction of Met-enk-NH_2 with the lipids due to its positive charge at the pH studied. Furthermore, both peptides reduced Cs^{-1} values with a greater effect for the modified Met-enk, especially for Cho film. In general, lower values of the compressional moduli most probably are due to a penetration of the opioid molecules into the monolayers. This leads to disordering of the lipid tails and consequently to increased fluidity of the respective mixed model membranes. The results of the Cho films confirm our speculation regarding the dislocation effect of the peptides on cholesterol monolayers.

More detailed analyses of the interaction between synthetic peptides and raft-forming lipids were provided by BAM at $\pi \approx 10$ and 30 mN/m (Fig. 2). The surface pressure values were chosen to represent a "diluted" state of the lipid films (lower π), and surface pressure of ca. 30 mN/m, near to the native membranes (Ege and Lee 2004; Evans and Skalak 1980; Ishitsuka et al. 2006; Marsh 1996; Seelig 1987). Brewster angle microscopy is a well-established method for characterization the morphology of ultra-thin surface films on aqueous subphases. BAM images show contrast within the film resulting from differences in reflectivity of the *p*-polarized light incident at the Brewster's angle from the monolayer as compared with the pure air–water interface (Henon and Meunier 1991; Hönig and Möbius 1991). Without a surface film the reflection is zero and the film is black, but when the thin film forms the reflective index changes. Our data showed a noticeable effect of the addition of both peptides only at lower surface pressure, except for POPC monolayers, which was logical since in the other two cases (for SM, and Cho) at $\pi \approx 30$ mN/m there is no enough free area for the peptide molecules, whereas POPC at this surface pressure requires a bigger area per molecule, suggesting that there is more space for penetration of the enkephalins as compared with Sm and Cho. However, based on the analyses of the respective isotherms and compressional moduli we suggest that the peptides changed their conformation at the dense monolayers, leading to structure reorganization of the lipid molecules at the air–water interface. At the lower values of surface pressure, the domains in the mixed peptide–lipid films formed suggest that there is an insertion of the peptides into the monolayers. Moreover, we expect that whereas in the case of the zwitterionic Met-enk the interaction with the lipids was mainly due to hydrophobic forces, in the case of Met-enk-NH_2 the positive charge of the neuropeptide resulted in a different folding and consequently in a different type of interaction with the lipid molecules. These results are in agreement with our previous report on the interaction of both peptides studied with dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) monolayers, which showed that the surface interactions between the neuropeptides and both phospholipids were predominantly due to hydrophobic interactions. Electrostatic forces probably play a role only at compact ordered lipid monolayers for the blocked peptide C-terminus by the amino-group of Met-enk-NH_2 (Tsanova et al. 2012). Moreover, the amidation of Met-enk results in converting the zwitterionic Met-enk into a cation and, therefore, facilitates its penetration into the membrane lipid monolayers.

The comparison between the effects of the injection of the enkephalins under the pure lipid monolayers and the double- and triple-component monolayers showed a different affinity of the peptides to the Cho-containing lipid mixtures. Our data on the π/A-isotherms of the lipid mixtures alone confirmed the results of other authors (Wydro 2012). The pressure/area isotherms and the compressional moduli of the films revealed again insertion of the neuropeptides into the monolayers at low surface pressure, i.e. loose film, especially in case of the cationic Met-enk-NH_2.

This effect was confirmed by the BAM experiments that showed the formation of more homogenous lipids–enkephalinamide films. Met-enk, however, most probably prefers the POPC-containing liquid-disordered part of the monolayer, since the most visible film morphology change was found at the equimolar mixture of POPC and Cho (Fig. 4). This effect was logical since Sm and Cho have a strong affinity for each other, leading to the formation of domains of liquid-ordered phase state (Wydro 2012).

At more dense packing of the lipids, both peptides led to reorientation of the molecules of the film, manifested by lowering the area per lipid molecule in case of the double lipid mixtures, especially at Sm/Cho monolayers (Fig. 3, panel b_1) because of the formation of domains of liquid-ordered state (Wydro 2012). This tendency was observed for the amidated peptide in the triple raft mixture (Fig. 3, panel c_1). However, the zwitterionic Met-enk had an opposite effect, suggesting again a different affinity for the lipid monolayer. Most probably in this case the peptides interact with POPC-containing liquid-disordered part of the monolayer between Sm/Cho liquid-ordered domains in a different way.

The expected increase in surface density of the monolayers containing cholesterol was evidenced by the compressional moduli of all double and triple mixtures, showing maximum values of Cs^{-1} higher than 100 mN/m (Fig. 3, panels with index 2). The addition of Met-enk increased the elasticity (lower Cs^{-1}) of the monolayers in lower degree as compared with its amidated derivative, suggesting that the positive charge of the peptide plays an important role for the interaction of Met-enk-NH_2 with the raft-forming lipid mixtures. Our results were in agreement with the findings of Jaikaran et al. (1995) who observed that gramicidin-derivates with C-terminal amino groups incorporate readily and refold quickly when added to dioleoylphosphatidylcholine lipid vesicles. They suggested that the amino groups at C-terminus of gramicidin interact with lipid phosphate groups (more strongly than carboxylates interact with phospholipid head groups) and provide some sort of anchor for the C-terminal end of the peptides during the refolding process. In addition, according to our data the compressional moduli calculated showed unnoticeable liquid-expanded to liquid-condensed phase transition confirming the strong interaction between both enkephalins and the lipids.

Conclusions

Based on the results obtained we suggest that the studied enkephalins insert into the lipid model membranes at low π values (liquid-disordered phase state), whereas at high surface pressure the interaction of peptides with the raft-forming lipids lead to reorientation of the molecules at the monolayers. Moreover, the amidation of Met-enk results in converting the zwitterionic Met-enk into a cation and, therefore, suggests a different type of folding and interaction with the lipids. In addition, most probably at the raft triple mixture both peptides interact with POPC-containing liquid-disordered part of the monolayer between Sm/Cho liquid-ordered domains. Our results demonstrated as well that Langmuir's monolayer technique in combination with Brewster angle microscopy can be successfully used as elegant and simple methods for enlightening the lipid–novel peptide analogs interactions at the plane of biomembranes.

Conflict of interest The authors declare that they have no conflict of interest.

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