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Partitioning of vasoactive intestinal polypeptide into lipid bilayers

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Abstract

Incubation of radiolabeled vasoactive intestinal polypeptide (VIP) with preformed lipid vesicles composed of phosphatidylcholine, phosphatidylglycerol and cholesterol resulted in reversible and saturable association of the peptide with the lipid bilayer. The pH-optimum for the reaction was in the physiological range. The vesicle-associated peptide displayed enhanced stability to proteolytic digestion, it was efficiently released into the supernatant by detergent-solubilization of the vesicles but remained vesicle-associated during treatment with agents that disrupt ionic interactions. Peptide binding by electrically neutral vesicles was lower than that by negative vesicles. Electron spin resonance studies with 5-doxylstearic acid or 16-doxylstearic acid labeled vesicles suggested that VIP decreased the fluidity close to the surface of the bilayer and increased the fluidity in its hydrophobic core. These observations suggest that VIP can bind and penetrate lipid bilayers.

Key words: Vasoactive intestinal polypeptide; Lipid bilayer; Amphipathic peptide

1. Introduction

Vasoactive intestinal polypeptide (VIP), a 28-amino acid neuropeptide, displays a broad profile of biological actions and activates multiple signal transducing pathways (reviewed in [1,2]). A Schiffer-Edmundson projection of VIP as a π-helix reveals segregation of apolar and polar residues onto the opposite faces of the helix and amphipathic character is also evident when VIP is modeled as a distorted α-helix [3]. A correlation between the helix-forming tendency of VIP analogues and their biological activity is described [4]. In pure water, the spectral characteristics of VIP are consistent with those of a random coil, but organic solvents and anionic lipids induce helix-formation in the molecule [5–7].

Short peptides capable of forming amphipathic helices are known to bind and penetrate lipid bilayers [8,9]. Examples include model peptides like (L-K-K-L-L-K-L-)$_2$ [10] and the 26-residue bee venom peptide, melittin [11]. Possible mechanisms for the binding include: (a) alignment of peptide monomers parallel to the surface of the bilayer mediated by electrostatic interactions between polar animo acids and phospholipid headgroups, and (b) insertion of peptide aggregates into the apolar bilayer core, stabilized, in part, by the hydrophobic effect [9]. The goal of the present study was to investigate the association of VIP with protein-free lipid vesicles. Saturable, reversible and pH-dependent partitioning of the peptide into lipid bilayers in which the hydrophobic effect as well as electrostatic interactions may play a role was observed. The possible biological significance of these observations is evaluated.

2. Materials and methods

Lipid vesicles. Phosphatidylcholine (PC) and phosphatidylglycerol (PG) purified from egg yolk were from Sigma. The fatty acid composition of the phospholipids determined by the supplier was: (PC, 16:0, 35%; 18:0, 12%; 18:1, 31%; 18:2, 14%); (PG, 16:0, 30%; 18:0, 13%; 18:1, 30%; 18:2, 16%). Unilamellar vesicles were prepared by reverse-phase evaporation [12] from

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a mixture of PG/PC/CH (molar ratio 1:4:5) or PC/CH (1:1) dissolved in chloroform. The lipid solution was taken to dryness using a rotary evaporator, dissolved in 3 ml diethyl ether (phospholipid and cholesterol concentrations, 12 mM each), mixed with 1 ml 50 mM Hepes (pH 7.3) and sonicated for 2 min in ice using a bath sonicator (Branson). The suspension was evaporated under reduced pressure for 20 min at 20–25°C, diluted with 10 ml of 50 mM Hepes (pH 7.3), centrifuged at $12500 \times g$ (7 min), the supernatant was discarded and the vesicle pellet was resuspended in buffer. The vesicles were stored at 4°C in buffer containing 0.02% sodium azide and used within ten days of preparation. Microscopic observation did not indicate aggregation or disintegration of the vesicles over this period. The phospholipid content of the vesicles was measured by colorimetric determination of inorganic phosphate (P_i) using the modified microassay method of Bartlett described in [13]. Vesicle concentrations are expressed in P_i units. Electron microscopy (Philips 410 LS) on a formvar-coated grid after negative staining of vesicles with an equal volume of 1% ammonium molybdate (w/v, in 50 mM Hepes (pH 7.3)) [14] revealed that > 90% of the vesicles were between 200 nm and 1000 nm in diameter.

Peptide binding by vesicles. Synthetic VIP (HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2; peptide content 81%, Bachem) was labeled with ^{125}I using chloramine-T. [*Tyr*^{10}-^{125}I]VIP was separated by reversed-phase high performance liquid chromatography (RP-HPLC) and identified by N-terminal radiosequencing [15]. The specific activity of the peptide was 2000 Ci/mmol. Unlabeled VIP synthesized at the University of Florida, Gainesville and purified by preparative RP-HPLC on a C-18 column was used in some of the experiments. The peptide content of this preparation was 83% and its purity was confirmed by amino acid analysis and automated N-terminal sequencing at the University of Nebraska Protein Structure Core Facility.

In preliminary studies, several brands of polypropylene microfuge tubes were observed to adsorb up to 30% of available [*Tyr*^{10}-^{125}I]VIP. Adsorption of the [*Tyr*^{10}-^{125}I]VIP by the polypropylene tubes (Sarstedt) was reduced to acceptable levels (< 3%), by: (a) pretreatment of the tubes with 0.3% PEI (1.5 ml, 60 min; Sigma) followed by five washes with 0.2 ml 50 mM Hepes (pH 7.3), and (b) inclusion of an excess concentration (0.5% w/v) of the unrelated protein bovine serum albumin (BSA; Sigma) in the buffer used to assay vesicle-peptide association. The conditions of incubation were: reaction volume, 0.2 ml; buffer, 50 mM Hepes (pH 7.3), 0.5% BSA (w/v), [*Tyr*^{10}-^{125}I]VIP and vesicle concentrations as indicated, temperature 23°C, incubation time, 60 min. The vesicles were pelleted ($12000 \times g$, 10 min; Beckman Microfuge™), the supernatants were aspirated and vesicle-associated radioactivity determined at 70% efficiency (Beckman model 5500 gamma counter). There was minimal loss of the vesicles in the supernatant, since more than 90% of the inorganic phosphate present in the reaction mixture was recovered in the pellet. The vesicles did not pellet firmly in HCl, NaOH and NaCl. In experiments with these reagents, the reaction mixtures were centrifuged, the supernatants passed through 0.2 μm polycarbonate filters (Nucleopore; prewetted with 0.01% poly-(L-lysine)) and free [*Tyr*^{10}-^{125}I]VIP was measured in aliquots of the filtrates. The binding data were corrected for peptide adsorption by the reaction tubes determined in parallel incubations. Dissociation and saturability data were analyzed by means of Kinetic and Enzfitter (Elsevier Biosoft). Binding of carboxyfluorescein (CF; 100 nM; Eastman Kodak) was measured using experimental conditions identical to those used for VIP. The vesicles were solubilized with 20% acetonitrile (final concentration) for 10 min at 23°C and CF fluorescence was determined (λ_{em} 520 nm, λ_{ex} 490 nm; Perkin-Elmer LS50 fluorimeter). In a control experiment, the fluorescence intensities of vesicles (2.3 mM P_i) mixed with known concentrations of CF in the absence and presence of 20% acetonitrile were found to be essentially identical.

Peptide hydrolysis. Treatment of free or vesicle-bound [*Tyr*^{10}-^{125}I]VIP with trypsin (Sigma) was in 50 mM Hepes (pH 7.3). The vesicles were centrifuged to separate released radioactivity, solubilized in 20% acetonitrile, BSA was added to 0.1% (w/v) as carrier and the TCA-insoluble radioactivity (undegraded VIP) was determined according to [15]. The levels of VIP hydrolysis determined by this method correlate with those observed by separation of the reaction mixtures by RP-HPLC ($r > 0.9$) [16]. Hydrolysis of Pro-Phe-Arg-MCA (Peptides International) was determined as the fluorescence of the coumarin leaving-group (λ_{em} 460 nm, λ_{ex} 370 nm).

ESR. Lipid vesicles were prepared as described above, except that 5-doxylstearic acid (5-DS) or 16-doxylstearic acid (16-DS) (Sigma) was included in the phospholipid solution in ether to give a spin label concentration of 0.2 mM (spin label/phospholipid molar ratio, 1:60). The labeled vesicles were washed, incubated with VIP in 0.2 ml 50 mM Hepes (pH 7.3), 0.5% BSA (w/v) (see above for conditions), centrifuged at 12000 rpm for 15 min and the pellet was resuspended in 100 μl of 50 mM Hepes (pH 7.3). The ESR spectra were recorded as described previously [17] at 27 ± 0.5°C using a JES-FE1XG ESR spectrometer (JOEL, Tokyo) with instrumental parameters as follows: 9.060 GHz microwave frequency, 0.2 mT modulation width at 100 kHz modulation frequency, response time 0.3 or 1.0 s, sweep time 10 mT/2 min, microwave power 8 mW. The polarity-corrected order parameter

S was calculated from the hyperfine splitting pattern according to [18] from the 5-DS spectra and the motion parameter τ_o, corresponding to the rotational correlation time, from the 16-DS spectra according to [19].

3. Results

Peptide-lipid vesicle binding

Unilamellar lipid vesicles were prepared by reverse evaporation and assayed for binding of radiolabeled VIP. BSA was included in the assay diluent to saturate nonspecific polypeptide binding sites in the lipid vesicles and the polypropylene surface of the reaction tubes. Increasing concentrations of preformed lipid vesicles (2.5–45.6 mM P_i) displayed increasing binding of [Tyr^{10}-^{125}I]VIP up to 56.6% of available peptide (0.42 nM). Under conditions identical to those used for VIP, the vesicles took up very small amounts of carboxyfluorescein (Table 1), a small polar molecule (376 daltons) often used to study the integrity of lipid vesicles. On the other hand, vesicles formed in the presence of VIP and CF contained somewhat greater concentrations of CF than VIP. The latter values represent encapsulation of VIP and CF within vesicles. Taken together, the data show that the observed association of VIP with the vesicles does not represent entrapment due to vesicle breakage and resealing.

Incubation of [Tyr^{10}-^{125}I]VIP-vesicle complexes in the presence of excess unlabeled VIP (2 mM) resulted in release of approx. 90% of the initial vesicle-associated peptide and steady-state conditions were reached at the earliest time-point examined (30 min), showing that the peptide was not irreversibly sequestered in the lipid bilayers. In the absence of exogenous unlabeled peptide, slow release of approx. 50% of the vesicle-associated peptide over 24 h was observed (Fig. 1).

The partitioning of [Tyr^{10}-^{125}I]VIP into the vesicles was competitively inhibited by increasing concentrations of unlabeled VIP (IC50 560 μM; Fig. 2). A plot of bound versus available peptide could be fitted to the equation for a rectangular hyperbola typical of a saturation isotherm [$y = (B_{max} \cdot x)/(1/K_{p,obs} + x)$, where x and y are available and bound peptide, respectively]. The values of B_{max} and $K_{p,obs}$ obtained from this curve were 0.079 mol VIP/mol P_i and 1.3×10^{-3} M^{-1}, respectively.

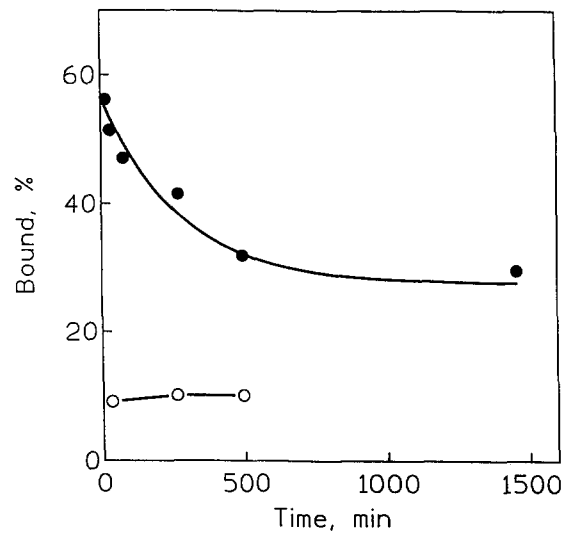

Fig. 1. Reversibility of binding of VIP by lipid vesicles. Vesicles (3.3 mM P_i) were labeled with [Tyr^{10}-^{125}I]VIP (0.11 nM), washed to remove free peptide and incubated in 0.2 ml buffer in the absence (○) or presence of (●) of 2 mM unlabeled VIP for varying time-periods (23°C). The remaining vesicle-associated radioactivity was determined and expressed as % of initial bound peptide (35410 cpm).

In a control experiment performed to eliminate the possibility of artefacts due to use of radiolabeled peptide, lipid vesicles (2 mM P_i) were permitted to bind unlabeled VIP (0.2 mM), the vesicles were solubilized with 1% SDS and extracted on a C-18 cartridge and analyzed by RP-HPLC on a C-18 column according to [15]. A peptide peak displaying the characteristic retention time of authentic VIP was observed. The amount of peptide recovered in this peak, estimated by its absorbance at 214 nm, was 76% of the value predicted from radiolabeled VIP-binding experiments.

The binding of VIP (100 nM) by electrically neutral and negative vesicles prepared from PC/CH and PG/PC/CH, respectively, was compared. In each case, the levels of peptide binding increased with increasing concentration of the vesicles. The values of % VIP bound ± S.D./μmol P_i were, PG/PC/CH vesicles, 26.2 + 3.4; PC/CH vesicles, 12.6 ± 1.5. Provided that minor differences in fatty acid composition of the phospholipids are not an interfering factor, these data are consistent with a stabilizing role for electrostatic interactions between acidic lipid headgroups and VIP, which is a basic peptide rich in Lys and Arg residues. The inclusion of cholesterol, an agent that can be expected to decrease bilayer fluidity at the tempera-

Table 1
Binding and encapsulation of VIP and carboxyfluorescein (CF) by lipid vesicles

Probe	Binding (nM)	Encapsulation (nM)
VIP	20.5 ±1.0	37.5±5.0
CF	0.01±0.001	82.5±7.5

Values are means ± S.D. Probe-binding was with pre-formed vesicles (2.3 mM P_i) and 100 nM CF or 0.2 nM [Tyr^{10}-^{125}I]VIP mixed with 100 nM unlabeled peptide. Encapsulation was by making vesicles from lipid solutions (2.6 mM P_i) containing 2.5 μM CF or 1.7 nM [Tyr^{10}-^{125}I]VIP mixed with 2.5 μM unlabeled VIP. Free probes were removed by three washes with 50 mM Hepes (pH 7.3), containing 0.5% BSA. CF values were determined by fluorimetry after solubilization of vesicles, and VIP values, by measurement of vesicle-associated radioactivity.

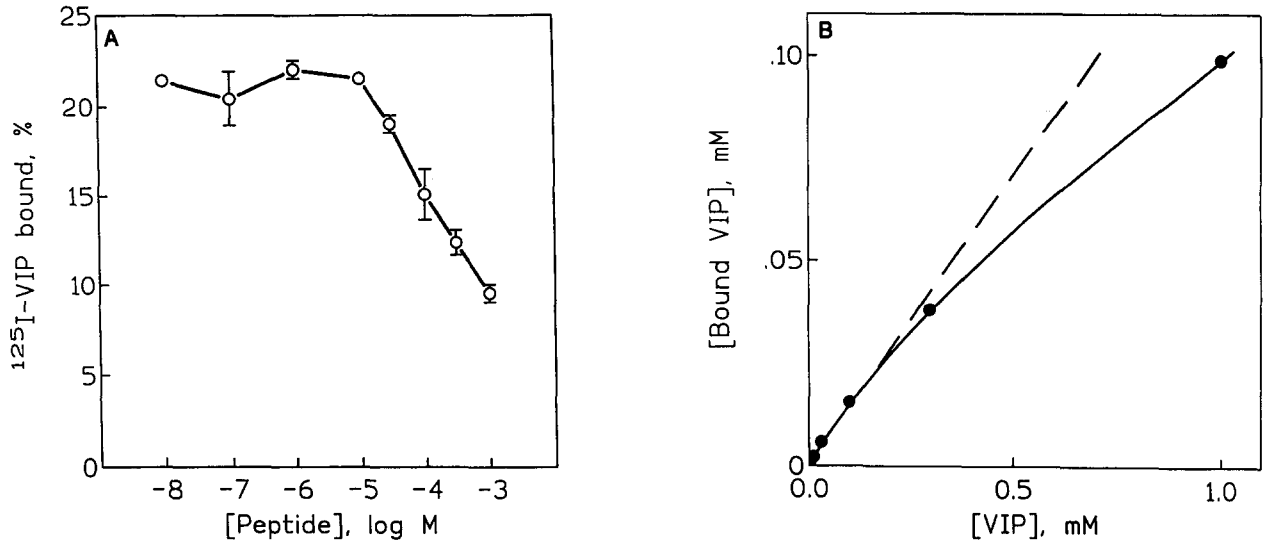

Fig. 2. (A) Competitive inhibition of binding of [*Tyr*^{10}-^{125}I]VIP (0.26 nM) by the vesicles (2.4 mM P_i) by unlabeled VIP (10 nM–1 mM). Data are means ± S.D. expressed as % of available radiolabeled peptide. (B) A saturation isotherm constructed from the data in A (solid line). The dotted line shows the levels of binding expected in a non-saturable reaction.

tures used in this study [20], did not influence the binding of VIP by the vesicles (26.2% and 24.9% VIP bound/μmol P_i PC/PG/CH and by PC/PG vesicles respectively).

Solvent effects

[*Tyr*^{10}-^{125}I]VIP binding by the PC/PG/CH vesicles was assayed at several pH values in a constant ionic strength buffer [21] (Fig. 3). The binding was low at the extreme pH values and optimal binding was observed at near-neutral pH (pH 6–8).

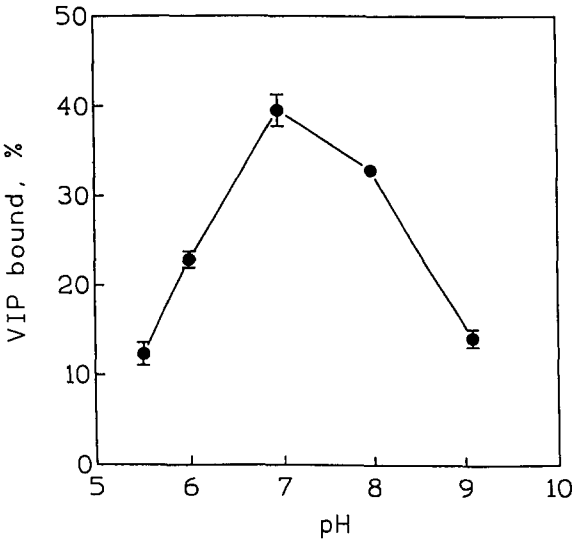

Fig. 3. pH-dependent binding of [*Tyr*^{10}-^{125}I]VIP (0.14 nM) by lipid vesicles (2.1 mM P_i) assayed in a constant ionic strength buffer (25 mM ethanolamine, 25 mM Tris, 50 mM morpholinoethanesulfonic acid). Data are means ± S.D. from a representative experiment.

Treatment of [*Tyr*^{10}-^{125}I]VIP-vesicle complexes with 25 mM EDTA, 1 mM HCl, or 1 M NaCl caused little or no release of the bound peptide (Table 2). At alkaline pH (1 mM NaOH), a small but significant proportion (16%) of the bound radioactivity was reproducibly released. Dissolution of the vesicles with SDS produced near-complete peptide release into the supernatant, reflecting, presumably, uptake of the peptide into mixed detergent-lipid micelles.

Degradation of bound VIP

With increasing trypsin concentration, increasing hydrolysis of free [*Tyr*^{10}-^{125}I]VIP after 45 min of incubation (up to 80% of available peptide) was evident (Fig. 4). In the case of vesicle-bound [*Tyr*^{10}-^{125}I]VIP, most of the radioactivity remained associated with the vesicles treated with 1 nM, 5 nM and 26 nM trypsin (82, 83 and 73% of initial radioactivity, respectively). Compared to

Table 2
Release of vesicle-bound VIP

Treatment	% VIP released
25 mM EDTA	8.1 ± 10.0
1 mM HCl	3.0 ± 3.5
1 M NaCl	2.7 ± 3.0
1 mM NaOH	15.9 ± 4.0
1% SDS	81.1 ± 9.6

Lipid vesicles (4 mM P_i) were permitted to bind [*Tyr*^{10}-^{125}I]VIP (1 nM), washed extensively to remove unbound radioactivity and then treated twice with 1 ml of the indicated solutions for 10 min (23°C). Released radioactivity was determined in the pooled supernatants obtained by centrifugation. Values are % of initial vesicle-bound radioactivity (76300 cpm), corrected for peptide release in control incubations performed in 50 mM Hepes (pH 7.3) (4.2 ± 3.4%).

the free peptide, a smaller proportion of the vesicle-associated radioactivity recovered after the trypsin-treatment was TCA-soluble (Fig. 4), suggesting a reduced susceptibility to proteolysis. To test for nonspecific inhibition of enzyme activity, a methylcoumarinamide (MCA) conjugate of a basic peptide (Pro-Phe-Arg-MCA) (15 μM) was incubated with trypsin (10 nM) in the absence and presence of the vesicles (1 mM P_i) for 30 min, followed by removal of the vesicles by centrifugation. The observed increases in fluorescence intensity in the presence and absence of the vesicles were similar (868 FU and 885 FU, respectively).

VIP effects on bilayer fluidity

ESR spectra of 5-DS and 16-DS labeled lipid vesicles were obtained. These spin labels are commonly used to measure the fluidity close to the surface (5-DS) and core (16-DS) of lipid bilayers (e.g., [17,22]). With increasing VIP/lipid molar ratios, progressively decreasing values of the motion parameter τ_0 were evident using 16-DS labeled vesicles (Table 3). In contrast, the order parameter S computed using the 5-DS probe tended to increase by exposure to VIP, with the effect reaching statistical significance at a concentration of 500 μM VIP. These data suggest a pronounced VIP-induced increase of fluidity in the hydrophobic core of the bilayer and a relatively small but significant decrease in fluidity close to the bilayer surface.

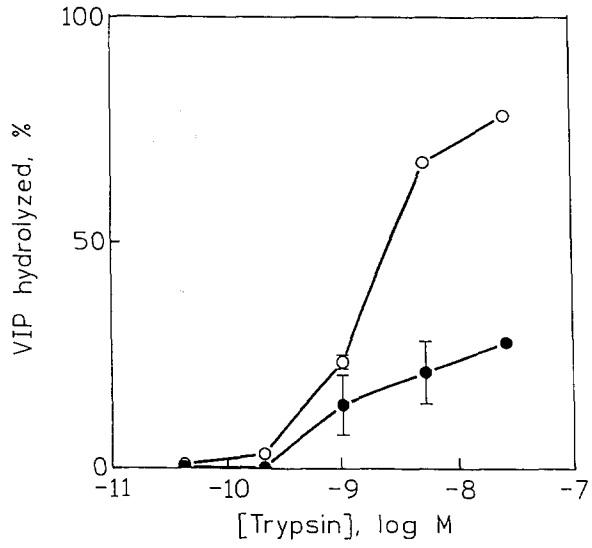

Fig. 4. Decreased proteolytic hydrolysis of vesicle-bound VIP (•) compared to free VIP (○). Vesicles (4.2 mM P_i) were labeled with [Tyr^{10}-^{125}I]VIP (0.37 nM), washed with buffer to remove free peptide and treated with varying concentrations of trypsin for 45 min in 50 mM Hepes (pH 7.3). Released radioactivity was removed by centrifugation and aspiration of the supernanant. Vesicles were solubilized with acetonitrile (30%), TCA was added to 10% and BSA to 0.1%, and acid-soluble radioactivity was measured to determine the degree of hydrolysis. Data are means ± S.D. expressed as % of initial vesicle-associated peptide (28290 cpm) corrected for TCA-soluble radioactivity observed in the absence of trypsin (10%).

Table 3
Effect of VIP on bilayer fluidity in 5-DS and 16-DS labeled vesicles

VIP (μM)	S (5-DS)	τ_0 (10^{-10} s) (16-DS)
0	0.751 ± 0.014	136.06 ± 22.01
200	0.755 ± 0.012	121.71 ± 32.41
300	0.771 ± 0.017	91.85 ± 9.31 *
500	0.801 ± 0.031 *	88.43 ± 2.65 *

Concentrations of PC/PG/CH vesicles were 2.8 mM P_i (5-DS) and 3.7 mM P_i (16-DS). Values of S and τ_0 for 5-DS and 16-DS labeled vesicles, respectively, are means ± S.D. from three (5-DS) or four (16-DS) experiments.
* $P < 0.05$ versus control values (vesicles in the absence of peptide) calculated by ANOVA.

4. Discussion

The general conclusion from this work is that VIP can bind and penetrate the hydrophobic core of model lipid bilayers. The binding was saturable and reversible at neutral pH. The nominal values of the partition constant derived from the binding isotherm ($1.4 \cdot 10^{-3}$ M^{-1}) was in the same range as that reported for another amphiphilic peptide, melittin [23]. The apparent binding capacity at saturating VIP concentrations was 1 mol VIP/12.6 mol phospholipid.

Electrostatic interactions probably play a role in the observed VIP binding by PG-containing negatively-charged vesicles, since VIP is a basic peptide. However, several considerations indicate that electrostatic binding alone cannot explain the observed interaction: (a) Neutral vesicles also displayed VIP binding activity, in contrast to observations that several other basic peptides are bound only by negatively charged vesicles [24]. (b) VIP bound by PG/PC/CH vesicles was released minimally or not at all by acid, alkali and high ionic strength solvent. (c) The optimal pH for VIP binding by negatively charged vesicles was in the neutral range, unlike alkaline pH optima observed for the binding of basic proteins like lysozyme by similar vesicles [25]. (d) Treatment with VIP produced significant changes in the mobility of nitroxide spin-labeled stearic acid probes incorporated in the lipid vesicles. These considerations are consistent with the hypothesis of electrostatic binding of VIP by to phospholipid headgroups combined with penetration of a hydrophobic region(s) of the peptide into the bilayer.

Estimates of the fluidity at different depths in the bilayer can be obtained by measuring the motion of spin labels placed at varying distance from the carboxyl group of fatty acid probes (e.g., 5-DS and 16-DS used in the present study). Binding of polypeptides by membranes can lead to qualitatively similar or opposing effects on fluidity at different depths in the bilayer (see

Ref. [26] for review). In the present study, the association of VIP with the vesicles was accompanied by a small but significant increase in the order parameter S for 5-DS and a striking decrease in the motion parameter τ_0 for 16-DS, suggesting decreased fluidity close to the surface of the bilayer and increased fluidity in its core. Myelin basic protein [27] has been previously described to produce similar effects on the fluidity characteristics in the bilayer core and surface. The decrease in 5-DS mobility following exposure to VIP may derive from electrostatic binding at phospholipid headgroups. In analogy with the mechanism proposed for myelin basic protein, penetration of a hydrophobic region of VIP (see below) into the bilayer may produce a larger volume increase in the core of the bilayer compared to its surface, accounting for the increased mobility (decreased τ_0) of the 16-DS probe.

The lipid binding properties of VIP are not entirely unexpected in the context of its known structural characteristics. Modeled as a π-helix (4.4 residues/turn) or a twisted α-helix [3], the cationic and apolar residues of VIP segregate onto the opposite faces of the helix. α-helical amphiphilic peptides are well-documented to bind lipid bilayers. For example, depending upon the experimental conditions, melittin and alamethicin may bind along the surface of lipid bilayers or form bilayer-spanning aggregates [9]. Similarly, peptides in which α-helix distortions permit segregation of apolar and polar residues are known to bind lipid bilayers [28]. In the case of VIP, the central segment spanning residues 12–21 contains cationic residues interspersed with apolar ones, has a high propensity for helix formation [5–7] and could be important in formation of peptide aggregates with membrane binding activity. All of the trypsin-sensitive bonds in VIP are located in this region (R^{12}-L^{13}, R^{14}-K^{15}, K^{15}-Q^{16} K^{20}-K^{21}, K^{2}-Y^{22}). The observation of diminished trypsinolysis of vesicle-associated VIP is consistent with burial of this segment into the lipid bilayer.

The observations described here do not directly address the question of the biological significance of partitioning of VIP into lipid bilayers. However, since VIP was bound by both types of bilayers examined in the present study (negative and neutral vesicles) and optimal binding was observed in the neutral pH range, it is reasonable to speculate that the peptide may interact with biological membranes in a receptor-independent manner. The biological actions of VIP are remarkably diverse and this neuropeptide activates apparently unrelated signal transducing systems (reviewed in [1,2]). VIP is a known modulator of synaptic transmission, smooth muscle tone, transmembrane water and ion flux, neuroendocrine secretion and T- and B-lymphocyte immunological activities. There are several ways in which VIP-lipid bilayer interactions may be important. In the absence of definitive evidence concerning the mechanism of removal and inactivation of VIP, it is commonly assumed that proteolytic degradation is responsible for termination of the biological effects of the peptide. In the present study, we have shown that within the limits of saturability of the system, large proportions of the radiolabeled VIP (up to 60%) were bound by lipid vesicles, indicating that partitioning into membranes could be a factor governing the availability of soluble peptide in extracellular fluids. Second, binding of VIP by lipid particles or soluble lipids may stabilize the peptide to proteolysis and permit its delivery to distant target cells. Third, partitioning of VIP into lipid bilayers may restrict the peptide into a specific conformation(s) and thus modify its interaction with membrane receptors. Finally, local concentration of VIP within neuronal storage vesicles and at the site of release from nerve endings may be sufficiently great to directly modulate membrane function via changes in bilayer fluidity.

Acknowledgements

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