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Bicelle composition-dependent modulation of phospholipid dynamics by apelin peptides

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Abstract
Apelin peptides are cognate ligands for the apelin receptor, a G-protein coupled receptor (GPCR). The apelinergic system plays critical roles in wide-ranging physiological activities including function and development of the central nervous and cardiovascular systems. Apelin is found in 13-55 residue isoforms in vivo, all of which share the C-terminal portion of the preproapelin precursor. Characterization of high-resolution structures and detergent micelle interactions of apelin-17 led to a two-step membrane-catalyzed binding and GPCR activation mechanism hypothesis recapitulated in longer isoforms. Here, we examine interactions of the apelin-13 and -17 isoforms with isotropic zwitterionic and mixed zwitterionic-anionic lipid bicelles to test for hallmarks of membrane catalysis in a more physiological membrane-mimetic environment than a micelle. Specifically, $^1$H and $^{31}$P relaxation and diffusion solution-state NMR techniques demonstrate that both apelin isoforms interact with both types of isotropic bicelles. Bicelle hydrodynamics were observed to be differentially modulated by apelin peptides, although these effects were minimal. Phospholipid headgroup $^{31}$P spin relaxation behaviour was, conversely, clearly perturbed. Perturbation of this nature was also observed in magnetically-aligned bicelles by $^{31}$P solid-state NMR spectroscopy and spin relaxation experiments. This behaviour is consistent with an apelin-bicelle binding process allowing significant peptide mobility, facilitating membrane-catalyzed GPCR encounter.

Key words
Phospholipid bicelles; peptide-bicelle interactions; nuclear spin relaxation; pulsed-field gradient diffusion NMR spectroscopy; membrane catalysis.
Introduction

The apelin peptides are 13-55 amino acid C-terminal fragments of the 77 amino acid preproapelin precursor (Tatemoto et al. 1998). The 13, 17, and 36 residue apelin peptides have been the most frequently identified isoforms in body fluids (reviewed in (Shin et al. 2018)), with the 55 residue isoform identified in milk (Mesmin et al. 2011). All of these isoforms are bioactive (Shin et al. 2018), with the 12 C-terminal amino acids being key for binding to and activation of a class A G-protein coupled receptor (GPCR) (Lee et al. 2005; Tatemoto et al. 2001), the apelin receptor (previously known as APJ, or angiotensin-like receptor 1 (O'Dowd et al. 1993)). Length-dependent variations in potency and activity have also been shown for apelin (Shin et al. 2018). A second cognate peptide ligand, apela (also known as ELABELA or toddler) (Chng et al. 2013; Pauli et al. 2014), has recently been identified for this GPCR. Like apelin, apela appears to have multiple bioactive isoforms ranging in length from 11 (Murza et al. 2016) to 32 (Chng et al. 2013) residues.

Apelin acts as a circulating hormone or paracrine hormone, depending on context, and has attributed adipokine and neuropeptide functions (recently reviewed in (Chapman et al. 2014; Shin et al. 2018)). Activation of the apelin receptor by apelin is known to affect cardiac contractility, vascular tone, glucose metabolism, behaviour, and homeostasis. Furthermore, the apelinergic system has been implicated in neoangeogenesis during tumour formation, in atherosclerotic plaque formation and the apelin receptor is known to be a CD4 glycoprotein co-receptor for human immunodeficiency virus 1 (HIV1) cell fusion. The variety and import of apelinergic system function have, therefore, naturally led to a wide range of medicinal chemistry studies probing structure-activity relationships in this system (Narayanan et al. 2015). Less is known about apela (patho)physiology. Although it was first associated with key rolls in cardiovascular system development (Chng et al. 2013; Pauli et al. 2014), a number of recent studies show that apela is also expressed following development (Shin et al. ...
All bioactive isoforms of apelin from apelin-12 through -55 interact with phospholipid membrane-mimetic detergent micelles in a similar headgroup-dependent manner (Langelaan and Rainey 2009; Shin et al. 2017b). Namely, binding is highly favoured to micelles with anionic headgroups (sodium dodecyl sulfate (SDS) or lyso-palmitoylphosphatidylglycerol (LPPG)), while zwitterionic (dodecylphosphocholine (DPC)) micelles exhibit a lesser degree of binding. Anionic micelles, correspondingly, induce a similar structuring in the shared C-terminal portion of all of the apelin peptides regardless of length, while induced structuring is not observed in DPC micelles (Shin et al. 2017b). Although such preferential binding appears logical from the cationic nature of the apelin peptides (net charge at pH 7 of ~+4 to +11; pI ~12.5-13.4), apela is similarly cationic (net charge +2 (apela-11 monomer; +4 if disulfide linked dimer) to +11, pI 8.6-12.6) but favourably binds to both zwitterionic and anionic micelles (Huang et al. 2017). Interestingly, unlike apelin, apela shows isoform-dependent differences in structuring (Huang et al. 2017).

The behaviour of both of these ligands is consistent with membrane catalysis of peptide-receptor binding (Sargent and Schwyzer 1986). In the membrane catalysis mechanism, peptides are held to encounter and bind to the phospholipid bilayer as a precursor to receptor binding. This process restricts the degrees of freedom of diffusional search for the receptor (e.g., two instead of three spatial dimensions), increases local ligand concentration assuming favourable binding, and may involve membrane-induced structuring of the ligand allowing receptor recognition, with evidence coming from a number of studies (reviewed in (Langelaan and Rainey 2010)). Following from these arguments, membrane-catalyzed ligand-receptor binding appears likely in the apelinergic system.

While detergent micelles are a popular membrane-mimetic environment (Qureshi and Goto 2012), examples of protein structural or functional perturbation in a micellar
environment (Qureshi and Goto 2012; Zhou and Cross 2013) imply that these are not uniformly applicable. As an alternative, bicelles formed upon mixture of a long-chain lipid with a short-chain lipid or detergent provide an environment that better mimics the lipid bilayer (Marcotte and Auger 2005; Qureshi and Goto 2012). Modulation of the ratio of long-chain to short-chain species (q) allows for fine-tuning of the environment from an isotropically tumbling discoidal species (at low q, q < ~1) through to larger discoids or perforated extended lamellae. These larger structures spontaneously align with a magnetic field, providing an environment with natural orientational anisotropy. Bicelles, hence, are applicable membrane mimetics both for solution-state NMR spectroscopy (q < 1) or for oriented sample solid-state NMR (q > 1).

Herein, we characterize interactions of two apelin isoforms (apelin-13 and -17) with bicelles containing either uniformly zwitterionic phosphatidylcholine (PC) headgroups or mixtures of zwitterionic PC and anionic phosphatidylglycerol (PG) lipids. These particular isoforms were chosen for study herein due to (i) the high potency and very widespread in vivo detection of apelin-13 (Shin et al. 2018) and (ii) the extensive biophysical data available for apelin-17-micelle interactions (Langelaan and Rainey 2009). \(^1\)H and \(^{31}\)P solution-state, nuclear spin relaxation, and pulsed field gradient (PFG)-based diffusion ordered spectroscopy (DOSY) NMR techniques were used to characterize apelin-bicelle interactions for isotropic bicelles. It should be noted that q=0.5 bicelles exhibit mixing between long- and short-chain lipid constituents with a resemblance to mixed micelles (Beaugrand et al. 2014). In contrast to this, hallmarks of DMPC bilayer formation have been demonstrated in a q=0.5 bicelle-solubilized integral outer membrane protein (Lee et al. 2008), implying the potential for isotropic bicelles to modulate their behaviour to accommodate membrane proteins. Here, to test for potential differences in apelin interaction behaviour with bicelles having a better-defined planar bilayer surface area than q=0.5 bicelles, \(^{31}\)P solid-state NMR experiments and
nuclear spin relaxation time measurements were carried out using magnetically-oriented bicelles with \( q=3 \).

**Materials and Methods**

**Materials**

Apelin-13 (QRPRLSHKGPMPF) and apelin-17 (KFRRQRPRLSHKGPMPF) were chemically synthesized and purified (> 95% purity; both with a free amine at the N-terminus and a free carboxylic acid at the C-terminus) as detailed previously (Langelaan et al. 2009). Peptide identities were confirmed by matrix-assisted laser desorption ionization mass spectroscopy (C-CART Facility, Memorial University of Newfoundland, St. John’s, NL). Phospholipids for bicelle production, 1,2-dihexanoyl-\( sn \)-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-\( sn \)-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-\( sn \)-glycero-3-phospho-(1’-\( rac \)-glycerol) (DMPG), were purchased from Avanti Polar Lipids (Alabaster, AL). \( D_2O \) was purchased from C/D/N isotopes (Pointe Claire, QC) and 2,2-dimethyl-2-silapentane-5-sulfonate sodium (DSS) salt from Wilmad-LabGlass (Vineland, NJ)

**Bicelle preparation**

Bicelle preparation followed general protocols and sample condition considerations outlined previously (De Angelis and Opella 2007). Specifically, two types of isotropic bicelles with \( q=0.5 \), where \( q \) is the molar ratio of long-chain to short-chain lipids, were prepared for solution-state NMR studies: (1) zwitterionic bicelles composed of 50 mM DMPC and 100 mM DHPC (“PC bicelles”); and, (2) mixed zwitterionic-anionic bicelles composed of 40 mM DMPC, 10 mM DMPG and 100 mM DHPC (“PC-PG bicelles”). Zwitterionic bicelles with \( q=3 \) were also prepared for solid-state NMR studies, employing 120 mM DMPC and 40 mM DHPC. In all cases, bicelle stocks were prepared by dissolving
lipids in 90% H$_2$O + 10% D$_2$O in the presence of 150 mM NaCl and subjecting them to 7 to 11 cycles of heating (at 42 °C; 15 min) and cooling (on ice; 15 min), with vortexing (2 min) between each cycle. The pH of each bicelle stock was adjusted to 7.0 using HCl and NaOH on ice. For samples containing apelin-13 or apelin-17, peptide was added to a given bicelle stock solution rather than being present throughout the bicelle formation procedure.

**Solution-state NMR spectroscopy**

Samples were prepared containing each type of isotropic bicelle (150 mM total lipid) with or without addition of either apelin-13 or apelin-17 (0.5 mM). Experiments were performed at 11.74 T (500 MHz $^1$H frequency; Avance, Bruker Canada, Milton, ON) using a double-resonance BBFO SmartProbe with a z-axis gradient (Bruker Canada). NMR spectra were acquired at 37 °C using standard pulse programs from the Bruker TopSpin 2.1 pulse program catalogue and processed using TopSpin 3.1. 1D $^1$H experiments were acquired using excitation sculpting with gradients for water suppression ((Hwang and Shaka 1995); 16 scans, 2 s relaxation delay, 14 ppm spectral width, 2 s acquisition time). After comparing experimental data with and without $^1$H decoupling, subsequent 1D $^{31}$P experiments were acquired without $^1$H decoupling (pulse program “zggd” with decoupler power at 0 W, 16 scans, 3 s relaxation delay, 40 ppm spectral width, 2 s acquisition time with 32,360 acquired points). $^{31}$P T$_1$ measurements were performed using the inversion-recovery experiment (pulse program “t1ir”, 64 scans, 10 s relaxation delay, 40 ppm spectral width, 2 s acquisition time with 32,768 acquired points). The variable delay list for inversion-recovery experiments contained 16 delays of 0.01, 0.05, 0.10, 0.16, 0.24, 0.34, 0.46, 0.60, 0.78, 1.00, 1.25, 1.55, 1.90, 2.40, 3.00, 4.00 s. $^{31}$P T$_2$ measurements were performed using Carr-Purcell-Meiboom-Gill spin echo experiments (pulse program “cpmg”, 64 scans, 10 s relaxation delay, 40 ppm spectral width, 2 s acquisition time with 32,768 acquired points). The variable counter list for
spin echo experiments produced 16 delays of 2, 6, 12, 20, 30, 44, 60, 80, 104, 130, 160, 200, 250, 320, 400, 500 ms. $T_1$ (longitudinal) and $T_2$ (transverse) $^{31}$P relaxation time constants were determined from single-component exponential fits to the intensities observed over each series of experiments with varying delays using the T1/T2 Relaxation module within TopSpin 3.1. Time constants were extracted from the relationships:

\[
I(t) = I(0)(1 - 2Ae^{-t/T_1})
\]

(1)

\[
I(t) = I(0)e^{-t/T_2}
\]

(2)

where $I(t)$ is the observed signal intensity at delay time $t$, $I(0)$ is the intensity of the full signal without decay, and $A$ is a normalization factor. Rotational correlation time ($\tau_C$) values were inferred from the ratio of $T_1/T_2$ using the field-strength dependent polynomial expressions for $^{31}$P relaxation from Carper and Keller, with polynomial coefficients employed as appropriate for a given observed $T_1/T_2$ ratio (Carper and Keller 1997).

**Solid-state NMR spectroscopy**

Samples were prepared containing pure PC bicelles (160 mM total lipid concentration; q=3) without and with apelin-13 (1.2 mM). Experiments were performed at 16.4 T (Bruker Avance) using a two-channel Efree static 5 mm solenoid probe (Bruker BioSpin, Billerica, MA). NMR spectra were acquired at 37 °C using standard pulse programs with some necessary in-house modifications and processed using TopSpin 3.1 with 5 Hz of line-broadening applied. 1D $^{31}$P data were collected using single pulse excitation with proton decoupling (8.2 µs pulse lengths at 30 kHz rf field strengths, 64 scans, 5 s relaxation delay, and continuous wave proton decoupling). $^{31}$P $T_1$ measurements were performed using the saturation recovery experiment (using 80 saturation pulses with 1 µs pulse separation, 64 scans, and continuous wave proton decoupling). The variable delay list for $T_1$ contained 16 delays of 30µs, 100ms, 500ms, 750ms, 1s, 1.25s, 1.5s, 1.75s, 2s, 2.5s, 3s, 5s, 10s, 15s, 20s,
measurements were performed using a Hahn echo sequence (using 8.2μs and 16.4μs for the 90 and 180 degree pulses, respectively, 64 scans, 3.5 s relaxation delay, and continuous wave proton decoupling). The variable delay list for T₂ contained 16 delays of 27.51μs, 77.51μs, 177.51μs, 327.51μs, 527.51μs, 777.51μs, 1.1ms, 1.5ms, 2.5ms, 5ms, 10ms, 20ms, 50ms, 100ms, 300ms, 500ms. T₁ and T₂ 31P relaxation time constants were determined from single-component exponential fits to the series of experiments with varying delays using the T1/T2 Relaxation module within TopSpin 3.1. The time constants were extracted from the relationships

\[ I(t) = I(0) + P e^{-t/T_1} \]  
\[ I(t) = I(0) + P e^{-t/T_2} \]

where P is an iterative fitting parameter.

**PFG diffusion NMR spectroscopy**

Hydrodynamic properties of isotropic bicelle samples were measured using PFG diffusion experiments. Pseudo-2D ¹H DOSY experiments (Morris and Johnson 1992) were performed using stimulated echo with presaturation for water suppression (pulse program “ledbpgpppr2s”, 256 scans, 2 s relaxation delay, 12 ppm spectral width, 1 s acquisition time with 12,016 acquired points). Similarly, stimulated echo pseudo-2D 31P DOSY experiments (Morris and Johnson 1992) were performed (pulse program “ledbpgp2s”, 128 scans, 3 s relaxation delay, 40 ppm spectral width, 1.24 s acquisition time with 19,998 acquired points). In both ¹H and 31P DOSY experiments, the signal envelope was attenuated by increasing the gradient strength from 2% to 95% in 16 steps. According to the Stejskal-Tanner relationship (Stejskal and Tanner 1965), modified as appropriate for the employed DOSY experiment (Wu et al. 1995), the observed signal decay as a function of gradient strength is exponentially related to the translational diffusion coefficient (Dₜ):
$I = I(0) \exp[-D_C \times (2\pi\gamma g\delta)^2 \times (\Delta - \delta/3) \times 1e4]$  \hspace{1cm} (5)

where $I$ is the observed signal intensity, $I(0)$ is the unattenuated signal intensity, $\gamma$ is the gyromagnetic ratio (4257.7 Hz/G for $^1H$, 1723.56 Hz/G for $^{31}P$), $g$ is the gradient strength (maximum amplitude 50 G/cm), $\delta$ is the gradient pulse length (8 ms for both $^1H$ and $^{31}P$), and $\Delta$ is the diffusion time (200 ms for both $^1H$ and $^{31}P$). $D_C$ under a given condition was thus determined through a the fit of the observed ratios of $I/I(0)$ as a function of $g$ using the T1/T2 Relaxation module within TopSpin 3.1.

**Results and discussion**

**Apelin conformation in the presence of isotropic bicelles.**

Apelin-13 and -17 (0.5 mM) were both readily soluble in isotropic (q=0.5) bicelle solutions containing 150 mM total lipid (150 mM NaCl, pH ~7) prepared either with DMPC:DHPC (1:2 eq., PC bicelle) or DMPC:DMPG:DHPC (4:1:10 eq., PC-PG bicelle). 1D $^1H$ NMR spectra are indicative of changes in peptide conformation and/or protection of a subset of exchangeable amide protons for both apelin-13 and apelin-17 regardless of bicelle type. This is evident in the amide-aromatic region of the $^1H$ NMR spectrum (Fig. 1), with other regions obscured by lipid resonances (Fig. S1 in the Supplementary Material). Notably, both apelin isoforms exhibit differences in both numbers of observed resonances and in chemical shift patterns from each other and between the normal PC bicelle vs. mixed PC-PG bicelle conditions. This is consistent with the trends in binding and interaction observed with detergent micelles for apelin isoforms ranging from apelin-13 to -55, where binding to zwitterionic DPC micelles was clear from PFG-based diffusion NMR experiments but led to relatively minimal conformational change whereas highly favourable binding was observed with anionic SDS and LPPG micelles coupled to a significant conformational change and structuring in the C-terminal region of the apelin peptides (Langelaan and Rainey 2009; Shin...
et al. 2017b).

**Effects of apelin binding on lipid dynamics in isotropic bicelles.**

Using solution-state $^{31}$P NMR spectroscopy, the lipid constituents of both zwitterionic and mixed isotropic bicelles were monitored in the presence and absence of apelin-13 and apelin-17 (Fig. 2). In bicelles comprising only PC lipids (DMPC:DHPC), a single $^{31}$P peak consisting of multiple overlapping resonances was observed. In many instances, separate $^{31}$P resonances are observed for DMPC and DHPC (Glover et al. 2001) (as indeed is seen in our $q=3$ solid-state NMR experiments, vide infra), although this is not universally the case (Sanders and Schwonek 1992). In mixed PC-PG (DMPC:DMPG:DHPC) bicelles, two $^{31}$P resonances are observed: a deshielded peak at a relative area of 1:14 to a more shielded peak. The deshielded peak, thus, arises from the PG headgroup of DMPG while the more shielded peak corresponds to the DHPC and DMPC headgroups. The PC chemical shift is unchanged between zwitterionic and mixed bicelles. This behaviour is consistent with the relative resonances frequencies expected for bicelles (Crowell and Macdonald 1999) and, more generally, for a PG vs. PC headgroup (Cullis and De Kruyff 1976).

Neither of the apelin peptides affected the $^{31}$P NMR spectral behaviour of either type of bicelle. This is consistent with the 1:300 apelin:lipid stoichiometry employed, where even a strong binding interaction between apelin and a bicelle would only be able to affect a small proportion of the total lipid population. Furthermore, given that apelin-micelle binding for a variety of apelin isoforms led to chemical shift averaging in the fast exchange regime (considered on the NMR timescale (Arnold 1956; Keeler 2010)) rather than distinct populations of bound vs. unbound peptides in slow exchange (Langelaan and Rainey 2009; Shin et al. 2017b), apelin-bicelle interactions may also occur in the fast exchange regime. Detection of binding through headgroup $^{31}$P chemical shift perturbation would thus be
confounded both by a relatively small perturbed population at any given time and by the potential for fast exchange with the unperturbed state.

To better characterize effects of apelin upon bicelles, if any, $^{31}$P spin relaxation measurements were performed. The rates of longitudinal and transverse nuclear spin relaxation are characterized by first order decay time constants $T_1$ and $T_2$, respectively. These first order processes are influenced by mechanisms arising from dipole-dipole interactions and chemical shift anisotropy through rotational motion (e.g., as reflected in $\tau_c$, the rotational correlation time), local motion, and the surrounding environment of nuclear spins (Keeler 2010). Correspondingly, perturbation of global tumbling and the local environment around a subpopulation of spins upon apelin-bicelle binding may be anticipated to perturb $T_1$ and/or $T_2$. A change of global tumbling, or in $\tau_c$, is more likely to manifest in an observable perturbation, given that the same arguments as for minimal chemical shift perturbation would apply in terms of local environmental contributions to spin relaxation.

In examining the effects of both forms of apelin upon bicelles, the $T_1$ of the $^{31}$P nuclei is practically unperturbed in all cases (Fig. 3A). For DMPC:DHPC bicelles, where the observed $^{31}$P peak is a convolution of the DMPC (i.e., bilayer forming) and DHPC (i.e., bicelle rim-forming plus micellar and free species, if any) signals, in all cases the observed $T_1$ is ~1.07 s. In DMPC:DMPG:DHPC bicelles, the DMPG $^{31}$P resonance is distinct, and is reflective only of the bicelle state rather than of a lipid that is expected to be in fast exchange with states that have greater degrees of solvent exposure and/or mobility. Correspondingly, this exhibits a lower $T_1$ (~840-850 ms).

Contrasting with $T_1$, the effects of apelin upon $T_2$ are pronounced. Namely, $T_2$ is decreased for the DMPG resonance both with apelin-13 and, to a greater degree, with apelin-17 (Fig. 3B). The PC headgroup $^{31}$P nuclei exhibit an almost unperturbed $T_2$ with apelin-13 in PC bicelles, but a clear attenuation in both PC and PC-PG bicelles in the presence of apelin-
17 with the degree of attenuation scaled similarly in both cases (Fig. 3B).

Corresponding to the above-noted dependence of $T_1$ and $T_2$ upon rotational motion, the observed ratio of $T_1/T_2$ can be used to provide an estimate of $\tau_c$ (Navon and Lanir 1972), with polynomial expressions available to estimate $\tau_c$ for nuclei undergoing self-relaxation and proximal to $^1$H nuclei (Carper and Keller 1997). Using $T_1$ and $T_2$ of the $^{31}$P resonance of the DMPG headgroup, a clear trend of slower tumbling is observable as a function of apelin peptide addition (Table 1). For mixed bicelles, the $T_1$ and $T_2$ observed for the convoluted DMPC and DHPC resonances provide $\tau_c$ estimates smaller than the corresponding DMPG-based values (Table 1), as would be anticipated from a linear combination of DHPC in monomeric, micellar, and bicellar forms. Considering $\tau_c$ estimates based upon the convoluted PC resonances, the same trend of attenuated tumbling upon apelin incorporation is generally observed for both PC and mixed bicelles with one exception (Table 1). Namely, the estimated $\tau_c$ for PC bicelles in the presence of apelin-13 is effectively unchanged in the presence of apelin-13. In all instances, however, apelin-17 accentuates the decrease in bicellar tumbling relative to apelin-13. These findings will be revisited in the context of translational diffusion measurements (vide infra).

**Effects of apelin binding on lipid dynamics in magnetically-oriented bicelles.**

To characterize the interactions of apelin with bicelles having a substantial planar bilayer surface relative to $q=0.5$ isotropic bicelles, $^{31}$P solid-state NMR spectroscopy was employed. Namely, PC bicelles with $q=3$ were employed. At this stoichiometry of long-chain (DMPC) to short-chain (DHPC) lipids at ~160 mg/mL, 150 mM NaCl, and 37 °C, lamellar structures are expected (Nieh et al. 2002). The DMPC is expected to form a bilayer with normal perpendicular to the static magnetic field ($B_0$) while the DHPC lines the rim of holes in lamellar DMPC sheets (e.g., a “Swiss cheese-like” morphology (Prosser et al. 1998)).
Separate resonances are expected for DHPC (~isotropic $^{31}$P resonance from a PC headgroup) and DMPC (PC headgroup $^{31}$P resonance for a bilayer with normal oriented perpendicular to the static magnetic field) (Sanders and Schwonek 1992). This is indeed observed for our $q=3$ bicelle preparation (Fig. 4), fully consistent with formation of well-oriented bicelles (Picard et al. 1999).

Magnetically-aligned $q=3$ PC bicelles in the presence of apelin-13 exhibit practically indistinguishable $^{31}$P NMR spectral characteristics (Fig. 4). This is in contrast to membrane-perturbing peptides, where $^{31}$P spectra are visibly modulated as a function of peptide addition (e.g., (Marcotte et al. 2006)). To test for longer-term sample stability and peptide-induced perturbation, $^{31}$P spectra were also acquired for samples incubated for 9 and 12 days without any perturbation to resonance positions or linewidths (Fig. S2 in the Supplementary Material). This is consistent with the fast-exchange bicelle-apelin binding implied in solution-state NMR studies of isotropic bicelles.

Measurement of $^{31}$P $T_1$ and $T_2$ relaxation time constants was performed for the DHPC and DMPC resonances (Fig. 5). Interestingly, the DMPC resonance exhibited a distinct increase in $T_1$ in the presence of apelin-13, while that for DHPC is slightly decreased. $T_2$, conversely, decreases in the presence of apelin-13 for both DMPC and DHPC. This is consistent with a situation where apelin-13 is affecting dynamics of the PC headgroup of the DMPC bilayer phase in a more pronounced manner than of the rim/micellar phase DHPC. That this is not also reflected in chemical shift perturbation (Fig. 4 and Fig. S2 in the Supplementary Material) is consistent with apelin-13-DMPC interactions on a relatively transient basis that decrease lipid translation without tight and long-lived binding leading to significant changes in the observed chemical shift.
Effects of apelin upon bicelle diffusion.

Using PFG-based diffusion NMR spectroscopy, bicelle hydrodynamics with and without apelins were investigated. Specifically, $D_C$ values of PC and PC-PG isotropic ($q=0.5$) bicelles were determined in the presence and absence of apelin-13 and -17. $^1$H DOSY experiments were employed to distinguish between DMPC and DHPC species through the distinct methyl resonances of DMPC vs. DHPC ((Andersson and Maler 2005; Lu et al. 2012); Fig. S3 in the Supplementary Material), with $^{31}$P DOSY experiments employed to distinguish between the PC and PG components (e.g., Fig. 2B). These values are reported with an estimated solution viscosity corresponding to 90% H$_2$O/10% D$_2$O (Fig. 6; representative data fitting illustrated in Figs. S4-S5 in the Supplementary Material). It should be noted that apelin-13 and -17 in buffer at 37 °C exhibit $D_C$ values of 3.49 ($\pm$ 0.07) $\times 10^{-10}$ m$^2$/s and 3.37 ($\pm$ 0.03) $\times 10^{-10}$ m$^2$/s, respectively (Fig. S6 in the Supplementary Material); hence, the observed bicelle diffusion is much slower than that of either apelin isoform in its free state. As would be anticipated, the $D_C$ values coming from $^1$H and $^{31}$P DOSY for overlapped DHPC+DMPC resonances are highly consistent for any given bicelle condition (Fig. 6C-D). Correspondingly, the bilayer phase DMPC ($^1$H DOSY; Fig. 6B) and DMPG ($^{31}$P DOSY; Fig. 6D) $D_C$ values are also highly consistent with each other for any given PC-PG bicellar condition. It should be noted, however, that the PG $^{31}$P DOSY fits exhibit a greater degree of data scatter than any of the other fits (Fig. S5 in the Supplementary Material) based upon the relatively low intensity of the resonance (Fig. 2B); these $D_C$ estimates are, thus, less reliable despite good general agreement with the DMPC $D_C$ values. Although the $D_C$ values observed are similar to some previous studies (e.g., range of reported values reviewed in (Liebau et al. 2017)), these should not be considered absolute $D_C$ values. Instead, we compare values observed for a single type of bicelle, with the rationale for doing so detailed below.

At the employed lipid concentration of 150 mM, the volume fraction of lipids would
be estimated to be ~8% (Andersson and Maler 2006). This, in turn, should have a minimal effect upon $D_C$ strictly upon the basis of solute crowding (Andersson and Maler 2006; Jönsson et al. 1986) in terms of the timescales for translational diffusion of uniformly structured bicelles. As a probe viscosity, the $D_C$ of the chemical shift standard DSS was monitored, with the intention of employing this as an internal standard for solution viscosity. However, it was apparent that DSS bound to some degree to the bicellar species, as the observed reduction in its $D_C$ (Fig. S7 in the Supplementary Material) was too extreme to be simply caused by a solution viscosity increase noted in previous studies of bicelles (comprehensively reviewed in (Liebau et al. 2017)). This actually prompted us to examine DSS behaviour in simpler micellar environments, with the finding that DSS favourably binds to zwitterionic DPC and anionic SDS micelles (detailed in (Morash et al. 2018)).

The $D_C$ observed for DSS in the presence of each class of bicelle was similar within experimental error (Fig. S7 in the Supplementary Material). Assuming a lack of apelin-DSS interaction (e.g., no evidence of such interaction was observed in any of our solution-state NMR studies of various apelin isoforms in buffer (Langelaan et al. 2009; Shin et al. 2017a)), the $D_C$ observed for DSS may be inferred to be a population-weighted average of free and bicelle-bound species. Assuming, in turn, that this partitioning varies primarily as a function of headgroup (Morash et al. 2018), each class of bicelle exhibits similar hydrodynamics when taking into account DSS partitioning between solution and bicelle. Comparison of $D_C$ values obtained for each bicelle type and each lipid species are thus strictly compared between bicelles of a given type with and without an apelin species but otherwise identical in conditions to avoid issues in overestimate of $D_C$ that may arise due to elevated viscosity.

Comparing across a given class of bicelle, some clear trends become apparent. In the case of PC bicelles, $D_C$ is uniformly attenuated in the presence of apelin-13, whether considering DHPC, DMPC, or DHPC+DMPC species. Converse to this, the $D_C$ observed for
all PC species is uniformly elevated in the presence of apelin-17. In contrast to this, for PC-
PG bicelles, the general trend is an observed increase in $D_C$ to a less pronounced manner than
noted for PC bicelles for all lipid species except DMPG in the presence of apelin-17
(~unchanged within error, with the caveat as noted above that the PG DOSY fits are less
reliable).

These trends are consistent with a case where apelin-13 interactions with PC bicelles
lead to an observable population of bicelle-peptide complex that is slightly increased in size.
Conversely, in all other instances, apelin-bicelle interactions are leading to an apparent
increase in phospholipid translational diffusion. This may be due to a bicellar rearrangement,
perhaps including a change in the partitioning of DHPC between a micellar state and the
bicelle rim upon peptide interaction. As a whole, the observed changes in $D_C$ are relatively
small, indicative of surface interactions of apelin peptides with bicelles rather than a
penetration into the hydrophobic core, regardless of the composition of the bicelle. This, in
turn, is consistent with our prior studies of apelin-micelle interactions, where peptides were
observed to associate with micelles (Langelaan and Rainey 2009; Shin et al. 2017b), but
without any significant protection from solvent indicative of penetration into the micellar
core (Langelaan and Rainey 2009).

Conclusions

Considering the spin relaxation behaviour in light of the hydrodynamic behaviour, the
observed $D_C$ values (Fig. 6) are not consistent with a situation where $^{31}$P nuclear spin
relaxation (Fig. 3) is being modulated strictly by a change in $\tau_C$ (e.g., as assumed in Table 1).
Were apelin-bicelle binding indeed leading to pronounced increase in $\tau_C$ for the lipid species
(i.e., a peptide-bicelle complex that tumbles more slowly than the free bicelle), $D_C$ would be
expected to decrease. With the exception of apelin-13-PC bicelle complexes, this was not the
situation. Instead, it appears that apelin-bicelle interactions lead to an observable modulation of spin relaxation primarily through effects upon the local environment around the phospholipid headgroup and/or perturbation to the bilayer-phase motional freedom of the lipid upon bicelle binding without a concomitant overall decrease in translational diffusion. This perturbation is particularly pronounced for the PG headgroup, implying a preferential interaction of apelin with the PG species within mixed PC-PG bicelles. An electrostatic apelin-PG interaction would be favourable given that both apelin isoforms are highly cationic and is consistent with our prior observation of a higher binding propensity of apelin isoforms to anionic than to zwitterionic micelles (Langelaan and Rainey 2009; Shin et al. 2017b). As a whole, apelin-bicelle interactions are manifest through relatively subtle perturbations to the bicelle. Such interactions would be beneficial to allow for membrane-catalyzed receptor binding (Sargent and Schwyzer 1986) since this would allow for a rapid 2D diffusional search upon membrane interaction, unlike a tight binding of or bilayer penetration by the peptide. Examining the corresponding effects of bicellar interaction upon apelin through, e.g., \(^{15}\)N-labelling of apelin (Shin et al. 2017b) and/or perdeuteration of lipid species (e.g., (Whiles et al. 2001)) to facilitate NMR spectroscopic observation of the relatively dilute apelin species in the presence of high lipid concentration would be beneficial for future study. Any such potentially experimentally confounding effects arising from apelin-lipid interactions must be considered as part of the overall context for in-depth characterization of the likely highly dynamic processes of apelin-receptor recognition, binding, and activation.

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References


Table

Table 1. Ratio of $T_1/T_2$ determined for $^{31}$P signal from indicated headgroup type in either DMPC:DHPC (1:2; labelled PC) or DMPC:DMPG:DHPC (4:1:10; labelled PC-PG) ($q=0.5$) under indicated conditions and resulting inferred (Carper and Keller 1997) rotational correlation time ($\tau_c$) on the basis of a $^{31}$P nucleus undergoing both self-relaxation and proximal to $^1$H nuclei.

<table>
<thead>
<tr>
<th>Condition</th>
<th>PC bicelles</th>
<th>PC-PG bicelles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_1/T_2$ (PCs)</td>
<td>$\tau_c$ (ns)</td>
</tr>
<tr>
<td>Bicelle only</td>
<td>6.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Bicelle + apelin-13</td>
<td>5.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Bicelle + apelin-17</td>
<td>10.5</td>
<td>3.4</td>
</tr>
</tbody>
</table>
Figure Captions

**Fig. 1.** Amide and aromatic $^1$H regions of 1D NMR spectra of (A) apelin-13 (Ap13) and (B) apelin-17 (Ap17) in isotropic bicelles composed of DMPC:DHPC (1:2; labelled PC) or DMPC:DMPG:DHPC (4:1:10; labelled PC-PG) relative to in water. The corresponding full 1D $^1$H NMR spectra are provided in Fig. S1 in the Supplementary Material.

**Fig. 2.** 1D $^{31}$P NMR spectra of q=0.5 (A) DPMC:DHPC (PC) and (B) DMPC:DMPG:DHPC (4:1:10; PC-PG) bicelles in the absence or presence of apelin-13 (Ap13) or -17 (Ap17) acquired at 37 °C. Resonances arising from PC vs. PG headgroup $^{31}$P nuclei are annotated.

**Fig. 3.** $^{31}$P (A) $T_1$ (longitudinal) and (B) $T_2$ (transverse) relaxation times of q=0.5 DHPC:DMPC (PC) or DMPC:DMPG:DHPC (4:1:10; PC-PG) bicelles in absence or presence of apelin-13 (Ap13) or -17 (Ap17) recorded at 11.7 T and 37 °C.

**Fig. 4.** 1D $^{31}$P solid-state NMR spectra of magnetically-oriented q=3 DPMC:DHPC (PC) bicelles in absence or presence of apelin-13 (Ap13) at 16.4 T.

**Fig. 5.** $^{31}$P (A) $T_1$ (longitudinal) and $T_2$ (transverse) relaxation times determined for magnetically-oriented DMPC:DHPC bicelles in the absence or presence of apelin-13 (Ap13) at 16.4 T and 37 °C.

**Fig. 6.** Translational diffusion coefficient ($D_C$) estimated for indicated lipid type(s) in isotropic (q=0.5) bicelle samples composed of DMPC:DHPC (PC) or DMPC:DMPC:DHPC (4:1:10; PC-PG) in the absence or presence of apelin-13 (Ap13) or -17 (Ap17) determined using (A-C) $^1$H or (D) $^{31}$P pulsed-field gradient DOSY at 37 °C. In (D), it should be noted that $^{31}$P PG signal decay fits show much more data scatter (Fig. S5 in the Supplementary Material).
Fig. 1

182x72mm (300 x 300 DPI)
Fig. 2

PC Bicelle + Ap17
PC Bicelle + Ap13
PC Bicelle

PC-PG Bicelle + Ap17
PC-PG Bicelle + Ap13
PC-PG Bicelle

182x72mm (300 x 300 DPI)
Fig. 3

86x86mm (300 x 300 DPI)
Fig. 5

86x29mm (300 x 300 DPI)
Fig. 6

182x117mm (300 x 300 DPI)