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ARTICLE

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Molecular dynamics of lipid association at the hydrophobic interface of gramicidin S

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Abstract Gramicidin S was incorporated into dimyristoylphosphatidylcholine dispersions and the observed two-component EPR spectra of spin-labelled lipids at 30 °C were analysed by a two-stage algorithm, including spectral subtractions and two-site exchange simulations. A limited range of temperatures around 30 °C was found suitable for such measurements. It has been found that negatively charged labelled lipids display a selectivity towards the intramembranous part of the peptide. The relative association constants for spin-labelled stearic acid (14-SASL) and phosphatidylserine (14-PSSL) were $K_r = 2.08 \pm 0.10$ and 1.18 ± 0.08, respectively, when compared with the zwitterionic phosphatidylcholine label (14-PCSL, $K_r \equiv 1$). The lateral diffusion of spin-labelled lipids in peptide-free regions causes exchange between those labels in the bulk fluid lipid phase and motionally restricted boundary labelled lipids at the apolar interface of gramicidin S. Owing to exchange, the spectral anisotropy of labelled lipids giving rise to the slow-motion spectral component was gradually decreased, and there was an augmentation of spectral intensity in between the motionally restricted (slow motion) and the fast tumbling (motionally averaged) labelled lipid components. Two-component exchange simulations allowed the determination of off-rates of labelled phospholipids, showing an inverse proportionality with lipid-protein selectivity. Spin-labelled procaine exhibited limited selectivity and fast exchange similar to the on-coming non-specifically associated lipids.

Key words Gramicidin S · Molecular dynamics · Lipid association · Hydrophobic interface · Spin labelling electron paramagnetic resonance (EPR)

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Abbreviations *DMPC* dimiristoylphosphatidylcholine · *14-SASL* 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; spin-labelled stearic acid · *14-PCSL* 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine; spin-labelled phosphatidylcholine · *14-PSSL* 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoserine; spin-labelled phosphatidylserine · *PSL* procaine spin label · *GS* gramicidin S

Introduction

Gramicidin S (GS) is an antibiotic cyclodecapeptide, cyclo(Val-Orn-Leu-^{D}Phe-Pro)$_2$, isolated from *Bacillus brevis*, but unlike other related antibiotics (e.g. valinomycin, eniantin) GS does not possess an internal cavity capable of shielding metal cations. It has been suggested that the most significant role of GS is the formation of β-pleated sheets (Graciani et al. 1994; Katayama et al. 1994). The Orn residues are certainly involved in the formation of amino group/lipid complexes since electrostatic interaction between the δ-amino groups of Orn residues and negatively charged parts of the membrane can modulate the gating mechanism of ionic channels in plasma membranes (Tamaki et al. 1996; Bendahhou et al. 1997; Benitah et al. 1997).

Non-covalent peptide-lipid associations in the membrane result in motional coupling between fluid lipids and peptides owing to frictional forces arising between the colliding molecules of different mobilities, leading to the formation of at least one shell of motionally restricted lipids at the hydrophobic surface of peptides (Saffman and Delbrück 1975; Cornell et al. 1978). Amongst the available spectroscopic methods, spin-label EPR spectroscopy has an optimal timescale to resolve such motionally restricted boundary lipids from fluid lipid molecules which undergo rapid unrestricted motion in membrane phases devoid of peptide molecules (Jost et al. 1973; Devaux and Seignereut 1985; Marsh 1985).

The central aim of the present paper is the description of molecular dynamics of lipids and a representative of a

local anaesthetic, namely procaine, at the polar interface of GS using spin-label ESR. The enhanced selectivity of GS towards negatively charged lipids and a local anaesthetic is characterised by the membrane thermodynamics.

Materials and methods

Dimyristoylphosphatidylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland) and buffer solutions of 0.2 M Tris.HCl (pH 6, 7 and 9 adjusted with HCl) were used. Gramicidin S was obtained from Sigma (Munich, Germany). A spin-labelled positional isomer of stearic acid (14-SASL) was used for the preparation of spin-labelled phospholipids (1-acyl-2-spin-labelled stearic acid resulting from 14-PCSL and a small amount of lysoPC). 14-PGSL and 14-PSSL were obtained by phospholipase D-mediated head group exchange of 14-PCSL in the presence of glycerol and L-serine, respectively (Marsh and Watts 1982). Spin-labelled procaine (PSL) was prepared as described previously (Mihailescu et al. 1993).

For spin labelling, 0.5 mg of DMPC was suspended in 0.5 ml buffer and 10 µl of 1 mg/ml spin-label solution in ethanol was added while mixing the sample with a vortex mixer (Marsh 1982, 1989). The sample was incubated at room temperature for 60 min and repeatedly mixed with a vortex mixer. The spin-labelled samples were then filled into 1 mm i.d. EPR sample capillaries and pelleted in a bench top centrifuge.

EPR spectra were recorded using a Bruker ECS 106 (9 GHz) spectrometer with rectangular TE_{102} cavity and equipped with a computer-controlled nitrogen gas-flow thermostat (ER 411VT). Typical spectrometer settings: microwave power, 10 mW; scan range, 10 mT; modulation amplitude, 0.12 mT; gain, 2×10^5; A/D resolution, 16 bit. Spectral subtractions were done using the software provided by ECS 106 and spectral files were transferred to an IBM compatible environment by the Bruker software Doswrite. Further spectral analyses, including subtractions and two-component spectral simulations, and plotting were performed on IBM machines using software written by one of us (Horváth et al. 1988; Horváth 1996).

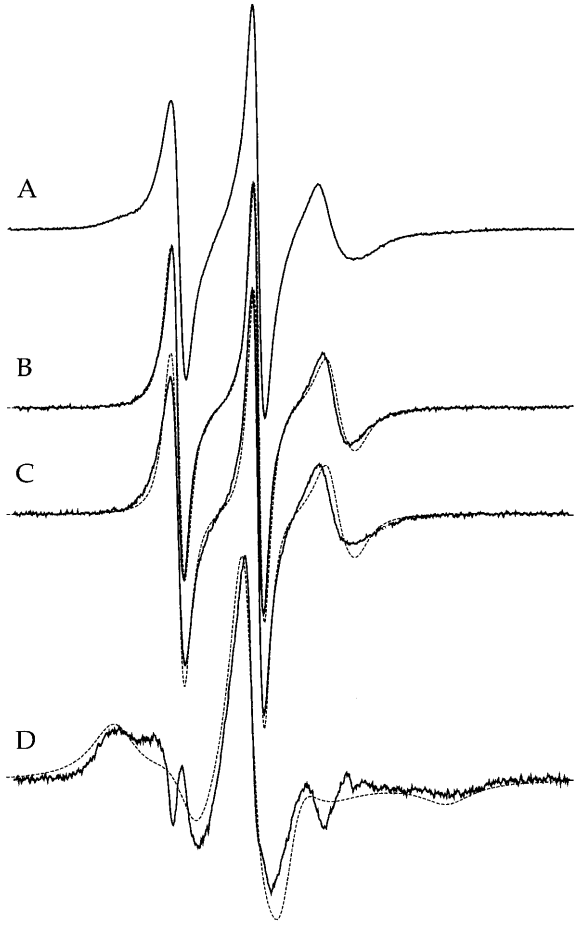

Fig. 1 EPR spectral simulations of the two components of spin-labelled phosphatidylcholine (14-PCSL) in DMPC/gramicidin S complexes. *A* Two-component EPR spectrum of the spin label in DMPC/GS complexes of 7 mol/mol; *B* single component. EPR spectrum of 14-PCSL in DMPC at 30 °C (*solid line*) shown together with the best-fitting single component simulated EPR spectrum for DMPC (*dashed line*); *C* single component EPR spectrum of 14-PCSL in DMPC at 25 °C (*solid line*) shown together with the best-fitting single component simulated EPR spectrum for DMPC at 30 °C (*dashed line*); *D* lineshape of the boundary component subtracting the spectrum of 14-PCSL in DMPC at 25 °C from the two-component EPR lineshape recorded at 30 °C (*solid line*) shown together with the best-fitting simulated slow motion lineshape (*dashed line*). Total scan width = 10 mT

Results

The EPR spectrum of spin-labelled phosphatidylcholine (14-PCSL) in DMPC/gramicidin S complexes consists of two spectral components, indicating lipid domains devoid of peptide molecules and motionally restricted lipid molecules at the hydrophobic interface of incorporated peptides (Fig. 1, trace A). The presence of these two components can be illustrated by subtracting the spectrum of 14-PCSL in pure DMPC vesicles recorded at 30 °C and 25 °C (Fig. 1, traces B and C), giving an endpoint lineshape of slow motion character (Fig. 1, trace D). This is similar to data published for several membrane proteins and other intramembranous peptides (Jost and Griffiths 1976; Marsh and Watts 1982; Devaux and Seignereut 1985; Marsh 1985). It should be emphasised that the lineshape of the fluid component due to lipid domains free of peptides is not the same as the same label in pure DMPC vesicles recorded at the same temperature. The observed differences can be accounted for by subtracting spectra recorded at lower temperatures by ΔT = 4–5 °C, i.e. having greater linewidths (Fig. 1, traces B and C). However, this temperature correction was a first indication of exchange-induced line broadening.

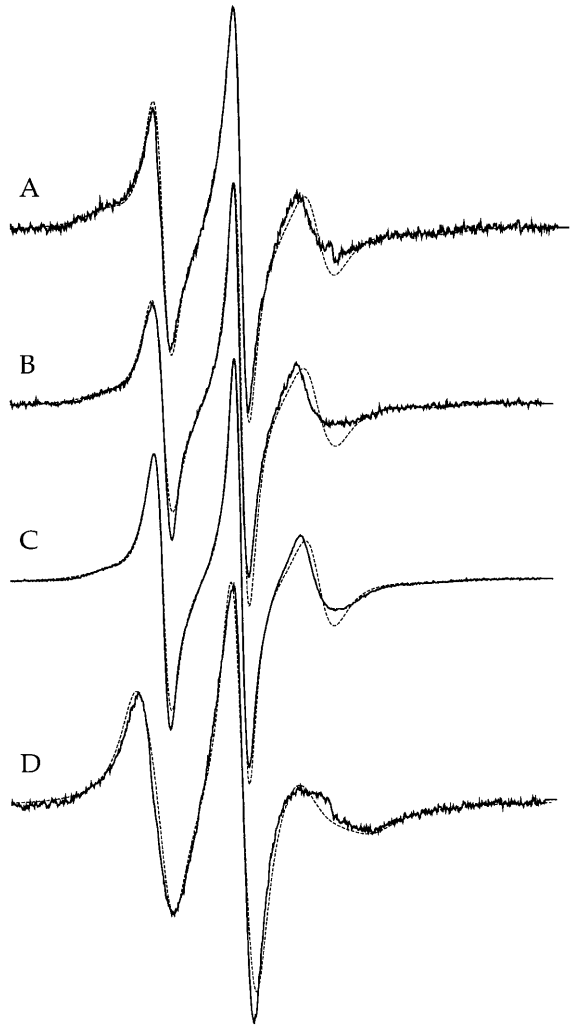

Fig. 2 Two-component simulated EPR spectra of spin-labelled phospholipids and procaine in DMPC/gramicidin S complexes of 7 mol/mol. *A* Spin-labelled stearic acid (14-SASL, with a fitting error of ε^2 = 1.9%); *B* spin-labelled phosphatidylserine (14-PSSL, ε^2=2.9%); *C* spin-labelled phosphatidylcholine (14-PCSL, ε^2= 1.1%); *D* spin-labelled procaine (PSL, ε^2 = 2.8%). Total scan width = 10 mT

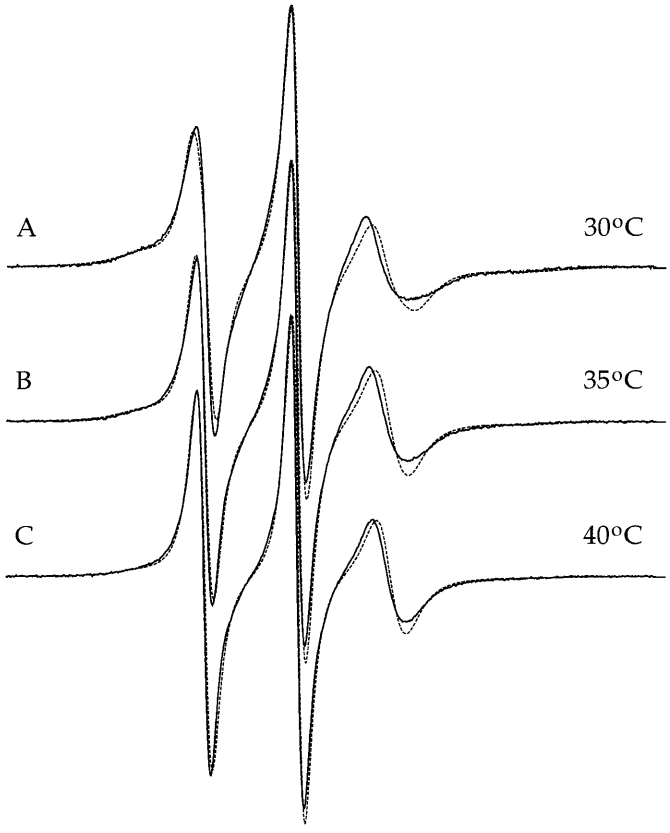

Fig. 3 EPR spectra of spin-labelled phosphatidylserine (14-PSSL) in dispersions of DMPC/gramicidin S complexes (7 mol/mol) recorded at various temperatures (*solid line*) shown together with best-fitting two-component simulated EPR spectra (*dashed line*): *A* at 30 °C (with a fitting error of ε^2 = 1.03%); *B* at 35 °C (ε^2 = 1.02%); and *C* at 40 °C (ε^2 = 0.87%). Total scan width = 10 mT

A series of spin-labelled phospholipids, namely phosphatidylcholine (14-PCSL), phosphatidylserine (14-PSSL) and stearic acid (14-SASL), were incorporated into DMPC/GS complexes of 7 mol/mol ratios and in every case a two-component lineshape was observed (Fig. 2). In each case the spectrum of 14-PCSL was used as a reference for host lipids (DMPC). The integrated intensities of the motionally restricted boundary and the fluid lipid component were different in these cases. The negatively charged 14-SASL, as in other cases (Marsh 1995), was incorporated to a greater extent into the boundary layer as judged by integrated intensities, while the zwitterionic 14-PCSL was observed mostly in DMPC domains devoid of peptide; the 14-PSSL was in between these two cases. The specificity of gramicidin S towards these phospholipids was measured by the specific binding constant (K_r), comparing the ratio of the integrated intensities of the motionally restricted and fluid components with respect to the non-specific phosphatidylcholine (Marsh 1985). For 14-SASL and 14-PSSL labels, association constants of K_r(SASL) = 2.08 ± 0.10 and K_r(PSSL) = 1.18 ± 0.08 were obtained, respectively.

A rather similar pattern was observed in the case of spin-labelled procaine (Mihailescu et al. 1993; PSL): labelled anaesthetic molecules were either at one of the peptide/lipid interfacial sites, and consequently motionally restricted, or underwent rapid anisotropic motion together with non-associated lipids, and, in such cases, two spectral components could be observed. However, the fluid component was not isotropic and a small anisotropy ($2A_{max}$ = 2.0 mT) was obained, indicating slight inclination of the nitroxyl-carrying piperidinyl ring with respect to the coordinate axes of the doxyl groups of the lipid labels (Fig. 2). The relative binding constant in the case of PSL at the interface of GS was close to 14-PSSL: K_r(PSL) = 1.28 ± 0.12.

On increasing the amount of fluid lipids, i.e. on increasing the lipid/peptide ratio from 7 to 20 mol/mol, the spectral intensity of the motionally restricted component was linearly decreased. A preliminary titration gave an approximate intercept of ~6 mol/mol in DMPC/GS complexes (data not shown; Brotherus et al. 1980, 1981; Marsh 1985).

From two-component lineshape simulations, the exchange rate between motionally restricted interfacial and bulk fluid lipid sites can be determined (Davoust and Devaux 1982; Horváth et al. 1988; Marsh and Horváth 1989). Optimised simulated lineshapes are shown together with experimental spectra in Fig. 2. It should be noted that two-site exchange leads to line broadening of the fluid lipid component; this spectral change was taken into account in spectral subtractions by selecting pure lipid lineshapes recorded at lower temperature by 4–5 °C. It should be noted that line broadenings due to exchange and temperature were not identical (Fig. 2). In the above cases the following off-rates were found: 7.0×10^6 s^{-1} for 14-SASL, 1.2×10^7 s^{-1} for 14-PSSL, 1.3×10^7 s^{-1} for 14-PCSL and 1.15×10^6 s^{-1} for PSL. Clearly, motionally restricted lipids at the hydrophobic interface, which are associated specifically, are exchanged less frequently, similarly to lipid/protein interaction between charged phospholipids and the myelin proteolipid protein (Horváth et al. 1988; Marsh and Horváth 1989; Marsh 1995).

As the temperature range was extended to 40 °C, two-component EPR spectra were observed (Fig. 3) throughout. The fraction of the intensities of the two spectral components changed slightly from $f = 0.47$ to 0.43 as the temperature was increased from 30 °C to 35 °C (Fig. 3, traces A and B) and then decreased markedly to a value of $f = 0.30$ (Fig. 3, trace D) at 40 °C.

Discussion

On incorporating gramicidin S into DMPC vesicles, a second spectral component in the EPR spectra of spin-labelled lipids has been observed similar to all the membrane proteins and other intramembranous peptides studied (Marsh 1985, 1995; Devaux and Seignereut 1985). The two spectral components were assigned to motionally restricted lipids in the boundary layer at the apolar surface of the peptide/lipid interface and to fluid lipids in peptide-free domains. Such two-component EPR spectra in the case of DMPC/GS complexes, although with significantly decreasing fractions of the boundary component, were observed in a temperature region of 30–40 °C (Fig. 3). As an explanation of the decreasing fractional intensity of the boundary component, it has to be noted that boundary lipids in the first layer of the fast moving GS will undergo rapid averaging in the temperature range of $T \geq 40$ °C owing to the small molecular weight of GS. However, it should be noted that the intensity of the narrow isotropic component, assigned to pure fluid lipid domains devoid of peptides, increased significantly on the incorporation of gramicidin S without any change of temperature (Fig. 2). This change in linewidth was allowed for in subsequent spectral subtractions by lowering the temperature where pure lipid reference spectra were recorded, typically by $\Delta T = 4$–5 °C, with respect to the temperature at which the DMPC/GS complexes were recorded. Such a lowering of the temperature is known to increase the linewidths of EPR spectra of spin-labelled lipids in the fluid phase to a similar extent as the incorporation of GS.

The observed line broadening is primarily due to lipid exchange between boundary and fluid lipid sites (Horváth et al. 1988; Horváth 1996). Further consequences of exchange are the observed changes in the anisotropy of spin-labelled lipids in the boundary layer and the build-up of EPR intensity between the two components owing to slow exchange which overlaps with new boundary components (Horváth et al. 1988).

With all these alterations in the EPR spectra of spin-labelled lipids on incorporating intramembranous peptides in mind, the following two-stage algorithm including spectral subtraction and lineshape simulation strategy was used. In the first stage, the fraction of the intensities of the two spectral components was determined by subtraction using a temperature correction for the fluid component. It should be noted, however, that even with appropriate linewidth corrections the subtraction endpoint of the so-called boundary component is strongly influenced by line broadening and decreasing anisotropy due to exchange (Fig. 2). In the second stage of the lineshape analyses, the off-rates were determined by two-component exchange simulations by fitting the fluid component to pure DMPC recorded at the same temperature, i.e. no temperature correction was applied. In order to reduce computational requirements, only the above two spectral components, namely boundary and fluid lipid components, were taken into account. Consequently, any new components of low intensities predicted by the multisite model were neglected (Ge and Freed 1993). It should be noted that these new components have different spectral anisotropies corresponding to other orientations of the nitroxyl group at the hydrophobic surface (Horváth et al. 1988) and site-specific off-rate profiles. The deviation between the experimental spectrum and simulated lineshapes were measured by the error function as given by

$$\varepsilon^2 = \Sigma_i (y_{\mathrm{sim},i} - y_i)^2 / y_i^2 \quad (1)$$

which was minimised by least-squares optimisation (Horváth et al. 1988; Horváth 1996). Using this two-stage algorithm, best fits could be found according to objective criteria within reduced computational time.

Different spin-labelled lipids are associated with the hydrophobic surface of gramicidin S to different extents. Their relative binding constant is defined as (Marsh 1985)

$$K_\mathrm{r} = [\mathrm{L}]/[\mathrm{PL}] \times [\mathrm{PL}^*]/[\mathrm{L}^*] \quad (2)$$

where the concentrations of labelled (L*) and unlabelled (L) lipids competing for boundary sites (PL and PL*) are given as mole fractions of lipids. If the amount of labelled lipids is <1%, the specificity of lipids can be determined from relative fractions of labelled lipids in fluid and boundary interfacial sites. In the case of DMPC/GS complexes, a trend towards negatively charged lipids was found: $K_\mathrm{r} = 2.08 \pm 0.10$ for 14-SASL and $K_\mathrm{r} = 1.18 \pm 0.08$ for 14-PSSL compared to the zwitterionic 14-PCSL.

Lateral diffusion of lipid molecules leads to exchange between bulk fluid lipids and motionally restricted boun-

dary lipids at the apolar interface of transmembranous peptides. The rate of molecular transfer, namely the onrate constant $1/\tau_{on}$ and off-rate constant $1/\tau_{off}$ of lipids, is determined by the respective populations of these two environments. At equilibrium there is no net transfer (Marsh 1985)

$$(1/\tau_{off})/(1/\tau_{on}) = f/(1-f) \quad (3)$$

where f denotes the fraction of motionally restricted lipids. The combination of mass balance and equilibrium association gives inverse proportionality between off-rate and relative association constant data (Marsh 1985; Brotherus et al. 1980, 1981)

$$(1/\tau_{off})/(1/\tau_{on}) = (n_t/N_b - 1)/K_r \quad (4)$$

where n_t and N_b denote the lipid/protein ratio and the total amount lipids at motionally restricted sites, respectively. In agreement with Eq. (4), the off-rate constants of selectively spin-labelled charged lipids, namely stearic acid (14-SASL) and phosphatylserine (14-PSSL), are significantly slower than the exchange rate for non-selective phosphatidylcholine (14-PCSL; $K_r \equiv 1$), and a reciprocal proportionality was found between the relative binding constant K_r and the off-rate constant $1/\tau_{off}$ (Fig. 4). The non-selective zwitterionic phosphatidylcholine, 14-PCSL, exhibited a significantly faster off-rate.

Owing to exchange-induced broadening and the use of unoriented samples (macroscopically disordered; Ge and Freed 1993), the linewidths of conventional EPR spectra were broadened, thereby reducing the spectral resolution. The EPR spectral simulation was based on the line broadening due to fluid-to-boundary exchange (Horváth et al. 1988); other line broadening mechanisms were not treated in the present simulation programme. The initial linewidth was adjusted with pure DMPC spectra (~0.1 mT in the low field region, Fig. 1B), i.e. free of gramicidin S, and the broadening due to lipid exchange (~0.01–0.02 mT) was treated as described (Horváth et al. 1988). The contributions of the various line broadening mechanisms can quantitatively be studied by special pulse sequences in conjunction with two-dimensional Fourier-transform EPR (Patyal et al. 1997).

Spin-labelled procaine has an identical efficiency on nerve fibre as the normal procaine (Mihailescu et al. 1993) and so the details of the interactions of PSL could directly be extrapolated to the behaviour of an ordinary local anaesthetic. Regarding spin-labelled procaine, it has been found that there has been only a slight specificity of procaine towards gramicidin S.

In conclusion, two-component EPR spectra were observed on intercalating spin-labelled lipids into DMPC/gramicidin S complexes of 7–20 mol/mol. The presence of motionally restricted boundary labelled lipids was confined to a limited range of temperature because of the similar motional rate of lipids and peptides. The selectivity of negatively charged lipids, like stearic acid and phosphatidylserine, was correlated with the off-rates of these lipids at the apolar interface of transmembrane peptides. From a two-stage analysis, including spectral subtractions and exchange simulations, an inverse proportionality has been observed between the fast off-rates and the relative association constants.

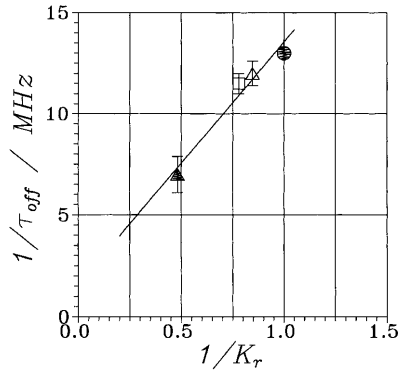

Fig. 4 Correlation diagram between the reciprocal of off-rate constants, $1/\tau_{off}$, and the reciprocal of the relative association constants, $1/K_r$, for different spin-labelled lipids in DMPC/GS complexes of 7 mol/mol using temperature corrected spectral subtractions and two-component exchange simulations. ▲, spin-labelled stearic acid (14-SASL); △, spin-labelled phosphatidylserine (14-PSSL); ●, spin-labelled phosphatidylcholine (14-PCSL); □, spin-labelled procaine (PSL)

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