Lactadherin binds to phosphatidylserine-containing vesicles in a two-step mechanism sensitive to vesicle size and composition ☆

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ABSTRACT

Lactadherin binds to phosphatidylserine (PS) in a stereospecific and calcium independent manner that is promoted by vesicle curvature. Because membrane binding of lactadherin is supported by a PS content of as little as 0.5%, lactadherin is a useful marker for cell stress where limited PS is exposed, as well as for apoptosis where PS freely traverses the plasma membrane. To gain further insight into the membrane-binding mechanism, we have utilized intrinsic lactadherin fluorescence. Our results indicate that intrinsic fluorescence increases and is blue-shifted upon membrane binding. Stopped-flow kinetic experiments confirm the specificity for PS and that the C2 domain contains a PS recognition motif. The stopped-flow kinetic data are consistent with a two-step binding mechanism, in which initial binding is followed by a slower step that involves either a conformational change or an altered degree of membrane insertion. Binding is detected at concentrations down to 0.03% PS and the capacity of binding reaches saturation around 1% PS (midpoint 0.15% PS). Higher concentrations of PS (and also to some extent PE) increase the association kinetics and the affinity. Increasing vesicle curvature promotes association. Remarkably, replacement of vesicles with micelles destroys the specificity for PS lipids. We conclude that the vesicular environment provides optimal conditions for presentation and recognition of PS by lactadherin in a simple binding mechanism. This article is part of a Special Issue entitled: Protein Folding in Membranes.

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1. Introduction

The 409-residue protein lactadherin (also known MFG-E8) binds the lipid phosphatidylserine (PS) in a stereospecific and calcium-independent manner [1]. Lactadherin was initially identified by its association with bovine milk fat globules, appearing as two glycosylation variants of 47 and 52 kDa, respectively [2]. Besides mammary epithelial cells [3], lactadherin is also secreted by epididymal epithelial cells [4], vascular cells [5], stimulated macrophages [6] and stimulated endometrium cells [7]. Bovine lactadherin comprises two N-terminal epidermal growth factor homology domains (EGF1 and EGF2), and the C-terminal region consists of two C domains (C1 and C2), which share homology with the lipid-binding "C" domains of blood coagulation factor VIII and factor V. These C domains, and especially the C2 domains, confer the PS-binding ability to the coagulations factors and

lactadherin [8–10]. The EGF2 domain contains an Arg-Gly-Asp (RGD) motif that confers binding to the αvβ5 and αvβ3 integrins [9]. It was hypothesized that lactadherin by the two-sided binding affinities could operate as an opsonin-facilitating PS-dependent phagocytosis of apoptotic cells [2,9,10], which was subsequently confirmed *in vivo* [6].

The crystal structure of the bovine lactadherin C2 domain reveals a distorted β-barrel formed by eight of the 19 antiparallel β strands [11]. Equivalent β-barrel cores are found in the C2 domains of factors V and VIII, and have been conserved across the family of discoidin-type homology domains. In analogy with the C2 domains of factors V and VIII, the membrane interacting part consists of three relatively large loops termed "spikes" [11]. Mutagenesis studies demonstrated that solvent-exposed hydrophobic residues, Trp26, Leu28, Phe31, and Phe81, and/or Gly residues of spikes 1 and 3 most likely participate in membrane binding [11]. Even though the C2 domain of lactadherin closely resembles the corresponding domains in factors V and VIII, there are small differences which may explain the more efficient PS-binding of lactadherin [11].

The phospholipid binding characteristics of lactadherin were initially examined by coating microplate wells with individual pure phospholipids. In this assay, lactadherin showed affinity toward PS,

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phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidic acid, phosphatidyl-glycerol (PG) and cardiolipin, but not to phosphatidylcholine (PC) and sphingomyelin [9]. In the same work, implications for selective binding to negatively charged phospholipids were also given, as only 2% PS mixed with PC effectively supported lactadherin binding. Exclusivity for PS-binding of lactadherin was demonstrated shortly after in a study using a slightly different setup [12]. Lactadherin also displays low affinity binding to the abundant anionic phospholipid phosphatidylinositol in extruded vesicles [13]. This might, in turn, explain why binding could be achieved with pure phospholipids other than PS. The binding of factor VIII to PS is markedly enhanced by PE [14]. Similarly, lactadherin binding was firstly shown to be stimulated with 20% PE at 2% PS, though it hardly had any effect at 4% PS [9]. However, successive investigations implied no specific effect on lactadherin PS-binding [13].

Other membrane-binding features of lactadherin include Ca^{2+}-independent stereoselective affinity for the L-form of PS and preference for membranes with high surface curvature. Lactadherin displays an almost linear relationship between PS content and the number of binding sites at low (0–2%) PS levels [13], reaching saturation around 8% PS content. The PS-threshold for lactadherin binding to membranes has not been determined, but it has been suggested that lactadherin will bind to cell membranes with a PS content above 0.5% [15]. Lactadherin thus shows promise as a new tool for Ca^{2+} independent PS imaging, and might allow experimental setups where the benchmark PS-recognizing protein annexin V cannot be utilized. The use of lactadherin as a PS-localization tool is accelerating, with the most prominent examples in the field of hematology (e.g. [16–18]).

Motivated by the central importance of lactadherin–PS interactions, we have carried out a kinetic in vitro investigation to elucidate steps involved in the binding reaction using purified bovine lactadherin and isolated phospholipid vesicles of different compositions and sizes. We show that the binding pattern fits to a two-step binding mechanism involving an initial binding and a second rearrangement step. This binding step is entirely associated with the C2 domain and does not involve the EGF domains. Our kinetic assay is sensitive enough to allow us to investigate binding at very low PS concentrations, and demonstrates significant binding at PS contents around 0.03%. Although there is saturation of the degree of binding around 1% PS, the rate constant of binding continues to increase at higher PS concentrations and is promoted by increased vesicle curvature but relatively unaffected by increasing PE. Remarkably, the signature specificity for PS is abolished in dodecylphosphocholine micelles, where other anionic phospholipids elicit the same response as PS. This indicates that the vesicular environment is essential for appropriate presentation of the PS moiety.

2. Materials and methods

2.1. Materials

Lactadherin (409 residues, 45,590 Da), in both glycosylation forms, was prepared as described previously [2]. Purity was checked by SDS-PAGE and N-terminal amino acid sequencing and shown to be >97%. Protein concentrations were determined by amino acid analysis based on o-phthaldialdehyde derivatization. Discoidin C2 domain of lactadherin (residues 252–409) was produced as described in Ref. [11]. 1,2-Diacyl-sn-glycero-3-phospho-L-serine (Sigma, P7769) (denoted PS) and 3-sn-phosphatidyl-ethanolamine (Sigma, P7693) (PE) were from bovine brain, and L-α-phosphatidylcholine (Sigma, P3556) (PC) was from egg yolk. These lipids had a mixture of different chain lengths and were used for experiments involving bilayer vesicles, where all lipids are incorporated homogeneously into vesicles. Given that lactadherin is believed to bind to the interfacial region rather than penetrating to the hydrocarbon layer, the existence of

different chain lengths in the hydrocarbon layer is unlikely to affect binding to any significant degree. Dodecylphosphocholine (DPC), dilauroylphosphatidylserine (DLPS), dilauroylphosphatidyl glycerol (DLPG) and dilauroylphosphatidylcholine (DLPC) were from Avanti (Alabaster, AL). These lipids all had the same 12-carbon chain length and were used for experiments involving micelles, where matching chain length was important to facilitate appropriate mixing of different components. Chemicals were of the highest analytical grade and from Sigma-Aldrich or Merck unless specified.

2.2. Preparation of mixed micelles and vesicles

Mixed micelles were prepared by dissolving solid phospholipids (w/v) in chloroform:methanol (95:5) to obtain the desired phospholipid weight ratios. All concentrations are given as mg/ml because the mixture of different chain lengths precludes calculation of molar concentration. The solvent was evaporated by a stream of dry nitrogen for at least 2 h followed by exposure to vacuum for the same length of time until a dry lipid film was observed. This was done prior to suspension (10 mg/ml) of the phospholipids in 75 mM NaH_2PO_4–Na_2HPO_4, pH 7.4. Small unilamellar vesicles of phospholipids were prepared by sonication using a Bandelin Sonopuls (Bandelin Electronics GmbH, Berlin, Germany) with 50% power and 10% cycles, typically for a few minutes while keeping sample on ice and ensuring it did not heat up beyond room temperature, until no further optical clearing of the suspension was observed. Samples were flushed with nitrogen prior to use to minimize oxidation of unsaturated bonds. The sonicated samples were centrifuged to remove titanium particles before use. Unless otherwise stated, all vesicles were sonicated just before the experiment and were used within 2 days of preparation. Extrusion of vesicles with filters of pore sizes 200, 100 and 50 nm was carried out using a 15 mL thermobarrel extruder (Northern Lipids, Vancouver) using two stacked filters as described [19]. Dynamic light scattering confirmed that the average vesicle size was within the expected range for each extrusion fraction, though there was some overlap between the different fractions [20].

2.3. Steady state fluorescence experiments

Steady state fluorescence experiments were performed utilizing a Photon Technologies, Inc. cw spectrofluorometer equipped with a Peltier heating and cooling sample chamber. Experiments were performed in 10×10×40 mm quartz cuvette. A U330 filter was placed in the excitation pathway to minimize 2nd order and stray light. Emission spectra were recorded for 40 nM lactadherin in Tris-buffered saline pH 7.4 at 20 °C. Spectra were recorded approximately 5 min after addition of vesicles. Excitation wavelength was 280 nm and bandpass for excitation and emission was set at 2 nm. Displayed results are the average of 3 separate experiments each recorded in triplicate.

2.4. Stopped-flow experiments

Kinetic fluorescence measurements were performed on an Applied Photophysics (Leatherhead, U.K.) SX18MV stopped-flow apparatus, using an excitation wavelength of 280 nm and emission through a 320 nm glass filter. All experiments were carried out at 25 °C in 75 mM sodium phosphate pH 7.4. Vesicles or micelles of a given composition were mixed 1:1 with protein solution (either full-length lactadherin or the C2 domain) to give a final protein concentration of 0.66 μM (based on $\varepsilon_{280}^{1mg/ml} = 1.72$). Data typically fitted to two exponential relaxation phases according to the following equation:

$$\text{Signal}(t) = \text{Amp}_1 * \exp(-k_1 * t) + \text{Amp}_2 * \exp(-k_2 * t) + c \qquad (1)$$

where Amp_1 and Amp_2 are amplitudes, k_1 and k_2 are rate constants for the first and second phase and c is an experimental offset. To avoid bias, ratios between Amp_1 and Amp_2 were not fixed.

The rate constant for the first (fast) relaxation phase was fitted to the following linear equation:

$$k_1 = k_{ass} * [vesicle] + k_{diss} \qquad (2)$$

where k_{ass} is the second-order association rate constant of binding and k_{diss} is the first-order dissociation rate constant of binding.

The rate constant for the second (slow) relaxation phase was fitted to the hyperbolic equation:

$$k_2 = k_{slow} * [vesicle]/(K_D + [vesicle]) \qquad (3)$$

where k_{slow} is the slow conformational change associated with the formation of the final bound state in Scheme 1 and $K_D = k_{diss}/k_{ass} = [Lact][vesicle]/[Lact:vesicle]$ is the equilibrium dissociation constant describing the formation of the initial (Lact:vesicle)* complex.

2.5. Determination of critical micelle concentration (cmc) of mixed micelles

This was done using the pyrene assay as described [21,22]. Briefly, we determine the ratio between the emission intensity at two wavelengths ($I_1 = 372.5$ and $I_3 = 383.5$ nm) upon excitation at 355 nm. I_3/I_1 is around 0.6 in the absence of surfactant but increases to a plateau value of 0.85–0.9 in the presence of micelles, depending on the specific surfactant headgroups. We operationally define the cmc as the surfactant concentration at which the ratio reaches the plateau value.

3. Results

3.1. Fluorescence change of lactadherin upon binding to phosphatidylserine-containing vesicles

The crystallographic structure of the lactadherin C2 domain indicates an exposed Trp residue on the putative membrane-binding interface [11]. This suggests that membrane interactions may lead to altered intrinsic fluorescence. To test this possibility, lactadherin was incubated with sonicated vesicles containing PS (Fig. 1A). The results indicated that binding to these vesicles causes an overall fluorescence of increase of approximately 10% and a blue shift of approximately 2 nm in the emission spectrum. These results are consistent with a membrane-binding change in which at least one Trp residue becomes
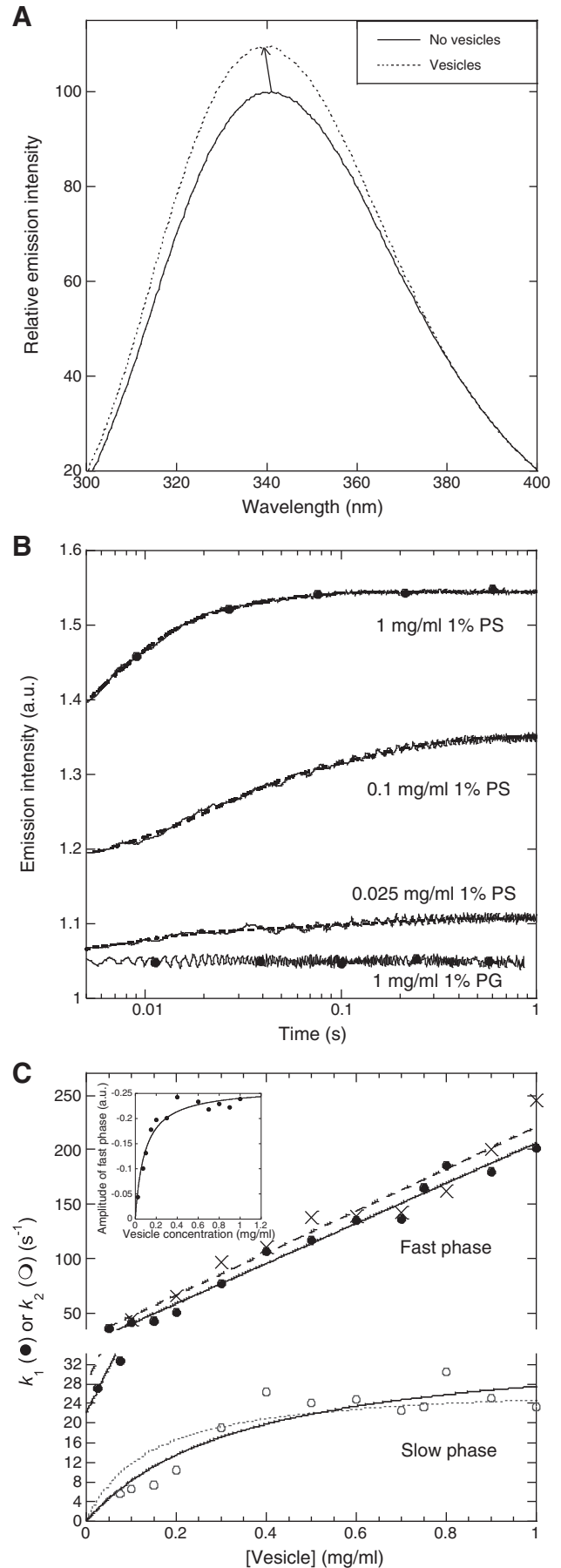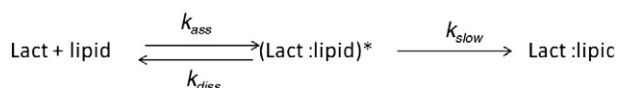

Fig. 1. (A) Lactadherin fluorescence emission spectra obtained without (joined lines) and with (stippled lines) sonicated phospholipid vesicles (composition 4PS:20PE:76PC 4:20:76). (B) Time profiles for the binding of lactadherin to vesicles containing 99% PC and either 1% PS or 1% PG, monitored by the change in lactadherin Trp fluorescence. The time profiles with PS are fitted to two exponential decays (Eq. 1). Note the complete lack of fluorescence change when PS is replaced by PG, emphasizing the specific binding of lactadherin to the PS group. (C) Dependence of the fast (filled circles) and slow (empty circles) rate constants on vesicle concentration for binding of lactadherin to vesicles containing 1% PS and 99% PC. Data for the fast rate constants for binding of the recombinant C2 domain to the same type of vesicles (crosses) are shown for comparison. Joined lines indicate best fits to full-length lactadherin data. Fast rate constants are fitted to a linear equation (Eq. 2) to yield a slope (k_{ass}) of 184 ± 6 (mg/ml)$^{-1}$ s^{-1} and an intercept (k_{diss}) of 22.0 ± 3.5 s^{-1}, while the slow rate constants are fitted to a hyperbolic equation (Eq. 3) to give a plateau value (k_{slow}) of 37 ± 6 s^{-1}. Eq. (3) yields an apparent equilibrium constant $K_D = 0.34 \pm 0.13$ mg/ml while Eq. (2) predicts an equilibrium constant $K_D = k_{diss}/k_{ass} = 0.12 \pm 0.02$ mg/ml. The stippled hyperbolic curve indicates the fit to the slow rate constants using a fixed equilibrium constant of 0.12 mg/ml. The dashed straight line is the best fit to the fast rate constants for the C2 domain, which yields a slope of 193 ± 16 (mg/ml)$^{-1}$ s^{-1} and an intercept of 28 ± 10 s^{-1}, values which within error are identical to those of full-length lactadherin. *Inset*: Change in the amplitude of the fast phase with increasing lipid concentration. The joined line is the best fit to Eq. (3).

better shielded from water. This effect may be primarily from immersion in the membrane or may be due, in part, to a conformational change. This change in intrinsic fluorescence provides a useful signal for measuring the kinetic properties of membrane interaction.

3.2. Binding of lactadherin can be described by a two-step binding mechanism and only involves the C2-domain

When full-length lactadherin is mixed with synthetic phospholipid vesicles containing 99% PC and 1% PS in a stopped-flow apparatus and the reaction is followed by changes in Trp fluorescence, we obtain an increase in fluorescence over time which can be fitted to two exponential decays (Fig. 1B). This signal is completely absent when PS is replaced by the phospholipid phosphoglycerol (PG), which like PS has an overall negative charge of −1 but lacks the serine group. There is no signal when the amino acid Trp is used instead of lactadherin, irrespective of which lipid is used (data not shown). Both these sets of data indicate that there is no significant contribution of light scattering to the reaction, which therefore must reflect change(s) that occur upon membrane binding, and is entirely consistent with lactadherin's specificity for PS [9,13]. The signal change associated with the fast phase increases significantly with increasing vesicle concentration, reaching a plateau level above ~0.5 mg/ml vesicle (insert to Fig. 1C) while the signal change for the slow phase is smaller, determined with greater error and shows more fluctuation (data not shown). The rate constant for the fast phase increases linearly with lipid concentration while that of the slow phase reaches a plateau in approximately the same concentration range as the amplitude of the fast phase (Fig. 1C). As we measure total emission above 320 nm, we cannot determine whether the two phases lead to changes in the emission spectrum in overall intensity. Rather, we use the data as evidence for two distinct processes associated with vesicle interaction. The simplest interpretation of the kinetic data is a two-step binding mechanism between protein (Lact) and lipid with the following parameters:

$$\text{Lact} + \text{lipid} \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} (\text{Lact}:\text{lipid})^* \xrightarrow{k_{slow}} \text{Lact}:\text{lipic}$$

Here k_{ass} is the association rate constant describing the binding of lactadherin to lipid, k_{diss} the rate constant for the dissociation of this complex and k_{slow} a conformational or membrane insertion change from the initial complex to the final bound state. In this model, the slope of the linear plot in Fig. 1C (Eq. (2) in Materials and methods) corresponds to $k_{ass} = 184 \pm 6 \, (\text{mg/ml})^{-1} \, \text{s}^{-1})$ and the intercept is $k_{diss} = 22.0 \pm 3.5 \, \text{s}^{-1}$. The hyperbolic concentration dependence of the slow phase reflects the fact that the conformational change of this step is not directly dependent on protein concentration but rather on the fraction of the protein that is complexed as (Lact:lipid)*. Once we have reached vesicle concentrations where essentially all lactadherin complexes to vesicles, the rate limiting step becomes the conformation change described by k_{slow}. This concentration dependence is described by Eq. (3). Strictly speaking, a hyperbolic fit of the slow rate constant versus vesicle concentrations in this scheme presupposes a rapid equilibrium between free and bound lactadherin which is not affected by the slow phase, in other words that $k_{slow} < k_{diss}$. This is not quite correct,

Fig. 2. (A) Dependence of the fast rate constants on vesicle concentrations at different proportions of PS. The remaining lipid is PC. Straight lines are best linear fits to the data. (B) k_{ass} at different proportions of PS, obtained from data in panel A. Notice the steep rise in k_{ass} with PS up to 1% PS. (C) Dependence of the maximal amplitude of binding (the plateau value of the total amplitude when plotted versus vesicle concentration for a given vesicle composition) on the proportion of PS. Data are fitted to a binding isotherm similar to Eq. (3) to yield an apparent binding constant of $0.15 \pm 0.03\%$ PS, meaning that vesicles containing 0.15% PS show half the maximal binding capacity.
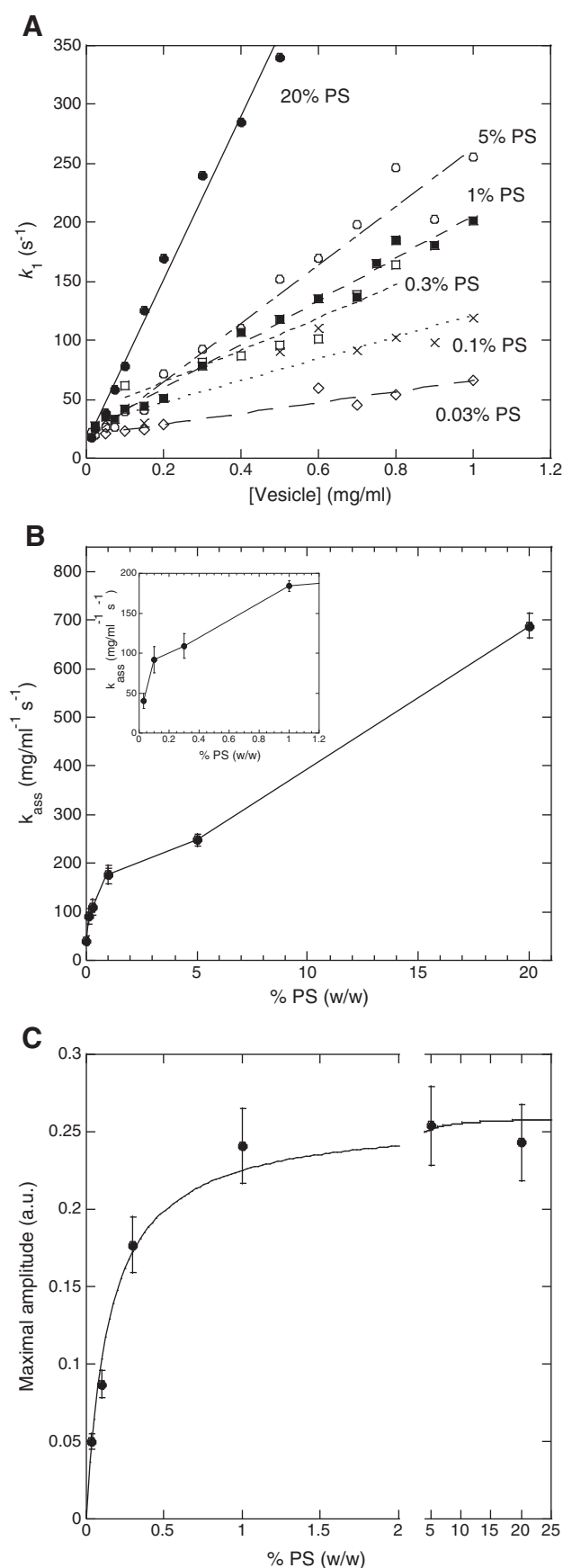
as the hyperbolic fit in Fig. 1C predicts a k_{slow} value of 37 ± 6 s^{-1}, which is almost twice as large as k_{diss}. However, in practice the discrepancy is not very large. The ratio $k_{diss}/k_{ass} = 0.12 \pm 0.02$ mg/ml (derived from the fast phase; concentration refers to vesicles) describes the equilibrium constant for binding of lactadherin to vesicles in the first step, and this is in the same range as the equilibrium constant $K_D = 0.35 \pm 0.14$ mg/ml (derived from the slow phase). As shown in Fig. 1C, the two equilibrium values lead to quite similar hyperbolic fits. Further, the model assumes that lipids are in excess, leading to pseudo-first order binding kinetics in the first step. We justify this assumption on two grounds. Firstly, the final protein concentration (after mixing) is 0.03 and 0.012 mg/ml (full lactalbumin and C2 domain, respectively) which is well below the final lipid concentration for most of our data points. Secondly, a three-fold reduction in protein concentration does not lead to any significant change in kinetics (data not shown).

Thus we conclude based on these data that lactadherin binds in a two-step mechanism to vesicles, in which the first step is a binding event while the second step involves a change in Trp fluorescence that can be ascribed to either a conformational change in lactadherin or a change in the extent of insertion into the phospholipid membrane. It is not possible based on the present data to distinguish between these two possibilities.

Having established the specificity for PS lipids, we next investigated whether the binding was associated with the C2 domain. A recombinantly expressed C2 construct was subjected to the same studies and yielded identical results within error (data for k_{fast} shown in Fig. 1C). We therefore conclude that the binding reaction monitored by stopped-flow can entirely be attributed to the interaction between the C2 domain and PS-containing vesicles.

3.3. As little as 0.03% PS leads to detectable binding, and the dynamics (but not affinity) of interactions are promoted by smaller vesicles but not significantly by inclusion of PE lipids

Previous studies have shown that lactadherin can bind to cell membranes with a PS content above 0.5% [15]. We decided to investigate the lower limits of binding by monitoring the binding reaction using vesicles containing 0.03–20% PS. It was possible to detect binding over the entire interval, though the signals and rate constants diminished with decreasing PS content. Nevertheless, linear correlations between the fast rate constant and vesicle concentration were detected in all cases (Fig. 2A), and these made it possible to determine the k_{ass} values with good precision (Fig. 2B). The k_{diss} values and k_{slow} values stayed essentially constant with decreasing PS content, but the decreasing signal made it difficult to determine them with the same precision, so we focus on k_{ass}. This increases steeply with the percentage of PS up to around 1% PS, after which there is a more measured increase (Fig. 2B). Interestingly, the maximal amplitude associated with each PS value (determined as in the insert to Fig. 1C) also increases with PS but only up to 1% PS, after which it stays constant. In fact the maximal amplitude data can be fitted to a binding isotherm to yield an apparent binding constant of $0.15 \pm 0.03\%$ PS (Fig. 2C), meaning that vesicles containing as little as 0.15% PS have half the maximal binding capacity of PS-containing vesicles. Further increases in PS content promote the kinetics of binding (and by implication the affinity of binding, since the dissociation constant does not appear to change) but does not to any significant extent affect the *capacity* of binding (i.e. the amount of protein that can bind to vesicles). We conclude that lactadherin is remarkably sensitive to PS, detecting it at concentrations at least an order of magnitude lower than previously reported using equilibrium conditions [15].

Lactadherin has also been reported to be sensitive to the degree of membrane curvature [13], and our kinetic model provides a sensitive means of investigating this in further detail. We prepared different sized vesicles by extruding them successively through filters of decreasing size to yield vesicles of diameter 200, 100 and 50 nm. Finally
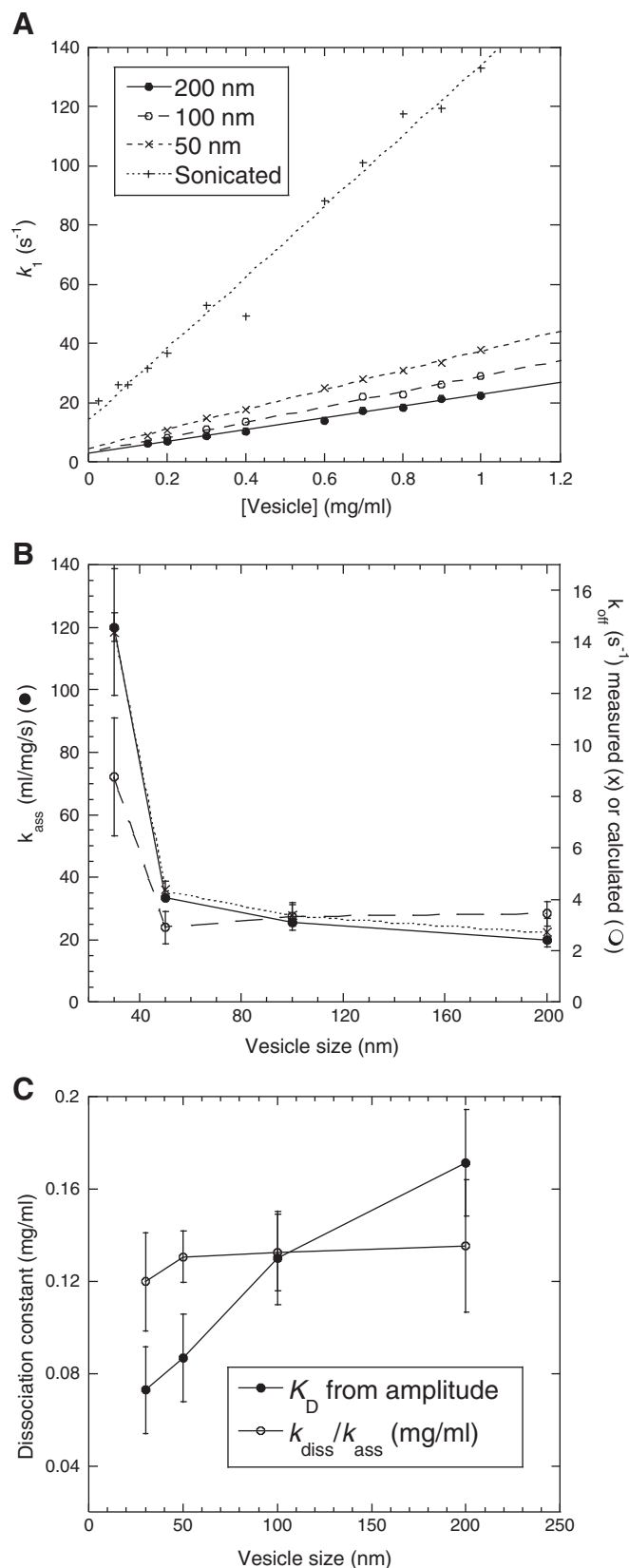
Fig. 3. Interaction of lactadherin with vesicles of different sizes. (A) Dependence of the fast rate constant on vesicle concentration for vesicles of different sizes. Lines indicate best linear fits. (B) Dependence of k_{ass} and k_{diss} (from data in panel A) on vesicle size. (C) Dependence of the dissociation constant $K_D = k_{diss}/k_{ass}$ on vesicle size.

the remaining portion of vesicles was sonicated to yield vesicles of around 30 nm. Note that although extrusion clearly leads to the formation of vesicles with different size distribution ranges as determined by dynamic light scattering [20], there is some degree of overlap between the different fractions. We therefore focus on the general trends in the results and do not attempt to make quantitative predictions. Although all vesicles yielded the customary linear plots when the fast rate constant was plotted versus vesicle concentration (Fig. 3A), there was a clear decrease in both k_{ass} and k_{diss} with increasing vesicle size (Fig. 3B). This parallel decrease meant that the dissociation constant $K_D = k_{diss}/k_{ass}$ remained essentially constant irrespective of vesicle size (Fig. 3C). However, as is common for many such ligand-binding experiments, the dissociation constant is determined with considerable error because of the large error on k_{diss}. An alternative approach is therefore to determine affinity using the amplitude of the major binding phase as in the insert to Fig. 1C. Although the K_D values for individual vesicle sizes determined by the two approaches are similar, there is a much clearer trend with the amplitude data that the affinity decreases with increasing vesicle

size (Fig. 3C), in nice agreement with our previous observations [13]. When we use these K_D values to back-calculate k_{diss} using the already determined k_{ass} values, k_{diss} remains essentially constant for extruded vesicles of size 50–200 nm but is significantly larger for the sonicated vesicles (Fig. 3B).

In view of the conflicting data on the effect of PE on lactadherin binding [9,13], we also investigated the kinetics of binding of lactadherin to vesicles with 1% PS and different concentrations of PE. Once again linear plots were observed for the fast rate constants (Fig. 4A), and the k_{ass} values increased linearly with the PE content (Fig. 4B). However, the slope of this plot was around 8.6 ± 1.6, which is around a third of the value (27.3 ± 2.3) for corresponding changes in PS in the range of 1–20% PS (Fig. 2B). The k_{diss} values changed much less (Fig. 4B). Clearly PE increases the affinity of lactadherin for membranes, although this effect is not as strong as observed for PS and is also wholly dependent on PS; no binding was observed in the absence of PS (data not shown). Just as remarkable was the change in amplitude. In the absence of PE, the amplitude of binding is negative, corresponding to an increase in fluorescence upon binding and conformational change.
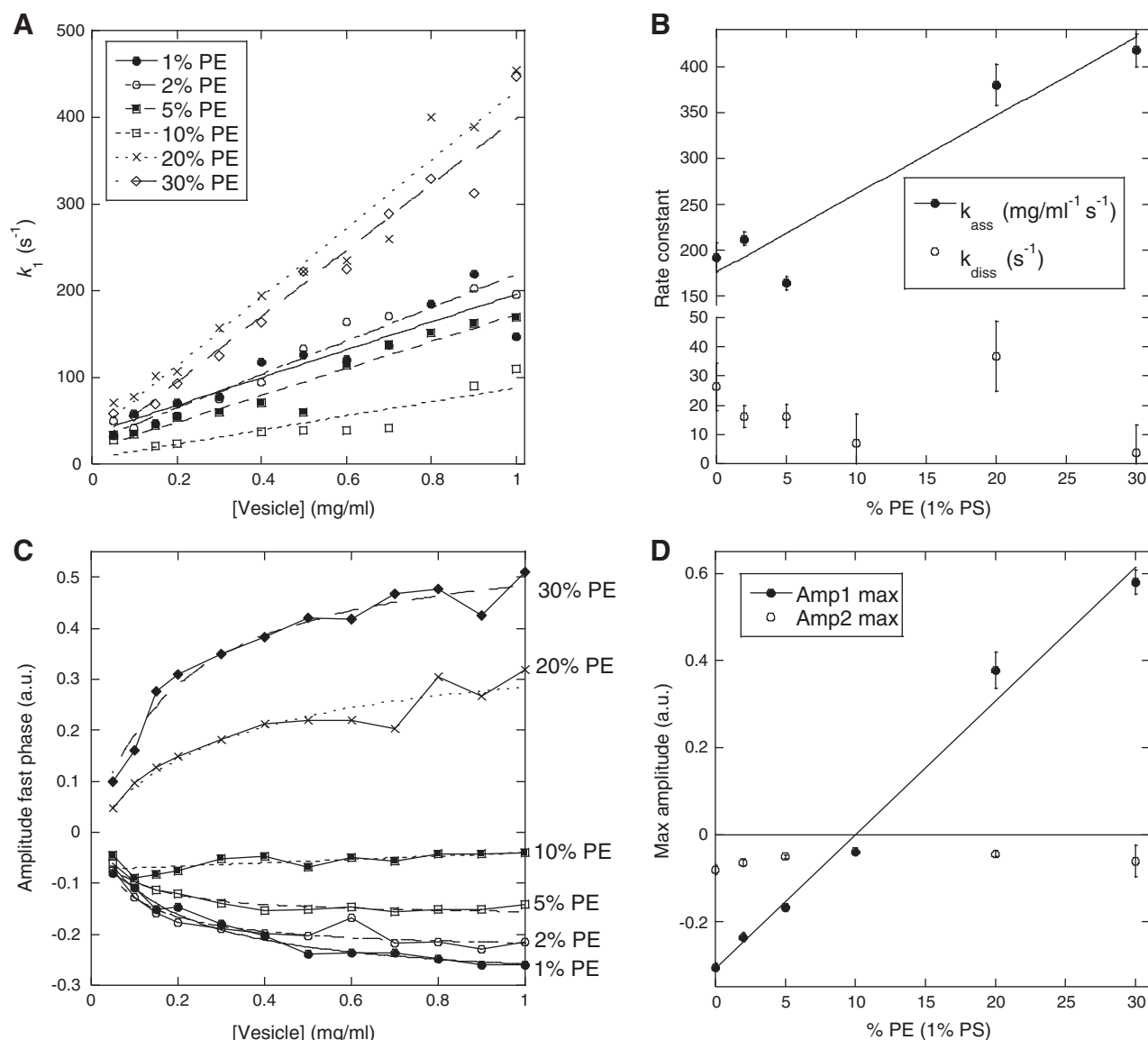

Fig. 4. Effect of PE on lactadherin binding kinetics. (A) Dependence of the fast rate constant on vesicle concentration for vesicles containing different amount of PE. Lines indicate best linear fits. (B) Variation of k_{ass} and k_{diss} (from data in panel A) with vesicle size. Line indicates best linear fit to k_{ass}, yielding a slope of 8.6 ± 1.6 and an intercept of 176 ± 25 s^{-1}. (C) Amplitude of the fast phase as function of vesicle concentration for vesicles containing different amount of PE. Lines indicate best fits to a hyperbolic binding equation (similar to Eq. 3). (D) Change in maximal amplitude of the fast and slow phases with PE content. Line indicates best linear fit.
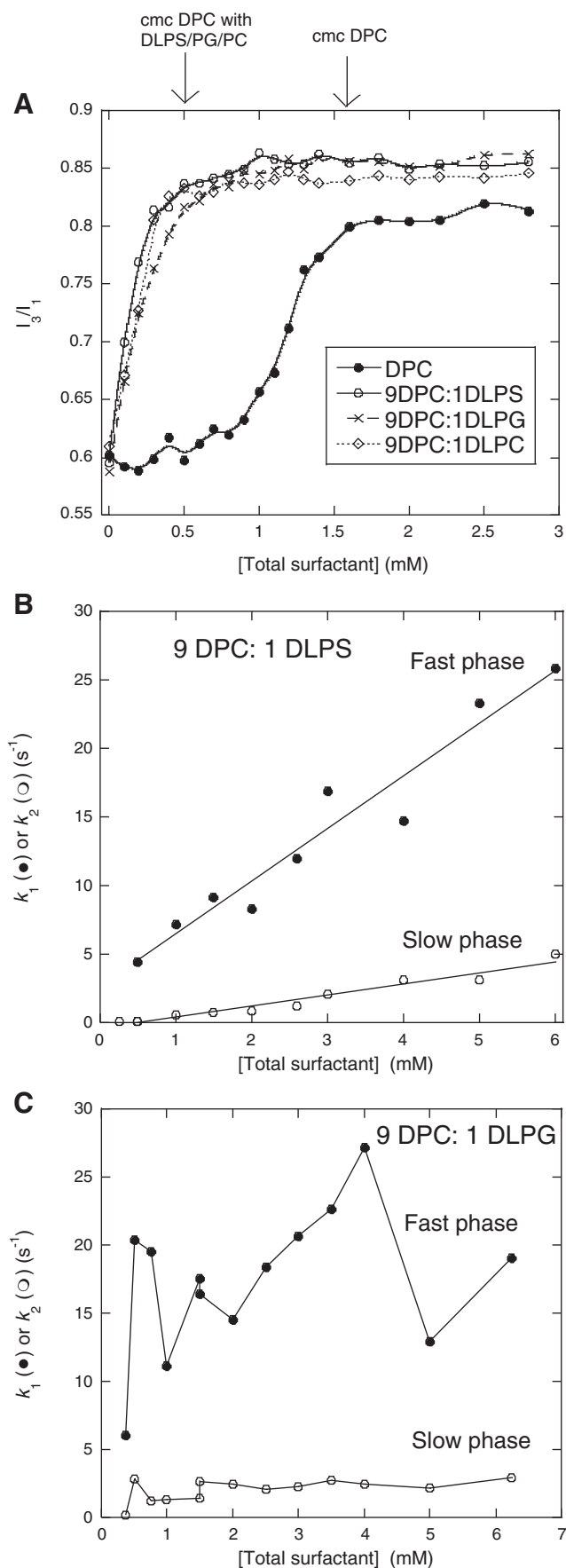
However, with increasing PE, the amplitude of the fast phase becomes increasingly less negative and switches to positive around 10% PE (Fig. 4C), while that of the slow phase remains negative throughout (Fig. 4D). There is a clear linear relationship between the maximal amplitude and the PE content. This suggests a simple change in the fluorescence properties of the vesicle which means that the more PE is present, the more the Trp fluorescence is quenched by the vesicle upon binding.

3.4. Binding of lactadherin to micelles containing PS

The increased interaction with smaller vesicles suggests that lactadherin–vesicle interactions are promoted by increased curvature, *i.e.* increased exposure of PS residues. A simple way to increase this exposure is to replace vesicles with micelles, where the individual components are less closely packed, more surface exposed and in a more rapid exchange with surroundings [23]. We do this by replacing the diacylated phosphatidyl choline with the single chain surfactant dodecylphosphocholine (DPC) which forms micelles rather than vesicles. The critical micelle concentration for DPC is around 1.5 mM. However, upon adding the diacylated phospholipids DLPS, DLPG or DLPC (which all have an alkyl chain length of 12 atoms like DPC), micelles form at significantly lower concentrations (Fig. 5A), and micelle formation is complete around 0.5 mM total surfactant. This is not surprising, given that the diacylated phospholipids have a much stronger tendency to associate than their single-chain counterpart and will therefore promote mixed micelle formation. Therefore we can safely assume that micelles are present from 0.5 mM surfactant and up.

When lactadherin is mixed with micelles containing 10% DLPS at micellar concentrations, we observe two exponential decays as for the corresponding vesicles (Fig. 5B). However, there are several differences compared to their closest counterpart, namely sonicated vesicles containing 10% PS. Firstly, the rates are much lower. The slope of the plot is around $4 \, \text{mM}^{-1} \, \text{s}^{-1}$ or $\sim 12 \, (\text{mg/ml})^{-1} \, \text{s}^{-1}$ while the corresponding slope for vesicles with 10% PS is interpolated from Fig. 2B to be $\sim 400 \, (\text{mg/ml})^{-1} \, \text{s}^{-1}$. Secondly, the slow rate constant for micelle interactions does not follow a hyperbolic relationship, unlike that of the vesicles (cfr. Fig. 1C), suggesting that we do not have a simple two-state binding but possibly two parallel binding steps. Thirdly and most importantly, similar exponential decays are observed when DLPS is replaced with the anionic phospholipid DLPG and even with the zwitterionic lipid DLPC (data shown for DPC:DLPG in Fig. 5C; similar data for DPC:DLPC). The data are more scattered and do not show a clear linear dependence, but there is nonetheless a marked contrast to vesicles where DLPG failed to elicit any response (Fig. 1B). Using a completely different pair of surfactants (SDS instead of DLPS/DLPG and dodecyl maltoside instead of DPC) also gives rise to the same two-exponential decay with rate constants in the same range (data not shown), while DPC alone leads to a single exponential decay similar to the slow phase in Fig. 5B. Below the cmc, the kinetics in both DPC:DLPS and DPC:DLPG micelles are even more complex, giving rise to three exponential decays where the fast phase has rate constants up to $400 \, \text{s}^{-1}$ (data not shown). All this indicates that lactadherin–micelle binding involves more than simple recognition of the PS moiety. The signals probably reflect more general protein–surfactant interactions (for a more detailed account of these phenomena, see Ref. [24]). This nicely illustrates that the intact vesicle structure is vital for the correct presentation of PS for recognition by lactadherin.

Fig. 5. Interaction of lactadherin with micelles containing PS and other phospholipids. (A) Determination of the critical micelle concentration of DPC with and without diacylated phospholipids using pyrene fluorescence. (B) Rate constants for binding of lactadherin to micelles containing 90% DPC and 10% DLPS (molar ratio). The linear fit for the fast phase yields a slope of $3.85 \pm 0.37 \, \text{mM}^{-1} \, \text{s}^{-1}$ and an intercept of $2.59 \pm 0.23 \, \text{s}^{-1}$. The corresponding values for the slow phase are $0.80 \pm 0.06 \, \text{mM}^{-1} \, \text{s}^{-1}$ and $-0.38 \pm 0.18 \, \text{s}^{-1}$. (C) Data as in panel B using DLPG instead of DLPS. The lines are shown to guide the eye.

4. Discussion

It is recognized that redistribution of PS to the outer leaflet of the plasma membrane is crucial to recruit numerous signaling molecules or create an environment that facilitates specific enzymatic and/or biological activities. For example, PS exposed on activated platelets or long term stored red blood cells serves as procoagulant surfaces supporting the generation of factors VIIIa and Xa, which lead to conversion of prothrombin to thrombin by coagulation factor Xa in the presence of calcium and factor Va [25]. Lactadherin has been shown to be a highly suitable probe for PS exposure. Accordingly, the present study provides detailed data for the binding kinetics of lactadherin to PS-containing membranes. Based on these data, we conclude that lactadherin binds in a two-step mechanism to vesicles, a fast initial binding event followed by a slower step that reflects either a change in lactadherin conformation or the extent of binding to the phospholipid membrane. With the present data it is not possible to distinguish between these two possibilities.

It has previously been shown that factors VIII and V bind to PS-containing vesicles via a multistep process with a rapid and a successive slower association step [26]. Both the C1 and C2 domains of factor VIII participate in membrane binding [8,27]. In fact, the C2 domain of factor VIII differs from the C2 domain of lactadherin in that membrane binding of the isolated domain is of very low affinity and does not have PS specificity [28]. Thus, a plausible multi-step mechanism binding mechanism for factor VIII involves sequential membrane engagement of the C1 and C2 domains. A similar model may be appropriate for lactadherin, though it appears more likely that the C2 domain makes the primary membrane engagement and may be followed by membrane engagement of the C1 domain. The differences between the membrane binding patterns of the C2 domain of lactadherin and the C2 domain of factor VIII illustrate that the membrane interactions are more complex than the overall structural homology had suggested.

Upon comparison with annexin V, it has been suggested that use of lactadherin may enable a more accurate description of the degree of PS exposure when studying blood platelet activation and/or apoptosis [16]. The present work adds new knowledge to the lower limits of PS-detection lactadherin. Even at a PS content of 0.03%, binding was still observed. Sensitivity at this level might enable collection of more information about the timeline of PS-exposure during the apoptosis and studies of phospholipid asymmetry during normal and pathological processes and provide more insight into the physiological role of lactadherin. We have previously shown that lactadherin has a preference for curved membranes, with a four-fold difference in affinity between small and large unilamellar vesicles [13]. This feature indicates a preference for certain membrane structure, e.g. pseudopods, filopodia-like extensions, membrane vesicles, small milk fat globlets or platelet-derived microvesicles. Our kinetic data nicely reproduce this curvature preference, showing that the affinity approximately doubles as we go from large (200 nm) to small sonicated (30 nm) vesicles. Furthermore, the data suggest that both association and dissociation kinetics are affected as we go from the 30 nm vesicles to the next size class (50 nm), while subsequent size increases have a smaller kinetic effect but mainly decrease association kinetics. This strong sensitivity to small vesicles seems reasonable as sonicated vesicles are inherently unstable compared to larger vesicles [29] and harbor a significantly greater degree of membrane defects associated with increased curvature compared to even the smallest extruded vesicles.

Lactadherin's membrane-binding requirements parallel those of factor VIII to great extent. However, when it comes to the impact of PE the picture for lactadherin is less clear in the literature [9,13]. The present stopped-flow experiments show two things: firstly, PE does increase the affinity of lactadherin for vesicles (by increasing the association rate constant) but only in the presence of PS and not

as much as this lipid on a molar basis. We interpret this to mean that PE acts as an ancillary lipid that can promote binding to whatever PS is present. Secondly, there is a clear linear relationship between the maximal amplitude and the PE content. This suggests a simple change in the fluorescence properties of the vesicle which means that the more PE is present, the more the Trp fluorescence is quenched by the vesicle upon binding. That this does not affect the slow phase indicates that the second step does not involve any changes in the interaction with the PE moiety (either formation or dissolution of interactions), in other words the second phase is the same in the absence or presence of PE. Thus both observations strongly indicate that PE only affects the very first part of the docking of lactadherin onto PS-containing vesicles.

To investigate whether the increased lactadherin–vesicle interactions are stimulated by augmented exposure of PS residues due to increased curvature, we substituted vesicles with micelles comprising single and dodecylphosphocholine and diacylated phospholipids. The experiments, which also employ use of SDS and dodecyl maltoside, indicate that lactadherin–micelle binding involves more than simple recognition of the PS moiety. The loss of specificity probably reflects more general protein–surfactant interactions, of which there is an almost overwhelming variety of different types (for a more detailed account of these phenomena, see Ref. [24]). This nicely illustrates that the intact vesicle structure is vital for the correct presentation of PS for recognition by lactadherin.

In conclusion, lactadherin demonstrates an even greater sensitivity toward PS than previously reported, detecting PS in vesicles at a level at least an order of magnitude lower than previously reported [15]. Lactadherin binds to PS vesicles in a manner that is promoted by content of both PE and increased membrane curvature. Lactadherin and PS interaction is mediated by the discoidin C2 domain and is critically dependent on vesicle structure since specificity is completely destroyed in micelles.

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