SUPPLIMENTAL MATERIAL:

Membrane Modulates Affinity for Calcium Ion to Create an Apparent Cooperative Binding Response by Annexin a5

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Materials:

Both 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC or 16:0,18:1PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS or 16:0,18:1PS) were from Avanti Polar Lipids, Inc. (Birmingham, AL). Potassium chloride (KCl) was Puriss-grade and 3-(N-morpholino)propanesulfonic acid (MOPS) was Biochemika grade from Fluka Chemical Corp. All buffers used were decalcified using Chelex-100 ion-exchange resin from Bio-Rad Labs.

Methods:

PROTEIN PURIFICATION

Purification of wild-type *Rattus norvegicus* annexin a5 was based on Ca^{2+}-dependent binding to membrane. cDNA encoding rat annexin a5 was kindly provided by H. Sohma (Sapporo Medical University School of Medicine) and was transformed into *Escherichia coli* Bl-21 cells. Overexpression was initiated by the addition of 1mM isopropyl-ß-D-thiogalactopyranoside at optical densities between 0.4–0.8. After a 5hr induction, cells were harvested by centrifugation and lysed by sonication in 20mM 4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid (HEPES), 100mM KCl, 10mM calcium chloride, 1mM phenylmethanesulfonylfluoride and 1 mM 2-mercaptoethanol (βME) at pH 7.5. Cellular debris was pelleted by centrifugation at 16,000rpm for 30min. The pellet, containing annexin, was then resuspended in 20mM MOPS, 100mM KCl, 20mM ethylenediaminetetraacetic acid (EDTA), 20mM ethylene glycol-bis(2-aminoethyl-)-N,N, N',N'-tretraacetic acid (EGTA) and 1 mM βME at pH 7.5, and gently stirred overnight to release annexin. The mixture was then centrifuged and sterile-filtered through a 0.45µm Millipore PES filter, dialyzed against 20mM HEPES at pH 8.0, and incubated with 5 mM magnesium chloride and benzonuclease (Novagen 90% purity) at a concentration of 10units/ml for 16hrs at 4°C. The protein solution was applied to a pre-equilibrated Q-Sepharose Fast Flow anion exchange column and eluted using a linear gradient of sodium chloride containing 20mM HEPES and 1mM βME at pH 8.0. Fractions containing annexin were pooled and filtered using a 0.20µm Nalgene PES disposable filter unit. Recombinant annexin was then extensively dialyzed in 20mM MOPS, 100mM KCl (in excess of 8L) and passed through Chelex-100 resin to remove contaminating Ca^{2+}. Final purity of annexin was >95% as determined by both SDS-PAGE densitometry and a Nanodrop A260/A280 ratio <1. The protein was concentrated using an Amicon Ultra 15 Centrifugal Filter Unit (30,000 MW) from Millipore, and the final concentration was determined using a Nanodrop (Thermo Scientific) at 280 nm (21,050 $M^{-1}cm^{-1}$).

PREPARATION OF LIPID VESICLES

Large unilamellar vesicles (LUVs) composed of POPC:POPS (60:40) and POPC:POPS (80:20) were prepared by aliquotting stock solutions of lipid in chloroform into borosilicate culture tubes using gastight syringes (Hamilton Co., Reno, NV). Samples were dried to a thin film under a gentle stream of argon and dried for 4 hours under a vacuum of less than 20 mTorr. Samples were then lyophilized by dissolving the lipids in benzene/methanol (19/1, v/v) plunging the samples in liquid nitrogen, and placing them under vacuum (less than 20 mTorr) until the solvent was completely removed (approximately 8 hours). Lipids were hydrated in the dark above the gel-fluid phase transition temperature with decalcified 20mM MOPS, 100mM KCl, pH 7.5, under argon. LUVs were prepared by extruding a multilamellar vesicle dispersion through a sandwich of prefilters around a 0.1µm pore size polycarbonate filter (Avanti Polar Lipids, Inc.) at least 31 times.

CARBOXYFLUORESCEIN RELEASE ASSAY

To determine whether or not the binding of annexin disrupted the liposomes, the fluorescence signal of LUVs composed of 60% POPC 40% POPS, and hydrated with carboxyfluorescein (CF) buffer (20mM MOPS, 0.02% NaN_3, and 50 mM 5-(and 6)-carboxyfluorescein (mixed isomers) (Invitrogen), pH 7.5) was measured using an excitation wavelength of 492 nm and an emission wavelength of 515 nm. The experiments were run for one hour as the temperature of the water bath was brought from 20°C to -3°C; the lowest temperature attained within the cuvette itself was 1.1°C. Samples used in the study were 333µM lipid, 6 µM Annexin a5, 500 µM Ca^{2+}. Concentrations were adjusted using a column equilibration buffer (20 mM MOPS, 100 mM KCl, 0.02% NaN_3, pH 7.5). Triton X-100 detergent was added after each scan to confirm that CF was present within the liposomes and to determine the maximum efflux possible. All fluorescence measurements were performed on a Fluorolog 3 double excitation and double emission monochromoter (Horiba Jobin Yvon) in a quartz cuvette.

Temperature change within the cell was regulated by a Pharmacia Biotech Multitemp III water bath set to decrease from 20°C to -3°C. To determine the change occurring, the quartz cuvette was filled with the MOPS KCl column equilibration buffer and the temperature change was monitored with a Fluke 51II Thermometer. Temperature was recorded every 30 seconds in both the water bath and the cuvette for a 60 minute time period.

ISOTHERMAL TITRATION CALORIMETRY

Isothermal titration calorimetry experiments to determine the binding of Ca^{2+} and varying compositions of POPC:POPS containing lipids to annexin a5 were performed on a TA Instruments Nano ITC at both 15 and 18°C. The Ca^{2+} and lipid titrant solutions were prepared in the protein dialysate that was saved after concentrating the protein. This consisted of 20mM MOPS and 100mM KCl at pH 7.5 that was passed through Bio-Rad 100 Chelex resin to remove cation impurities and filtered using a 0.2µm Nalgene PES disposable filter unit. The Ca^{2+} concentrations used in the experiments were verified through the use of BAPTA (Invitrogen/Molecular Probes) and Ca^{2+} electrode (Fisher). The titrant lipid concentrations were verified by phosphate assay as described in Kingsley and Feigenson [1]. The annexin concentrations were directly measured using a Nanodrop (Thermo Scientific) (extinction coefficient of 21,050$M^{-1}cm^{-1}$ at 280nm) prior to the experiment, but after loading the calorimeter cell. For all experiments, the stir speed was 250rpm and the interval between injections was 300s. In addition, all solutions (macromolecule-containing, heat of dilution samples and dialysate) were thoroughly degassed prior to rinsing and loading both the titration and sample cell.

For the Ca^{2+} titrations into annexin in the absence of membrane, 9mM Ca^{2+} solution was injected with a 1µl injection to displace air from the syringe followed by 27 x 9µL injections. The titrations were completed at 15°C with the cell concentration of protein being 0.09mM. Higher protein concentrations for this particular experiment often resulted in precipitation.

For experiments in which Ca^{2+} was injected in the presence of both annexin and membrane, a 1.5mM Ca^{2+} stock was added to 0.024mM annexin by a 1µl injection to displace air from the syringe followed by 27 x 9µL injections. These titrations were completed at 18°C. The total lipid

concentration of 2.0mM was the same in both the cell and the syringe so that only the enthalpy of binding between annexin and Ca^{2+} in the presence of membrane could be measured. The acidic phospholipid content was 40% to provide the signal necessary to deconvolute the complex binding isotherms in the presence of lipid. The heats of dilution were collected by injecting Ca^{2+} and lipid into buffer containing the same concentration of lipid without the protein. To obtain the enthalpy of binding for Ca^{2+} to annexin in the presence of membrane, these resulting heats of dilution were subtracted from the raw integrated heats in the presence of protein. The possibility exists that a fraction of the heat of dilution is due to interactions between Ca^{2+} and membrane. However, the association constant of divalent cation for acidic phospholipid is about 10 M^{-1}, so contributions from Ca^{2+} and membrane interactions are negligible [2]. Furthermore, the resulting heats of dilution were small and comparable to that observed previously.

For lipid titration experiments, the sample cell was filled with a solution consisting of 0.029mM annexin and 0.75mM Ca^{2+}, while the syringe was loaded with 250μL of 30mM total lipid (LUVs composed of a 60:40 mixture of POPC:POPS) and 0.75mM Ca^{2+}. Previous attempts of injecting only POPC containing lipid in the presence of calcium into a solution of annexin a5 saturated with calcium resulted in no detectable heat of binding. The use of the same Ca^{2+} concentration in both the cell and the syringe allowed the enthalpy of membrane binding to be measured with minimal contribution from Ca^{2+} binding. This Ca^{2+} concentration represents 74% saturation of annexin a5 by Ca^{2+} that corresponded to a specific free calcium ion concentration (see Equation 2 for K_{app}). Due to the high protein concentration necessary for signal, the very high Ca^{2+} levels that would correspond to near complete saturation would not be compatible with acidic-containing membrane. Precipitation does result. Lipid titrant was added in a 1μL injection to displace air from the syringe followed by 27 x 9μL injections. The first two data points were removed due to subsaturation of the protein by lipid (determined using the lipid binding affinity (K_L) as derived from Equation 2). For membrane titrations, raw heats were integrated and the heat of dilution subtracted by injecting the lipid and Ca^{2+} into buffer containing the same concentration of Ca^{2+} without protein. Also, a buffer-buffer titration was conducted as a control ensuring that interfering heats in the buffer were not present.

Between experiments, the sample cell was cleaned by rinsing 5 times with 250 mL of an aqueous solution of 15% Contrad-70 and 15% methanol, followed by 5 L of exhaustive rinsing with Milli-Q water to remove any contaminating lipids. Titration and injection syringes were rinsed with methanol, followed by extensive rinsing with de-ionized water to remove any protein or lipid contaminants. Syringes were dried under argon prior to storing.

Analysis of the ITC binding data was undertaken using a partition function approach. In each case, the relative likelihood of existing in any possible state of the protein was described according to the partition function stated in Equation 1. The overall heat of binding produced by a given distribution of ligated states can be modeled as the statistical likelihood of existing in each state (θ_i) multiplied by the response corresponding to each state, in this case heat of binding (ΔH_i), summed over all states. In an ITC experiment, however, the overall heat of binding is not measured; rather, it is the heat associated with shifting distributions of states from one set of conditions to another (i.e. changes in protein and ligand concentrations resulting from each injection). This heat of redistribution is simply the difference between the overall heats

associated with the distribution before and after an injection. In this way, the partition function approach is well suited for modeling binding equilibria monitored by ITC.

Each set of ITC experiments was repeated from 3-8 times under differing conditions. In order to determine the error inherent within the data itself, all replicate ITC experiments would need to be run under the exact same conditions and concentrations. Though repetition of the same titration using the exact same parameters is useful in determining error in the data, it does not ensure that the shape of the isotherm is not an artifact of the titration parameters. Thus we measured the binding curve over a range of relevant experimental parameters in order to optimize the binding isotherm. Once the data sets are normalized, their fits should yield the same results regardless of the experimental parameters used to achieve them as long as the isotherms reach saturation, and any competing equilibria are accounted for. As such the major component of the error reported here is the error associated with the fit.

It is also important to note that under some conditions we were not able to reach saturation. Given that all of the binding parameters fit by the model are dependent upon this, these titrations cannot be analyzed. However, the shape of these scans still overlaps with those that did reach saturation lending further support to our findings.

ITC Fitting:

FITTING PROTOCOL

To analyze the annexin a5 binding data, a stepwise approach was taken. This allowed the numerous parameters necessary to describe the system to be determined in a manner that allowed the fewest parameters to float during a given fit. This procedure was as follows:

1. The binding of Ca^{2+} in the absence of membrane was fit first. Note that in the absence of membrane ([L] = 0), the full partition function (Equation 1) simplifies to $Q = (1 + K_0[X])^n$. The results of this fit, n_0, K_0, and ΔH_0, were fixed for all other fitting routines.

2. The binding of Ca^{2+} in the presence of membrane was fit to the full partition function (Equation 1) in a piecewise, iterative fashion. It was assumed that the two trends in the data were due to two sets of Ca^{2+} binding sites, one endothermic with high affinity and one exothermic with low affinity. The expected exothermic heat of binding due to the low affinity sites was fit to determine n_{1b}, K_{1b}, and ΔH_{1b}. The heat contributed from these sites was then subtracted from the total measured signal. The small, yet significant heat of binding from annexin a5 in solution binding Ca^{2+} was accounted for using the fit parameters determined in Step 1. The remaining heat was then fit to the high affinity, endothermic set of sites to determine n_{1a}, K_{1a}, and ΔH_{1a}. An added assumption of conservation of sites ($n_{1a} + n_{1b} = n_0$) further constrained the fits for the POPC:POPS (60:40) data. This process was repeated for several iterations until the reported parameter values converged.

3. The binding of membrane in the presence of Ca^{2+} was fit last. Note that at constant high Ca^{2+} concentration, only two states would dominate (Ca^{2+} saturated annexin a5 in solution or Ca^{2+} saturated annexin a5 on the membrane). This is also reflected in the

overall partition function. For a given Ca^{2+} concentration, it would simplify to $Q = 1 + K_{app}[L]$ where the apparent affinity for membrane, K_{app}, would be constant (Equation 2).

ADDITIONAL CRITERIA FOR SELECTING A MODEL

The proposed binding model was arrived at by systematically fitting the presented data sets with binding models of increasing complexity (multiple sets of independent sites, sequential binding sites, and various combinations of each). One particular alternative model of note predicts that association of protein with membrane could be entirely reliant upon the protein being in a calcium ion ligated state. In such a sequential model, binding is ordered and would proceed from being bound to calcium ion in solution to being membrane associated [3]. If binding were to occur in this order, the expected heats would be of opposite sign to what was measured toward calcium ion in the presence of membrane. Regardless of this prediction, such models were tested against the data and shown to be insufficient to describe the binding data shown in Figure 4.

Additional ITC Experimental Results/Analysis:

To illustrate the limited window of experimental viability, the K_{app} was evaluated in the presence of 0.50, 0.75 and 1.00mM calcium ion (Figure S1 and Table S1). As annexin becomes increasingly saturated with calcium ion, the K_{app} increases. This is a consequence of the term $K_{app} = K_L\ (1 + K_{1a}[Ca^{2+}])^2\ (1 + K_{1b}[Ca^{2+}])^3 / (1 + K_0[Ca^{2+}])^5$ where $[Ca^{2+}]$ is the unbound or free calcium ion. When ITC data is presented as heat versus the total ligand/total protein ratio, the slope is a manifestation of the equilibrium constant. This slope (K_{app}) visibly increases as the protein approaches calcium ion saturation. However, as the system approaches saturation, the experimental sensitivity plummets, as seen in the abrupt change of slope that is itself characterized by few experimental data points. In contrast, with the sub-saturating calcium ion concentration of 0.5mM, a much more gradual slope is obtained and greater experimental sensitivity is achieved. But because the 0.5mM calcium ion concentration does not saturate the system, the $(1 + K_{1a}[Ca^{2+}])^2\ (1 + K_{1b}[Ca^{2+}])^3 / (1 + K_0[Ca^{2+}])^5$ ratio is not constant over the course of the lipid titration. This has a consequence such that, as lipid is added, the membrane-associated affinity for calcium ion is greater than the solution state calcium ion affinity. In this scenario, as lipid is titrated into the suspension of calcium ion and protein, the distribution of lipid-associated and solution state calcium ion binding sites will change, giving rise to a heat profile that has contributions from both lipid binding to calcium-bound annexin and calcium binding to annexin. This complicates the heat profile, as the measured heat is now a compilation of multiple binding events. To retain experimental sensitivity and limit overlap of different ligand binding heats, a compromising calcium ion concentration of 0.75mM was used. At this concentration, annexin saturation is 74% compared to 77% with 1mM calcium.

Table S1[1]**:** Thermodynamic parameters for annexin a5 titrations with 60:40 POPC:POPS and varying Ca^{2+}

	Calcium Ion Binding			Membrane Binding[2, 3]	
	Without Membrane	With Membrane, High Affinity	With Membrane, Low Affinity	With Saturating Ca^{2+} Present (0.75 mM)	With Saturating Ca^{2+} Present (1 mM)
N	5.0 ± 0.4	2.0 ± 0.1	3.0 ± 0.1	z = 46.5 ± 0.1	z = 47.5 ± 0.1
K (M^{-1})	3100 ± 300	410000 ± 120000	5800 ± 100	78000 ± 4000	790000 ± 185000
K_D (μM)	330 ± 40	2.4 ± 1.0	170 ± 10	13.0 ± 1.0	1.3 ± 1.0
ΔH (kcal/mol)	-2.4 ± 0.2	3.8 ± 0.2	-13.4 ± 0.4	-17.3 ± 0.1	-18.3 ± 0.1
$T\Delta S$ (kcal/mol)	2.2 ± 0.2	11.2 ± 0.3	-8.5 ± 0.4	-10.9 ± 0.1	-10.6 ± 0.1
ΔG (kcal/mol)	-4.6 ± 0.1	-7.4 ± 0.2	-4.9 ± 0.1	-6.4 ± 0.1	-7.7 ± 0.1

[1] Reported error represents 95% confidence intervals.

[2] The affinity for annexin to bind membrane in the absence of calcium ion (using the 0.75mM Ca^{2+} titration K_{app} value) was calculated to be K_L = (2.1 ± 0.3) x 10^1 M^{-1} ($K_{D,L}$ = 50 ± 20 mM).

[3] The affinity for annexin to bind membrane in the absence of calcium ion (using the 1mM Ca^{2+} titration K_{app} value) was calculated to be K_L = (16 ± 0.4) x 10^1 M^{-1} ($K_{D,L}$ = 6 ± 4 mM)[3]

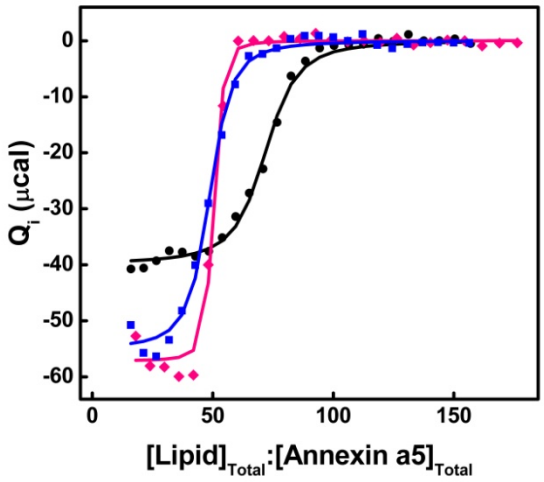

Figure S1: Results of the titration of 30 µM annexin a5 with lipid as LUVs made of a 60:40 mixture of POPC:POPS in the presence of 0.5mM Ca^{2+} (solid black circles), 0.75mM Ca^{2+} (solid blue squares), and 1mM Ca^{2+} (solid pink diamonds) at 15°C. Integrated heats of binding are displayed as a function of ligand to protein ratio.

Because K_{app} and K_L are directly proportional, to define K_L, we then varied K_L to define the range over which K_{app} described the data. The range over which K_L could vary and thus still globally describe the binding data in the presence of lipid is shown in Figure S2. In order to accurately capture the inflection point of the binding curve (Figure S2) in addition to the nuances of the inset regions highlighted, correct calculation of the K_L value within a statistical range is necessary. The sensitivity of the K_L value is illustrated by the two additional K_L values (shown in red and light blue respectively) used to simulate the additional fits shown. The inflection point of the curve is reasonably satisfied by all K_L values shown (including the additional fits). However, the insets clearly emphasize the slight variation of the additional K_L values' fits with respect to the integrated raw data. It is evident that the K_L range that describes the data is limited.

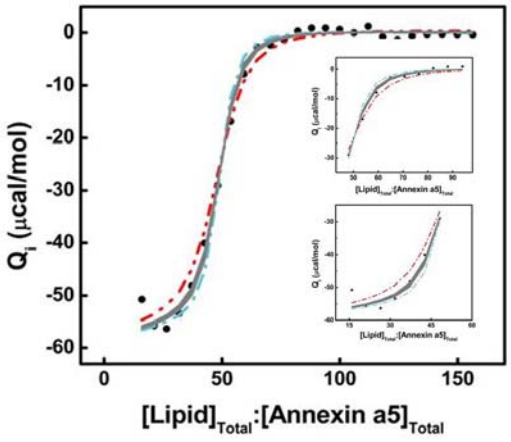

Figure S2: Results of the titration of 30 µM annexin a5 with lipid as LUVs made of a 60:40 mixture of POPC:POPS in the presence of 0.75mM Ca^{2+} (solid black circles). The solid grey line depicts the range of the calculated K_L value with 95% confidence. The simulated fits of calculated K_L values of 10 M^{-1} (dashed red line) and 35 M^{-1} (dashed light blue line) are shown to display the narrow experimental window of K_L values that capture the integrated heats of binding. Both inset graphs highlight the difference in fit with varying K_L values (dashed red and dashed light blue lines) in comparison to the 95% confidence interval of the calculated fit which is $K_L = (2.1 \pm 0.3) \times 10^1$ M^{-1}(solid grey line).

As a final test of our fitted values of enthalpy (ΔH), association constants (K) and number of binding sites (n) for the thermodynamic cycle, the fractional distribution of states at each point in the experiments were multiplied by the associated heats and overlaid with the data (Figure S3).

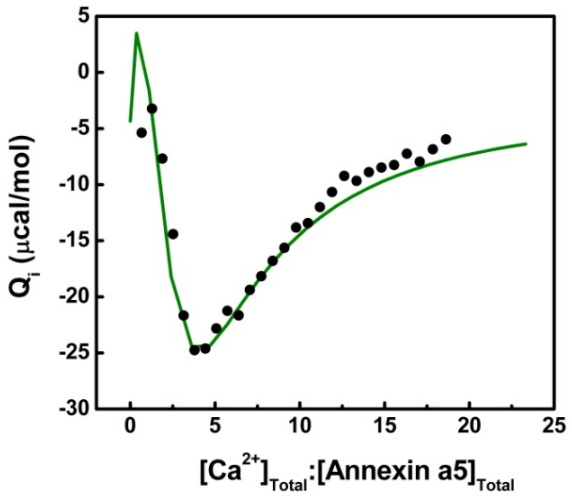

Figure S3: Calculation of heats through the use of partition functions and the fit parameters of enthalpy, equilibrium constants and binding stoichiometries simulating the integrated heats obtained by the titration depicted in Figure 4. The thermodynamic fit parameters including the enthalpies (ΔH), association constants (K), and number of binding sites (n_{1a}, n_{1b}, and n_o) were multiplied by the corresponding fractional distribution of states at each injection of the titration. These total heats for each state were then summed and the change in total heat between each point in the titration was determined. The integrated heats of binding as a function of ligand to protein ratio from Figure 4 (solid black circles) are overlaid with the total heats simulated by the heat partition function (green line).

Carboxyfluorescein Assay to Evaluate Potential Membrane Disruption upon Annexin Binding:

As a control, to verify that the measured heats from each titration were due to binding and not to disruption of the membrane, carboxyfluorescein efflux studies were conducted as in Gauer et.al [4]. Disruption of the membrane upon annexin binding will correlate with a fluorescent increase as CF dye is released from the liposomes. CF is a self-quenching dye and has a relatively low fluorescent signal when encapsulated in liposomes due to its high concentration. Upon release of the dye from the liposomes, the diluted dye has a much higher fluorescent signal compared to the encapsulated signal. Carboxyfluorescein-containing LUVs were measured for fluorescent efflux as the temperature of the system was lowered from 20˚C to 1.1˚C. As the temperature is lowered and the temperature approaches the phase transition of the lipid mixture, membrane disruption is enhanced. A change in efflux of the CF from the LUVs in the presence of annexin with respect to the control in the absence of annexin, would imply ability of annexin to impact membrane permeability.

The lowest efflux of CF and thus the lowest permeability of the membrane occur when the LUVs are incubated in the presence of both annexin a5 and Ca^{2+} (Fig S4). However, a slight decrease in permeability still occurs when annexin a5 is incubated with LUVs in the absence of Ca^{2+}. This slight drop in the magnitude of the efflux of CF in the annexin-LUV scans, compared to the LUV-only control acts to further illustrate the weak membrane binding affinity of annexin in the absence of Ca^{2+} that was unable to be directly measured via ITC due to its athermal profile. Thus, the larger drop in efflux of the annexin-LUV-Ca^{2+} scans compared to the LUV-Ca^{2+}-only scans is consistent with the enhanced binding affinity of annexin in the presence of Ca^{2+}. Additionally the apparent decrease in CF permeability of the LUVs in the presence of Ca^{2+} without annexin

implies membrane rearrangement upon Ca^{2+} binding that can also be seen in the shape of the heat of dilution for Figure 4. We conclude that annexin does not disrupt the membrane upon binding, and in fact, appears to reduce membrane permeability as also recently shown in Creutz et.al [5].

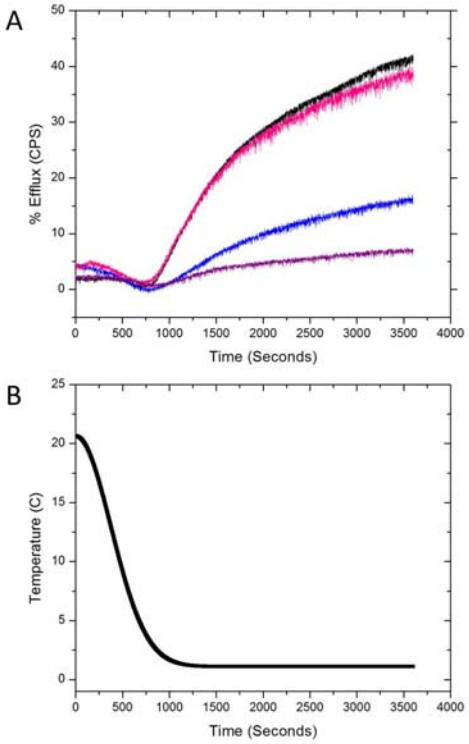

Figure S4: The binding of annexin a5 in the presence of calcium ion shows a decrease in efflux of CF compared to lipid alone. (A) Percent fluorescence signal change of CF leaking from POPC:POPS (60:40) lipid vesicles upon cooling over 60 minutes. All samples contained 333 μM lipid and all plots are an average of three trials. Black (top): lipid alone; blue (2nd from bottom): lipid and Ca^{2+}; pink (2nd from top): lipid and Annexin a5; purple (bottom): lipid, Annexin a5, and Ca^{2+}. (B) Temperature change within the cuvette over 60 minutes with the water bath set to decrease from 20°C to -3°C. The baseline temperature achieved within the cuvette was 1.1°C.

Supporting References:

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