AD_____

Award Number: W81XWH-10-1-0777

TITLE: Repair of Nerve Cell Membrane Damage by Calcium-Dependent, Membrane-Binding Proteins

PRINCIPAL INVESTIGATOR: Carl E. Creutz, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22903

REPORT DATE: September 2012

TYPE OF REPORT: Revised Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
September 2012	Revised Annual	1 September 2011 - 31 August 2012

4. TITLE AND SUBTITLE
Repair of Nerve Cell Membrane Damage by Calcium-Dependent, Membrane-Binding Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0777

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carl E. Creutz

E-Mail: creutz@virginia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, VA 22903

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the second year of this contract calcium-dependent, membrane-binding proteins of the annexin class were found to be effective at preventing damage and repairing damage to membranes caused by osmotic shock or other chemical and physical stresses using an in vitro assay involving synthetic membrane vesicles. These proteins may therefore be promising agents for protecting or promoting the repair of cell membranes in the case of spinal cord injury.

15. SUBJECT TERMS
Spinal cord injury, membrane repair

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

In acute spinal cord injury the plasma membranes of spinal neurons are torn allowing high concentrations of calcium to enter the cytoplasm, activating proteolytic cascades and leading to neuronal cell death. Membrane repair mechanisms have evolved that protect cells from this type of damage by repairing the cell membrane as soon as the increase in intracellular calcium is sensed by calcium-binding proteins. If these repair mechanisms can be strengthened either before or after spinal cord injury it may be possible to reduce cell damage resulting from the injury. In this project we are testing the hypothesis that the action of copine, a human calcium-dependent-membrane-binding protein, in model systems can promote a stable repair of broken membranes that could preserve cell viability. Preliminary data obtained using a novel imaging technology, atomic force microscopy, suggested that calcium-dependent, membrane-binding proteins of the copine class can repair membranes through direct binding to the edges of torn membranes and promoting sealing of the edges.

BODY OF REPORT

Research accomplishments associated with tasks (Aims) described in the statement of work.

In the previous annual report progress towards accomplishment of the first four Specific Aims was described. In the current year we have focused on continuing the fourth Specific Aim: developing and using a liposome model for membrane damage caused by osmotic shock or other physical and chemical stresses and the ability of calcium-dependent, membrane-binding proteins to protect or repair the liposome membrane. These studies demonstrated that annexins were the most effective proteins of this type in promoting protection and repair and therefore became the focus of research. This Aim is now essentially completed and the results are detailed in the sections below.

Methods used to establish a liposome model for membrane damage and repair

Unilamellar CF-loaded liposomes were prepared by extrusion through 100 nm Nuclepore polycarbonate filters using an extruder from Avanti Polar Lipids [1,2]. The lipids were mixed in appropriate ratios from stocks in chloroform, dried under a stream of argon and overlaid with a solution of 100 mM CF adjusted to pH 7.4 with NaOH. After extrusion the liposomes were separated from free dye on a Sephadex G-25 column equilibrated in 100 mM KCL, 50 mM HEPES-NaOH, pH 7.4.

Measurement of the dequenching of CF upon release from the liposomes as initially described by Weinstein and colleagues [3,4] was performed in a SPEX Fluorolog 111c spectrofluorometer with excitation at 495 nm and emission at 525 nm. Samples were incubated in a 5 by 5 mm quartz cuvette in a volume of 300 µl at 37 degrees C in 100 mM KCl, 50 mM HEPES-NaOH pH 7.4, 1 mM $CaCl_2$ ("Assay Buffer"). Fluorescence intensity was recorded as counts per second (cps) which represents actual photomultiplier counts per second divided by the value of the current from the reference detector (0.01 µA). Experiments were initiated by adding 10 µl of a liposome suspension containing 1 mg per ml of lipids to the final volume of 300 µl (final lipid concentration 33 µg/ml). Total releasable CF was determined after experiments by adding 10 µl 10% Triton X-100 and typically produced a fluorescence intensity of 0.5 to 1.0 X 10^8 cps. The initial percentage of free CF in the vesicle preparations was typically 5% to 10% of the total CF and experiments were designed so that not more than 50% of the CF was released during the time course of observation. For critical titrations and the osmotic shock experiments the Triton X-100 intensity values were used to normalize the data.

For the osmotic shock experiments 10 µl of the 1mg/ml vesicle suspension was incubated in the bottom of the fluorometer cuvette with 5 ul 100mM KCl 25 mM HEPES-NaOH, 1mM EGTA (FPLC fraction buffer) containing various amounts of annexin protein and 3 µl of 7.5 mM $CaCl_2$ (final free Ca^{2+}

concentration 1.0 mM) and then diluted with Assay Buffer containing 3.5 or 5 mM $MgCl_2$ or the same buffer without KCl to remove osmotic support.

Measurements of the turbidity of vesicle suspensions were performed at 350 nm in a Beckman DU7 recording spectrophotometer in a volume of 1 ml.

Recombinant human annexin A5[5] and A6[6] were prepared by expression in yeast and isolation by calcium-dependent binding to multilamellar liposomes prepared from bovine brain Folch Fraction I lipids (Sigma-Aldrich) and ion exchange chromatography on Poros Q medium using a Pharmacia FPLC system[5].

Strategy for establishing the membrane leakage model

The strategy employed in these studies was to encapsulate carboxyfluorescein (CF) in liposomes at a self-quenching concentration and to monitor leakage by continuous measurement of the fluorescence increase associated with CF leakage and dequenching. The liposomes were monitored for "baseline" leakage and were exposed to a number of agents expected to perturb the membrane permeability barrier. Annexins were added to the exterior medium in the presence of Ca (1 mM) to promote binding of the annexin to the liposome in order to assess the effects this had on membrane permeation of CF. Since CF is a large (molecular weight 376 Da), negatively charged compound it does not readily pass through the bilayer and the leakage reflects significant disruption to bilayer structure.

Most members of the annexin family exhibit a "bivalent" activity resulting in the aggregation of membranes coincident with the binding of the annexin to the membrane [7,8]. Such membrane aggregation could potentially make the leakage data more complicated to interpret. The free liposome surface area exposed to the external medium would be reduced and therefore an annexin that promoted membrane aggregation might appear to reduce leakage for this reason. Alternatively, membrane-membrane aggregation might be associated with distortion of the liposome geometry introducing regions of high curvature that might be more permeable, thus leading to greater leakage. In addition, in the presence of some of the membrane perturbants used in this study, membrane-membrane aggregation caused by the annexin might be followed by membrane fusion [9] and such fusion may be associated with a transient increase in membrane permeability. For these reasons the studies described here focused on annexins A5 and A6 since they do not promote membrane aggregation. To confirm that these annexins do not aggregate membranes in the conditions of the experiments the turbidity of the vesicle suspensions (absorbance at 350nm) was measured and found to be stable during the time course of the experiments, except in certain cases as described in the sections below. In contrast, in control experiments with annexin A1, which does promote membrane aggregation, the turbidity of the vesicle suspension increased 3 to 4 fold during the same time period.

The liposomes used for these studies were prepared from a mixture of lipids in order to reflect the complexity of lipids in the cytoplasmic leaflet of the plasma membrane: PS, PC, PE, cholesterol in a ratio of 1:1:1:1 by weight (approximately 1:1:1:2 molar ratio). For each condition that was examined in the sections below, titrations of critical parameters were performed in order to establish conditions under which sustained leakage of CF could be observed, compatible with the time course of the hand-mixing experimental techniques. The concentration of Mg^{2+} was found to have a significant influence on the rate of CF leakage from these negatively charged liposomes and on the apparent ability of some of the agents used to permeabilize the membranes. Standard Mg^{2+} concentrations of 1, 3.5, and 5 mM were tested. For different perturbing agents a single Mg^{2+} concentration was typically selected that provided a significant, sustainable leak during the course of the experiments. Calcium was tested at the single concentration of 1 mM, reflecting the high levels of calcium that might be anticipated at a site of damage at the plasma membrane of a cell (or the membrane of a calcium-containing organelle). In some cases high

concentrations of the added permeabilized agents promoted vesicle aggregation, therefore the leakage measurements were limited to lower concentrations of the agents at which this was not detected in turbidity measurements. Some poorly soluble agents were added in ethanol or DMSO as solvent. The amounts of these solvents were kept to a minimum to reduce leakage due to the solvent. Leakage rates due to the solvent are reported in the experiments below if they were above baseline leakage in the absence of solvent.

Effects of annexins on baseline leakage of CF from liposomes

When incubated at 37 degrees C in 100 mM KCl, 50 mM HEPES-NaOH pH 7.4, 1 mM $CaCl_2$ ("Assay Buffer"), the liposomes exhibited a "baseline" leakage of CF that was enhanced by increasing the concentration of Mg^{2+} (Figure 1). When the liposomes were added to the cuvette with the annexin already present in solution in the cuvette the slopes of the CF release curves were analyzed after one to two min (Figure 1A). At 1 mM Mg^{2+} leakage was low and only slightly affected by the binding of either annexin which caused a 0 to 10% decrease in slope during the time course of the experiment (up to 20 min). At 3.5 mM Mg, binding of annexin A5 resulted in a very significant 43% reduction in leakage (Figure 1A) and annexin A6 a more modest 12% reduction in leakage. At 5 mM Mg^{2+}annexin A5 caused a 42% reduction in slope; the effect of annexin A6 was not determined at this Mg^{2+} concentration. When the liposomes were preincubated in the cuvette first and then the annexin was added it was possible to observe an initial burst of release of CF apparently caused by the initial binding of the annexin to the liposome membrane (Figure 1B). Subsequent to this event, the rate of release was reduced by the presence of the annexin. In the experiment illustrated in Figure 1B annexin A5 reduced the rate of release 11% at 1 mM Mg, 65% at 3.5 mM Mg, and 84% at 5 mM Mg. The size of the initial burst of CF release caused by the binding of the annexin, as seen in the figure, was larger in the presence of higher Mg^{2+} concentrations: This burst of CF release in 3.5 mM Mg^{2+} was 8 times higher than at 1 mM Mg^{2+}, and 12 times higher at 5 mM Mg^{2+}than at 1 mM Mg^{2+}.

When liposomes were added to Assay Buffer containing very high concentrations of divalent cations (10 mM Ca and 10 mM Mg^{2+} together) the liposomes rapidly aggregated giving a large increase in turbidity (absorbance at 350 nm) of 300% in 6 min and the CF was completely released during the first minute. At 1 mM Mg, 1 mM Ca no turbidity increase was seen. However, at both 3.5 and 5 mM Mg^{2+} in the presence of 1 mM Ca a small increase in turbidity was detectable (initial rate .026% per sec, compared to 10% per second in the presence of 10 mM Ca, 10 mM Mg) suggesting a slow process of vesicle aggregation may have been occurring, although other changes in membrane organization such as divalent cation-induced lipid phase separation may also have contributed to this small increase in turbidity. To determine whether the increased rate of baseline CF efflux at the higher Mg^{2+} concentrations was dependent upon vesicle-vesicle aggregation, the release rate was analyzed as a function of the vesicle concentration, anticipating that the rate would be second order relative to vesicle concentration if vesicle-vesicle interaction was required to promote the release of CF. However, with 5 mM Mg^{2+}, 1 mM Ca in the buffer, doubling the vesicle concentration resulted in an increase in the rate of release of a factor of 1.91+/- 0.15 (average of two independent experiments) suggesting a first order dependence on the vesicle concentration. The higher Mg^{2+} concentrations (3.5 and 5 mM) therefore appeared to be directly influencing the intrinsic permeability of the liposome membranes and the protective effect of the annexins was evidently not due to suppression of vesicle aggregation.

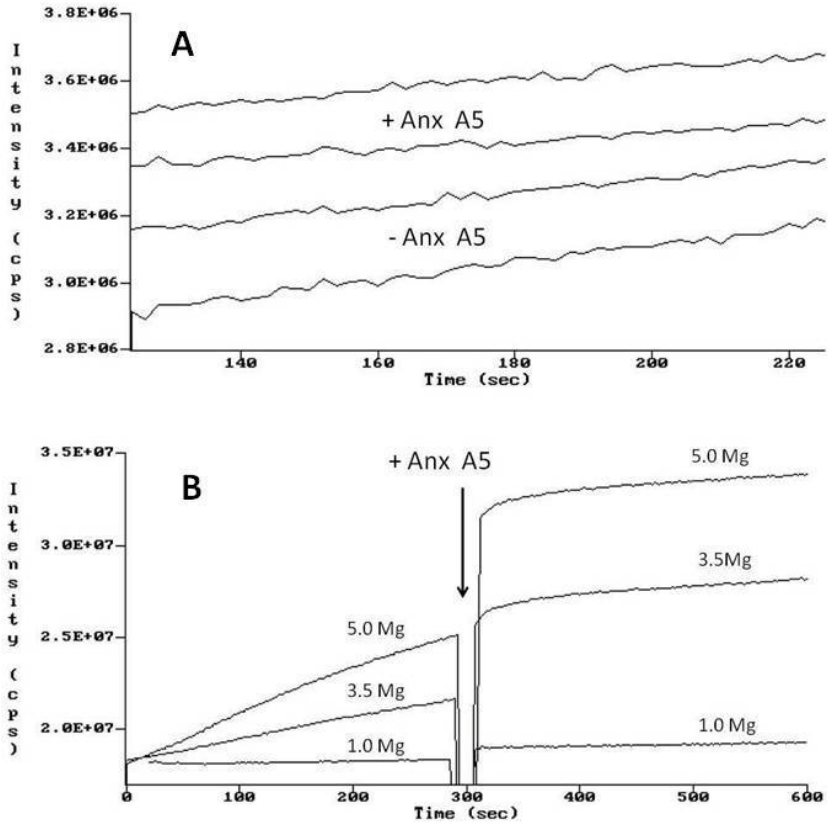


Figure 1: Reduction of baseline leakage of CF from liposomes by annexin A5. ***Part A****: Duplicate samples of liposomes (33µg lipid/ml) in the presence or absence of 4.8 µg/ml annexin A5 were incubated in Assay Buffer with 3.5 mM $MgCl_2$. Fluorescence intensity (cps – photomultiplier counts per second) is plotted as a function of time. Leakage (slope of the fluorescence trace) from samples without annexin is 2480 +/- 110 cps/sec, and with annexin A5 is 1420 +/- 160 cps/sec (a 43% reduction in slope due to the annexin). The individual traces have been translated along the vertical axis to separate them for clarity. Maximum fluorescence after adding Triton X-100 was 7.41 X 10^7 cps.* ***Part B****: Addition of annexin A5 to liposomes undergoing baseline leakage causes a burst of CF release after which the leakage rate is reduced. Liposomes were incubated in Assay Buffer with 1, 3.5, or 5 mM $MgCl_2$ as indicated. The annexin (4.50 µg/ml) was added at the arrow, at which point the fluorescence signal is lost during closure of the fluorometer shutters. Maximum fluorescence after adding Triton X-100 was 1.01 X 10^8 cps.*

Effects of annexins on disruption of the membrane permeability barrier by arachidonic acid

Arachidonic acid is an important precursor for lipidic signaling molecules and is liberated by phospholipases from membrane phospholipids. As seen in Figure 2 when free arachidonic acid was added in ethanol as a vehicle it caused an increase in the liposome leakage rate (3.5 fold increase in slope). An initial burst of release of CF occurred when the arachidonate was added, but a burst of similar magnitude also occurred with an ethanol control and so may be due to a transient action of ethanol on a small number of vesicles before it is diluted. After this initial burst there was no increase in leakage due to the ethanol alone above the initial baseline rate. When the ethanol/arachidonate stock mixture was diluted three fold with Assay Buffer immediately before adding the arachidonate, there was no increase in slope due to the arachidonate and the initial burst was also almost eliminated. Therefore, it appears that if the arachidonate is allowed to form micelles in buffer before addition to the liposomes its transfer to the liposome membrane may be blocked on the time scale of the experiment.

When the liposomes were pre-incubated with 4.8µg/ml annexin A5 the leakage rate due to the addition of arachidonate was strongly suppressed as shown in Figure 2A. Similarly, when the same amount of annexin A5 was added after the arachidonate, it caused an initial burst of release of CF then it strongly inhibited the leak reducing it to a rate similar to that seen before addition of the fatty acid (Figure 2B). Annexin A6, however, added before the arachidonate at a similar concentration (4.9 µg/ml) caused an enhancement of the leakage due to arachidonic acid by 24%. At a two-fold higher concentration of annexin A6 (9.8 µg/ml) this stimulating effect on leakage was lost, but no protection from the permeability loss due to arachidonate was observed with annexin A6 at any concentration of protein. If added after the arachidonate, 4.9 µg/ml annexin A6 also caused an enhancement of the CF release rate by 38%.

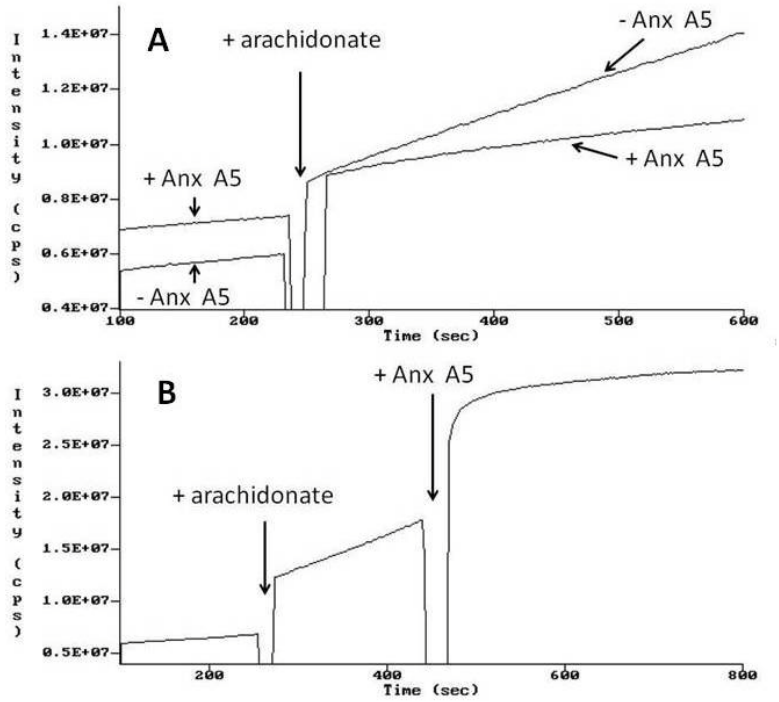


Figure 2: Annexin A5 protects membranes from leakage due to arachidonic acid. ***Part A****: Liposomes were preincubated in Assay Buffer with 5 mM $MgCl_2$ ("-* ***Anx A5****"). At 250 sec 2 µl of 0.4 mg/ml arachidonic acid dissolved in ethanol was added giving a final arachidonic acid concentration of 2.7 µg/ml ("+* ***arachidonate****"). This resulted in a burst of release of CF followed by continued leakage of CF at an increased rate as shown. When the liposomes were pre-incubated with 4.8 µg/ml A5 ("+* ***Anx A5****") the initial burst of CF release and the subsequent increase in release rate were inhibited as shown.* ***Part B****: Liposomes were pre-incubated in the absence of annexin A5. At 250 seconds 2.7 µg/ml arachidonic acid was added causing an increase in the rate of CF release. At 450 seconds 4.8 µg/ml annexin A5 was added which resulted in a burst of CF release followed by a suppression of the release rate to a level comparable to the initial rate.*

Effects of annexins on disruption of the membrane permeability barrier by lysophosphatidic acid (LPA)

Lysolipids such as lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) are generated by the action of phospholipase A2 in the process of liberating arachidonic acid as a precursor to prostaglandin and leukotriene signaling molecules. Both agents have significant detergent-like properties. As illustrated in Figure 3A the addition of 1-palmitoyl LPA to liposomes promoted an initial burst of release of CF followed by a high rate of continued release. Similar to its action with arachidonic acid, annexin A5

was found to virtually completely protect the liposomes from this leakage, whether added before or after LPA (Figure 3A,B).

In contrast to the results with annexin A5, when annexin A6 under these conditions was bound to the liposomes it caused a 3.6 fold greater initial burst of CF release when the LPA was added, and a 2.8 fold elevated leakage rate subsequent to the initial burst. When the annexin A6 was added after the LPA it also caused a slight increase (1.2 fold) in the release rate.

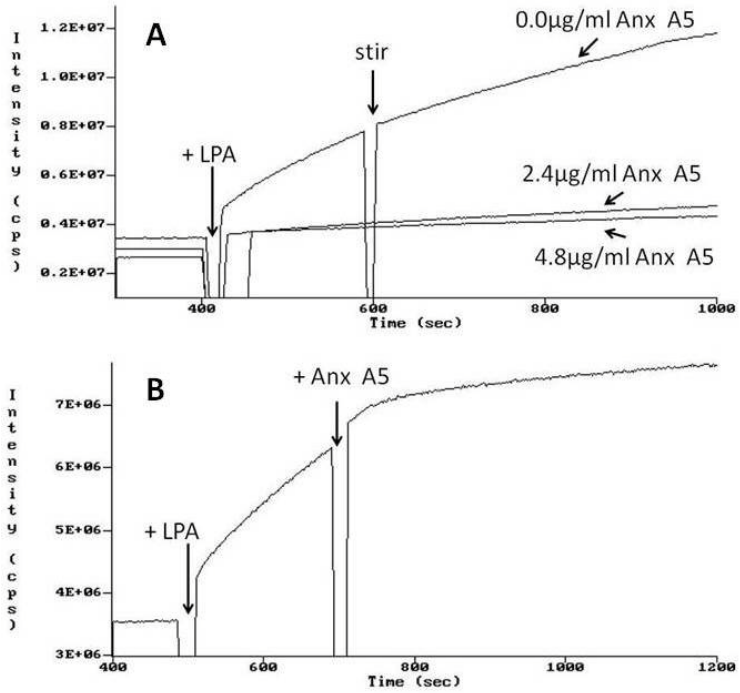


Figure 3: Annexin A5 inhibits the increase in CF leakage from liposomes due to addition of 1-palmitoyl lysophosphatidic acid (LPA). ***Part A****: LPA in 10 µl water was added to liposomes in Assay Buffer with 1 mM $MgCl_2$ at 450 seconds ("****+LPA****", final LPA concentration 6.7 µg/ml) causing an increase in the rate of CF release. When the liposomes were preincubated with 2.4 or 4.8 µg/ml annexin A5, as marked, both the intial burst of CF release as well as the subsequent increase in release due to LPA were inhibited. At 600 seconds the LPA alone sample was stirred ("****stir****") as a control for the effects of stirring (compare with the annexin addition at 700 seconds in Part B).* ***Part B****: After addition of 6.7 µg/ml LPA at 500 seconds ("****+LPA****"), 4.8 µg/ml of annexin A5 was added at 700 seconds ("****+ Anx A5****") which reduced the rate of CF leakage.*

Effects of annexins on disruption of the membrane permeability barrier by lysophosphatidylcholine (LPC)

In contrast to LPA, LPC is zwitterionic and has a net neutral charge. It has been studied extensively for its effects on model membrane structure and has been attributed roles in membrane permeabilization and membrane fusion. In these experiments the addition of 1-oleoyl LPC in aqueous buffer or ethanol had only modest effects on liposome permeability at 1 mM Mg^{2+} concentration, acting to slightly enhance leakage (Figure 4A). At higher Mg^{2+} concentrations it appeared that larger amounts of LPC were able to interact with and/or enter the lipid bilayer. The initial interaction was associated with a large and rapid release of CF followed by stabilization of the membrane as CF permeability was then reduced below the original baseline (Figure 4A). This may have been due to a reduction of the surface charge of the bilayer as the neutral lipid was incorporated and to an alteration of lipid domain structure.

In order to establish a model for determining whether the annexins could protect against membrane permeabilization by LPC, attention was therefore focused on experiments with the lower concentration of Mg^{2+} (1 mM) in which the addition of LPC enhanced membrane leakage rather than reducing it. When pre-associated with the liposomes, both annexins A5 and A6 reduced the initial burst of CF release associated with mixing LPC with the liposomes, and caused a 44% (A5) or 38% (A6) reduction in the leakage rate (Figure 4B). If the annexins were added after the LPC, the leakage rate was reduced by 28% by annexin A5 and 51% by annexin A6.

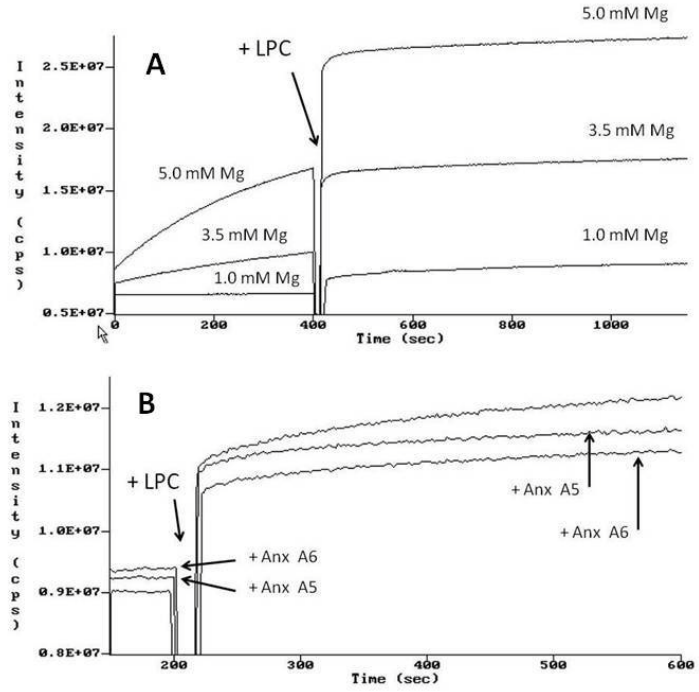


Figure 4: Effects of 1-oleoyl lysophosphatidylcholine (LPC) and annexins on liposome permeability. ***Part A****: LPC reduces liposome permeability at high magnesium concentrations. Liposomes were incubated in Assay Buffer with 1.0, 3.5, or 5.0 mM $MgCl_2$ as marked. At 400 sec LPC in 5 µl of water was added to give a final concentration of 16.7 µg/ml ("**+LPC**"). As shown, CF release was increased by LPC at 1 mM $MgCl_2$ but reduced at the higher levels of $MgCl_2$.* ***Part B****: Annexins A5 and A6 reduce the leakage of CF from liposomes caused by the addition of LPC. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$ in the absence of annexin or in the presence of 4.5 µg/ml annexin A5 ("**+Anx A5**") or 4.3 µg/ml annexin A6 ("**+Anx A6**"). At 200 sec LPC was added to a final concentration of 8.4 µg/ml ("**+LPC**").*

Effects of annexins on disruption of the membrane permeability barrier by diacylglycerol

Diacylglycerols are important intermediates in the biosynthesis and degradation of triglycerides, glycerophospholipids, and glyceroglycolipids and are known to act as second messengers in cell signaling through the activation of protein kinase C. Diacylglycerol has been found to cause alterations of membrane curvature, modification of surface charge, and promotion of bilayer to nonbilayer phase transitions. The addition of diacylglycerol (diolein) to the liposomes from an ethanol stock in the presence of 3.5 mM Mg^{2+} caused a significant, sustained leakage of CF (Figure 5). When annexin A5 was prebound to the liposomes it effectively blocked the increase in the release rate of CF due to the diacylglycerol after an initial burst of release that may have been due in part to the ethanol vehicle (Figure 5A). When the diacylglycerol was added first, the annexin A5 was effective in blocking the diacylglycerol-dependent leak, although the initial interaction of the annexin with the membrane caused a small burst of CF release before the release rate was returned to baseline levels (Figure 5B).

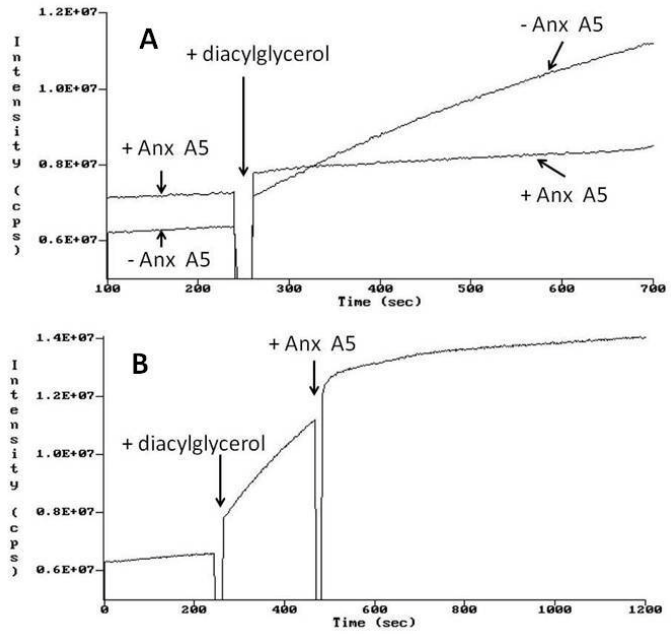


Figure 5: Annexin A5 inhibits membrane leakage induced by diacylglycerol (diolein). Liposomes were incubated in Assay Buffer with 3.5 mM $MgCl_2$. ***Part A****: At 250 sec diacylglycerol dissolved in 3µl of ethanol was added to give a final concentration of 1.0 µg/ml ("**+diacylglycerol**"). In one sample the vesicles were preincubated with 4.8 µg/ml of annexin A5 ("**+ Anx A5**") which blocked the increase in the rate of leakage after the initial burst of CF release that occurred when the diacylglycerol and ethanol were added.* ***Part B****: At 250 sec diacylglycerol was added as in part A to liposomes incubated in the absence of annexin. At 450 seconds annexin A5 was added to a final concentration of 4.5 µg/ml ("**+ Anx A5**"). After a burst of release of CF associated with the binding of the annexin to the liposomes, the subsequent rate of CF leakage was reduced to the baseline release rate in the absence of diacylglycerol.*

In contrast, annexin A6 at all levels tested caused an enhancement of the release due to diacylglycerol, although the effect was biphasic in that increasing the annexin A6 increased the release rate up to an annexin concentration of 2.5 µg/ml, but a lower release rate was promoted by 4.9 µg/ml annexin (Figure 6).

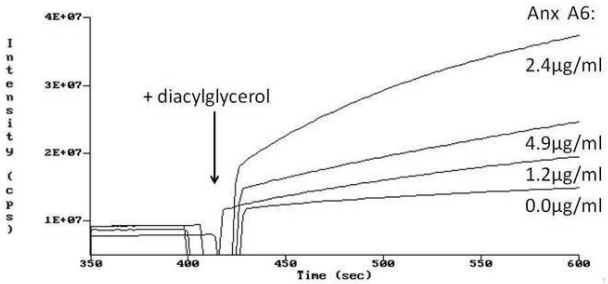


*Figure 6: Annexin A6 pre-bound to the liposomes enhances the leakage due to the addition of diacylglycerol (diolein) in a biphasic manner. Liposomes were pre-incubated with annexin A6 at the concentrations indicated. At 400 sec 3µl of diacylglycerol stock solution in ethanol was added to give a final diacylglycerol concentration of 1.0 µg/ml ("**+diacylglycerol**").*

Effects of annexins on disruption of the membrane permeability barrier by monoacylglycerol

Monoacylglycerols are generated through lipase action on triglycerides, and are also present as specific endocannabinoids such as 2-arachidonoylglycerol which function as signaling molecules. Similar to the

affects of annexins on diacylglycerol treated liposomes, annexin A5 slightly inhibited CF leakage due to monoacylglycerol (monolein) and annexin A6 exacerbated the leakage due to monoacylglycerol (Figure 7).

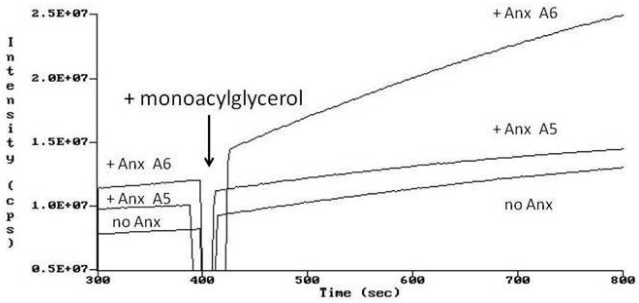


*Figure 7: Effects of annexins A5 and A6 on membrane permeabilization by monoacylglycerol. Liposomes were incubated in Assay Buffer with 3.5 mM $MgCl_2$. At 400 seconds monoacylglycerol (monolein) was added in 5µl of ethanol to a final concentration of 5.0 µg/ml ("**+monoacylglycerol**"). Preincubation of the liposomes with 4.5 µg/ml annexin A5 ("**+Anx A5**") reduced leakage, and with 4.3 µg/ml annexin A6 ("**+Anx A6**") increased leakage compared to monoacylglycerol alone ("**no Anx**").*

Effects of annexins on disruption of the membrane permeability barrier by spermidine.

The naturally occurring polyamines spermine, spermidine, and putrescine are polycations that are found at levels as high as millimolar in many cell types. Because of their cationic character polyamines bind to nucleic acids and also interact with anionic phospholipids in cell membranes. Some effects of polyamines on membrane properties have been described including membrane stabilization against osmotic stress, changes in membrane fluidity, changes in electrical conductivity, and effects on divalent cation-induced fusion of liposomes [10,11].

Annexin A5 pre-bound to the liposomes was highly effective at preventing leakage due to 2 mM spermidine in the presence of 1 mM Mg, reducing leakage to baseline levels at 4.5 µg/ml annexin and inhibiting leakage 50% at between 0.3 and 0.6 µg/ml (Figure 8A and Table 1). When annexin A5 was added after the spermidine it also suppressed leakage to baseline levels, but only after causing an abrupt and significant burst of CF release during the binding of the annexin to the liposomes (Figure 8B). This initial burst of CF release due to the initial binding of the annexin was much greater than seen with liposomes in the absence of spermidine at 1 mM Mg^{2+} (compare with Figure 1B).

In similar experiments annexin A6 also reduced the leakage due to spermidine in the presence of 1 mM Mg, although it was less effective. If added before 2 mM spermidine at a concentration of 4.3 µg/ml it reduced the leak due to spermidine by 59.2% and if added after the spermidine at the same concentration it reduced the leak by 57.1%. The binding of annexin A6 to the liposomes did not cause the significant burst of release of CF as seen with annexin A5 in Figure 8B.

The addition of 2 mM spermidine under these conditions (1 mM Mg^{2+}) was found to cause a slow increase of the turbidity of the liposome suspension at a rate of .05% per sec relative to the initial turbidity of the vesicle suspension. This rate of turbidity increase was 200 fold less than the rate of turbidity increase seen when the vesicles were incubated in a mixture of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (see section above on baseline release rates). This slow turbidity increase may have been due to vesicle aggregation caused by interaction of the positively charged spermidine with the negatively charged vesicles, or possibly reorganization of lipid domains in the membrane. In order to determine if vesicle aggregation may have contributed to the release of CF two independent experiments with different vesicle preparations were performed to determine the dependence of the rate of CF leakage upon vesicle concentration, similar to the strategy used to check for vesicle concentration effects on baseline leakage.

Doubling the amount of vesicles in the assays resulted in a 2.08 +/- 0.61 fold increase in release rate of CF indicating a first order dependence of the release rate on vesicle concentration. This suggests the spermidine was acting directly to alter the intrinsic permeability of the membranes independent of vesicle aggregation and the protective effect of the annexins was therefore unlikely due to the suppression of vesicle aggregation.

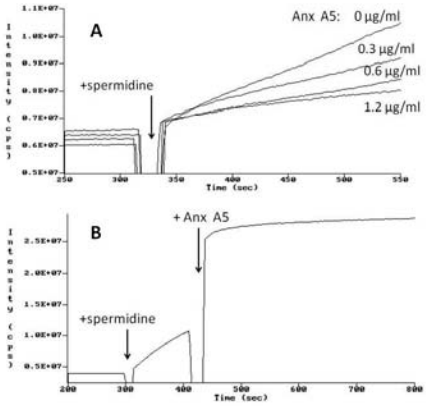


Figure 8: Annexin A5 suppresses CF leakage due to spermidine. ***Part A****: Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$ and with the concentrations of annexin A5 indicated. At 325 sec spermidine in water was added to a final concentration of 2 mM ("+**spermidine**"). Slopes of the traces after spermidine addition are given in Table 1.* ***Part B****: Addition of annexin A5 after spermidine caused a burst of CF release then stabilized the membrane leak. Spermidine was added at 300 sec to a final concentration of 2 mM ("+**spermidine**") . Annexin A5 was added at 425 sec to a final concentration of 1.9 µg/ml ("+**Anx A5**").*

Table 1: Effects of annexins A5 and A6 on the rates of release from liposomes treated with spermidine[a]

Annexin A5	Normalized Slope	Annexin A6	Normalized Slope
0.0 µg/ml	100	0.0 µg/ml	100
0.3 µg/ml	57.4	4.3 µg/ml	40.8
0.6 µg/ml	41.5		
1.2 µg/ml	28.4		
4.8 µg/ml	1.9		
0.0, µg/ml, no spermidine	1.5		

[a]Spermidine concentration was 2 mM, other conditions as described under materials and methods and the legend to Figure 8. The slopes of the fluorescence versus time curves were normalized to the value in the presence of spermidine but absence of annexins.

Effects of annexins on disruption of the membrane permeability barrier by amyloid-beta peptide 1-42 (A beta)

Alzheimer's disease is characterized by the buildup of fragments of the amyloid precursor protein, a neuronal plasma membrane protein, in extracellular spaces in the brain [12]. Soluble oligomers of the C-terminal fragment, amyloid-beta, or A-beta, have been demonstrated to be toxic to neurons so it has been hypothesized that A-beta contributes to the pathology of the disease [13]. A body of evidence suggests A-beta exerts its toxic effects through damaging the neuronal cell membrane and allowing excess calcium to enter nerve cells [13,14,15].

As shown in Figure 9, A-beta (12.8 µM) increased the release of CF from the liposomes and annexin A5 reduced this A-beta dependent leakage whether added to the liposomes before or after A-beta. The A-beta peptide was added to the vesicles in DMSO as a solvent. Control experiments indicated that DMSO alone had small but significant effects on the leakage rate and that this was suppressed by the annexin as well (Table 2). The effectiveness of the annexin had a very sharp dose-response titration providing no protection at 2.4 µg/ml and maximal protection at 3.6 µg/ml.

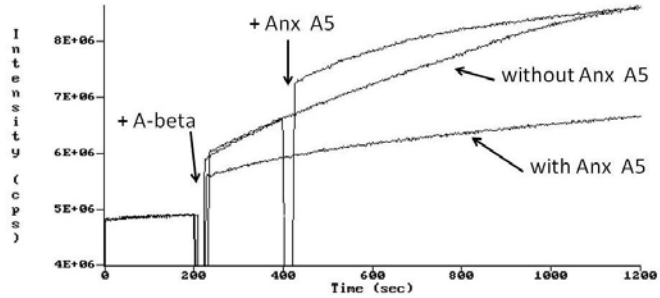
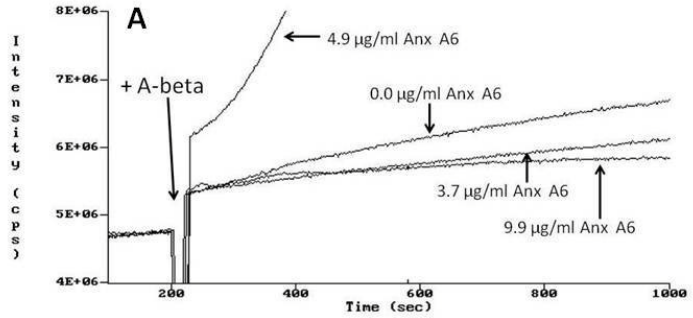


*Figure 9: Annexin A5 protects liposomes from permeabilization by A-beta. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$. At 200 sec A-beta was added in 7µl DMSO ("**+ A-beta**") to a final peptide concentration of 12.8 µM and stimulated CF efflux (trace marked "**without Anx A5**"). When 4.8 µg/ml annexin A5 was incubated with the liposomes prior to addition of A-beta, the rate of CF release due to A-beta was reduced (trace marked "**with Anx A5**"). When the same amount of annexin was added after A-beta (trace following "**+ Anx A5**" at 400 sec) the rate of CF release was subsequently reduced to the rate seen when the protein was added before the A-beta peptide.*

Annexin A6 also inhibited the release of CF due to A-beta (5.5 µM), but with a very dramatic anomaly at an annexin concentration of 4.9 µg/ml at which the annexin enhanced release due to A-beta (Figure 10). Annexin A6 inhibited the slope due to A-beta by up to 50% before this anomaly; at the anomaly it caused a large burst of CF release followed by a leakage rate increased 13 fold above leakage due to A-beta alone (Table 2). At concentrations above this anomalous point annexin A6 again reduced the leakage due to A-beta by 50%. This anomalous effect of annexin A6 was seen if the annexin was added before or after A-beta (Figure 10B). Annexin A6 at this concentration (4.9 µg/ml) did not enhance the baseline release in the presence of the vehicle DMSO alone.



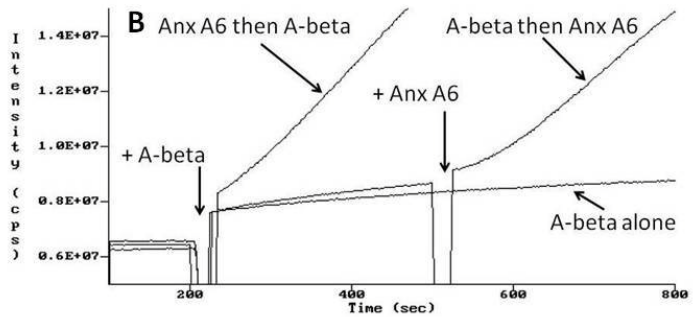


Figure 10: Effects of annexin A6 on CF leakage induced by A-beta. Liposomes incubated in Assay Buffer with 1 mM $MgCl_2$. ***Part A****: Liposomes were pre-incubated with 0, 3.7, 4.9, or 9.8 µg/ml annexin A6 and A-beta was added at 200 sec in 3µl of DMSO ("****+A-beta****") to a final peptide concentration of 5.5 µM. The top trace shows the enhanced leakage when the vesicles were incubated in 4.9 µg/ml annexin A6. The relative slopes of the traces are given in Table 2.* ***Part B****: Before 200 sec liposomes were preincubated in the absence (two samples) or presence (one sample) of 4.9 µg/ml annexin A6. At 200sec A-beta was added in 3 µl DMSO to give a final concentration of 5.5 µM peptide ("****+A-beta****") to all three samples. The sample containing annexin A6 exhibited a very high rate of release of CF ("****Anx A6 then A-beta"****). At 520 sec 4.9 µg/ml annexin A6 was added to one of the two samples without annexin that had A-beta previously added at 200 sec ("****A-beta then Anx A6****"). Note that the annexin strongly enhanced the leakage of CF induced by A-beta whether it was added before or after A beta.*

Table 2: Effects of annexins A5 and A6 on the rates of release of CF from liposomes treated with A-beta[a]

ANNEXIN A5		**ANNEXIN A6**	
Addition	Relative Slope	Addition	Relative Slope
None	100	None	100
DMSO	125	DMSO	157
A-beta	338	A-beta	504
A-beta + 1.2 µg/ml Annexin A5	345	A-beta + 1.2 µg/ml Annexin A6	357
A-beta + 2.4 µg/ml Annexin A5	375	A-beta + 2.5 µg/ml Annexin A6	264
A-beta + 3.6 µg/ml Annexin A5	108	A-beta + 3.7 µg/ml Annexin A6	407
A-beta + 4.8 µg/ml Annexin A5	62	A-beta + 4.9 µg/ml Annexin A6	6507
DMSO + 4.8 µg/ml Annexin A5	82	A-beta + 9.8 µg/ml Annexin A6	232

[a]A-beta was added at a final concentration of 5.5 µM from 3 ul of DMSO stock. DMSO controls were performed with 3 ul of DMSO alone. Slopes were normalized to 100 for the baseline slope in the absence of DMSO or A-beta. The slopes were determined 600 to 800 seconds after the addition of A-beta (corresponding to 800 to 1000 seconds in Figure 9). The experiments with annexin A5 and with annexin A6 were performed with different vesicle preparations. Other conditions were as described under Materials and Methods and in Figure 9 legend.

Effects of annexins on disruption of the membrane permeability barrier by amylin

In Type 2 diabetes there is a buildup of insoluble, fibrillar deposits of the peptide hormone amylin in the vicinity of the beta cells of the pancreas [16]. Soluble oligomers of amylin are apparently the precursors of these amylin fibrils and are in themselves toxic to the beta cells [17,18,19]. It has been hypothesized that these amylin oligomers contribute to the cause or severity of beta cell destruction in diabetes by a mechanism similar to that proposed for the action of A-beta on nerve cells, damaging the beta cell plasma membrane, allowing excess calcium to enter the cells.

When amylin was added to the liposomes it promoted the release of CF, and this release could be inhibited by annexin A5 whether the annexin was added to the liposomes before or after the addition of the peptide (Figure 11). At 4.8 µg/ml annexin A5 inhibited the enhancement of release by 6.4 µM amylin by 84%. When the same concentration of annexin A5 was added after the amylin (Figure 11B) the enhancement of release due to the amylin was reduced by 69%. Annexin A6 also provided protection of

the membrane from permeabilization by amylin, but it was somewhat less effective than annexin A5: at 4.9 µg/ml annexin A6 the increase in release rate due to 6.4 µM amylin was reduced by 32%. However, annexin A6 did not promote an anomalous enhancement of CF release as was seen with A-beta even though used at similar concentration and conditions, including the use of DMSO as a vehicle.

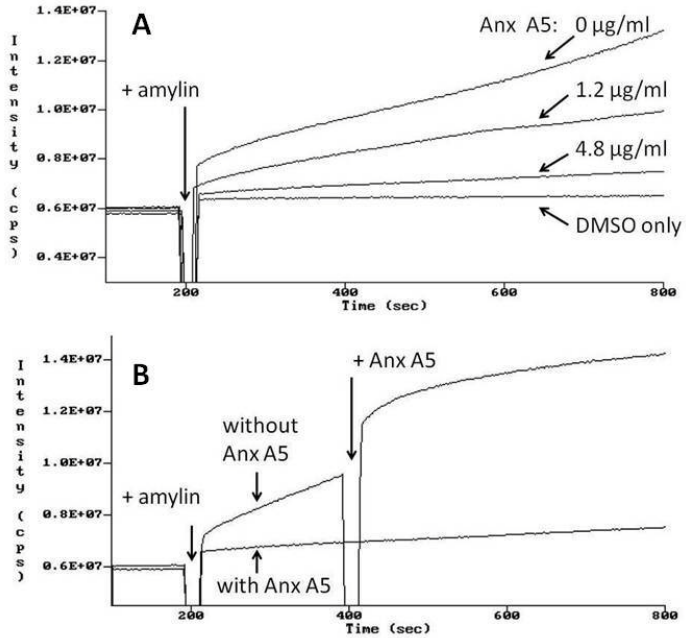


Figure 11: Annexin A5 inhibits leakage of CF induced by amylin. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$. ***Part A****: Liposomes were pre-incubated with 0, 1.2, or 4.8 µg/ml annexin A5, as marked, and amylin was added at 200 sec in 3µl of DMSO to a final peptide concentration of 6.4 µM ("**+amylin**"). Trace marked "**DMSO only**": no annexin, 3 µl DMSO alone added at 200 sec.* ***Part B****: Liposomes were preincubated in the absence ("**without annexin**") or the presence ("**with annexin**") of 4.8 µg/ml annexin A5. At 200 sec Amylin was added in 3 µl DMSO to give a final concentration of 6.4 µM peptide ("**+amylin**"). At 400 sec 4.8 µg/ml annexin A5 was added to the sample initially without annexin.*

Effects of annexins on disruption of the membrane permeability barrier by osmotic shock

The annexins were tested for the ability to suppress leakage due to osmotic shock of liposomes, a type of stress that may serve as a model for membrane disruption due to mechanical means such as may occur in **muscular dystrophies, spinal cord injury, or traumatic brain injury**.

Liposomes were incubated in a small volume (15 µl) of buffer with 150 mM KCl for osmotic support (see Materials and Methods for details). After 200 seconds 290 µl of isotonic buffer (Assay Buffer containing 3.5 or 5 mM Mg) or hypotonic buffer (the same buffer without KCl) was used to dilute the sample approximately 20 fold in the fluorometer cuvette and the fluorescence was subsequently monitored continuously (Figure 12). When diluted with isotonic buffer there was no significant leakage of CF associated with the dilution and mixing. When diluted with hypotonic buffer there was a burst of release of approximately 17 % of the encapsulated CF. Subsequent to this burst there was an increase in the leakage rate relative to the control not exposed to the osmotic shock suggesting that although the membrane resealed after the shock, the permeability remained altered to some degree (Figure 12A). When annexin A5 was present in the initial incubation period (at an amount such that the final concentration after dilution was 4.5 µg/ml) the release of CF during hypotonic shock was reduced to 9% of the encapsulated CF, a reduction of 47% of the leakage during osmotic shock which was 17% (Figure

12A). Subsequent to the shock the continued presence of the annexin reduced the leakage of CF to the control level seen with unshocked liposomes (Figure 12A).

Experiments performed with annexin A6 (4.3 µg/ml after dilution) also showed protection from osmotic shock, although less than with annexin A5 (Table 3). The degree of protection with annexin A6 was greater when the experiment was performed with 5 mM Mg^{2+} than with 3.5 mM Mg^{2+}, while the degree of protection with annexin A5 was similar at the two Mg^{2+} concentrations (Table 3). Since the osmotic shock occurred at 200 seconds into the time course of the experiment, but mixing was not complete and monitoring of fluorescence did not occur until approximately 220 seconds, Table 3 also includes data obtained by extrapolating the lines in the graphs to 200 seconds.

The ability of annexin A5 to provide protection against osmotic shock was calcium-dependent. When the osmotic shock experiment was conducted in the presence of EGTA instead of 1 mM $CaCl_2$ the presence of annexin A5 had no effect on the release of CF due to mixing with hypotonic medium (Table 3).
The protective action of annexin A5 occurred early in the process of osmotic shock. When the annexin was bound to the liposomes by the action of calcium in the pre-incubation step but the dilution was made with hypotonic buffer containing EGTA, which should promote the release of the annexin, suppression of the leakage due to the osmotic shock by 51% was observed (Figure 12B), although after the shock the rate of baseline release was not reduced by the annexin, as expected due to the removal of calcium (Figure 12 B). However, annexin A6 was not effective in this regard as it caused a slight increase in CF release that was not statistically significant upon osmotic shock with an EGTA containing buffer. The failure of annexin A6 to provide protection in this case may reflect a faster off rate for annexin A6 when the calcium was removed than occurs with annexin A5.

When the initial incubation was performed in 1 mM EGTA instead of 1 mM $CaCl_2$ and the dilution was performed in media containing 1 mM $CaCl_2$ there was no protection by annexin A5 from the osmotic shock, although the subsequent slow leakage was reduced by the annexin. Apparently the annexin was unable to move to the membrane quickly enough when the calcium-containing medium was added to provide protection from the initial impact of the osmotic shock.

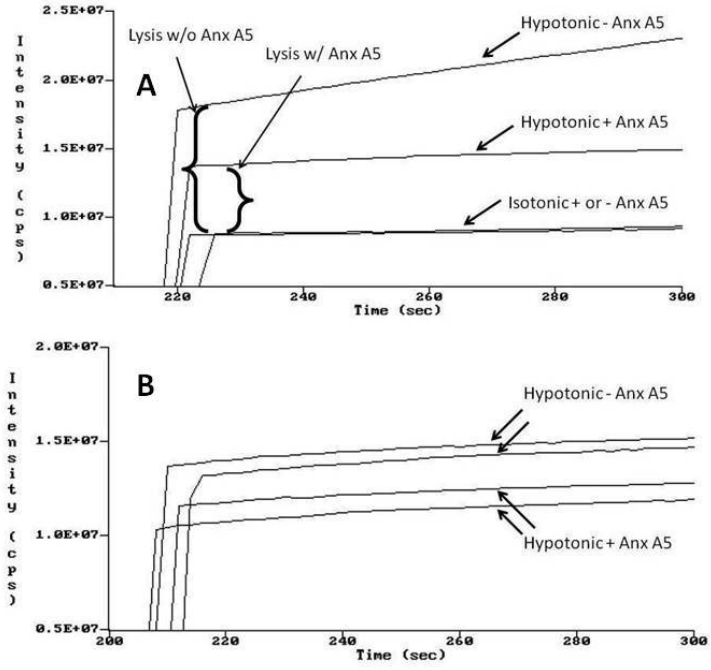


Figure 12: Annexin A5 suppresses the release of CF from liposomes exposed to osmotic shock. ***Part A****: Liposomes plus or minus annexin A5 were incubated in a small volume (15 µl) for 200 sec then were diluted to a volume of 305µl with isotonic or hypotonic buffer (see text for details). After the mixing was complete at approximately 220 sec the fluorometer shutters were opened and the fluoresence monitored continuously. Nearly coincident traces labeled "****Isotonic + or – Anx A5****" represent liposomes incubated with or without annexin A5 (4.5 µg/ml final concentration after dilution) and mixed with isotonic buffer. Trace "****Hypotonic - Anx A5****", lipsomes incubated without annexin A5 and mixed with hypotonic buffer; trace "****Hypotonic + Anx A5****", lipsomes incubated with annexin A5 and mixed with hypotonic buffer. The* ***brackets*** *mark the amount of CF released during the osmotic shock with or without annexin A5. Fluoresence intensity of suspensions after adding Triton was 6.7 X 10^7 cps.* ***Part B****: Annexin A5 provides protection against osmotic shock at an early time point. Liposomes were incubated in a small volume with or without annexin A5 and then diluted with hypotonic buffer containing 3.5 mM $MgCl_2$ and 2 mM EGTA in place of calcium. "****Hypotonic – Anx A5****", duplicate samples without annexin A5; "****Hypotonic + Anx A5****", duplicate samples with annexin A5. Fluorescence intensities at 220 sec: Without annexin 1.35+/-0.03 X 10^7 cps, with annexin 1.10+/-0.09 X 10^7 cps. Baseline intensity when diluted with isotonic buffer (not shown in the figure): 0.86+/-0.03 X 10^7 cps.*

Table 3: Effects of annexins A5 and A6 on the release of CF from liposomes subjected to osmotic shock

Annexin A5 or A6	**[Mg^{2+}]**	**% reduction in lysis[a]**	**% reduction extrapolated to 200 sec[b]**
A5	3.5 mM	46.9%	42.1%
A5	5.0 mM	47.3%	36.0%
A5 without Ca^{2+}	3.5 mM	0%	0%
A6	3.5 mM	16.5%	7.8%
A6	5.0 mM	28.6%	23.4%

[a] % reduction in lysis was determined by comparing the differences marked by the vertical brackets in the example in Figure 12. [b] % reduction extrapolated to 200 sec was determined by extending the fluorescence traces, as seen in the example in Figure 12, to the time of initial mixing of the liposomes with the hypotonic medium.

Summary

Annexin A5, and to a lesser extent annexin A6, was found in this study to stabilize the permeability barrier of complex liposomes against a wide variety of stresses, as summarized in Table 4.

Table 4: Summary of the effects of annexins A5 and A6 on the release of CF from liposomes subjected to stresses[a]

Agent or Action	**Annexin A5**	**Annexin A6**
None (Baseline)	Protection	Protection
Arachidonate	Protection	*Disruption*
Lysophosphatidic acid (LPA)	Protection	*Disruption*
Lysophosphatidylcholine (LPC)	Protection	Protection
Diacylglycerol	Protection	*Disruption*
Monoacylglycerol	Protection	*Disruption*
Spermidine	Protection	Protection
Amyloid-beta	Protection	Protection or *Disruption*[b]
Amylin	Protection	Protection
Osmotic Shock	Protection	Protection

[a]*The annexins either provided* ***Protection (****reduced the rate of release or CF) or caused* ***Disruption*** *(increased the rate of release of CF).*
[b]*Annexin A6 either provided protection or caused disruption in the presence of Amyloid-beta depending on the concentration of the annexin.*

KEY RESEARCH ACCOMPLISHMENTS

1. Establishment of an unsupported bilayer system consisting of liposomes for study of membrane repair.
2. Demonstration that annexins A5 and A6 promote repair of liposome membranes subjected to osmotic shock, excesses of amphiphilic molecules generated in metabolism or signaling, and cell damaging peptides involved in the pathogenesis of Alzheimer's disease and type 2 diabetes.

REPORTABLE OUTCOMES

1. Abstract presented at the Biophysical Society Meeting, San Diego, March 2012: C.E. Creutz, *Protection of the membrane permeability barrier by annexins.*
2. Publication: C.E. Creutz, J.K. Hira, V.E. Gee, and J.M. Eaton. Protection of the membrane permeability barrier by annexins. *Biochemistry* 51: 9966-9983 (2012).

CONCLUSION

To date this project has demonstrated that calcium-dependent, membrane-binding proteins of the annexin and copine classes have the potential to directly repair or stabilize lipid bilayers. They may therefore be promising agents for repairing damaged cell membranes in the case of spinal cord injury.

REFERENCES

[1] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim Biophys Acta 812 (1985) 55-65.
[2] B. Mui, L. Chow, M.J. Hope, Extrusion technique to generate liposomes of defined size, Methods Enzymol 367 (2003) 3-14.
[3] J.N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, W.A. Hagins, Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker, Science 195 (1977) 489-492.
[4] J.N. Weinstein, R. Blumenthal, R.D. Klausner, Carboxyfluorescein leakage assay for lipoprotein-liposome interaction, Methods Enzymol 128 (1986) 657-668.
[5] C.E. Creutz, N.G. Kambouris, S.L. Snyder, H.C. Hamman, M.R. Nelson, W. Liu, P. Rock, Effects of the expression of mammalian annexins in yeast secretory mutants, J Cell Sci 103 (Pt 4) (1992) 1177-1192.
[6] C. Freye-Minks, R.H. Kretsinger, C.E. Creutz, Structural and dynamic changes in human annexin VI induced by a phosphorylation-mimicking mutation, T356D, Biochemistry 42 (2003) 620-630.
[7] V. Gerke, C.E. Creutz, S.E. Moss, Annexins: linking Ca2+ signalling to membrane dynamics, Nat Rev Mol Cell Biol 6 (2005) 449-461.
[8] C.E. Creutz, The annexins and exocytosis, Science 258 (1992) 924-931.

[9] C.E. Creutz, cis-Unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin, J Cell Biol 91 (1981) 247-256.
[10] F. Schuber, K. Hong, N. Düzgünes, D. Papahadjopoulos, Polyamines as modulators of membrane fusion: aggregation and fusion of liposomes, Biochemistry 22 (1983) 6134-6140.
[11] F. Schuber, Influence of polyamines on membrane functions, Biochem J 260 (1989) 1-10.
[12] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol Rev 81 (2001) 741-766.
[13] A. Demuro, I. Parker, G.E. Stutzmann, Calcium signaling and amyloid toxicity in Alzheimer disease, J Biol Chem 285 (2010) 12463-12468.
[14] H.A. Lashuel, P.T. Lansbury, Are amyloid diseases caused by protein aggregates that mimic bacterial pore-forming toxins?, Q Rev Biophys 39 (2006) 167-201.
[15] N. Arispe, E. Rojas, H.B. Pollard, Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum, Proc Natl Acad Sci U S A 90 (1993) 567-571.
[16] P. Westermark, A. Andersson, G.T. Westermark, Islet amyloid polypeptide, islet amyloid, and diabetes mellitus, Physiol Rev 91 (2011) 795-826.
[17] R.A. Ritzel, J.J. Meier, C.Y. Lin, J.D. Veldhuis, P.C. Butler, Human islet amyloid polypeptide oligomers disrupt cell coupling, induce apoptosis, and impair insulin secretion in isolated human islets, Diabetes 56 (2007) 65-71.
[18] J. Janson, R.H. Ashley, D. Harrison, S. McIntyre, P.C. Butler, The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles, Diabetes 48 (1999) 491-498.
[19] M.F. Engel, Membrane permeabilization by Islet Amyloid Polypeptide, Chem Phys Lipids 160 (2009) 1-10.