THE MECHANICAL PROPERTIES OF SARCOLEMMAL VESICLES FROM RABBIT MUSCLE: THE EFFECTS OF INTERNAL CALCIUM AND MEMBRANE ACTIVE MOLECULES.

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SUMMARY

The technique of pipette aspiration was first developed by a biophysicist to study the mechanical properties of the red blood cells swollen into sperical form. In light of the discovery that skeletal muscle fibres can be induced to shed membrane vesicles, the technique has here been adapted to examine the mechanical properties of the muscle surface membrane and to provide novel evidence in the investigation of calcium-induced cell damage.

For these experiments, sarcolemmal vesicles (mean diameter 71 μm) were prepared from rabbit psoas muscle by treatment with collagenase in a fibre-swelling buffer solution of 140 mM KCl and 5 mM HEPES at pH 7.4. Vesicles prepared in this way had previously been examined by electronmicroscopic, biochemical and electrophysiological methods which confirmed their outside-out configuration and that they contained diluted muscle cytosol of similar composition to that found in intact muscle fibres. As far as is known, the preparation has retained the normal functional characteristics of the sarcolemma *in situ*, and represents a valid model for studying otherwise inaccessible properties of the muscle surface membrane.

Vesicles were stressed by partial aspiration into parallel bore micropipettes (mean diameter 19.3 μm) pulled from borosilicate glass capillaries. As the aspiration pressure was gradually increased, the membrane projection inside the pipette lengthened as the membrane expanded in response to the increased isotropic membrane tension until a critical point was reached and the vesicle ruptured. A precise value of the isotropic tension was calculated by applying the Law of Laplace to the hemispherical portions of the vesicle inside and outside the pipette. By measuring also the increase in projection length, the area elasticity of the membrane was determined. The stress-strain plot of membrane tension against corresponding area expansion was typically linear right up to

the lysis point, indicating that vesicles behaved as perfectly elastic structures. The maximum tension that vesicles could withstand was 12.43 mN m^{-1} (median) with 95% confidence limits of 12.29 to 12.67 mN m^{-1}, and the corresponding fractional increase in membrane area was 0.027 (median) with 95% confidence limits 0.0265 and 0.0274. The elastic modulus of area expansion, K, was 490 ± 88 (mean ± S.D.).

In accordance with previous biophysical studies on natural and artificial membranes, which were similarly conducted in nominally Ca^{2+}-free buffers, the tensile strength and elastic modulus of sarcolemma are considered properties of the cohesive forces between membrane lipids and the extent to which membrane proteins perturb the lipid matrix. That the addition of 500 μM Ca^{2+} to the bathing solution had no effect on the strength or elasticity of vesicles suggested that external Ca^{2+} does not influence membrane mechanical properties.

In studies on cell damage generally, much evidence pointed to the involvement of elevated intracellular Ca^{2+}, an effect that was often demonstrated by using the calcium ionophore A23187. Initial experiments conducted by Professor O.F. Hutter and Dr F.L. Burton in this laboratory, had shown that doping vesicles with A23187 in the presence of 500 μM Ca^{2+} resulted in their autolysis. A more detailed quantitative analysis of this effect was therefore required. Vesicles doped with 1 μM or 5 μM A23187 were aspirated in NTA-buffered solutions containing 0.8 to 20 μM Ca^{2+}. With 20 μM Ca^{2+}, vesicles were too weak to provide sufficient data on membrane mechanical properties. Between 2 and 10 μM Ca^{2+} there was a graded decrease in median lysis tension and an increase in the proportion of extremely weak vesicles bursting instantly on aspiration. For vesicles in 0.8 μM Ca^{2+}, median lysis tension was no different to that of untreated vesicles.

As this was the first demonstration of its kind, the authenticity of the effect required more rigorous examination. The reduction in membrane tensile strength was in no part

due to the presence of A23187 and its carrier DMSO. In addition, other divalent cations such as Mg^{2+} which also have a high affinity for the ionophore, did not produce a similar effect. When vesicles were preloaded with the cell permeant calcium chelator BAPTA-AM, a significant degree of protection was afforded to vesicles subsequently challenged by Ca^{2+}-overload. This deleterious and apparently specific effect of Ca^{2+} was unable to be reversed by addition of the ionic form of BAPTA to the bathing solution so as to reduce the concentration of Ca^{2+} to nanomolar levels.

Previous work on Ca^{2+}-mediated cell damage had suggested that possible mechanisms involved phospholipase and protease enzymes activated by Ca^{2+}. It had been demonstrated that putative inhibitors of the phospholipases A_2 and neutral protease calpain I were effective in reducing or preventing cell damage precipitated by the ionophore A23187. It therefore seemed appropriate to test whether or not the same inhibitors could protect Ca^{2+}-challenged sarcolemmal vesicles. In all cases the inhibitors themselves acted as membrane perturbing molecules, reducing median tensile strength and increasing the elasticity of vesicles. Despite this fact, all the PLA_2 inhibitors bar one still appeared to afford some degree of protection, as shown by an increase in the median tensile strength of vesicles pre-treated with an inhibitor prior to Ca^{2+}-loading. Further support for the involvement of PLA_2 came from experiments where lysolipids or arachidonic acid, both products of phospholipid hydrolysis, were similarly shown to have a powerful destabilizing influence on the membrane. No clear protective effect of the protease inhibitors could be seen over and above the weakening effects of the inhibitors themselves, suggesting that Ca^{2+}-activated proteases were less likely to be involved. That small amounts of exogenously added proteins can have a marked influence on membrane mechanical properties was incidentally shown in an attempt to use the pore-forming protein α-toxin to permeabilize vesicles to Ca^{2+}.

The involvement of any of the above mechanisms still requires biochemical proof that was beyond the scope of this project. Nonetheless, the pipette aspiration technique has

revealed for the first time, information on the mechanical properties of the cell membrane which may help to explain how raised intracellular Ca^{2+} ultimately leads to cell death. It has also been demonstrated that this technique can provide precise, quantitative information on membrane stability and the perturbing effects of intercalating molecules.

PART I

INTRODUCTION

The cell surface membrane as a permeability barrier and as a site of electrical potential has been studied exhaustively. By contrast, the mechanical properties of the cell membrane have scarcely been explored. The exception is the red blood cell membrane whose strength and elasticity have been determined by pipette aspiration (Rand, 1964; Evans, Waugh & Melnik, 1976; Waugh & Evans, 1979). In the present work, this technique has been adopted to study the mechanical properties of the surface membrane of the mammalian skeletal muscle membrane as presented in the form of sarcolemmal vesicles.

Properties of sarcolemmal vesicles

In a classical paper on "The Minute Structure and Movements of Voluntary Muscle", William Bowman (1840) described how the surface of a muscle fibre placed into water lifts off to form large blebs (Fig. 1.01). A similar process was described by Hogue (1919) in cultured cardiac tissue cells in hypotonic solution. In an early study with the phase microscope, Zollinger (1974) noted the formation of "blebs" on kidney, liver and other types of mechanically dispersed cells, and he described how such blisters become detached to form vesicles. With the more recent widespread use of enzymatically dispersed cells for electrophysiological experiments, membrane blebbing has become a commonplace observation and is generally regarded as indicative of a cell in less than perfect condition.

The liability of skeletal muscle to form blebs and vesicles was rediscovered by Standen, Stanfield, Ward & Wilson (1984) on frog *m. cutaneous pectoris*. In order to clean the muscle membrane for patch clamp experiments they first depolarized it in a high K^+ solution and then treated the muscle with collagenase and protease in sequence. By accident, they used 140 mM KCl as the depolarizing solution and so caused the fibres to swell, the membrane being permeable to both K^+ and Cl^- ions (Boyle and Conway, 1941). The combined effect of fibre swelling and enzyme treatment was a crop of membrane vesicles with a mean diameter of 80 μm. Burton, Dörstelmann & Hutter (1987) later developed the one step enzymatic digestion procedure for the production of vesicles from mammalian, including human, muscle fibres which is used in the present work. Vesicles can be produced also without the use of enzymes and swelling solutions by stretching isolated single muscle fibres to 5 - 8 times their resting length (Stein & Palade, 1989). Such treatment need not break the membrane because membrane folds and caveoli present at resting length supply the necessary extra surface (Dulhunty & Franzini-Armstrong, 1975), but the submembrane cytoskeleton is probably damaged by over-extension so that on subsequent shortening the membrane becomes uncoupled from the rest of the fibre. Some kind of disruption of normal membrane anchoring must presumably also occur in enzymatically treated swelling fibres to account for flotation of the membrane. It is interesting to note in this connection that whilst both spectrin and dystrophin are found in the submembrane region, only dystrophin remains attached to the membrane on vesicle formation (Zubrzycka-Gaarn *et al.*, 1991). The commonly observed vesiculation in a proportion of enzymatically dispersed liver, cardiac or similar small cells, is usually attributed to degradation of the submembrane cytoskeleton by proteases activated by a rise in intracellular Ca^{2+} (Nicotera, Hartzell, Davis & Orrenius, 1986). Sulphhydryl agents have also been used to induce membrane blebbing e.g. in myoblast and in red blood cells (Scott 1976; Smith & Palek, 1983) and in the latter case alteration in spectrin self-association has been demonstrated.

A muscle fibre undergoing vesiculation is shown in Fig. 1.02. As they bud off, vesicles enclose cytoplasmic ground substance, glycoprotein granules etc., but are devoid of organelles. The density of cytoplasmic material varies from vesicle to vesicle (Fig. 1.03). The reason for this variability is not precisely known but may be related to the sequence of formation of the vesicles. Thus the first vesicles to bud off would be expected to contain cytoplasm that is less diluted than is likely to be the case with the latter formed ones. Vesicles have been shown to contain the cytosolic enzyme creatine phosphokinase in amounts equal to intact muscle fibres when expressed in terms of total cytosolic protein (M.J. Jackson & O.F. Hutter, unpublished). All these findings clearly mean that the vesicle membrane remains in normal outside-out configuration. Electronmicrographs of muscle fibres which have undergone vesiculation show the transverse tubule and internal membranes to remain intact (D.L. Bovell, I. Montgomery & O.F. Hutter, unpublished). Heavily vesiculated fibres are denuded of outside membrane. In keeping with the above is that the total area of vesicle surface that can be produced by a muscle fibre does not exceed the area of surface membrane when allowance is made for membrane folds and caveoli. In view of their large size, the formation of vesicles must involve the disconnection of many transverse tubules from the surface membrane and the resealing of the membrane so disrupted. The possibility that some membrane from the upper part of the T-tubule is incorporated into the vesicle therefore cannot be ruled out. By way of further characterization of sarcolemmal vesicles as composed of cell surface membrane, they have been shown to contain Na^+-K^+-ATPase but not SR Ca^{2+}-ATPase (Zubrzycka-Gaarn *et al.*, 1991). The lactate transport system remains operational in sarcolemmal vesicles with kinetic parameters as for intact muscle (Juel, 1991).

Thanks to the use of collagenase in their preparation, sarcolemmal vesicles are devoid of basement membrane and so seal readily to patch pipettes. Advantage of this has been taken to study the delayed rectifyer K^+-channel (Standen, Stanfield & Ward, 1985) and the inwardly rectifying K^+-channel (Burton & Hutter, 1990). The behaviour of these channels in response to membrane depolarization provides additional, incontrovertible

evidence of the normal orientation of the vesicle membrane. Besides, patch clamp experiments give information on the ionic composition of sarcolemmal vesicles. That the single channel conductance of the inwardly rectifying K^+-channel is smaller for outward than for inward current (Burton, Dörstelmann & Hutter, 1988; Burton & Hutter, 1990) connotes that sarcolemmal vesicles contain enough Mg^{2+} to block that channel (Vandenberg, 1987). Similarly, the largely closed state of K_{ATP} channels in vesicle attached patches signifies that vesicles contain enough ATP to keep these channels inactive. High conductance K_{Ca} channels are readily found in inside-out patches exposed to Ca^{2+} containing solutions (Burton *et al.*, 1988), but in attached patches these channels open only on strong depolarization which signifies (Palotta, Magleby & Barrett, 1981) that the concentration of Ca^{2+} in vesicles is submicromolar i.e. near the levels found in normal resting muscle fibres.

In summary then, sarcolemmal vesicles consist of diluted cytosol enveloped by plasmalemma. In all so far tested respects the plasmalemma has retained its normal functional characteristics, despite its divestment from basement membrane and such disruption to the submembrane cytoskeleton as may be inevitable in vesicle formation. Sarcolemmal vesicles may be regarded as valid models for measuring otherwise inaccessible properties of the plasmalemma as it may be argued that the outer and inner appendages of the plasmalemma in intact muscle fibres will make little contribution to the mechanical properties under conditions of membrane area dilation such as hold with the pipette aspiration method as used here.

The pipette aspiration technique and its antecedents

The mechanical properties of a cell surface were first studied by K.S. Cole (1932) on the *Arbacia* egg. By compressing this large, spherical cell (diameter ≈100 μm) into a flattened shape of greater surface area, Cole showed the cell surface force to rise with surface area, but he did not attempt to localize the observed elasticity. Had this question

been broached it would have been possible, even before the advent of the electron microscope, to conclude on osmotic grounds (Lucké & McCutcheon, 1932) that the plasmalemma of sea urchin eggs is highly folded so that its true area will not be increased by the imposed deformation. Rather the observed elasticity was attributable to the components of the cell cortex which maintain the normally folded state of the plasmalemma.

The pipette aspiration method for studying the mechanical properties of the cell surface was introduced by Michison and Swann (1954), also working on sea urchin eggs. They deformed the egg by sucking a bulge into a pipette about half the diameter of the cell. From the suction pressure applied and the dimensions of the egg and the pipette, the tension created in the cell can be calculated. Typically 200 dyn cm^{-2} *, which is equivalent for a 100 μm egg to a membrane tension of 0.5 dyn cm^{-1}, sufficed to produce an increase in surface area of about 5%. Michison and Swann expressed their result in terms of a bulk modulus of elasticity, which involved an uncertain estimate of the thickness of the cell cortex. If instead their results are expressed as the ratio of force over fractional increase in area, they yield a modulus of area elasticity of 0.5 dyn cm^{-1} / 0.05 i.e. about 10 dyn cm^{-1}. Again, this must not be regarded as a measure of the elasticity of the plasma membrane, but rather of a cell cortex which, judging from the osmotic data (Lucké and McCutcheon, 1932), can increase in area by as much as 20% **.

The techniques developed for sea urchin eggs were later applied to red blood cells. These have the advantage of a membrane normally devoid of microvillous projections and infoldings and of a cell interior that may be treated as a concentrated homogeneous fluid; and though red blood cells are an order of magnitude smaller than the sea urchin eggs and therefore more difficult to handle they are just large enough to allow pipette aspiration experiments under the light microscope. Thus, using micropipettes of 2 μm diameter,

* The results here quoted are in the units used in older papers (dyn cm^{-1} = mN m^{-1}).
** The elastic modulus for osmotically swollen sea urchin eggs forced to undergo dilation of the plasmalemmal area has apparently not been measured hitherto.

Rand and Burton (1964) found that discoid or partly swollen red cells are at first easily pulled into the pipette, but when the part of the cell remaining outside the pipette became the portion of a sphere the cell membrane became 'rigid' and resisted the increase in membrane area that would be necessary, at constant cell volume, to draw more of the cell into the pipette. Rand and Burton viewed this increase in rigidity of the critically deformed cell as arising from some reorganization or change in membrane properties; and Rand (1964) shows that the 'rigid' membrane will burst at tensions of 10 - 20 dyn cm^{-1}, depending upon the temporal condition of the experiment.

With the recognition that the red cell membrane is a composite structure consisting of a fluid lipid bilayer containing proteins, some of which are linked to the submembrane cytoskeleton (Singer, 1974), it was realized that the observations of Rand and Burton did not really signify non-linear stiffening of the membrane with increasing stress. Rather, different material properties were being measured at different stages of a pipette aspiration experiment (Skalak, Tozeren, Zarda & Chien, 1973; Evans, 1973). So long as the cell is flaccid and aspiration produces a change in shape without an increase in membrane area, the elasticity of the submembrane cytoskeleton determines the mechanical properties of the cell. By contrast, the resistance of a rounded-up cell to area expansion is attributable to the strong cohesive force in lipid bilayers which arises from the hydrophobic effect (Tanford, 1973). By refining the vesicle aspiration technique, Evans and his colleagues were able to quantify the disparity between membrane deformation moduli i.e. shear and dilation. Thus the shear elasticity of the red cell is characterized by a modulus in the order of 10^{-2} dyn cm^{-1}, whereas the modulus of area expansion is in the order of 5×10^{2} dyn cm^{-1} (Waugh and Evans 1976, 1977). Unilamellar vesicles made artificially of known lipid composition, when subjected to pipette aspiration yield moduli of area expansion from 100 - 1000 dyn cm^{-1} (Evans and Needham, 1987). This emphasizes that membrane mechanical behaviour related to area expansion is largely the expression of the properties of the lipid bilayer.

In the experiments here to be described, the initial suction applied to a sarcolemmal vesicle was already adequate to render spherical the portion of the vesicle outside the pipette. Any subsequent increase in suction, and hence membrane tension, therefore produces membrane dilation. The results of the present measurements therefore provide information primarily about the cohesiveness of the lipid bilayer. However, owing to the high density of proteins in biological membranes, the mechanical properties of sarcolemmal vesicles here measured reflect also any perturbation of the lipid bilayer through its interaction with proteins. The mechanical properties of sarcolemmal vesicles prepared by a standard method are described in Part III. In subsequent parts the effect of Ca^{2+} and of various amphiphilic agents are described.

PHILOSOPHICAL

TRANSACTIONS

OF THE

ROYAL SOCIETY

XXI. *On the Minute Structure and Movements of Voluntary Muscle.* By WILLIAM BOWMAN, *Esq. Demonstrator of Anatomy in King's College, London, and Assistant Surgeon to the King's College Hospital. In a Letter addressed to* ROBERT BENTLY TODD, *M.D. F.R.S., Professor of General Anatomy and Physiology in King's College, London. Communicated by Professor* TODD.

Received June 18,—Read June 18, 1840.

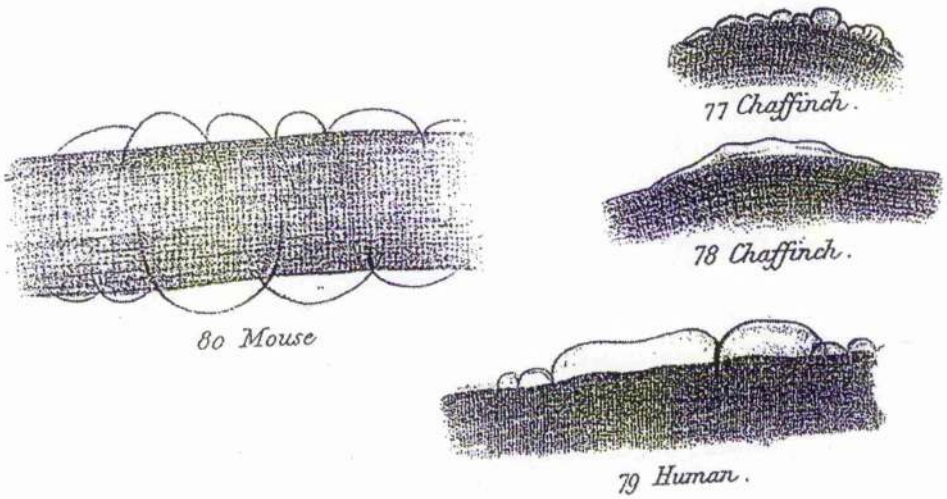

Figure 1.01 An early description of membrane blebbing in muscle fibres
Drawings, taken from a paper to the Royal Society by William Bowman (1840), describe the flotation of the surface membrane of mammalian and avian muscle fibres placed in water.

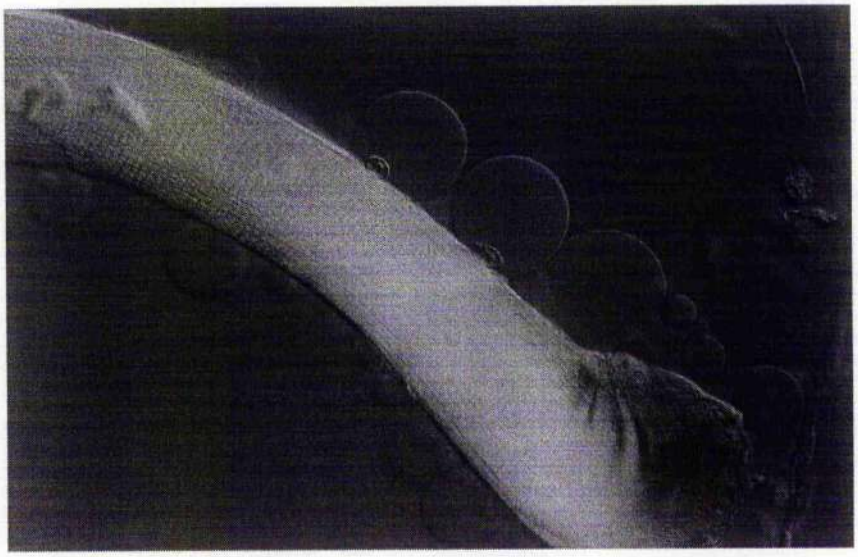

Figure 1.02 Vesiculation of a muscle fibre surface membrane

Photograph shows the formation of sarcolemmal vesicles on an isolated muscle fibre from rabbit psoas placed in a 140 mM KCl solution at pH 7.4 containing 100 units/ml type Ia collagenase.

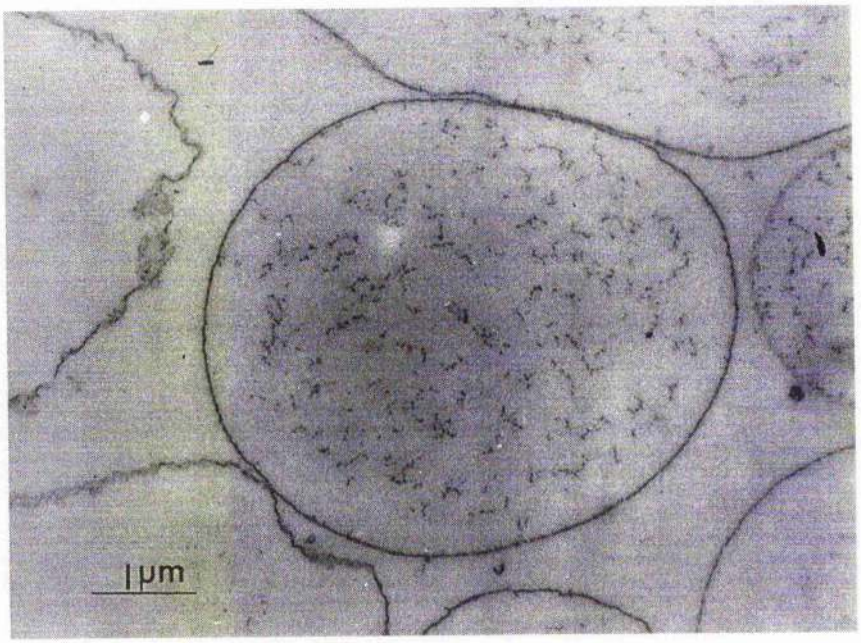

Figure 1.03 Electronmicrograph of sarcolemmal vesicles from rabbit psoas muscle

EM picture of small (crc. 10μm) vesicles in a pellet formed by light centrifugation showing the difference in density of cytosolic content between vesicles.

PART II

METHODS

Preparation of sarcolemmal vesicles

Mammalian muscles of all kinds will yield sarcolemmal vesicles (Burton *et al.*, 1988; Hutter, Burton & Bovell, 1991; Juel, 1991), but rabbit psoas muscle is particularly suitable on account of the length and size of its fibres and its relative freedom from interfascicular connective tissue and fat. NZW rabbits, both male and female, were killed by cervical dislocation or lethal injection of pentabarbitone and then bled out. After laparotomy, the peritoneum and fat overlying the psoas muscle was removed manually. A 3-4 cm length from the belly of the muscle was then freed from its dorsal attachment by blunt dissection, excised and placed in cold (4 °C) 140 mM KCl. When the cut muscle segment had relaxed, it was pinned out in a large petri dish under 140 mM KCl onto a layer of Sylgard. Strips of muscle were then separated and placed into 5 cm polystyrene dishes containing a layer of Sylgard for further dissection under 140 mM KCl at room temperature, the object being to arrive at an array of fascicles thin enough to allow microscope observation of vesiculation. The fascicles were kept lightly stretched by fine pins. To minimize debris, any obviously crushed ends were trimmed off with a razor blade and washed away with fresh 140 mM KCl. Preparation of a set of dishes containing fascicles usually took about 30 min during which time the fibres gradually swelled.

To induce vesiculation the preparations were exposed to 140 mM KCl containing 100 units ml^{-1} type Ia collagenase (Sigma Chemical Company) and the dishes placed on a hotplate at 34 °C for 10-15 min. Evaporation during incubation was minimized by covering the dishes with plastic lids to which moistened filter paper adhered. The final

step in the procedure was the transfer of the lightly drained fascicles into new dishes containing a measured volume of enzyme-free KCl solution, and the release of vesicles trapped between the fibres by gently teasing apart the muscle which was subsequently removed from the dish. Too early harvesting gives low yields because too few vesicles have yet formed; too late harvesting reduces the yield because too many vesicles have already been shed. With the enzyme solution here used, 10-15 min of incubation was usually best. At all events, periodic microscopic observation soon informs of the correct moment, and in practice the whole procedure is much simpler than its description suggests. In one experiment, designed to test whether muscle fibre type determines membrane mechanical properties, the above procedure was applied to rabbit soleus muscle with equal success.

Muscle segments kept for up to 2 h in cold 140 mM KCl solution before dissection yielded vesicles indistinguishable from those immediately prepared as above. However, delay in the excision of the muscle from the carcass prevented the formation of vesicles. Whether this is due to rapid autolysis of the membrane after death, possibly consequent upon a rise in intracellular Ca^{2+}, or whether incipient rigor extends to the submembrane cytoskeleton and prevents its disruption remains unexplored.

Vesicles as produced above vary in their refractivity. These differences in refractive properties are likely to be related to differences in the density of cytosolic content of the vesicles, as visible in EM sections of pelleted vesicles such as shown in Fig. 1.03 (D.L. Bovell, I.O. Montgomery & O.F. Hutter, unpublished). For the present experiments the more refractive vesicles between 30 and 120 μm diameter were used, (mean ± S.D. = 71.0 ± 11.5 μm).

In some experiments the muscle fascicles were teased apart whilst still in collagenase-containing 140 mM KCl solution. That solution, with the shed vesicles in it, was then transferred into a centrifuge tube with a plastic Pasteur pipette. Spinning for 2 min at low

speed was sufficient for the vesicles to settle into the tip of the tube. The supernatant was then sucked off and another batch of vesicles in collagenase solution added. By repetition, a pellet could be built up to desired size. Finally the pellet was taken up into enzyme-free KCl and a drop of the resultant vesicle suspension added to a known volume of solution in the experimental dish. This procedure was convenient in experiments involving experimental solution of diverse composition, but it had the disadvantage that large vesicles were harder to find: presumably, they were more liable to damage by transfer and spinning. Also, debris and erythrocytes accumulated in the pellet as much as did vesicles. Separation of vesicles from debris is possible by gradient layer separation (M.J. Jackson & O.F. Hutter, unpublished ; see also Juel, 1991), but again with loss of vesicles of the size preferred for aspiration.

Manufacture of pipettes

Pipettes were pulled from borosilicate glass capillaries (1.5 mm o.d., 1.2 mm i.d.) using a two stage electrode puller set to produce pipettes with a shank of uniform bore ranging from 9-25 μm diameter (mean = 19.3 μm) over a length of about 500 μm, before terminating in a tip of 1-2 μm open diameter. To break such pipettes cleanly towards the end of the uniform bore shank, the tip of the pipette, mounted on a micromanipulator, was brought into contact with a bead of solder glass (T_{soft} = 570 °C) kept molten on a loop of resistance wire. Thanks to the much higher melting point of the pipette glass, the pipette remained rigid as molten solder glass flowed into it from the bead by capillary action. When the solder glass had reached the uniform bore shank, the heating current was switched off and on cooling the solder glass in the pipette solidified with the bead. Quick withdrawal of the pipette from the bead then usually resulted in a clean break at the interface between the hollow and the solder-glass filled portions (Evans *et al.*, 1976; Zhelev, Needham & Hochmuth, 1994). Any jagged pipettes were rejected. Satisfactorily square-ended pipettes were finally bent to an angle of approximately 25-30 deg, at a point roughly 5 mm from the break, by reheating the glass under a loop of resistance wire.

This compensated for the slope of the pipette holder and allowed the working end of the pipette to lie parallel to the bottom of the dish. Pipettes were half-filled with filtered 140 mM KCl by back-filling through a fine tube.

Experimental apparatus

A diagram of the experimental set-up is shown in Fig. 2.01. A top-focussing microscope (Oxford Micro-instruments) fitted with long working distance x4 and x40 objectives was used. (With such a microscope the layer of Sylgard beneath the vesicles, or more importantly beneath pinned out preparations observed whilst undergoing vesiculation, presented no difficulty. With most inverted microscopes any layer of Sylgard needs to be kept thin). The eyepieces could be exchanged for a TV camera (Panasonic WV-BL200/B) coupled to a monitor and video recorder. The optical system was calibrated with a stage micrometer. The camera eyepiece magnified x2.5 and was chosen so as to record at high gain a field of view of approximately 120 μm, enough to contain the largest vesicles used. The entire optical assembly could be raised away to allow easy access to the experimental dish held in a mechanical stage. With the highly refractory vesicles used, phase contrast optics were unnecessary. Slight defocussing of the condenser improved visualization of vesicles.

The aspiration pipette was held in a patch-clamp type holder with its suction tube coupled to a water manometer. Pipette pressure was controlled by manual injection or withdrawal of air in the closed limb of the manometer with a 50 ml syringe. It could be measured visually from a scale with an accuracy of ± 1 mm H_2O and was read aloud into the voice channel of the video recorder. Zero pressure was determined as the reversal point of flow in or out of the pipette in the dish.

Aspiration technique

Before positioning the working end of the micropipette so as to lie level just above the bottom of the experimental dish, a slightly positive pressure was applied to keep debris away from it. The mechanical stage was manoeuvred so as to centre a settled vesicle in line with the axis and close to the mouth of the pipette. The pipette pressure was then reversed to between -0.5 and -1.0 cm H_2O. This gentle suction was enough to bring the vesicle towards the pipette and usually also to draw a projection into the latter. The ease with which this initial projection was usually formed indicates that it was due to a change in shape without an increase in membrane area. The pipette, with the vesicle attached to it, was next moved upwards away from the floor of the dish. In this way, any debris and erythrocytes lying at the bottom of the dish dropped out of the picture. The microscope was refocussed first for measurement of the diameter of the spherical portion of the aspirated vesicle, and next for optimum visibility of the vesicle projection within the pipette. The suction pressure was then increased to 2 cm H_2O and thereafter in steps of 2 cm H_2O until the vesicle ruptured. At the end of the experiment the videotape and accompanying soundtrack were replayed and the pipette and vesicle diameters and the length of the projection into the pipette measured from the screen, starting with the readings at 2 cm H_2O. (Evans recorded the suction pressure with a transducer and displayed it on the video screen in alphanumeric form. This is more elegant and less laborious, but only essential with the low pressures encountered in the measurement of shear modulus. For our purposes the simple and cheap technique here used served well, provided always that the voice channel was duly switched on!). The experiments were done at room temperature (19-25 °C).

Owing to their extreme weakness, many vesicles enriched with Ca^{2+} burst instantly on or soon after aspiration, so that no values for the modulus of dilational elasticity could be obtained. In an attempt to extract more information, pipettes of relatively narrow diameter i.e. 1/5 or 1/6 of the vesicle diameter were used in some experiments. This magnified the suction pressure which could be applied to a weak vesicle before it burst

and the length of the projection it yielded. Use of finer pipettes discriminated against vesicles which were unusually lax as these yielded an inconveniently long initial projection. But as laxity was not related to strength, this degree of selection seemed better than the loss of information on weak vesicles through early lysis when pipettes of larger relative diameter were used.

Analysis

In contrast to the patch clamp situation, in which much smaller-tipped, fire polished pipettes are used, no seal forms between vesicle membrane and the walls of the aspiration pipette. Rather, the membrane tension created by aspiration is transmitted to the portion of the vesicle outside the pipette i.e. an isotropic tension is set up. Following in the footsteps of Rand (1964) and Evans *et al.* (1976), the law of Laplace may therefore be applied to the hemispherical cap inside the pipette and to the spherical portion of the vesicle:

$$P_v - P_p = 4T/D_p \qquad (1)$$

$$P_v - P_o = 4T/D_v \qquad (2)$$

where P_v, P_p and P_o are the pressures in the vesicle, pipette and bathing solution respectively. D_p and D_v are the diameters of the pipette and spherical portion of the vesicle and T the isotropic tension keeping the system in equilibrium (Fig. 2.02). As D_p is smaller than D_v the pressure difference across the cap needs to be larger than the pressure difference across the major portion of the vesicle, which is achieved by the aspiration pressure

$$\Delta P = P_o - P_p \qquad (3)$$

ΔP is the measurable variable in the experimental situation and by subtraction of equations (1) and (2) comes to

$$\Delta P = 4T(1/D_p - 1/D_v) \qquad (4a)$$

or

$$T = \Delta P\, D_p / 4(1 - D_p/D_v)\,. \qquad (4b)$$

The membrane area of a vesicle aspirated into a pipette with light suction adequate to take up the membrane slack is given by

$$\pi D_v^2 - \pi D_p^2/4 + \pi D_p L \qquad (5)$$

where the first term is the area of a complete sphere of diameter equal to the aspired vesicle, the second term is the cross-sectional area of the pipette not covered by membrane, and the last term is the area of the projection, of length L, into the pipette. With increase in suction pressure and consequent dilation of the membrane, the projection can grow longer. The attendant movement of the vesicle content into the pipette provides at the same time additional area towards elongation of the projection. The increase in membrane area attributable to membrane dilation under stress, ΔA, will therefore be given by

$$\Delta A = \pi D_p \Delta L - \pi(D_{v0}^2 - D_{v1}^2) \qquad (6)$$

where ΔL is the increase in the length of the projection, D_{v0} is the initial diameter of the vesicle aspirated with light suction and D_{v1} the smaller diameter of the pressurized vesicle. As the membrane can suffer only a small area dilation, the volume displacement into the pipette is too small for D_{v1} to be measurably different from D_{v0}, but under assumption of constant total volume D_{v1} will be given by

$$D_{v1}^3 = D_{v0}^3 \{ 1 - [(6D_p^2 \Delta L)/4D_{v0}^3] \}. \qquad (7)$$

A computer programme which calculates this term and solves for ΔA by inserting it into equation (6) has been written by Dr. F.L. Burton and was used by Hutter *et al.*, 1991. The programme also calculates T from equation (4b) and the best-fit slope of the stress-strain relation from which the origin of the ΔA axis was established by extrapolation i.e. due allowance was made for the membrane area dilation caused by the initial suction pressure of 2 cm H_2O.

The assumption that the volume of vesicle plus projection remains constant during aspiration needs to be justified, as the pressurization of vesicles should, in principle, cause water to leave the vesicle until an osmotic pressure is set up to oppose the filtration pressures. For erythrocytes pre-swollen in hypotonic (135mOsm) solution to spheres of approximately 7μm diameter, as initially used by Evans *et al.*, (1976), the pressurization is in fact enough for appreciable water loss to occur: about 30% of the increase in the length of the projection into the pipette is due to extra membrane slack produced by pressure filtration (Evans & Waugh, 1977). However, with vesicles an order of magnitude larger and comparable membrane tensions, the filtration pressure will be an order of magnitude smaller; and the higher osmolarity of the content of the present vesicles will further help to reduce water loss during aspiration (see also Evans & Needham, 1987). In practice, no change in the length of the projection with maintained constant aspiration pressure was observed.

Equation (2) implies that the cap of the initial projection into the pipette is hemispherical i.e. that its length is at least equal to the radius of the pipette. For a vesicle to take up this configuration when first lightly aspirated with a pipette about 1/3 of its diameter, its membrane area needs to be about 1% greater than the minimum required to envelope its volume in perfectly spherical form. Many vesicles satisfied this criterion and a few were even laxer so that they formed initial projections several times the length of the pipette radius. Why vesicles vary in this respect remains unexplored. Unstressed phospholipid vesicles are known to show undulations of thermal origin (Sackmann, Duwe &

Engelhardt, 1986), but these constitute an excess surface area of less than 1% (Zhelev *et al.*, 1994). In sarcolemmal vesicles, small differences between the diameters of free vesicles in the horizontal and vertical planes, differences in the content of osmotically active material or incompletely unfolded membrane invaginations due to residual cytoskeletal elements are more plausible sources of excess membrane. A proportion of vesicles formed, on initial aspiration, only a small bulge into the pipette so that equation (2) was not applicable. Although it would have been possible to calculate membrane tension and area increase also in such vesicles, they were not used. In later experiments 16 mM sucrose was added to 140 mM KCl in the bath and in the pipette, in order to ensure that all vesicles were adequately deflated. The fact that vesicles were prepared at a higher temperature than that at which they were aspirated would tend to tighten vesicles as the membrane area shrinks more on cooling than does the vesicle content (Kwok & Evans, 1981). But when vesicles were allowed to form at room temperature they were not obviously laxer than those produced by the normal quicker procedure in which the enzyme treatment was conducted at ≈34 °C. The laxity of vesicles was an uncontrolled variable. It was quantified in terms of initial excess membrane area so that any relationship between it and another parameters might emerge. Laxity, Q, was defined as

$$Q = 100 \times [\,(A_o/A_{oR}) - 1\,] \qquad (8)$$

where A_o is the area of the spherical portion of the vesicle plus that of the projection formed on light aspiration (see equation (5) above) and A_{oR} is the area of a sphere of equal volume.

Solutions

140 mM KCl was always buffered with 5 mM HEPES and titrated with KOH to pH 7.4 at room temperature. No correction was made for the slight increase in pH to be expected when the solution was cooled to store muscle, or for the slight decrease in pH to be expected when the temperature was raised during enzyme action. When solutions containing < 20 μM Ca^{2+} were required, nitrilo-triacetic acid (NTA) was used as metal-buffer. The free acid was brought into solution by titration with 1N KOH and made up to give a 100 mM stock. The concentrations of $CaCl_2$ which were combined with 2 mM NTA to give various concentrations of Ca^{2+} are given in Table 2.1. The table holds for pH 7.4 and is based on a computer programme kindly supplied by Dr. G.L. Smith using the association constants for NTA given by Martell & Smith, (1974).

$$[H^+NTA^{3-}] / [H^+] [NTA^{3-}] = k_1 = 9.71$$

$$[H^+NTAH^{2-}] / [H^+] [H^+NTA^{3-}] = k_2 = 2.48$$

$$[H^+NTAH_2^{1-}] / [H^+] [H^+NTAH^{2-}] = k_3 = 1.80$$

$$[H^+NTAH_3] / [H^+] [H^+NTAH_2^{1-}] = k_4 = 0.80$$

$$[Ca^{2+}NTA] / [Ca^{2+}] [NTA] = k_{Ca1} = 6.41$$

$$[Ca^{2+}(NTA)_2] / [Ca^{2+}] [NTA]^2 = k_{Ca2} = 8.86$$

In practice, a solution containing 4 mM NTA in 140 mM KCl, 32 mM sucrose, twice the amount of Ca^{2+} given in Table 2.1 plus twice the final concentration of ionophore A23187 was prepared. This solution was then mixed with an equal volume of 140 mM KCl containing vesicles. An interval of at least 10 min was allowed before aspiration was begun.

Calcium ionophore A23187 was obtained from Sigma Chemical Co. in phials containing 1 mg. 1.9 ml of dry DMSO was injected into the phial to give a 1 mM stock solution, within the limits of accuracy of the amount of ionophore dispensed by the supplier. The stock solution was sonicated before aliquots were added to double strength Ca^{2+}-buffer to

give, on 1:1 dilution, a final concentration of 5 μM A23187 plus 0.5% v/v DMSO, or more often 1 μM A23187 plus 0.1% v/v DMSO. Buffered solutions of Mg^{2+} were made in similar fashion (Table 2.1). In this case the metal-ligand association constants used were:

$$[Mg^{2+}NTA] / [Mg^{2+}] [NTA] - k_{Mg1} = 5.41$$
$$[Mg^{2+}(NTA)_2] / [Mg^{2+}] [NTA]^2 = k_{Mg2} = 7.21$$

According to Chandler & Williams (1977), addition of A23187 (0.3-20 μM) to Ringer solution causes the formation of insoluble metal-ligand complexes which may be separated by centrifugation. After such centrifugation however, the rate of uptake of A23187 into erythrocyte ghosts, as measured fluorimetrically, is greatly reduced. This suggests that particulate A23187 acts as a reservoir for soluble ionophore. The present conditions differ from those of Chandler & Williams in that much lower concentrations of Ca^{2+} were used so that less precipitation of metal-ligand complex might be expected. Even so, the potentially heterogeneous physical state of A23187 in divalent cation containing solution could be a source of variability. In an attempt to standardize conditions, solutions containing A23187 were sonicated immediately before addition to vesicle-containing solution. With these precautions no obvious crystal formation occurred in the vesicle-containing solution to which Ca^{2+} or Mg^{2+} A23187 had been added. With Mn^{2+} and Co^{2+}, by contrast, progressive formation of crystals was evident (see p 56)

Ionomycin, purchased from Calbiochem, was dissolved in DMSO (1 mg in 1.33 ml DMSO) to give a 1 mM stock solution and then handled like A23187.

BAPTA-AM (Molecular Probes) was dissolved in dry DMSO to give a 10 mM stock solution. 0.2 ml aliquots were stored frozen. When diluted 1:500 with 100 ml KCl, a

cloudy solution resulted. Attempts made to prevent the precipitation of the ester are described in the text (p 59).

MDL 28,170 was kindly made available to us by Dr. E.H.W. Bohme of the Marion Merrel Dow Research Institute, Cincinatti, Ohio. 5 mg was dissolved in DMSO to give a 13 mM stock.

Lysophosphatidylglycerol (Sigma) 1 mg was taken up in 0.1 ml ethanol and diluted to 1 ml with 140 mM KCl to give a close on 2 mM stock solution in 10% ethanol. Lysophosphatidylcholine (LPC) 5 mg was taken up into 2 ml of ethanol to give close on 5 mM stock solution. The highest concentration of ethanol to which vesicles were exposed was 0.05% v/v with 10 μM LPG and 0.25% v/v with 12.5 μM LPC.

Manoalide (M.W. 416) was a gift by Dr. Barbara Potts, Scrips Institute of Oceanography, La Jolla, California. It was dissolved in methanol to give a 1 mg/ml stock solution (2.4 mM). 0.5 ml aliquots were dried down and stored at -80°C. For use they were re-dissolved in methanol. In the strongest concentration seen by vesicles, manoalide (1.2 μM) was therefore in 0.05% v/v methanol.

Nor-dihydroguoiateric acid (Sigma) was prepared as a freshly sonicated 200 μM suspension/solution and diluted as stated in the text.

Ro 31-4493 (M.W. 390) was kindly provided by Dr. M-S. Suleiman, Bristol Veterinary Physiology. 1 mg dissolved slowly in 1ml ethanol and was made up to 10 ml with KCl to give a 250 μM stock solution in 10% ethanol. Hence 12.5 mM Ro 31-4493 contained 0.5% v/v ethanol.

α-toxin was a crude α-lysin from *staphylococcus aureus* (strain Wood 46) kindly supplied deep frozen by the Department of Microbiology, University of Glasgow. Re-

assayed before present use, the potency of the batch was 3200 haemolytic units (H.U.) ml^{-1}. This would be equivalent to approximately 0.1 mg ml^{-1} of commercially available pure toxin (Calbiochem).

Arachidonic acid (Sigma) 10 mg was dissolved in 1 ml ethanol to give a 32 mM stock solution which was kept in a deep freeze. 0.1 ml of alcoholic stock dissolved in 100 ml 140 mM KCl then gave a 32 μM solution which was further diluted 1:1 with vesicle-containing 140 mM KCl solution to give a maximum concentration of 16 μM arachidonic acid in 1:2000 ethanol. Lower concentration down to 2 μM arachidonic acid was made by appropriate adjustment of the volumes of stock and 140 mM KCl used. To avoid the oxidation of arachidonic acid before its admixture to the vesicle-containing solution, 140 mM KCl was bubbled with N_2 for several minutes before addition of arachidonic acid then kept airtight. Dilution of arachidonic acid were freshly made up for each experiment.

Statistical Methods

The maximum vertical distance between cumulative lysis tension curves provided the statistic for determining the significance of the difference between them, in accordance with the Kolmogorov test. Skewness in a population was determined from the average value of the third moment about the mean.

Table 2.1. Composition of solutions containing $CaCl_2$ and $MgCl_2$ buffered with 2 mM NTA at pH 7.4.

Total Ca (μM)	Ca^{2+} (μM)	Total Mg (μM)	Mg^{2+} (μM)
345	20	480	181
187	10	344	126
116	6	117	41
79	4	41	14
40	2		
16	0.8		

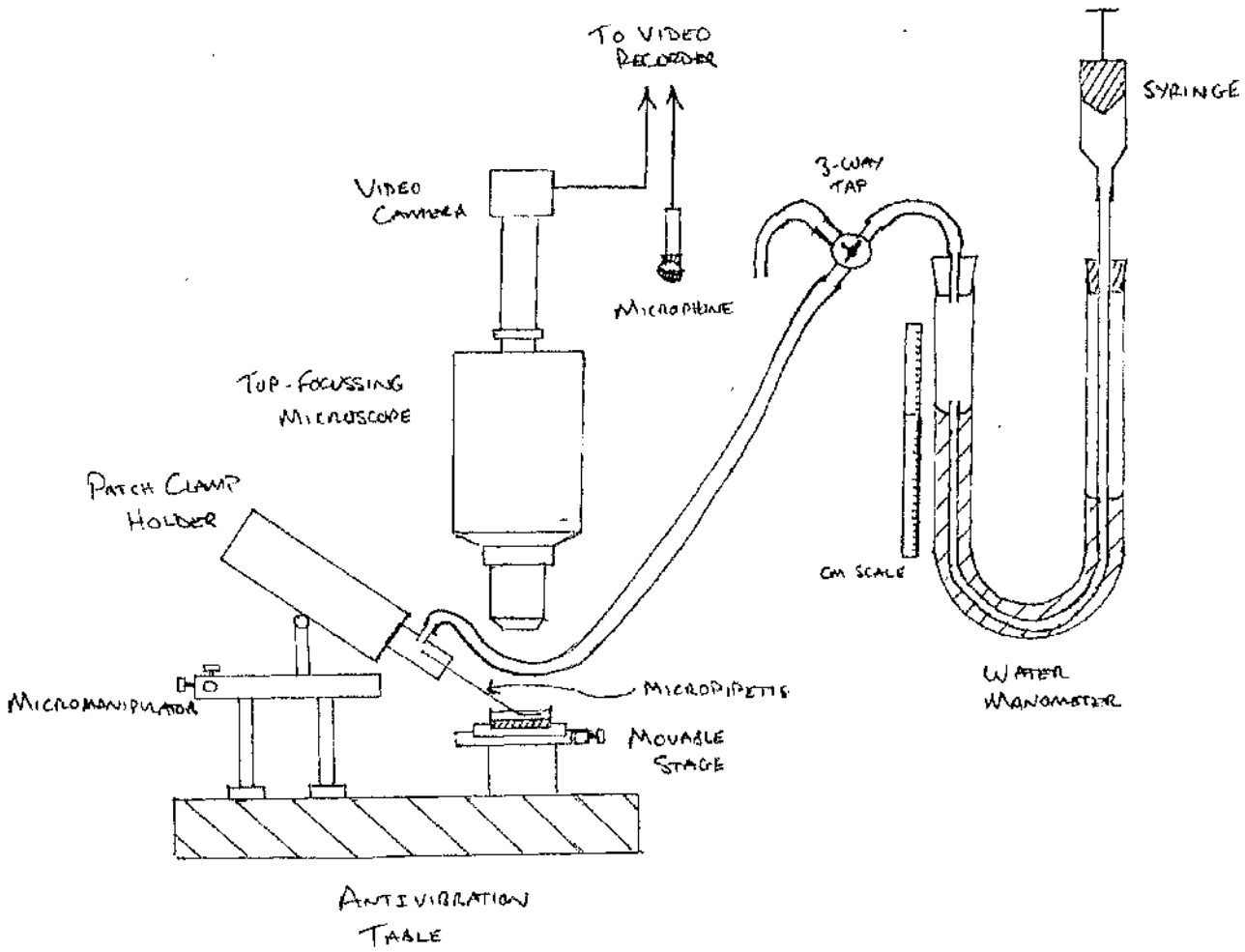

Figure 2.01 Diagram of pipette aspiration apparatus

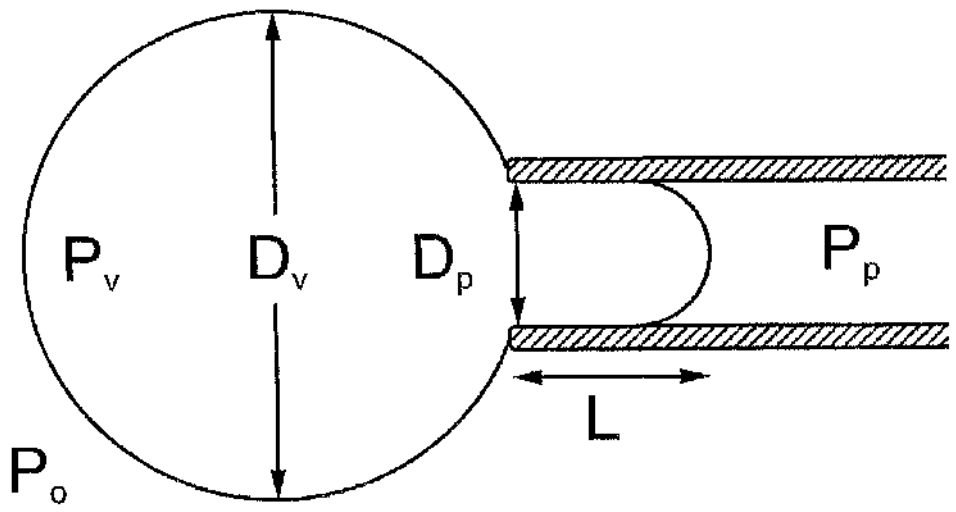

Figure 2.02 Diagramatic representation of the pipette aspiration technique

P_v, P_p, and P_o are the pressures in the vesicle, pipette and bathing solution respectively; D_p and D_v, the diameters of the pipette and spherical portion of the vesicle respectively, and L, the length of the projection into the pipette

As the aspiration pressure increases, the projection lengthens as the membrane area expands in response to the increase in isotropic membrane tension

PART III

MECHANICAL PROPERTIES OF UNTREATED SARCOLEMMAL VESICLES

Results

Samples of the video record of a typical pipette aspiration experiment are shown in Fig. 3.01. The first frame of the sequence shows a vesicle aspirated by a negative pressure of 2 cm H_2O, which was generally enough to take up any slack in the membrane. The subsequent frames show how with stepwise increases in aspiration pressure the projection of the vesicle into the pipette grows longer until sudden rupture occurs.

A plot of membrane tension, T, against the fractional increase in membrane area, ΔA, is shown in Fig. 3.02. Typically, the stress-strain relation is linear right up to the last measurable tension before the vesicle bursts, in this case 14.5 mN m^{-1}. This allows extrapolation of the stress-strain relationship to its origin and hence calculation of the unstressed membrane area. In the experiment of Fig. 3.02 the membrane area had increased by 0.03 of its original unstressed area when the vesicle burst. In subsequent descriptions the maximal sustainable membrane tension (or lysis tension) will be denoted as T^* and the maximum sustainable fractional increase in area as ΔA^*. The point representing T^* and ΔA^* on T *vs.* ΔA plots will at times be called the 'lysis-point'.

A linear stress-strain relationship signifies that the membrane of sarcolemmal vesicles behaves as a perfectly elastic structure whose distensibility can be defined by the slope of the stress-strain relationship

$$K = \frac{T}{\Delta A}$$

where K is the modulus of area dilation. In the experiment of Fig. 3.02 it amounts to 491 mN m^{-1}. The area under the stress-strain relation is

$$\frac{T \times \Delta A}{2}$$

and has the dimension of energy. It represents the energy stored per unit area in a vesicle just before it bursts. In the illustrated experiment it amounts to 0.21 mN m / m^2.

Only on a few early occasions did a days work consist of measuring the mechanical properties purely of 'normal' vesicles in 140 mM KCl. Usually, a variable was introduced into the experiment. One or two dishes of untreated vesicles were then also prepared and about 10 normal vesicles aspirated at intervals during the experiment to serve as controls. In this way, data on over a thousand normal vesicles was accumulated. It is summarized in Fig. 3.03 in which scattergram the lysis point for each normal vesicle is represented.

Fig. 3.03 is incomplete in the sense that it does not include vesicles which were so weak that they burst instantly on aspiration. A few such vesicles were usually present in each batch. A better way to represent the distribution in membrane tensile strength therefore is by means of a probability density function (p.d.f.). This shows that about 9% of vesicles burst at a lysis tension lower than could be readily measured under the experimental conditions normally used (Fig. 3.04). For the remaining 91% the p.d.f. shows a skewed distribution. An upper limit to the strength of the membrane is obviously imposed by the structure of the membrane. On the other hand, there is no limit to how weak a vesicle can be as a result, say, of faulty sealing during its original formation, or owing to other destructive influences.

A closely related way of representing the distribution of membrane tensile strength is to plot a cumulative distribution function (Fig. 3.05). This shows the proportion of vesicles bursting at or below a given membrane tension. For the present population of normal vesicles half had burst by 12.43 mN m^{-1} (median) with 95% confidence limits of 12.29 to 12.67 mN m^{-1}. The cumulative distribution curve is a compact form of data representation, and in later sections, changes in membrane tensile strength will be represented as shifts in this curve.

An interesting feature of Fig. 3.03 is the direct relationship between T^* and ΔA^*. Evidently, vesicles greatly differing in the maximum tension which they can sustain are distensible to a similar degree. Nevertheless, the area elasticity of the membrane varies to some extent from vesicle to vesicle even within the same batch. Fig. 3.06 gives a distribution of K for the control population of vesicles already characterized in Fig. 3.03. Bar a few outriders, K is distributed in approximately normal fashion with a mean value of 490 ± 88 mN m^{-1} (mean ± S.D.)

The distribution of the values of ΔA^* is shown in Fig. 3.07. As with the T^* distribution (Fig. 3.04), it is skewed because vesicles weakened for whatever reason can undergo only a small increase in area before bursting. The median of the distribution was 0.027 with 95% confidence limits 0.0265 and 0.0274 and the mode 0.028. Only a few of the strongest and most compliant vesicles were able to sustain a fractional increase in membrane area above 0.04.

To test whether the mechanical properties of sarcolemmal vesicles depended on muscle fibre type, vesicles were prepared also from the soleus muscle of a rabbit. The mean values for 10 vesicles from that source were T^* = 13 ± 1.6 mN m^{-1} and K = 511 ± 59 mN m^{-1} (mean ± S.D.) i.e. not distinguishable from the population of normal psoas vesicles.

Does $[Ca^{2+}]_o$ influence membrane tensile strength?

The results so far described relate to vesicles shed into 140 mM KCl to which no Ca^{2+} had been added and whose stray Ca^{2+} content was estimated to be $< 10^{-6}$ M. From the behaviour of K_{Ca} channels (Burton *et al.*, 1988), we may conclude that $[Ca^{2+}]_i$ in such vesicles is also sub-micromolar. Sarcolemmal vesicles contain ATP (Burton *et al.*, 1988) and are probably capable of glycolysis, but it is as yet unknown how well they can defend themselves against imposed ionic gradients. For the mechanical measurements here reported, vesicles were therefore normally left in the nominally Ca^{2+}-free KCl solution in which they formed. This also ensured comparability with the data on membrane mechanical properties of red blood cells which were similarly measured in saline without added Ca^{2+} (Evans *et al.*, 1976; Waugh & Evans, 1979). However, it was clearly necessary to test whether membrane mechanical properties are altered by lack of extracellular Ca^{2+}. 140 mM KCl to which $CaCl_2$ had been added was admixed to 140 mM KCl containing shed vesicles, so as to raise $[Ca^{2+}]_o$ to 0.1-1.0 mM. Up to 0.5 mM $[Ca^{2+}]_o$ no effect was detectable, but vesicles exposed to 1.0 mM $[Ca^{2+}]_o$ tended to become weaker if measurements were continued for longer than one hour (Fig. 3.08). The slow time course of this effect suggests that it probably arises from a failure of vesicles subjected to a Ca^{2+} gradient of physiological magnitude to maintain a normally low cytosolic Ca^{2+} level. Addition of 2 mM NTA to 140 mM KCl so as to lower stray Ca^{2+} to submicromolar levels had no detectable effect on membrane strength. That addition of up to 500 μM $[Ca^{2+}]_o$ had no effect accords with the view that membrane tensile strength is determined by the state of the lipid matrix; and that the zwitterionic phospholipids interspersed with cholesterol, which mostly comprise the external leaflet of cell surface membranes, have little tendency to bind Ca^{2+} (Shah & Schulman, 1967). On present evidence, membrane strength is thus distinct from membrane ionic permeability, where some proteinaceous membrane channels require $[Ca^{2+}]_o$ to retain their normal properties (Almers, McCleskey & Palade, 1984; Armstrong & Miller, 1990). As to the small reduction in membrane tensile strength observed on prolonged exposure to 1 mM

$[Ca^{2+}]_o$, our interpretation that this probably arises from accumulation of Ca^{2+} within vesicles is borne out by the results of Part IV.

Sources of variability

To test how far differences between muscle samples contributed to the total variance of K, the results from the 132 experimental days were subjected to one-way analysis of variance. This showed that 31% of the total variance was attributable to day-to-day variations. When the day-by-day results were plotted chronologically it also became evident that K tended to increase over the experimental period (Fig. 3.09). This trend, when approximated by a linear regression, explained 12% of the total variance. Possibly this trend was due to the overall faster conduct of the experiments which came with experience and which would have minimized any progressive deterioration of vesicles with time. No correction was made for the uncontrolled differences in room temperature from 19-25 °C which must also have contributed to the day-by-day variance. But as the Q_{10} for K measured between 15 and 30 °C is only 1.2 (E.A. Evans, W. Rawicz & O.F. Hutter, unpublished measurements on murine sarcolemmal vesicles) the variance from that source is unlikely to be more than 4% of the total variance (see above and also Kwok & Evans, 1981). Only about 15% of the total variance is thus attributable to differences between animals.

With 69% of the total variance in K attributable to scatter within each batch, it may be asked for how much measurement errors can account. This question was addressed by estimating the greatest likely S.D. for each of the measurements entering into K and then synthesizing values distributed normally around an appropriate mean. Values for K were then calculated and their distribution analysed. This procedure takes into account that the errors in the different measurements are not of equal weight, as some measurements enter more than once into the calculation of K. Starting with ΔP, this was considered accurate to $\pm$ 1mm H_2O. With typical lysis values of around 200 mm H_2O, the error from that

source was therefore negligible. The error in measuring D_v was also relatively small, say + 2%. The main sources of error in determining T^* and ΔA lie in the measurement of D_p and ΔL wherein imperfect optical definition limited accuracy probably to ± 10%. In arriving at K, the error in D_p, which enters both into T^* and ΔA (see page 24 equations 4b and 5) cancels out, but the error in ΔL is carried through. It probably accounts for about 50% of the S.D. of ± 88mNm^{-1} which is attached to the measurement of K (Fig. 3.06). The least accurate measurements entering into K were D_p, where an error of ± 5% i.e. ± 1 μm for a 20 μM pipette was conceivable, and ΔL where ± 5% again seemed an adequate allowance for error. On this basis at most 12% of the total variance can be attributed to measurement error. Therefore more than half the variance in K seems to be due to real but as yet unexplained differences between vesicles, or to unrecognized systematic error.

Discussion

Dilational elasticity

The pipette aspiration technique was first applied to red blood cells transformed into spheres by treatment with hypotonic saline solution (Evans *et al.*, 1976), as these present themselves singly in nature and may be readily transformed into spheres by treatment with hypotonic solution. For human red blood cells (n = 30) Evans, Waugh & Melnick found T^* to be 6-11 mN m^{-1} and K to be 288 ± 90 (S.D.) mN m^{-1}. Later the figure for K was uprated to 450 mN m^{-1} (25 °C) on taking into account an osmotic correction which was necessary owing to the small size of red blood cells and their low internal salt concentration when swollen in hypotonic solution (Evans & Waugh, 1977). According to Waugh & Evans (1979), the best figure for K based on about 200 red blood cells under various conditions is about 500 mN m^{-1} at 20 °C, i.e. substantially the same as found here for rabbit sarcolemmal vesicles. That an intact cell with a natural membrane displays membrane mechanical properties so similar to those of sarcolemmal vesicles argues in

favour of the view that sarcolemmal vesicles, though produced by an unphysiological procedure, represent a valid model for studying the muscle surface membrane.

In terms of material science, the area elasticity of the membrane is a continuum property which represents the behaviour of the constituent membrane molecules averaged over space and time (for a review see Bloom, Evans & Mouritsen, 1991). In a composite membrane made up of fluid lipid and relatively rigid integral protein molecules, as is here envisaged for the sarcolemma, the elastic properties will reside in the lipid component (Needham & Nunn, 1990). In the unstressed state the hydrophobic cohesive forces between the hydrocarbon chains will be in equilibrium with the repulsive forces, e.g. those between the head groups of the phospholipid molecules; and the area occupied by each phospholipid molecule will be the optimum area for minimum free energy (Israelachvili, 1985). When stress is applied to the membrane, each phospholipid molecule will come to possess more free energy and occupy a larger area. Israelachvili (1985) has shown that for a phospholipid bilayer expanding elastically the modulus of area elasticity K (mN m^{-1} or mJ m^{-2}) will be 4 times the minimum free energy due to hydrophobicity of each monolayer, of which the latter can be estimated to be about 50 mJ m^{-2}. For a phospholipid bilayer a value for K of about 200 mN m^{-1} would therefore be expected. Values of K in the order of 500 mN m^{-1}, as found in red blood cells and as here reported, must therefore be attributed to the presence in the sarcolemma also of (i) cholesterol which intercalates as a smooth, hard face between phospholipid molecules and so straightens acyl chains and improves cohesion (Bloom *et al.*, 1991) and (ii) of integral membrane proteins which are largely inextensible and so give rise to a higher value of K than would be obtained if the membrane were composed of lipids alone.

Of particular interest in this connection are the measurements of Evans & Needham (1987) and Needham & Nunn (1990) of the mechanical properties of artificial vesicles (20-40 μm diameter) formed from known mixtures of phospholipid and cholesterol. For vesicles composed purely from steoroyloleoylphosphatidyl choline (SOPC) they found

the lysis tension, T^*, to be 6-7 mN m^{-1} and K to be 260 mN m^{-1}. As the mole fraction of cholesterol was increased, the value for T^* rose to 30 mN m^{-1} and K to a plateau value of 1400 mN m^{-1}. This effect of cholesterol may be attributed to it filling spaces between phospholipid acyl chains, so that lipid-lipid interaction becomes stronger. For vesicles made from lipid extracted from red blood cells, which contain about 40 mole % cholesterol, K was found to be 470 mN m^{-1}, which agrees well with both the K value for SOPC/cholesterol vesicles of similar composition and with K as found for normal red blood cells (see above). At first sight, this agreement seems to suggest that the mechanical properties of the red blood cell membrane are explicable entirely by the behaviour of the lipid matrix of the membrane. However, note has to be taken of the fact that about 50% of the membrane surface area is occupied by protein molecules which are largely incompressible, and hence the lipid matrix must be more distensible than the composite membrane. On basis of aggregate theory, K for red blood cells should therefore be twice as high as the value obtained with protein free artificial vesicles of similar lipid composition. Needham & Nunn (1990) reconcile this argument by supposing that the proteins of the membrane confer enhanced distensibility upon the red blood cell lipid matrix by perturbing the lipid bilayer in the annular region.

The surface membrane of cells in general is known to be rich in cholesterol (Jain, 1988), and it seems reasonable to assume that integral membrane proteins will occupy a similar proportion of the total membrane area in sarcolemmal vesicles as they do in the red blood cells. By analogy, we may therefore conclude that the mechanical properties of sarcolemmal vesicles are determined (i) by the composition of the lipid matrix of the membrane and (ii) by the degree to which the presence of rigid membrane proteins on the one hand reduces the distensibility of the composite membrane, and on the other hand perturbs the lipid matrix of the membrane. In the interpretation of changes in the mechanical properties of sarcolemmal vesicles, such as will be described in the subsequent section, consideration will therefore be given to possible changes in the above factors.

The deformability and stability of a cell surface membrane under shear stress, i.e. deformation without area dilation, is a property of the membrane cytoskeleton and is distinct from membrane tensile strength and elasticity, as here measured, which resides in the cohesiveness of the membrane lipids. The evident similarity between the tensile strength and elasticity of the swollen red blood cell and the sarcolemmal vesicle probably reflects the similarity in lipid composition of plasmalemma in general. In the red blood cell, differences in fragility result from differences in cell shape and morphology and not tensile strength. The same is probably also true of skeletal muscle fibres which possess enough spare membrane in the folds and caveolae to protect them from damage by area dilation.

Lysis tension and critical areal strain

A consistent finding in the present work has been that untreated vesicles of similar distensibility may burst at greatly different stresses and hence area expansion. A likely explanation is that membrane rupture results from the development of a weakened spot and that this process is dominated by local fluctuations in membrane composition. Two mechanisms can be invoked (i) local thinning of membrane lipids (ii) lateral movement of membrane proteins floating in the fluid membrane into a configuration which reduces membrane strength. In view of the interaction between membrane lipids and proteins, these two processes are not necessarily distinct. Rather, lipid packing defects at lipid-protein interfaces may result from protein aggregation. At all events, membrane failure under tension probably results from stochastic processes (Evans *et al.*, 1976), so there would be a finite possibility of catastrophe occurring early during pipette aspiration. With progressive increase in the applied stress, a progressively smaller and hence more frequently occurring packing defect would then suffice to cause membrane rupture. If lysis tension were determined purely probabilistically i.e. if the population of vesicles were fully homogeneous, the probability density function for lysis tension should be perfectly bell-shaped, and the cumulative distribution function perfectly sigmoid. But in fact these functions were skewed with an excess of weak vesicles (Figs. 3.04 & 3.05). It must therefore be supposed that a proportion of vesicles are flawed for additional reasons, possibly related to their initial formation.

The general idea that rupture of a membrane under stress occurs at one weak spot receives support from what is known about the behaviour of cells and vesicles subjected to osmotic stress. Thus in red blood cells, membrane tension set up by osmotic pressure leads to the transient formation of a single large hole per cell, which may be large enough (> 1 μm) to be observable with light microscopy (Lieber & Steck, 1982). Similarly, in so called 'large unilamellar vesicles'(LUVs, diameter ≈200 nm) composed of phospholipid and choline, osmotic stress leads to a membrane defect whose diameter, as estimated by the release of dextrans, is large enough for a single hole per vesicle to

release the tension set up by the hydrostatic pressure (Mui, Cullis, Evans & Madden, 1993). In passing, it might here be mentioned that 85% of a normal sample of sarcolemmal vesicles survived dilution of the bathing medium from 140 mM KCl to 70 mM KCl (L-C. Tian & O.F. Hutter, unpublished). This is surprising as a vesicle of, say, 80 μm diameter lysing at a membrane tension of, say, 15 mN m^{-1} can sustain an internal pressure of only 7.5 x 10^2 mN m^{-2}, which is equivalent to the pressure set up by an osmotic gradient of only 0.33 mOsm. Sarcolemmal vesicles ought therefore to be extremely sensitive to osmotic lysis, unless other processes are at work. In principle, loss of KCl from sarcolemmal vesicles could abolish any osmotic gradient imposed by a reduction in the concentration of KCl in the bathing medium. But net loss of KCl through normal permeation channels in the sarcolemma is a slow process, especially in outward direction, owing to the inwardly rectifying nature of the K^+-channels (Adrian, 1960). We can therefore be confident that net salt movement across the membrane cannot keep pace with the osmotically driven inflow of water. The most likely explanation for the survival of osmotically stressed sarcolemmal vesicles therefore is that, as with LUVs (Mui *et al.*, 1993), osmotic lysis is not an all-or-none process causing disappearance of vesicles, but rather that it leads to the formation of transient defects through which some of the osmotic content of vesicles is discharged repeatedly. Determination of the number of vesicles surviving exposure to hypotonic solutions therefore gives no information on the tensile strength of the membrane. However, loss of vesicular content, (e.g. cytosolic enzymes) should be demonstrable on minimal dilution of the bathing fluid.

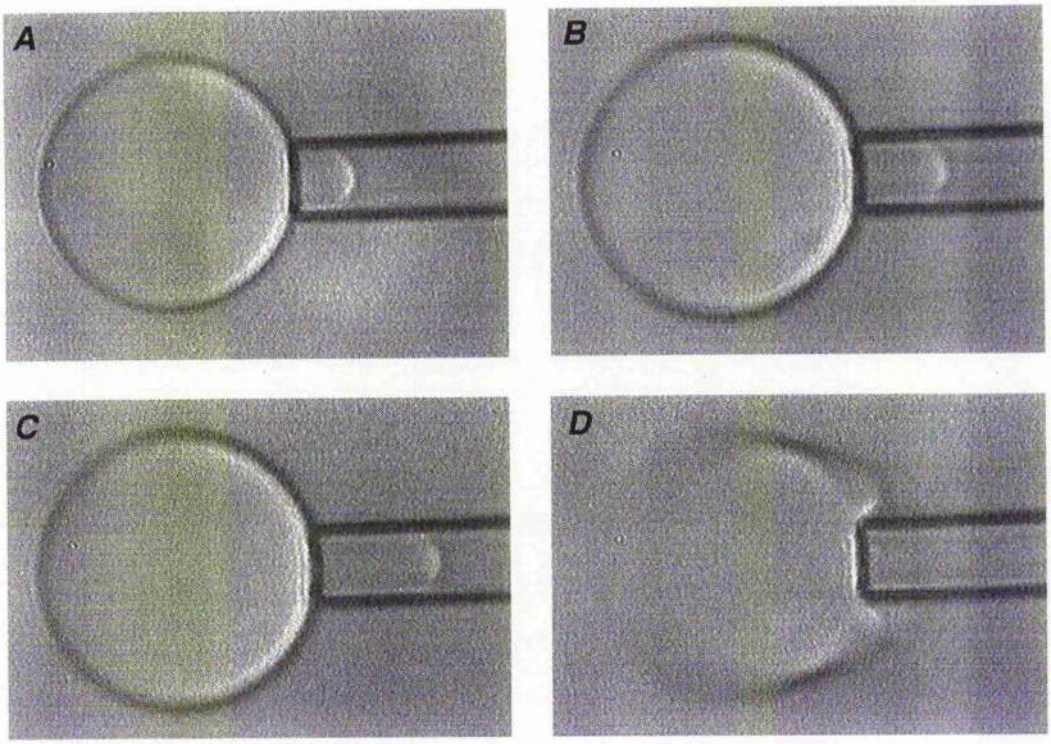

Figure 3.01 Stills from a video record of a sarcolemmal vesicle during aspiration into a parallel bore pipette

A, a vesicle aspirated with light suction (2 cm H_2O) sufficient to take up membrane slack and produce a small projection into the pipette (D_p = 19 μM). B, suction pressure 8 cm H_2O. C, suction pressure 20 cm H_2O. As the membrane is stressed, the projection lengthens upon expansion of the membrane area until a critical point is reached and the vesicle ruptures, D. Frames focussed for best visibility of the projection within the pipette. Spherical portion of vesicle (D_v = 66 μM) consequently out of focus.

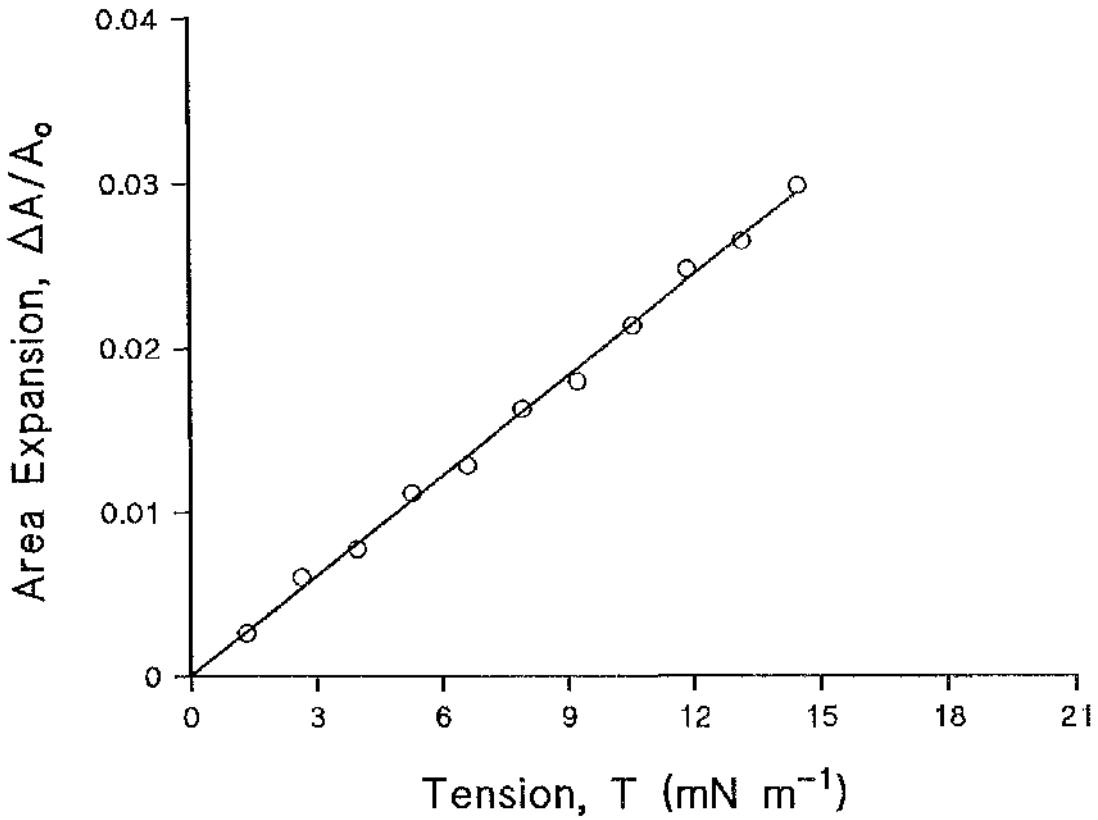

Figure 3.02 Stress–strain relationship for a typical vesicle.
Membrane tension (T) is plotted against fractional increase in area expansion ($\Delta A/A_o$), for every increment of 2 cm H_2O in suction pressure until vesicle ruptures. Uppermost point denotes lysis tension, T^*, and critical area expansion ($\Delta A/A_o$).

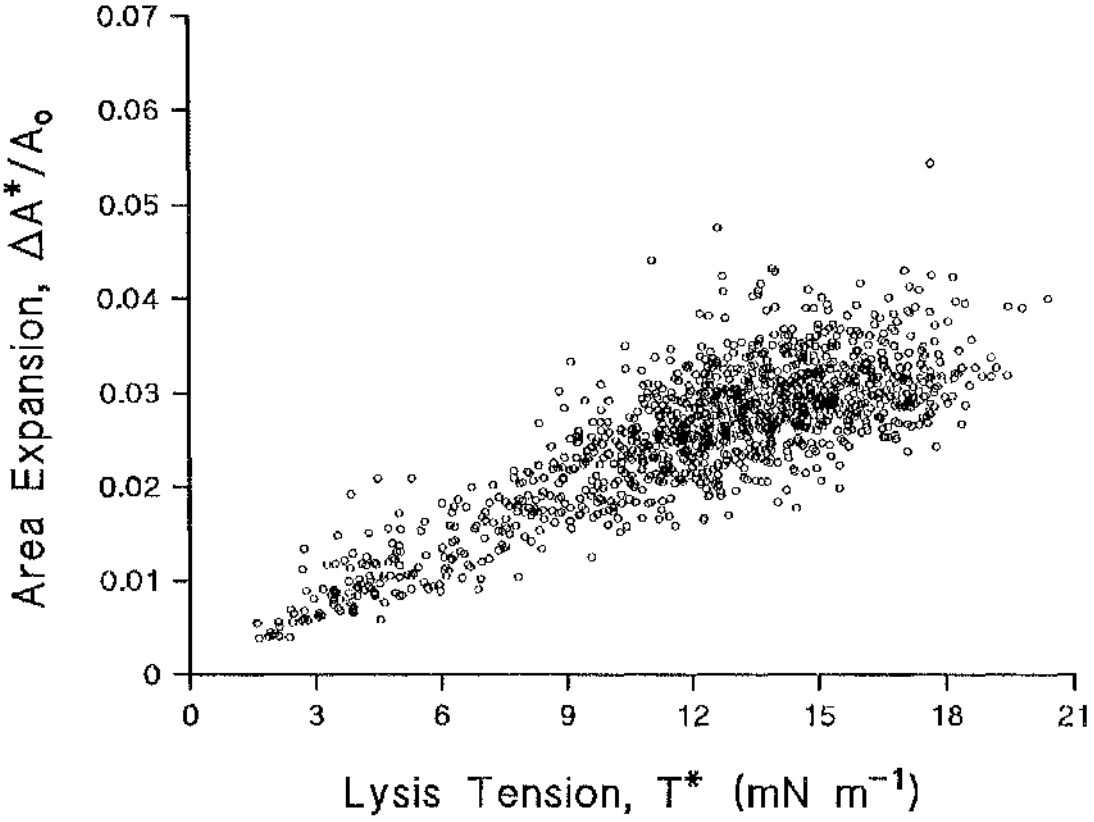

Figure 3.03 Distribution of lysis points for control vesicles

Points show the maximum sustainable tension, T^*, and the corresponding fractional increase in membrane area, A^*/A_o, for the 1301 control vesicles in 140 mM KCl, 5 mM Hepes, at pH 7.4, which yielded values of A^*/A_o under the experimental limits

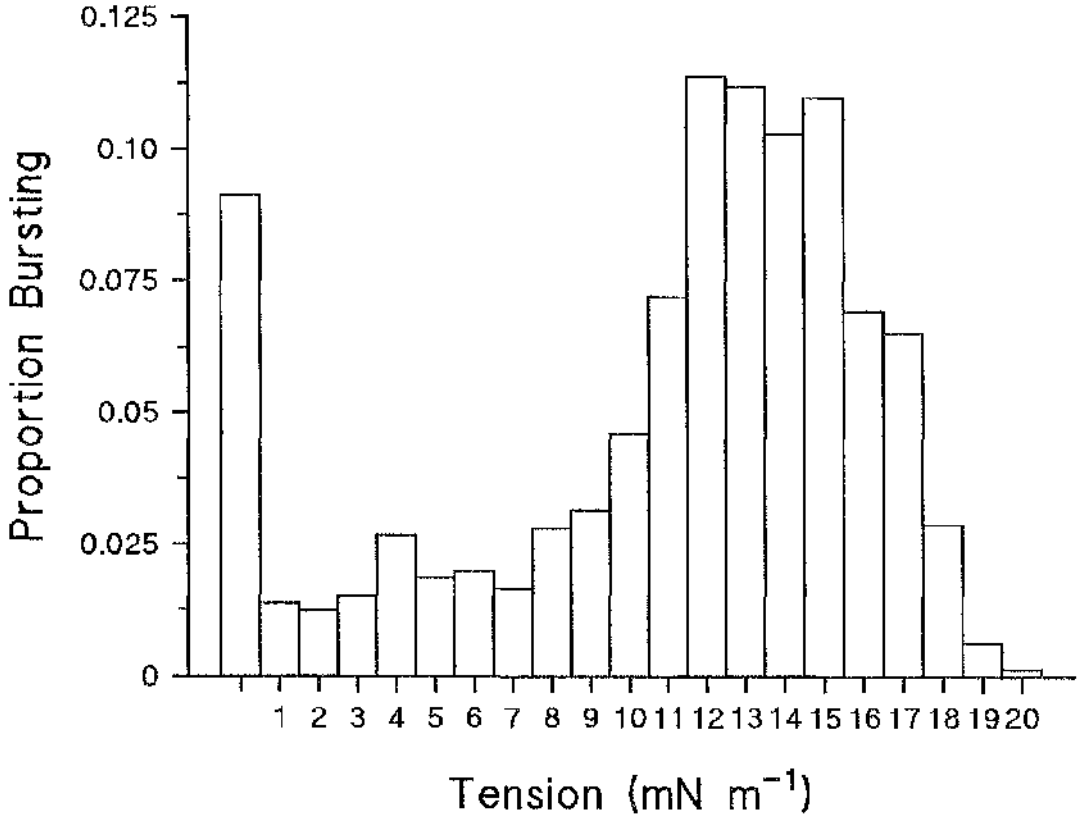

Figure 3.04 Probability density function of lysis tension (T^*) for control vesicles

Histogram shows the proportion of the 1459 control vesicles bursting for every increment of 1 mN m^{-1} membrane tension from 0.5 to 20.5 mN m^{-1}. The proportion of immeasurably weak vesicles (9.1%) is given by the extreme left–hand bar.

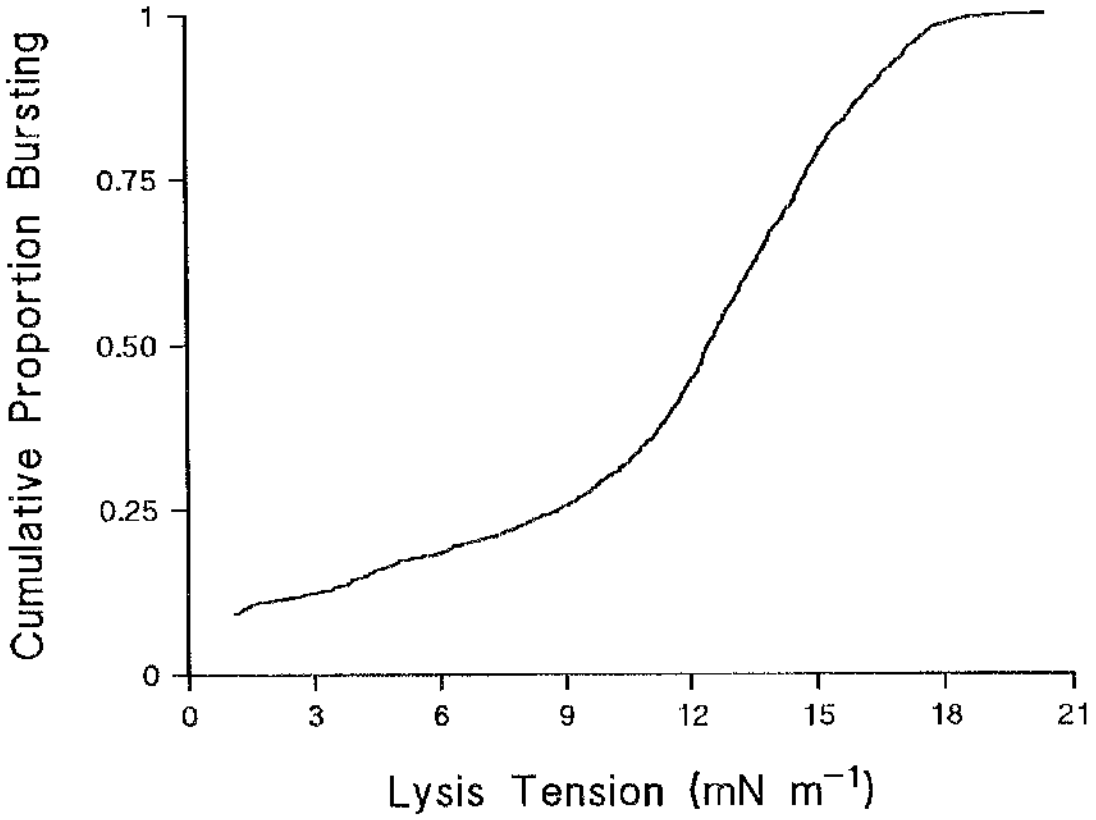

Figure 3.05 Cumulative distribution function of lysis tension (T^*) for control vesicles

Graph represents the proportion of vesicles burst at or below a given tension e.g. half of all vesicles measured burst at tensions below 12.8 mN m^{-1}. Proportion of immeasurably weak vesicles (9.1%) is given by the left–hand end of the curve.

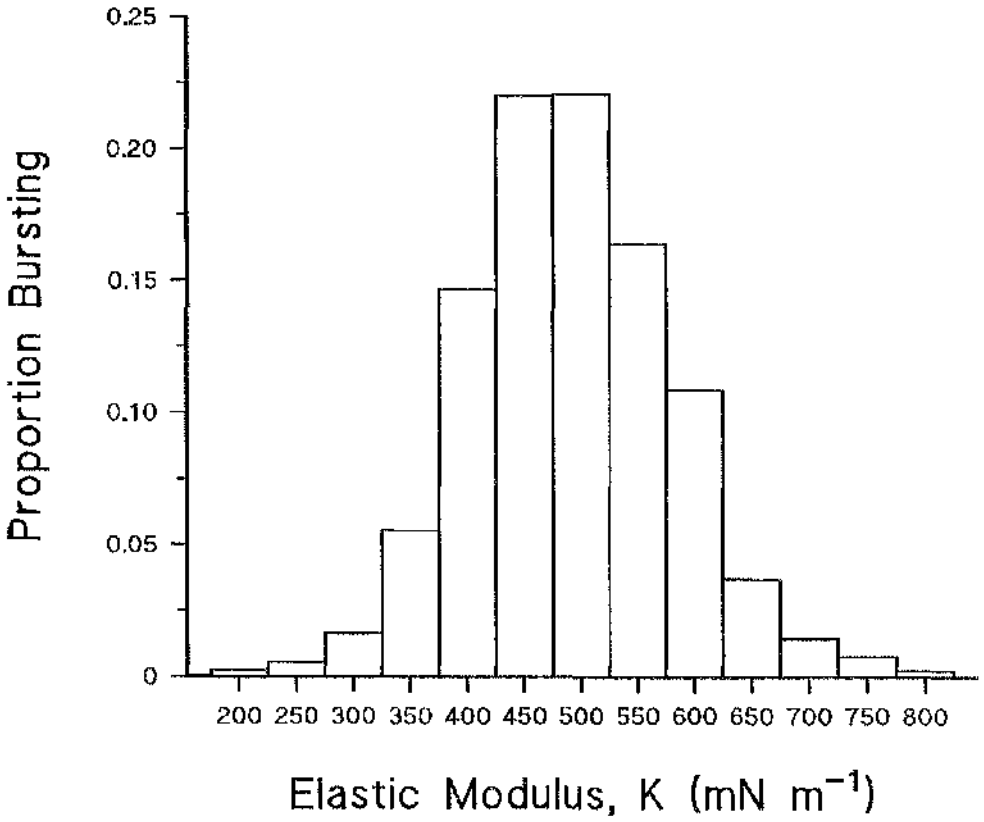

Figure 3.06 Probability density function of area elastic modulus, K, for control vesicles

Histogram shows the distribution of K values for 1301 control vesicles about the mean of 490 ± 88 mN m^{-1} and is consistent with a normally distributed function.

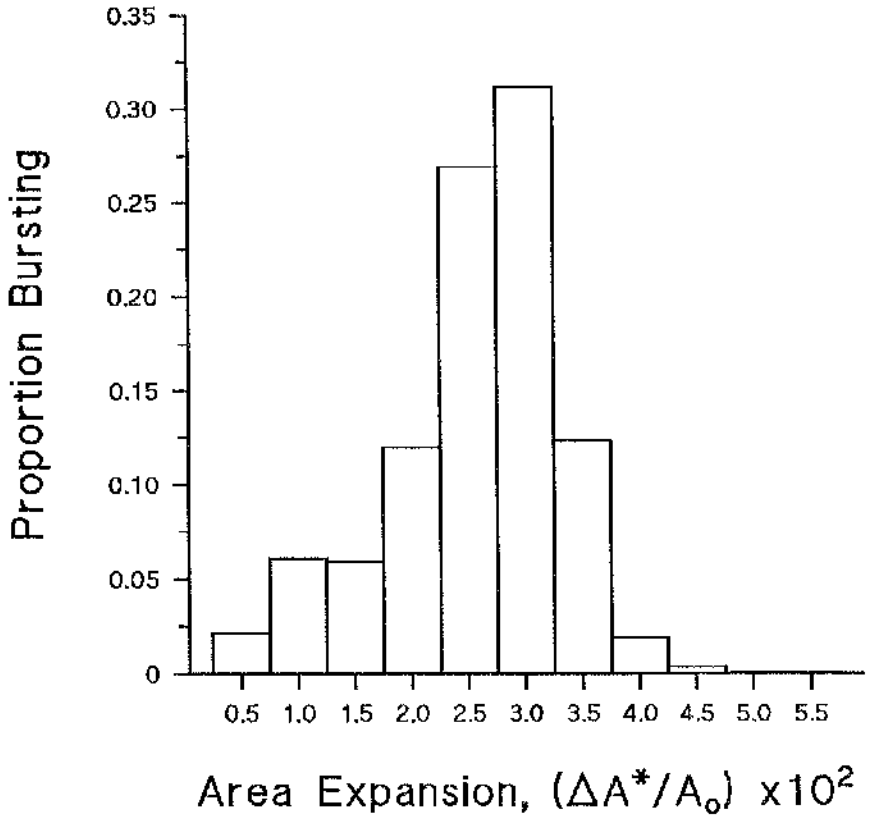

Figure 3.07 Probability density function of maximum fractional increase in area expansion, $\Delta A/A_o$, for all control vesicles

Histogram shows the proportion of control vesicles (n = 1301) bursting at an area expansion of between 0.25 and 5.75%.

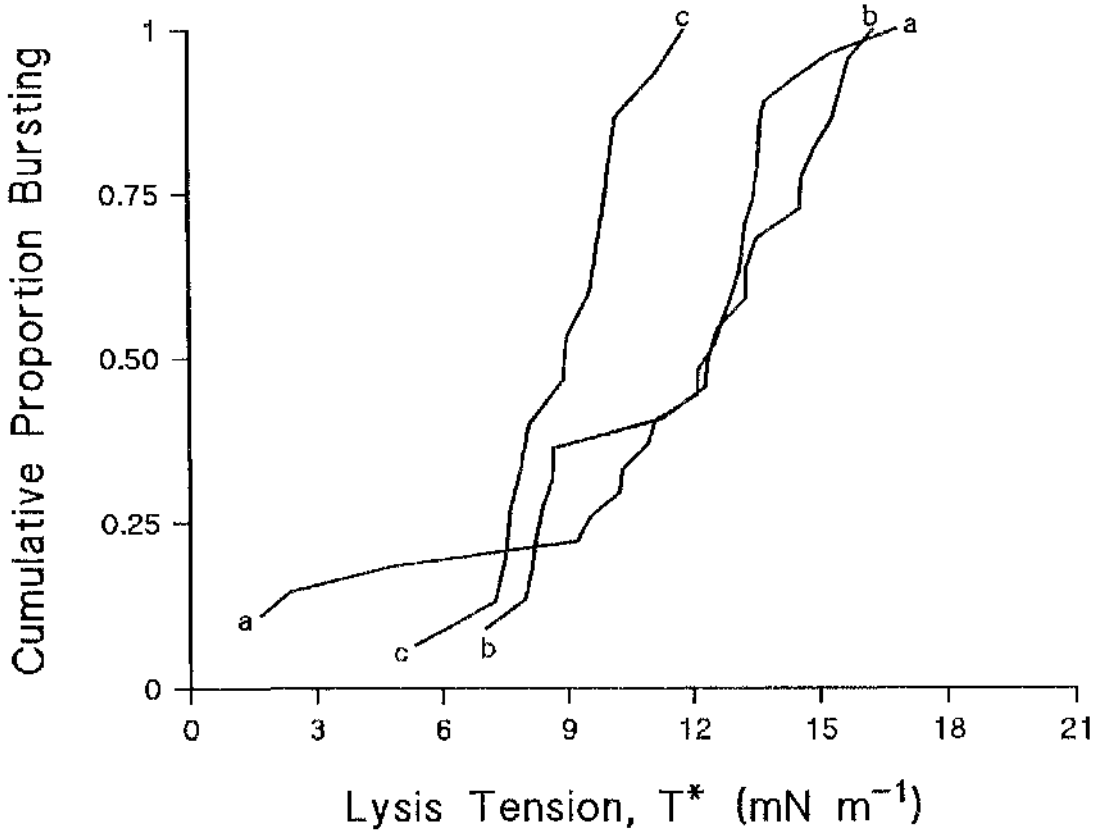

Figure 3.08 Effect on membrane strength of external calcium, $[Ca^{2+}]_o$. Cumulative curves of lysis tension showing: a, companion control vesicles (n = 25) in nominally Ca^{2+}–free 140 mM KCl; b, 0.5 mM $[Ca^{2+}]_o$; and c, 1 mM $[Ca^{2+}]_o$.

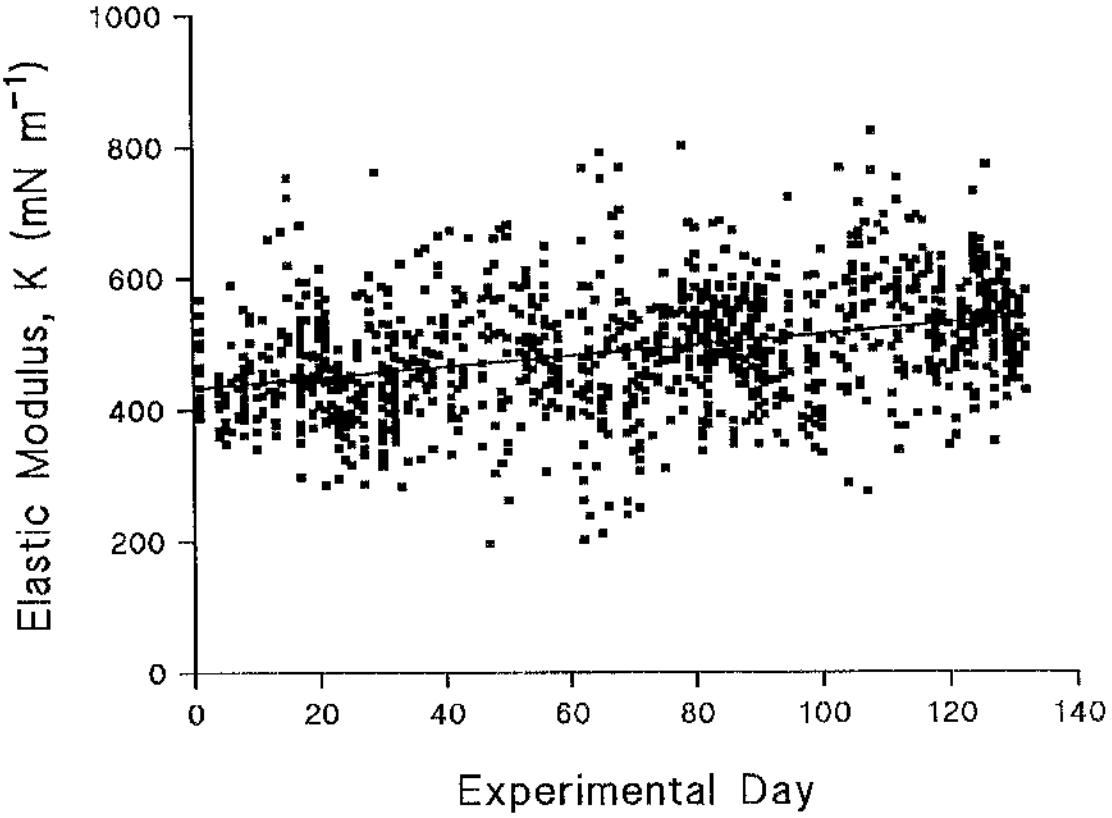

Figure 3.09 Linear regression analysis on K data for control vesicles
Values (n = 1301) of area elastic modulus, K, are plotted in chronological sequence, against the experimental day (n = 132) on which they were measured. Positive correlation between K and experiment number is shown by the least squares regression line y = 0.824x + 433.4.

PART IV

CA^{2+} LOADING REDUCES THE TENSILE STRENGTH OF SARCOLEMMA

Experiments with Ca^{2+} reported in the previous section were open to the interpretation that lack of added Ca^{2+} in the extracellular solution does not materially influence the mechanical properties of sarcolemmal vesicles, but that elevation of $[Ca^{2+}]_i$ might reduce the tensile strength of the membrane. In this section the clue is followed up by use of ionophores to elevate $[Ca^{2+}]_i$.

Results

Control experiments with DMSO and A23187

The poly-ether ionophore A23187 has been widely used to permeabilize cell membranes to Ca^{2+} (Pressman, 1976; Ferreira & Lew, 1976). The ionophore is usually first dissolved in DMSO and then dispersed in aqueous solution with a small volume of the solvent. In the experiments designed to elevate intravesicular Ca^{2+} to be described below, the maximum final concentrations so arising were 0.5% (v/v) DMSO and 5 μM A23187. To test for any direct effect of these agents on membrane mechanical properties control experiments were therefore necessary. 0.5% DMSO by itself was found to have no significant effect on membrane tensile strength (Fig. 4.01) but the distensibility was slightly increased by DMSO (Fig. 4.02). Similarly, with 5 μM A23187 in 0.5% DMSO no difference was found in membrane tensile strength, provided the solution bathing the vesicle contained also 2 mM NTA to minimize any stray Ca^{2+} (Fig. 4.03). As regards membrane distensibility, however, a more complex result again emerged: especially at higher membrane tensions a distinct increase in membrane area was observable (Fig.

4.04). Since tensile strength is not reduced by DMSO and A23187 by themselves, the effect on membrane strength of loading vesicles with Ca^{2+} will first be described. The more equivocal results of Ca^{2+} loading on membrane distensibility will then be presented.

Effect of elevated $[Ca^{2+}]_i$ on membrane strength

When Ca^{2+} as well as ionophore was admixed to 140 mM KCl solution containing vesicles, striking effects were observed. At above 20 μM $[Ca^{2+}]_o$ most vesicles became so weak that they burst instantly on aspiration. With continued exposure, their number decreased through autolysis: flocculent cytosolic protein accumulated in the bathing solution to give it an increasingly dirty appearance. As the above control experiments had shown no such drastic effects, it was concluded that these result from an increase in $[Ca^{2+}]_i$.

In order to characterize more closely the effect of raising intravesicular Ca^{2+} on membrane tensile strength, conditions giving a graded response were sought. Fig. 4.05 summarizes experiments in which 0.8-10 μM $[Ca^{2+}]_o$ was used in combination with 5 μM A23187. The results are presented as cumulative plots of the proportion of vesicles bursting at and below a given membrane tension, as in this way differences in the proportion of vesicles bursting immediately on aspiration are taken into account. With 10 μM $[Ca^{2+}]_o$, for instance, 178 out of 404 vesicles aspirated, i.e. 0.44 of the sample burst immediately on aspiration; and 0.75 by 3 mN m^{-1}. With 0.8 μM $[Ca^{2+}]_o$, by contrast, only 2 out of 51 vesicles burst immediately and the cumulative curve effectively overlaid the control curve, i.e. no significant reduction in membrane strength was detectable. Intermediate concentrations of $[Ca^{2+}]_o$ produced appropriately graded effects, but too few vesicles were tested for the results to be precisely quantifiable. In another set of experiments with only 1 μM A23187 (Fig. 4.06), 10 μM $[Ca^{2+}]_o$ produced a similar decrease in membrane strength as it did with the higher dose of ionophore; and with lower concentrations of $[Ca^{2+}]_o$ the median strength of vesicles increased progressively

towards that of the control distribution. Interestingly, 2 μM $[Ca^{2+}]_o$, the lowest concentration used with 1 μM A23187, still produced a highly significant reduction of membrane strength. Taken together with the lack of effect of 0.8 μM Ca^{2+} in the presence of 5 μM A23187, this suggests a steeply rising dose response relationship, such as is indicative of multiple binding of Ca^{2+} to its site of action.

Ionomycin is another ionophoric poly-ether antibiotic with a high affinity for Ca^{2+}. It differs from the mono-dentate A23187 in complexing and transporting Ca^{2+} in one-to-one stoichiometry (Liu & Hermann, 1978). Fig. 4.07 shows the cumulative lysis tension for vesicles exposed to 10 μM or 6 μM $[Ca^{2+}]_o$ in the presence of 5 μM ionomycin, together with a cumulative curve for control vesicles from the same batch of muscle. The clear shift in the curves produced by $[Ca^{2+}]_o$ also when carried by ionomycin rules out the off-chance that the effects observed above with A23187 were in some way singularly dependent on the presence of that ionophore. As ionomycin did not appear to be more effective than A23187 in the present situation, and as it is considerably more expensive, it was used only rarely in later experiments.

Mg^{2+} carried by A23187 does not mimic the effect of Ca^{2+}

Ionophore A23187 complexes not only with Ca^{2+} but also with Mg^{2+}. Two sets of experiments were done. In the first 5 μM A23187 was used and the bathing solution was buffered with 2 mM NTA so as to yield 181 μM Mg^{2+}. In contrast to the effect of Ca^{2+}, which in similarly high concentrations in combination with ionophore causes autolysis, vesicles survived well with Mg^{2+} and the proportion too weak to aspire was scarcely higher than for the untreated vesicles from the same batch. In the second set, in which only 1 μM A23187 was used, Mg^{2+} was buffered to yield 126 μM Mg^{2+}. In these experiments Mg^{2+} loaded vesicles were indistinguishable in strength from untreated vesicles, whereas vesicles from the same batches treated with 1 μM A23187 and 20 μM Ca^{2+} were strikingly weakened (Fig. 4.08).

In some systems Sr^{2+} can mimic Ca^{2+}. The point could not be put to the test in the present situation, owing to the low affinity of A23187 for Sr^{2+} (Table 4.1). Co^{2+} is rapidly transported into red cells by A23187 (Brown & Simonsen, 1985) and presumably this applies also to sarcolemmal vesicles. Mn^{2+} generally behaves similarly to Co^{2+}. With both these ions, no NTA was used to minimize stray Ca^{2+} in the bathing solution. Instead, we placed reliance on the much higher affinity of A23187 for Co^{2+} and Mn^{2+} (Dagher & Lew, 1988). In the presence of 2.5-5 μM A23187, neither 10 μM Co^{2+} nor 10 μM Mn^{2+} produced discernible reduction in membrane tensile strength in experiments of a duration comparable to that used with Ca^{2+}. However, these experiments presented a complicating feature not noticed with Ca^{2+} or Mg^{2+}: a crystalline precipitate formed and settled at the bottom of the dish. It presumably consisted of insoluble ionophore-metal complexes. Nevertheless, enough ionophore must have remained in association with the membrane for some inward transport of Co^{2+} and Mn^{2+}; for a striking precipitation of the intravesicular content developed gradually and eventually autolysis of vesicles occurred. Co^{2+} and Mn^{2+} are known to bind to cell content (Brown & Simonsen, 1985) and to be highly toxic, but since membrane strength was maintained for much of the time it would seem that the mechanism by which these ions cause eventual lysis may differ from that entrained by elevated $[Ca^{2+}]_i$.

Effects on membrane distensibility

Although doping of vesicles with either 0.5% v/v DMSO alone, or with the vehicle plus 5 μM A23187 had no obvious effect on lysis tension (Figs. 4.01 & 4.03), the fractional increase in membrane area at lysis was slightly higher in both conditions than in untreated vesicles (Figs. 4.02 & 4.04). Evidently, membrane distensibility is more sensitive to doping than is membrane strength. In order to decide whether an increase in $[Ca^{2+}]_i$ has any effect on membrane distensibility, vesicles doped with only 0.1 % v/v DMSO and 1 μM A23187 were therefore used; and as control, vesicles exposed to ionophore with Mg^{2+} rather than to ionophore alone, were considered more appropriate

because A23187 enters the membrane more readily when divalent cations are absent (Chandler & Williams, 1977).

Fig. 4.09 is based on the same population as the two right hand curves of Fig. 4.08. As was to be expected, the lysis points for vesicles doped with 1 μM A23187 in the presence of Mg^{2+} lie in the same tension range as those for the control vesicles, and on average the increase in membrane area was only a little higher i.e. the ionophore in combination with Mg^{2+} produces only a slight increase in membrane distensibility. Results from vesicles treated with 1 μM A23187 and 10 μM $[Ca^{2+}]_o$ are depicted in Fig. 4.10. Noticeable differences are: (i) the large proportion of vesicles lysing at low tension (ii) a proportion of vesicles of high distensibility. The abnormally distensible vesicles occurred seemingly randomly, but more frequently when narrow pipettes, that allowed determination of K from weak vesicles, were used. A possible explanation why only a proportion of Ca^{2+}-loaded vesicles were abnormally distensible will later be offered (p. 82).

Table 4.2 summarizes the data of Figs. 4.02, 4.04, 4.08 & 4.10 in terms of the mean values of the modulus of area expansion, K. Also given are values for vesicles doped with 1 μM A23187 and loaded in 2 or 4 μM $[Ca^{2+}]_o$, which were included in Fig. 4.06 but not in Fig. 4.10 to avoid overcrowding. The experiments of Figs. 4.02 & 4.04 were done early, those of Figs.4.09 & 4.10 late during this work, and the difference in K for the respective control groups reflects the drift illustrated in Fig. 3.08. As the controls for the Mg^{2+} and Ca^{2+} treated sets were similar, these sets may be compared directly. Only with 10 μM $[Ca^{2+}]_o$ was K significantly ($P = 0.0012$) lower than for the Mg^{2+} treated group. This was due to the score of abnormally weak vesicles which gave the probability density function of K for that group a distinctly skewed shape. Mean values for K for 4 and 2 μM Ca^{2+} were the same as for the Mg^{2+} treated set, although these concentrations of Ca^{2+} were high enough to reduce membrane strength (Fig. 4.06).

Protective effect of intracellular Ca^{2+} buffering

A now well-established method for buffering intracellular Ca^{2+} is to load cells with the acetoxymethyl (AM) ester of BAPTA, to which the membrane is permeable. Esterases within the cell then release the anionic form of BAPTA which chelates Ca^{2+}. This method was first developed by Tsien (1981) who showed that pretreatment of red blood cells with BAPTA-AM greatly delayed the onset of Ca-induced K^+ permeability increase caused by A23187. Preincubation of mast cells with BAPTA-AM was similarly able to stave off the normally immediate release of histamine induced by A23187 (Tsien, 1981). In hepatoma cells pretreatment with BAPTA-AM delayed cell death due to chemical anoxia attributed to increase in cytosolic Ca^{2+} (Nicotera, Thor & Orrenius, 1989); and injection of BAPTA into neurones lacking calcium binding proteins has been shown to protect them from prolonged stimulation, which otherwise leads to cell death through Ca^{2+} accumulation (Scharfman & Schwatzkroin, 1989).

In view of the above findings, it seemed interesting to test whether pretreatment with BAPTA-AM will also protect sarcolemmal vesicles against A23187 mediated influx of Ca^{2+}. To this end vesicles were teased out into 140 mM KCl solution to which BAPTA-AM dissolved in DMSO had been added to give a nominal concentration of 20 μM BAPTA-AM (sample A). At the same time, other vesicles were released into two other dishes (samples B & C) containing only 140 mM KCl, to serve as controls. After 30 min a solution containing A23187 and Ca^{2+} buffered with NTA was admixed to dishes A & B to give a final concentration of 10 μM Ca^{2+} and 1 μM A23187. Vesicles from the three dishes were then aspirated over the next 1-2 hours. The pooled results of these such experiments showed that vesicles pretreated with BAPTA-AM and then exposed to $[Ca^{2+}]_o$ plus 1 μM ionophore were stronger than the vesicles exposed to 10 μM $[Ca^{2+}]_o$ plus 1 μM ionophore in the ordinary way, but not as strong as the control vesicles from the same batches (Fig. 4.11). Under the conditions of the above experiment BAPTA-AM therefore protected vesicles partially against the effect of Ca^{2+} inflow. No time

dependence of this effect was observed i.e. weak vesicles in the BAPTA-AM pre-treated batch were found both early and late after addition of 10 μM $[Ca^{2+}]_o$ plus ionophore.

The acetoxy methyl ester of BABTA (and of Ca-sensitive fluorescent dyes e.g. Quin2-AM and Fura 2-AM) are of limited solubility in aqueous solution. They are usually added, dissolved in a small volume of DMSO, to relatively dense suspensions of cells kept stirred, in the hope that a proportion of the ester will associate with the cell membrane before precipitation occurs. Pluronic F127, foetal calf serum or bovine serum albumin have been recommended as dispersing agents to keep AM-esters in solution (Haugland, 1992; Tsien & Pozzan, 1989). In the above experiment dilute and fully transparent suspensions of sarcolemmal vesicles were used. So it was noticed that addition of that BAPTA-AM in DMSO produced a cloudy solution. Under the microscope formation of crystals was soon observed. It must therefore be supposed that most of the BAPTA-AM added came out of solution. With a view to improving the experimental conditions, the effect of pluronic F127 (0.05%) on sarcolemmal vesicles was tested. It behaved as a membrane active compound which reduced the strength and increased the distensibility of sarcolemmal vesicles. Although this effect was not large it was deemed an unacceptable complication for experiments in which the protective effect of BAPTA-AM was to be better studied. Addition of bovine serum albumin (4 μM) was found to delay precipitation of BAPTA-AM and the crystals which eventually formed were finer needles. However the use of albumin in the present situation is fraught with complications in that it binds both Ca^{2+} (Pedersen, 1971) and A23187 (Simonsen, 1980). Whilst it would have been possible to surmount these problems, it was not judged worthwhile; for the observed only partial protection by BAPTA-AM might have been due not so much to limited uptake of BAPTA-AM as to poor esterase activity in some vesicles.

Irreversibility of loss in membrane strength

The permeabilization of the membrane produced by A23187 should work in both directions and allow vesicles to lose $[Ca^{2+}]_i$, if $[Ca^{2+}]_o$ is lowered below $[Ca^{2+}]_i$. In some experiments the question whether the effects of elevated $[Ca^{2+}]_i$ is reversible was studied in that way. Several dishes of vesicles were prepared and treated with 10 μM $[Ca^{2+}]_o$ and 1 μm A23187. After a clear weakening of vesicles was established by aspirating some vesicles in each dish, 140 mM KCl containing BAPTA tetrasodium salt was added to one dish so as to produce a final concentration of 200 μM BAPTA. Owing to the high affinity constant of BAPTA for Ca^{2+} ($\approx 10^7\,mol^{-1}$), this should reduce $[Ca^{2+}]_o$ to sub-micromolar level. 30 min was then allowed for efflux of Ca^{2+} to proceed from the vesicles before further vesicles were aspirated. No return of membrane tensile strength was found (Fig. 4.12). The most likely interpretation of this finding is that elevated $[Ca^{2+}]_i$ produces an irreversible degradation of the membrane that persists after the increase in $[Ca^{2+}]_i$ has been reversed.

Discussion

The deleterious effect on the sarcolemma of raising intravesicular Ca^{2+} was obvious from the autolysis of vesicles exposed to >20 μM Ca^{2+} in the presence of A23187, and this phenomenon might be quantifiable by assaying the release of cytosolic enzymes from sarcolemmal vesicles. To bring the micro-pipette aspiration technique to bear on the problem, autolysis had to be minimized by lowering the concentration of Ca^{2+} to which vesicles were exposed. Considerable variation in the behaviour of vesicles then emerged: whilst the tensile strength of some vesicles in each batch was greatly reduced, other vesicles were little affected. One possibility is that vesicles differed in their sensitivity to $[Ca^{2+}]_i$. It seems improbable however, that vesicles from a given batch could have greatly differed in their membrane properties; for non-uniformities of molecular origin should be averaged out with vesicles as large as the ones here used. It is known however

from electronmicroscopic examinations (Fig. 1.03) that great differences exist between vesicles in the amount of cytosolic materials entrapped. So if a cytoplasmic factor were required for Ca^{2+} to produce its lytic effect, variability from vesicle to vesicle could arise in this way. Another possibility is that A23187, as here used, does not establish equilibrium between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$; and that $[Ca^{2+}]_i$ varies from vesicle to vesicle in the same batch, though some arguments against this possibility can be adduced.

The minimum effective $[Ca^{2+}]_i$

The unavoidable use of ionophore means that the relation between $[Ca^{2+}]_i$ and membrane strength cannot be precisely defined on present evidence. But there are grounds for arguing that the ionophore probably establishes equilibration between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$, or at least between $[Ca^{2+}]_o$ and the calcium ion concentration in the sub-sarcolemmal space: (i) no clear difference between the strength of vesicles of different diameter, aspirated after the usual delay allowed after the addition of ionophore and Ca^{2+}-containing solution (ii) no progressive weakening of vesicles on continued exposure to a given $[Ca^{2+}]_o$ (iii) the relatively small differences between the results obtained with use of 1μM or of 5μM A23187 (Figs. 4.05 & 4.06). Regarding the last point, it is relevant that 2 molecules of A23187 complex with each Ca^{2+} ion. According to the law of mass action, a 5-fold increase in the concentration of A23187 should therefore render the ionophore 25-times more effective. However, in view of the uncertain solubility of A23187 in Ca^{2+}-containing solution (Chandler & Williams, 1977) it cannot be taken for granted that a 5-fold increase in the dose of A23187 actually produces a 5-fold increase in ionophore concentration. The strength of argument (iii) above thus remains an open question. At all events, the minimum effective $[Ca^{2+}]_i$ will not be higher than the corresponding $[Ca^{2+}]_o$. To arrive at a fully quantitative analysis of the effect of elevated $[Ca^{2+}]_i$ on membrane tensile strength will require combination of pipette aspiration of vesicles with measurement of $[Ca^{2+}]_i$ by fluorescent indicators. On present evidence the

indication is that the threshold concentration of $[Ca^{2+}]_i$ for membrane weakening is in the order of 10^{-6} M.

In above connection, it is interesting to note that human red blood cells, uniformly permeabilized to Ca^{2+} with A23187, may greatly differ in Ca^{2+} content (García-Sancho & Lew, 1988 a, b) owing to the ability of some cells to sustain pump-leak balance with a low $[Ca^{2+}]_i$. While it seems unlikely that sarcolemmal vesicles pump Ca^{2+} as actively as do red blood cells, it would be surprising if they were devoid of all such activity. It is therefore conceivable that given a Ca^{2+} gradient of only a few μM those vesicles which contain enough energy source and metabolic machinery may be able to keep $[Ca^{2+}]_i$ below the level at which the membrane is appreciably degraded.

Table 4.1 **Specificity of ionophore A23187 for the divalent cations Ca^{2+}, Mg^{2+} and Sr^{2+}.**

Cation	Association Constant (M^{-1})
Ca^{2+}	$1.56 \pm 0.8 \times 10^5$
Mg^{2+}	$6.58 \pm 0.4 \times 10^4$
Sr^{2+}	$8.02 \pm 1.2 \times 10^3$

Table 4.2. Modulus of area elasticity for treated sarcolemmal vesicles and respective controls in 140 mM KCl.

Treatment	*n*	Mean ($mN\ m^{-1}$)	S.D. ($mN\ m^{-1}$)
controls	58	473	77
0.5% v/v DMSO	41	421	56
controls	32	463	72
5 μM A23187 + 0.5% DMSO	26	371	52
controls	24	521	90
126 μM Mg^{2+}; 1 μM A23187	19	429	56
controls	212	512	91
10 μM Ca^{2+}; 1 μM A23187	131	369	139
4 μM Ca^{2+}; 1 μM A23187	88	428	123
2 μM Ca^{2+}; 1 μM A23187	74	429	81

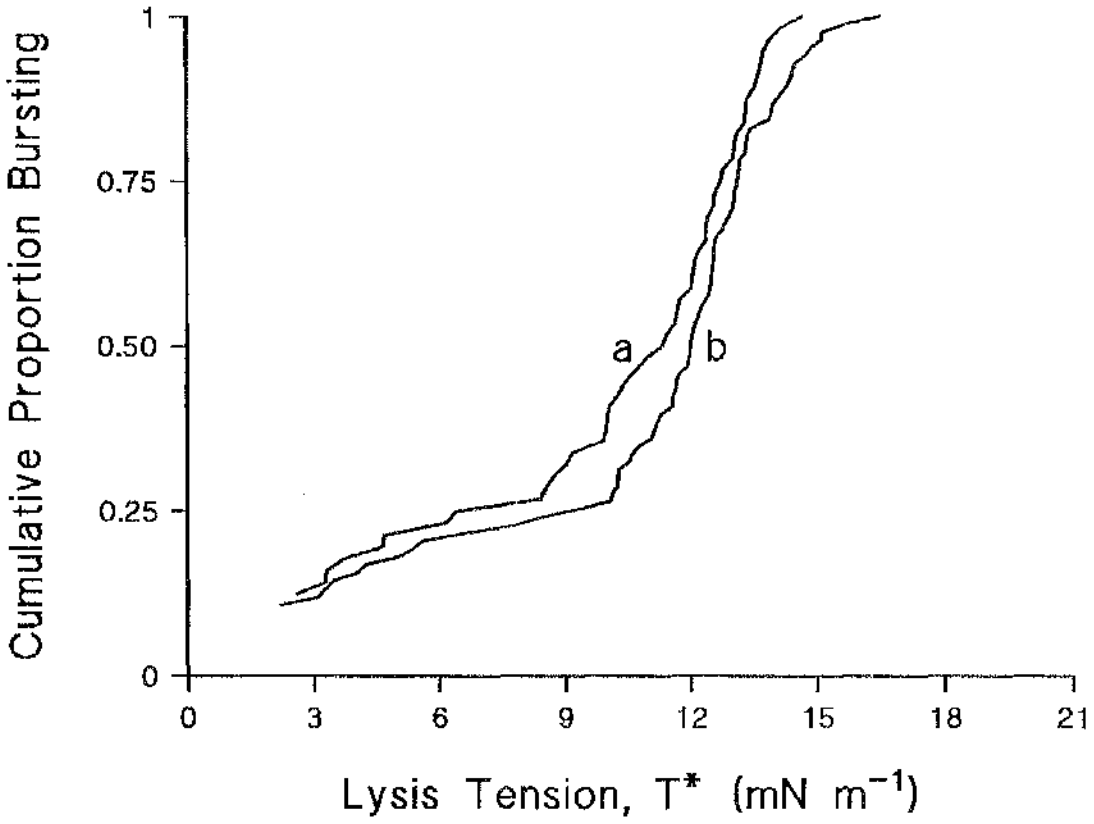

Figure 4.01 Control experiments on vehicle DMSO

Cumulative curves of lysis tension for vesicles in 140 mM KCl: a) containing 0.5% DMSO (n = 52) and b) untreated vesicles (n = 76) from the same batches. There is no significant difference between the curves

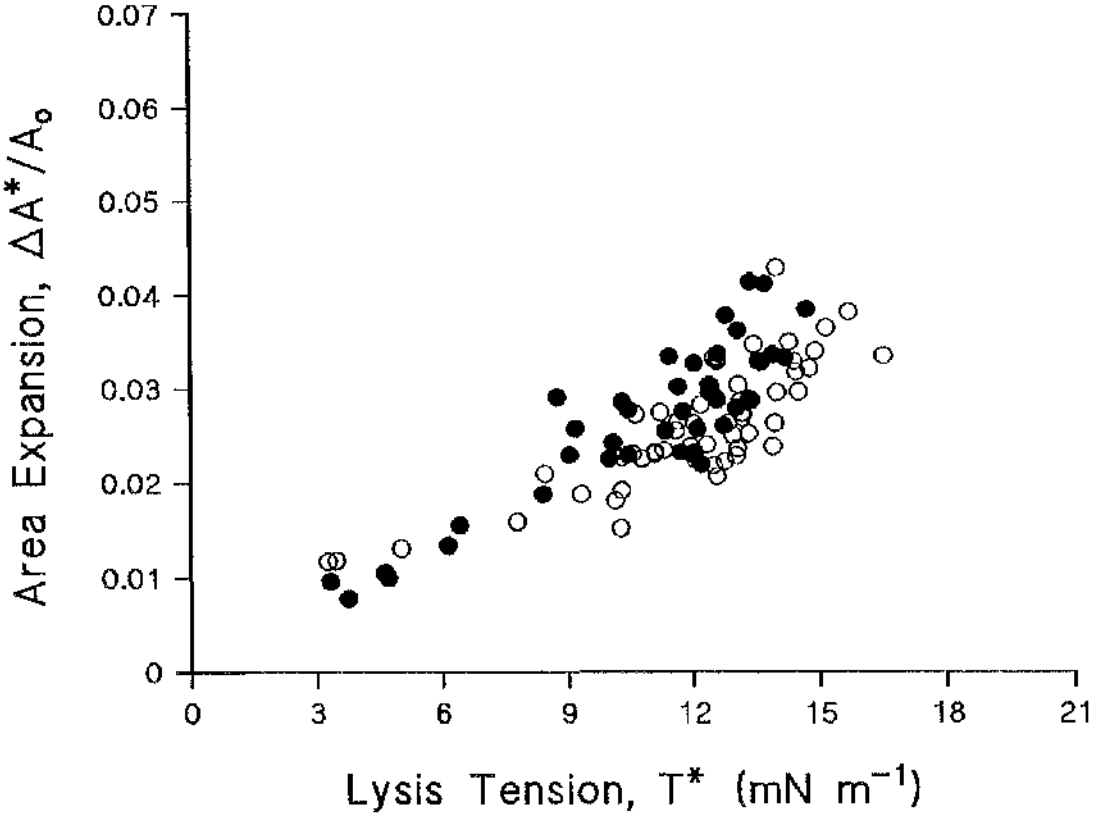

Figure 4.02 Lysis tension and corresponding area expansion for control experiments with DMSO

●, lysis points for vesicles in 140 mM KCl containing 0.5% DMSO (n = 52); ○, untreated companion vesicles in 140 mM KCl (n = 76).

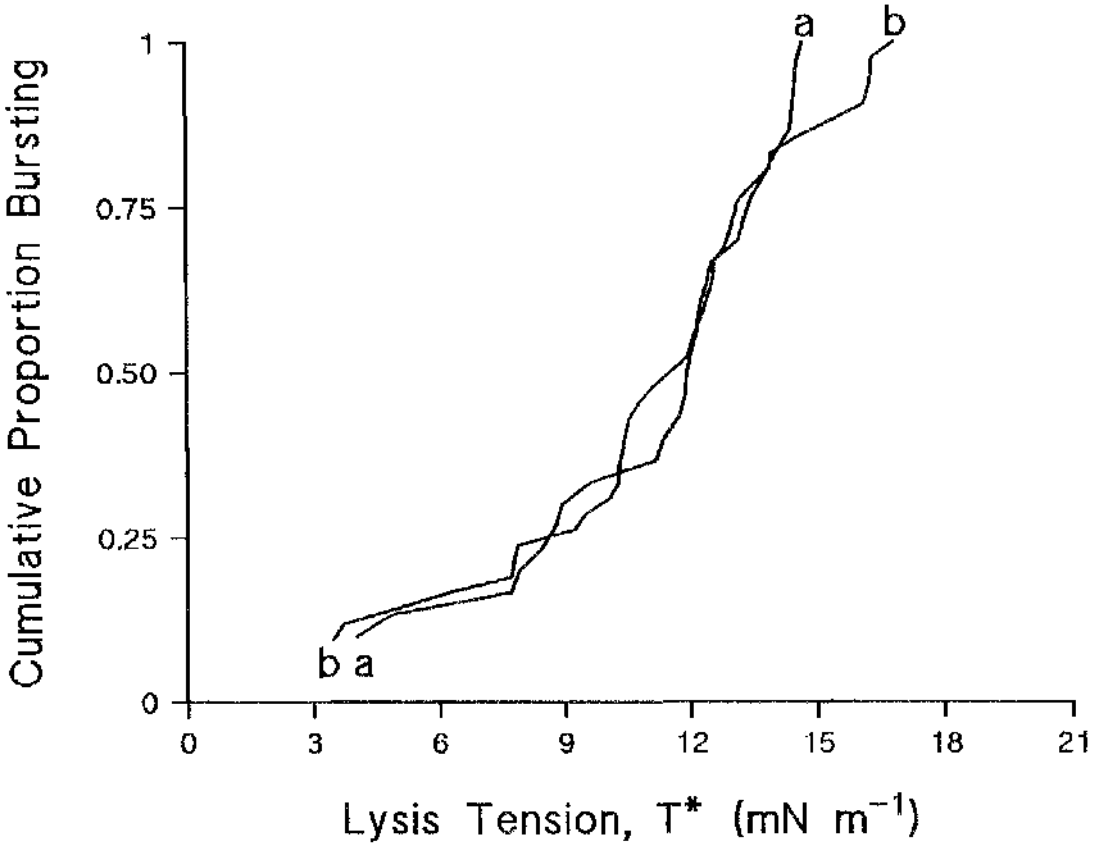

Figure 4.03 Control experiments on vehicle DMSO plus ionophore A23187 Cumulative curves of lysis tension for vesicles in 140 mM KCl: a) containing 0.5% DMSO, 5 μM A23187 and 2 mM NTA to buffer stray Ca^{2+} (n = 28); b) untreated vesicles (n = 39) from the same batches. Differences between the curves are not significant.

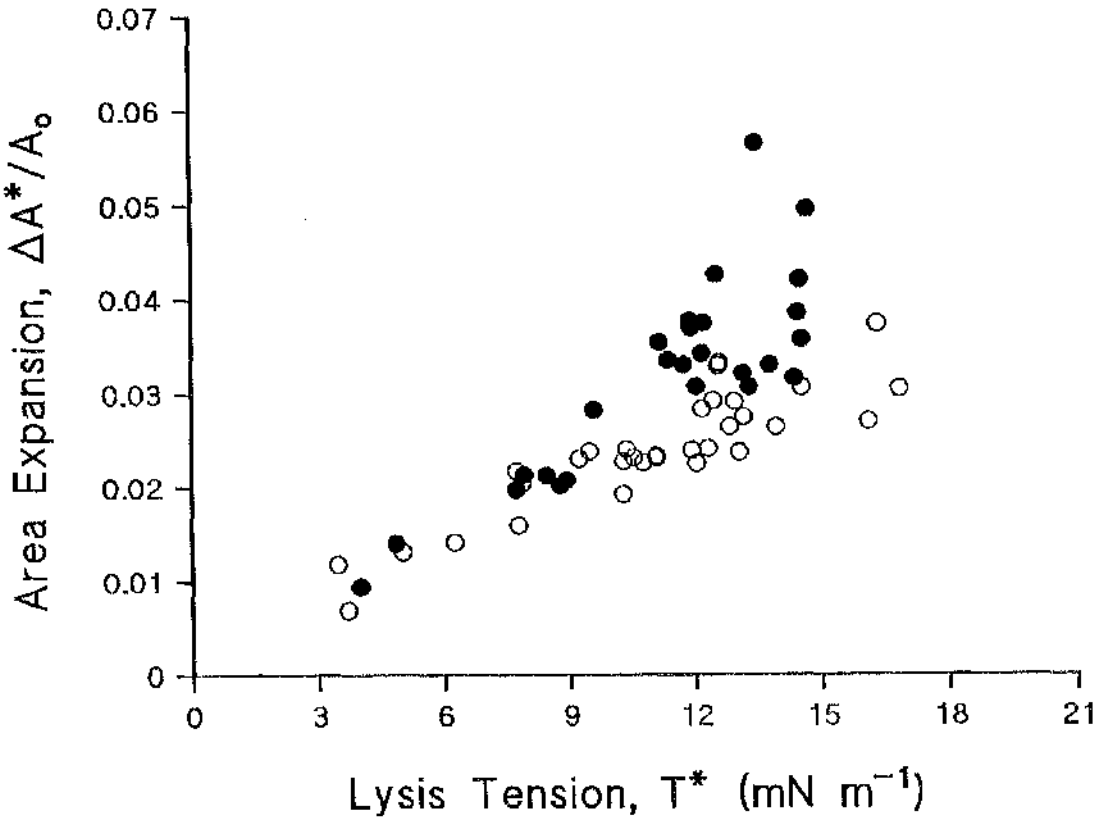

Figure 4.04 Lysis tension and corresponding area expansion for control experiments with ionophore A23187

●, lysis points for vesicles in 140 mM KCl containing 0.5% DMSO, 5 μM A23187 and 2 mM NTA (n = 28); ○, untreated companion vesicles in 140 mM KCl (n = 39).

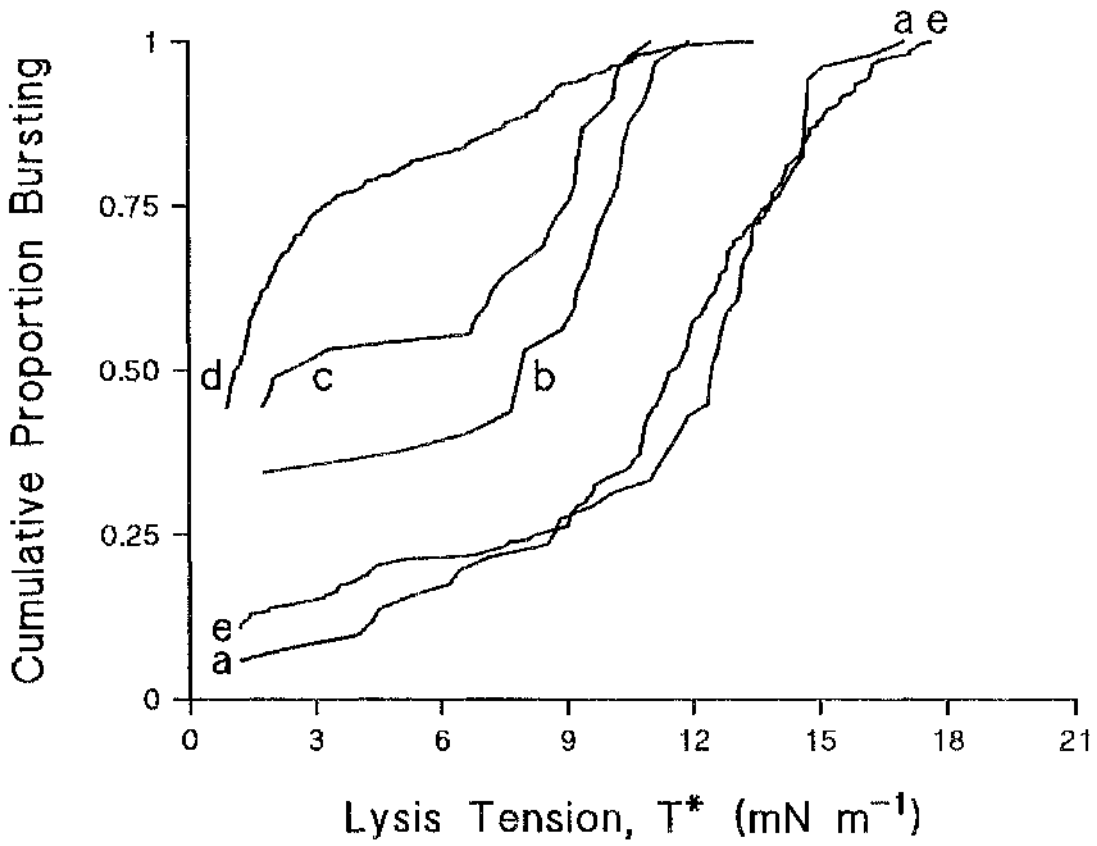

Figure 4.05 Effect on membrane strength of loading vesicles with Ca^{2+} using 5μM A23187

Cumulative curves of lysis tension for vesicles treated with 5 μM A23187 and loaded in: a), 0.8 μM (n = 49); b), 4 μM (n = 22); c), 6 μM (n = 26) and d), 10 μM Ca^{2+} (n = 226); e), companion untreated vesicles in 140 mM KCl (n = 180).

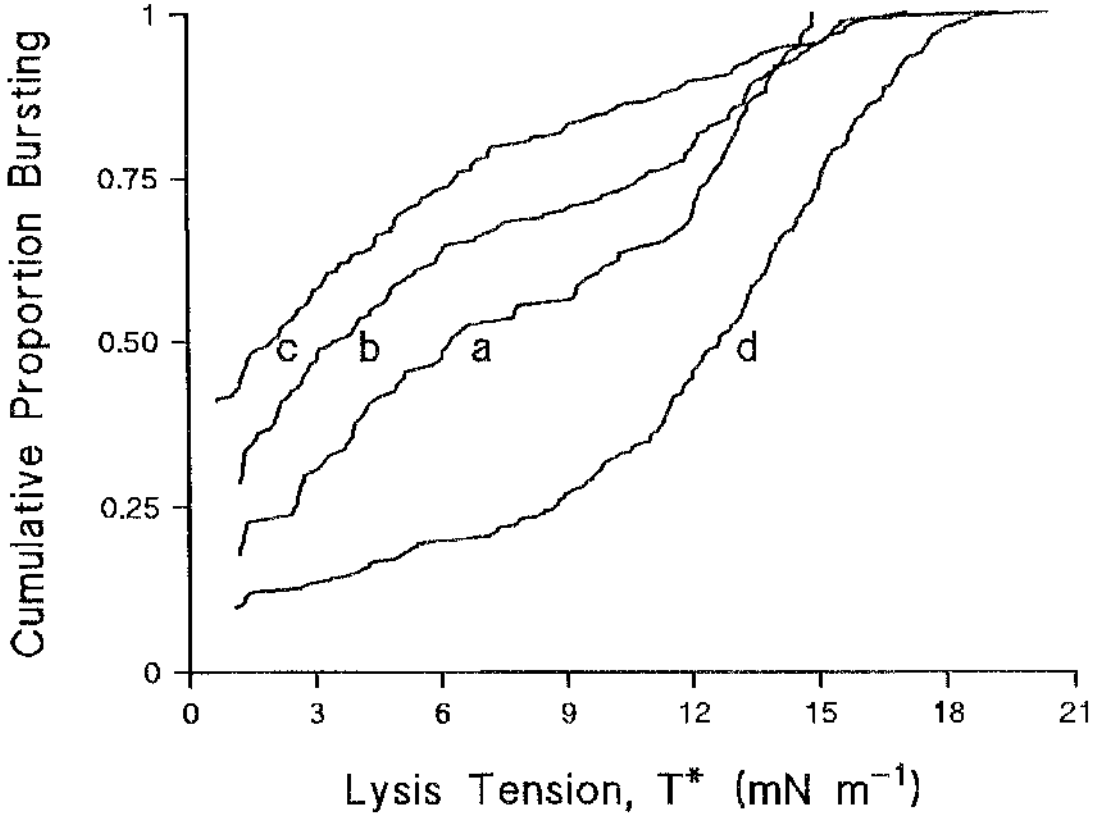

Figure 4.06 Effect on membrane strength of loading vesicles with Ca^{2+} using 1 μm A23187

Cumulative curves of lysis tension for vesicles treated with 1 μM A23187 and loaded in: a), 2 μM (n = 84); b), 4 μM (n = 125) and c), 10 μM Ca^{2+} (n = 160); d), untreated vesicles (n = 285).

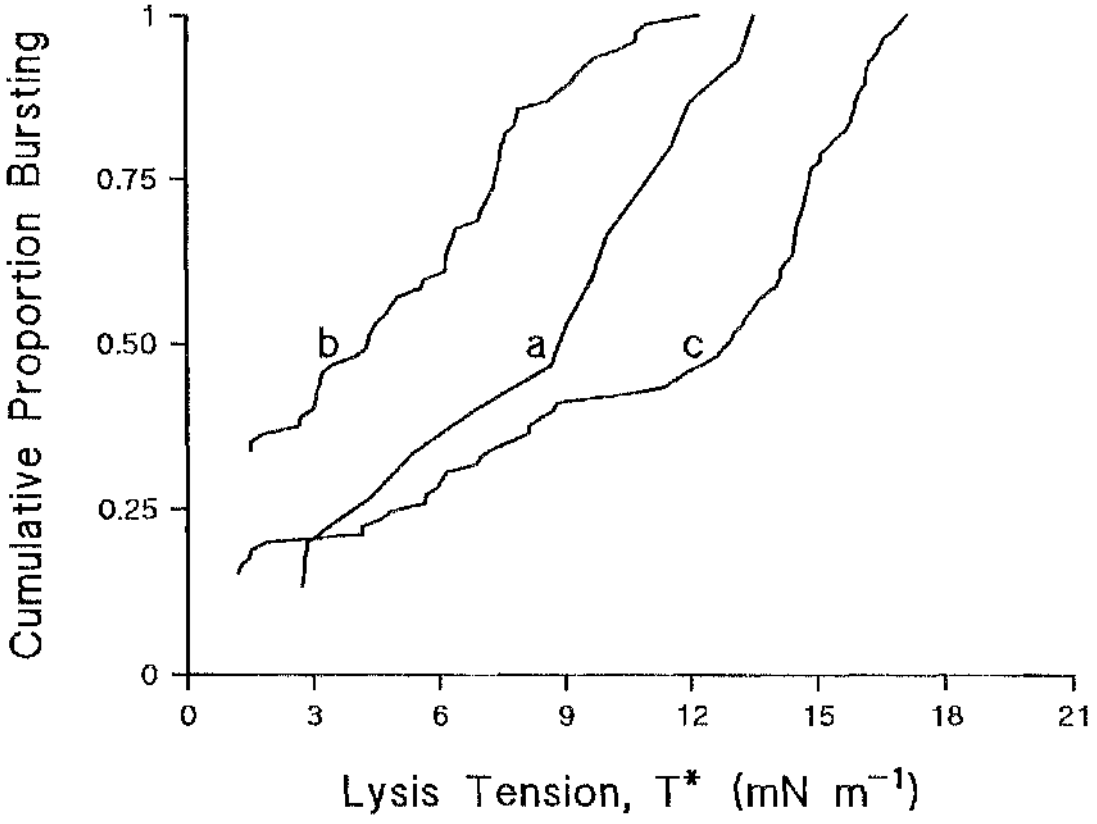

Figure 4.07 Effect on membrane strength of loading vesicles with Ca^{2+} using ionomycin

a), vesicles treated with 1 μM ionomycin and loaded in 6 μM Ca^{2+} (n = 14); b), vesicles treated with 5 μM ionomycin and loaded in 10 μM Ca^{2+} (n = 52); c), companion untreated vesicles (n = 73).

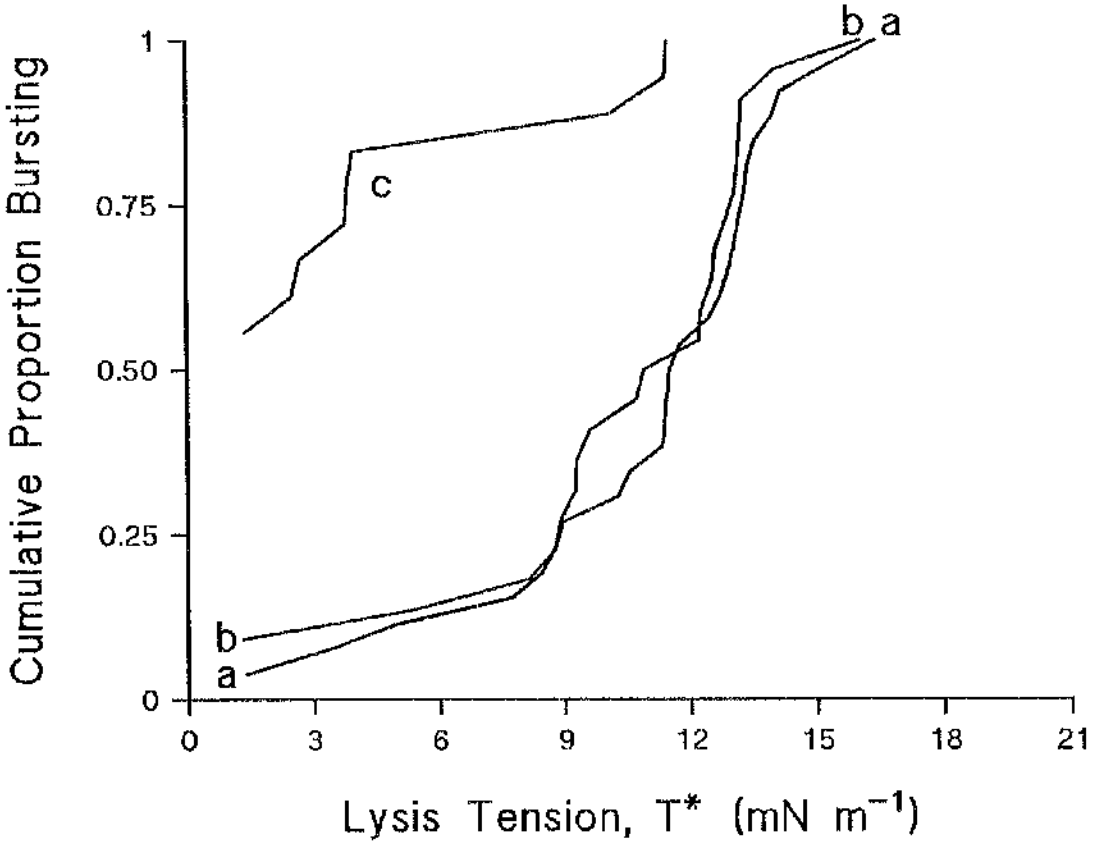

Figure 4.08 Comparison between the effects on lysis tension of internal Ca^{2+} and Mg^{2+}

Cumulative curves of lysis tension showing: a), control untreated vesicles (n = 29); and vesicles treated with 1 μM A23187 and bathed in solutions containing b), 126 μm Mg^{2+} (n = 21) and c), 20 μM Ca^{2+} (n = 9).

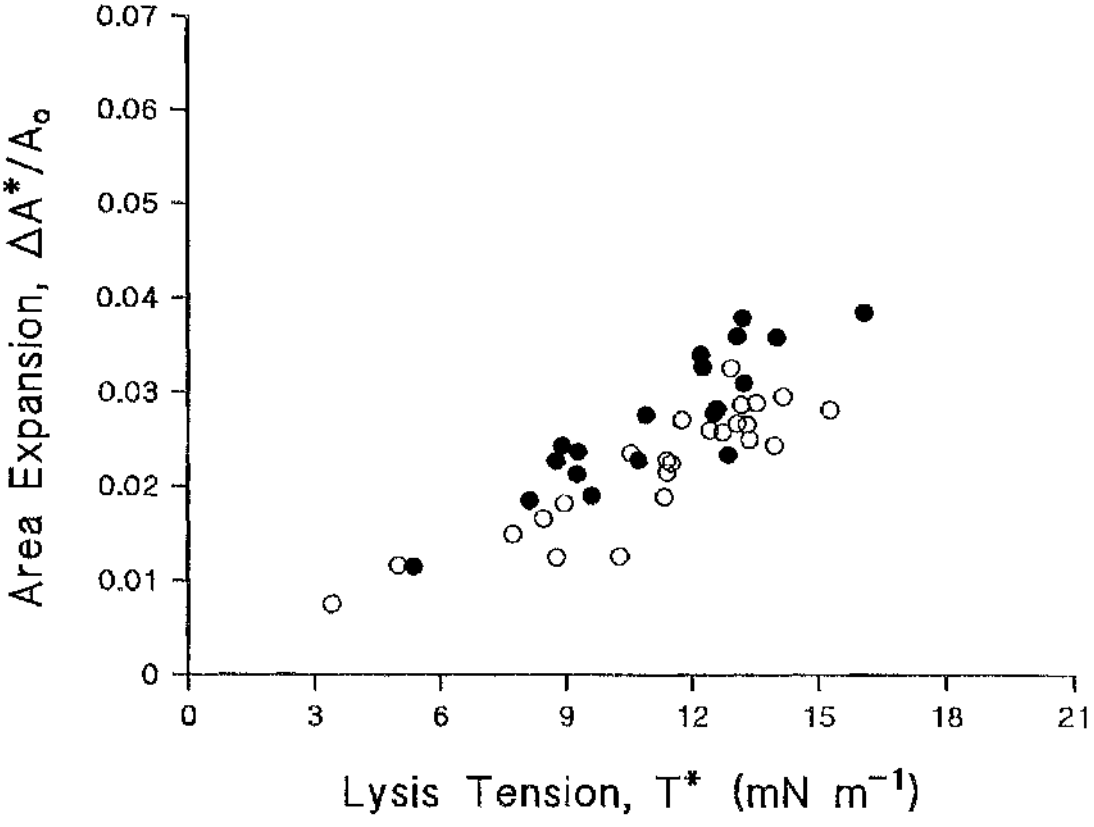

Figure 4.09 Lysis points for vesicles loaded in Mg^{2+}

●, lysis tension and corresponding area expansion for vesicles in 140 mM KCl containing 1 μM A23187 and 126 μM Mg^{2+} (n = 19); ○, untreated companion vesicles in 140 mM KCl (n = 24).

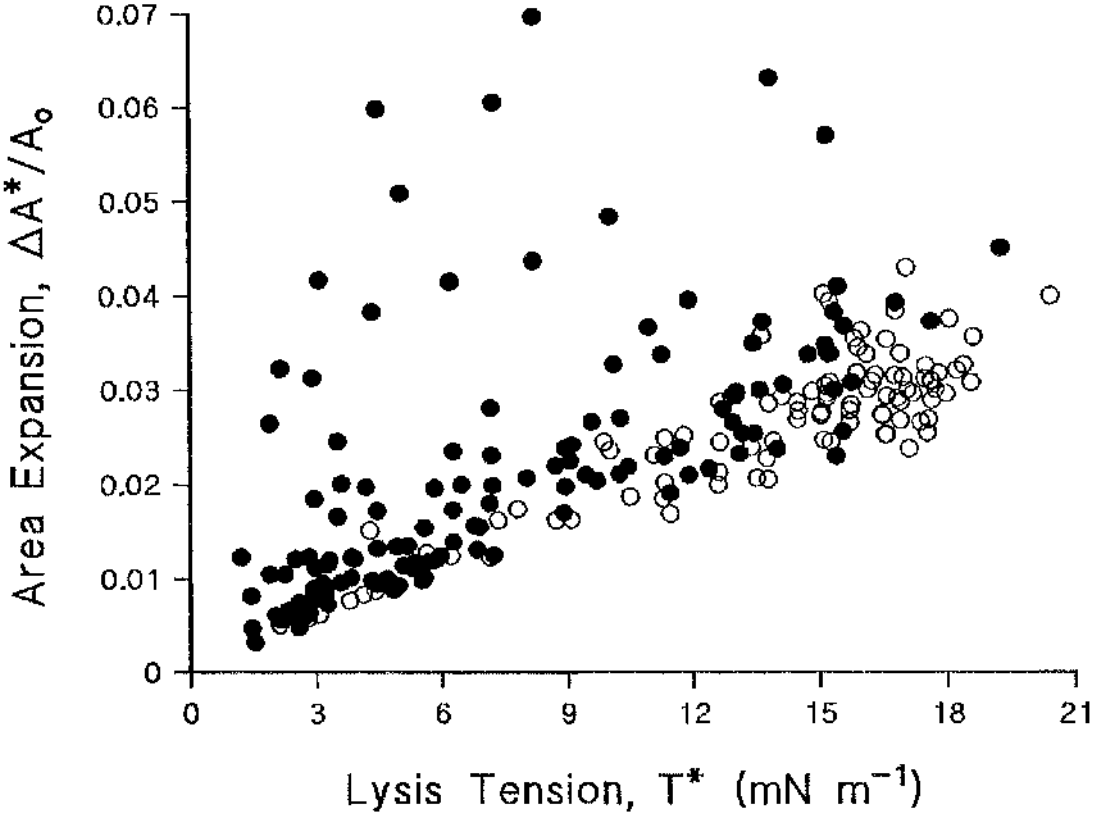

Figure 4.10 Lysis points for vesicles loaded in Ca^{2+}

●, lysis tension and corresponding area expansion for vesicles loaded in solutions containing 1 μM A23187 and 10 μM Ca^{2+} (n = 131); ○, untreated vesicles in 140 mM KCl from the same experiments (n = 98).

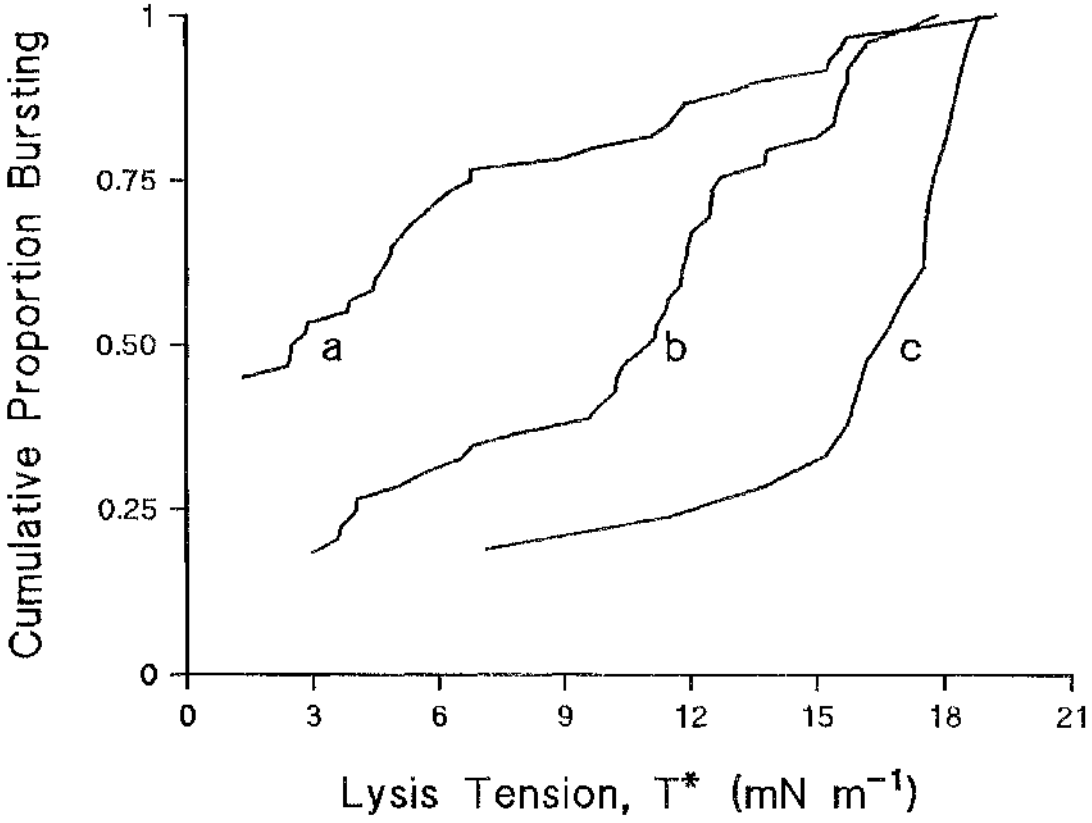

Figure 4.11 Protective effect of preloading vesicles with BAPTA–AM
Cumulative curves of lysis tension for sarcolemmal vesicles: a), loaded in a solution containing 10 μM Ca^{2+} and 1 μM A23187 (n = 34); b), preloaded with nominally 20 μM BAPTA–AM for 30 min and then loaded as in a), (n = 41); c), companion control vesicles in 140 mM KCl (n = 18).

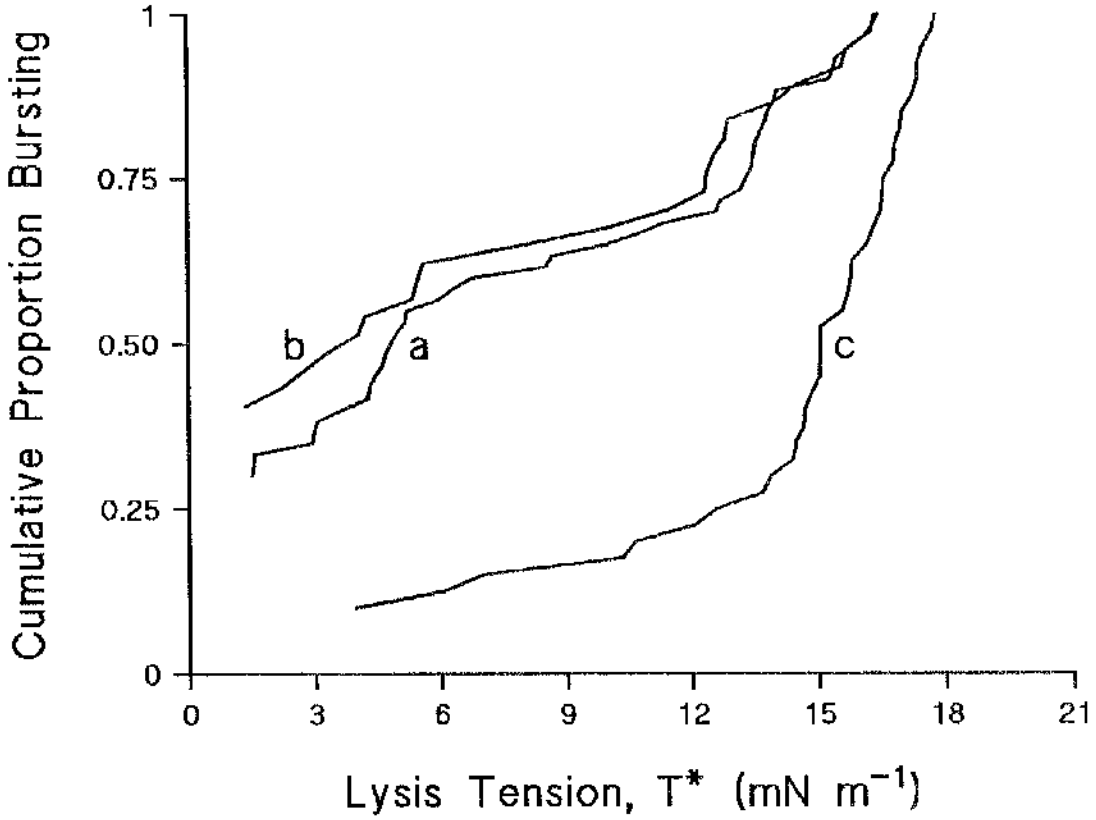

Figure 4.12 Reduction in membrane strength by Ca^{2+} loading is irreversible
Cumulative curves of lysis tension for vesicles: a), loaded in a solution containing 10 μM Ca^{2+} and 1 μM A23187 (n = 43); b), loaded as in a), and thereafter exposed for at least 30 min to the same A23187–containing solution with its Ca^{2+} content mopped up by 200 μM BAPTA tetrasodium salt (n = 23); c), companion controls in 140 μM KCl (n = 37).

PART V

POSSIBLE MECHANISMS BY WHICH ELEVATED CA^{2+} MAY REDUCE MEMBRANE STRENGTH

Much evidence points to an increase of cytosolic Ca^{2+} to micromolar levels as the final common path leading to cell death e.g. in brain ischaemia, excitatory neurotoxicity, ischaemic cardiac muscle, kidney and liver damage by cytotoxic agents etc. (Allshire, Piper, Cuthbertson, & Cobbold, 1987; Choi, 1988; Meldrum & Garthwaite, 1990; Nicotera, Bellomo & Orrenius, 1992). Segmental necrosis in skeletal muscle e.g. after unaccustomed eccentric exercise, or in myopathy has similarly been ascribed to elevated cytosolic Ca^{2+} (Jackson, Jones & Edwards, 1984). In above context, various mechanisms have been proposed through which Ca^{2+} overload may disrupt the cell membrane. These will now be considered as possible explanations for the reduction in membrane strength by elevated $[Ca^{2+}]_i$ here brought to light.

Lipid polymorphism and lipid scrambling

One hypothesis takes the asymmetrical distribution of different phospholipids between the two layers of the membrane as its starting point. In erythrocytes and cardiac myocytes, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are known to predominate in the inner layer of the membrane. It has been proposed that Ca^{2+} interacts directly with the negatively charged phospholipids and that a phase separation of lipids and a destabilizing aggregation of membrane protein is consequently engendered (Verkleij & Post, 1987). The chief arguments against the applicability of this proposition to the present situation are the relatively high concentration of Ca^{2+} required to induce change in phospholipid morphology, and that Mg^{2+} acts synergistically with Ca^{2+}

(Verkleij & Post, 1987). Even so, it would be desirable that lipid asymmetry and the conditions for its maintenance be studied in sarcolemma, and examination of sarcolemmal vesicles form that point of view could be rewarding.

Relevant in above connection is that in erythrocytes, elevation of $[Ca^{2+}]_i$ to 1 μM with A23187 inhibits aminophospholipid translocase almost completely (Zachowski, Favre, Cribier, Hervé & Devaux, 1986) and leads to exposure of phosphatidyl ethanolamine at the outer surface of the membrane (Williamson, Algarin, Bateman, Choe & Schlegel, 1985). Moreover, in erythrocytes an ionophore-induced increase in $[Ca^{2+}]_i$ also leads to accelerated passive transbilayer redistribution of all major lipids (Verhoven, Schlegel & Williamson, 1992; Williamson, Kulick, Zachowski, Schlegel & Devaux, 1992). Analogous lipid scrambling in similarly treated sarcolemmal vesicles seems therefore plausible. However, such lipid scrambling, if indeed demonstrable, is more likely to be a correlate of the changes in membrane mechanical properties wrought by $[Ca^{2+}]_i$ than a causal mechanism; for in pipette aspiration experiments on artificial giant vesicles of mixed phospholipid composition the nature of the headgroup seems to have little effect on membrane mechanical properties (Evans & Needham, 1986).

The low concentration in which Ca^{2+} is effective in the present situation is more consistent with activation of enzymes than with Ca-induced lipid polymorphism.

Activation of Ca^{2+}-dependent lipolytic enzymes

An accumulation of lysolipids in ischaemic Ca^{2+}-loaded tissue is well documented (Man, Slater, Pelletier & Choy, 1983) and points to the involvement of phospholipid deacylating enzymes. Of particular interest and possible relevance here is the high molecular weight (≈85,000 KDa) form of phospholipase A_2 (PLA_2) which is translocated from cytosol to membrane under the influence of low (< 10 μM) concentrations of Ca^{2+} and activated in the process (Clark *et al.*, 1991). The replacement of more or less cylindrical-shaped

diacyl phospholipid by cone-shaped lysolipid molecules would be expected to reduce the coherence of membrane lipids (Israelachvili, Marcelja & Horn, 1980). In turn this would be expected to reduce the tensile strength of the membrane and endogenous lysolipids in adequate proportions, through their detergent effect, could cause autolysis of vesicles such as observed at 20 μM Ca^{2+} and above.

The possibility that activation of phospholipase C, either directly by Ca^{2+} or via PLA_2 breakdown products, might contribute also deserves consideration. In lysosomes diacylglcerides have been shown to promote formation of inverted lipid phases (Siegel *et al.*, 1989). Provided such structures will also form in protein containing membranes, this may be an alternative path of arriving at destabilization of the bilayer configuration. Whether extraneous diacylglycerol influences membrane mechanical properties remains to be explored.

Ca^{2+}-dependent protease

Another enzyme often implicated in toxic and ischaemic cell injury owing to Ca^{2+}-overload is the neutral protease Calpain I. At micromolar $[Ca^{2+}]_i$, an inactive form is translocated from the cytosol to the membrane where the active form is produced by autoproteolysis (Croal & Demartino, 1991). Degradation of cytoskeletal proteins by Calpain causes membrane blebbing (Nicotera, Hartzell, Baldi, Svensson, Bellomo & Orrenius, 1986). This may be expected to alter the shear elasticity of the membrane surface, but not necessarily the cohesive properties of the sarcolemma. However, calpain also targets intra-membrane proteins. In red blood cells, for instance, the Ca^{2+}-pump and band-3 proteins are degraded (Salamino *et al.*, 1994). Zaidi & Narahara (1988) have demonstrated that calpain is present in skeletal muscle. The possibility therefore exists that by attacking intra-membrane proteins and thereby creating a lateral packing defect or mismatch in the transbilayer dimension (Lentz, 1988), Ca^{2+}-activated calpain may perturb the lipid matrix of the membrane so as to reduce its cohesive strength.

Peroxidation of membrane lipids

Recently, nitric oxide synthetase (NOS) has been found in skeletal muscle. The enzyme is restricted to the sarcolemma of fast (type II) fibres, like those comprising *m. psoas*, and is of the type which in neurones is activated by raised levels of $[Ca^{2+}]_i$ (Bredt & Snyder, 1990; Kobzic, Reid, Bredt & Stamler, 1994). If NOS remains associated with the membrane as the latter lifts off, as for instance is the case with dystrophin (Zubrzycka-Gaarn *et al.*, 1991), generation of NO in sarcolemmal vesicles would be expected when $[Ca^{2+}]_i$ is raised. Supposing further that the vesicle cytosol contains xanthine dehydrogenase and that Ca^{2+}-activated protease converts this to xanthine oxidase (McCord, 1985), formation of the superoxide anion would simultaneously occur. The stage would thus be set for peroxynitrite-induced peroxidative damage to membrane lipids (Radi, Beckman, Bush & Freeman, 1991) and the more so if the arachidonic acid cascade were also triggered through a Ca^{2+}-sensitive PLA_2.

As the above discussion illustrates, many causal mechanisms for the reduction in membrane strength produced by elevated $[Ca^{2+}]_i$ can be envisaged. Some involve both membranous and cytoplasmic components, some may work synergistically, and all could work additively. Further mechanisms such as a direct influence of $[Ca^{2+}]_i$ on membrane protein configuration are conceivable. Some of the mechanisms detailed are open to experimental test with the present method and the results of such explorations are described in the next sections. The effects of extraneously added lysophospholipids will be described first as these provide a good example of how membrane active compounds may alter membrane mechanical properties.

PART VI

A) EFFECT OF LYSOLIPIDS ON MEMBRANE MECHANICAL PROPERTIES

Results

Fig. 6.01 shows how activation of PLA_2 can be envisaged to alter the mechanical properties of the sarcolemma. The figure is oversimplified in as much as the membrane is represented as a bilayer of phospholipids only without representation of membrane proteins or cholesterol which intercalates between acyl chains giving the membrane added stability. But the main point is made: hydrolysis of the ester bond in the *C2* position and consequent release of fatty acid (often arachidonic acid) leaves a lysophospholipid in the membrane whose head group occupies at least the same volume at the surface of the membrane, but whose reduced acyl chain complement results in a loosening up of the membrane and hence a reduction of the cohesive forces in the lipid bilayer. It therefore becomes interesting to test what might be the effect on membrane mechanical properties of treating sarcolemmal vesicles with extraneous lysolipid. Most experiments were made with lysophosphatidylglycerol, LPG (Figs. 6.02 & 6.03) and Lysophosphatidylcholine, LPC (Figs. 6.04 & 6.05) as these proved readily miscible with aqueous solution. At above 10 μM, LPG produced lysis of the majority of vesicles, but at lower concentrations graded effects could be produced. At 2 μM LPG, vesicles were detectably weaker and more distensible. With 5 μM LPG the proportion of vesicles too weak to aspire rose above that for control vesicles and the increase in distensibility and decrease in strength were more marked. At both concentrations a few vesicles sustained an increase in membrane area above normal to between 0.04 and 0.05.

The increase in membrane distensibility, i.e the decrease in *K*, was a very regular feature of the action of lysolipids and a good measure of their potency as membrane active

compounds. In Fig. 6.06, LPG is shown to be about five times more effective in lowering K than is LPC. This is in accord with a larger head group volume of LPG. The latter is therefore a more wedge shaped molecule and hence more effective in disturbing the packing of membrane lipids (Israelachvili, 1985).

When an untreated vesicle is stressed, the membrane area expands promptly and then stays constant i.e. the stress-strain relation is time-independent. In presence of lysolipid, by contrast, the projection of the vesicle into the aspiration pipette grew slowly in length, although the suction was kept constant. In most experiments described above, the suction was increased stepwise in rapid succession, as with control vesicles, so that the slow 'creep' had little chance to express itself. In a few experiments, however, the time course of the slow 'creep' was deliberately studied (Fig. 6.07). With 12.5 μM LPC the membrane area increased progressively over three minutes and the fractional increase in membrane area eventually reached 0.04, before spontaneous lysis occurred. With 25 μM LPC the increase proceeded more rapidly and the membrane area increased by 0.05 before lysis.

Discussion

Both lysolipids and increased $[Ca^{2+}]_i$ cause membrane lysis and in lower concentrations a decrease in membrane strength and an increase in membrane distensibility, but with lysolipids the decrease in K manifested itself more regularly. Such a difference is not necessarily inconsistent with the hypothesis that elevated Ca^{2+} causes activation of PLA_2 and hence lysolipids to appear in the membrane: for added lysolipid might well become incorporated in the membrane in a uniform fashion which would tend to increase membrane distensibility, whilst activation of PLA_2 might be confined to limited areas of the membrane only, but nevertheless sufficient to weaken it. It has to be emphasized however, that the similarity between the effects of elevated $[Ca^{2+}]_i$ and of lysolipid on

membrane mechanical properties does not constitute proof of the involvement of PLA_2. At best, it increases the plausibility of that hypothesis.

Lysolipids derive their name from their ability to bring about lysis of red blood cells. Most of what is known about their action comes from experiment in which haemolysis was studied (for a review see Weltzien, 1979). The effect of lysolipids on membrane mechanical properties has not hitherto been studied. It is of broad interest because lysolipids have been shown to accumulate in ischaemic cardiac muscle, possibly as a result of activation of PLA_2 and/or from acylation of glycerophosphorylcholine by acyl-CoA that accumulates due to decreased fatty acid oxidation (Bergmann, Ferguson, & Sobel, 1981). When incorporated into the cardiac sarcolemma in concentration less than 1% of total membrane phospholipid, lysolipids produce electrophysiological changes which promote fibrillation (Gross, Corr, Lee, Selfitz, Crawford & Sobel, 1982). The action of lysolipids thus has many different facets.

In experiments on monolayers with a Langmuir film balance, it has been found that when a surfactant is added below the monolayer a decrease in surface pressure causes a slow increase in surface area due to the penetration of additional surfactant into the monolayer (Seelig, 1987). The here observed progressive increase in membrane area when a sarcolemmal vesicle is stressed by pipette aspiration in the presence of lysolipid can similarly be attributed to entry of more surfactant into the membrane. Eventually this reduces the interaction between lipids so much that the membrane cannot sustain even the low tension applied. Evidently, vesicle aspiration allows experiments on natural membranes of a kind hitherto possible only on monolayers.

B) EFFECTS OF ARACHIDONIC ACID ON MEMBRANE MECHANICAL PROPERTIES

Results

Arachidonic Acid is a tetraenoic fatty acid (20:4 *cis* 5,8,11,14) normally found esterified at the *sn*-2 position of glycerophosphatides, especially those composing the inner leaflet of the plasmalemma. Upon activation of PLA_2 it is therefore released together with lysolipid. There is evidence that arachidonic acid, like lysolipids, has a disordering effect on membrane lipids (Richieri, Mescher & Kleinfeld, 1990). Hence it may also alter membrane mechanical properties.

To test this point sarcolemmal vesicles were exposed to arachidonic acid in concentrations from 16 - 2 μM. With the highest concentration most vesicles lysed spontaneously. With 8 μM arachidonic acid surviving vesicles were clearly weakened and their distensibility greatly increased. The most consistent effect with 4 μM arachidonic acid was an increase in distensibility, K falling from 533 ± 54 in the control population to 242 ± 60 (mean ± S.D.) for the treated population. The maximum area increase at lysis was also clearly increased. With 2 μM arachidonic acid these effects were still detectable but smaller. The results are summarized in Figs 6.08 & 6.09 and Table 6.1. In the main, the potency of arachidonic acid in altering membrane mechanical properties was similar to that of lysolipids. Activation of PLA_2 thus causes a two-pronged attack on membrane integrity.

As regards its mode of action, it may be supposed that the charged carboxyl group of the freed acid will tend to "float" towards the hydrophilic surface of the membrane there to intercalate between the phospholipid headgroups. The fatty acid chain of arachidonic acid is known to adopt a hairpin-like configuration, owing to the multiple *cis* double bonds it contains. Consequently, the lipids in the deeper region of the membrane will be

packed more loosely which will weaken the cohesive forces within the membrane. Consistent with this view is the increase in membrane "fluidity" produced by arachidonic acid, as measured by fluorescence depolarization with DPH as the probe (Richieri *et al.*, 1990); and it is interesting to note that the concentration range of arachidonic acid required to produce changes in fluorescent polarization i.e. 2-10 μM is the same as that required to produce the changes in mechanical properties described above. This emphasizes that pipette aspiration of membrane vesicles provides a simple and certainly more functional alternative to other physical techniques for quantifying the effects of membrane perturbing agents.

In recent years, there have been many reports that arachidonic acid can regulate the activity of membrane ion channels. Indeed the activity of almost all channels which have been tested so far has been found to be altered, in one sense or another, by arachidonic acid in concentrations ranging from 1-100 μM (Meves, 1994). One hypothesis advanced as explanation is that arachidonic acid binds directly to the channel protein so as to alter its properties (Ordway, Singer & Walsh, 1991). In view of the fact that some proteins e.g. serum albumin are well known to possess fatty acid binding sites with K_d values that in many cases are in the micromolar range (Spector, 1975), and that water soluble enzymes such as protein kinase C can be activated by fatty acids, this hypothesis is clearly a strong contender. But another hypothesis is also in the field. It holds that changes in the bulk properties of the lipid bilayer are responsible for altering the behaviour of membrane ion channels (Takenaka, Horie & Hori, 1987; Takenaka, Horie, Hori & Kawakami, 1988). In this connection it has been proposed that the host bilayer can influence the function of integral membrane proteins because changes in protein configuration, like those associated with channel opening, may involve adjustments in the surrounding lipid bilayer (Lundbaek & Anderson, 1994). Changes in the deformation energy of the lipid bilayer could therefore contribute to the total energy barrier which has to be crossed for channel activity.

The two hypotheses considered above are clearly not mutually exclusive. Nor do the present results decide between them. But they show that an increase in lipid distensibility and hence a decrease in deformation energy is sure to accompany any direct action on channel properties that arachidonic acid may possess.

Table 6.1. Effects of arachidonic acid on membrane strength and elasticity.

[AA] (μM)	T^* (mNm^{-1})			K (mNm^{-1})	
	n	median	95% C.I.	n	mean ± S.D.
0	74	14.8	(14.3, 15.2)	70	535 ± 54
2	30	13.2	(12.5, 13.5)	30	358 ± 70
4	50	10.7	(9.9, 11.2)	47	242 ± 60
8	20	5.87	(4.4, 7.2)	17	159 ± 54

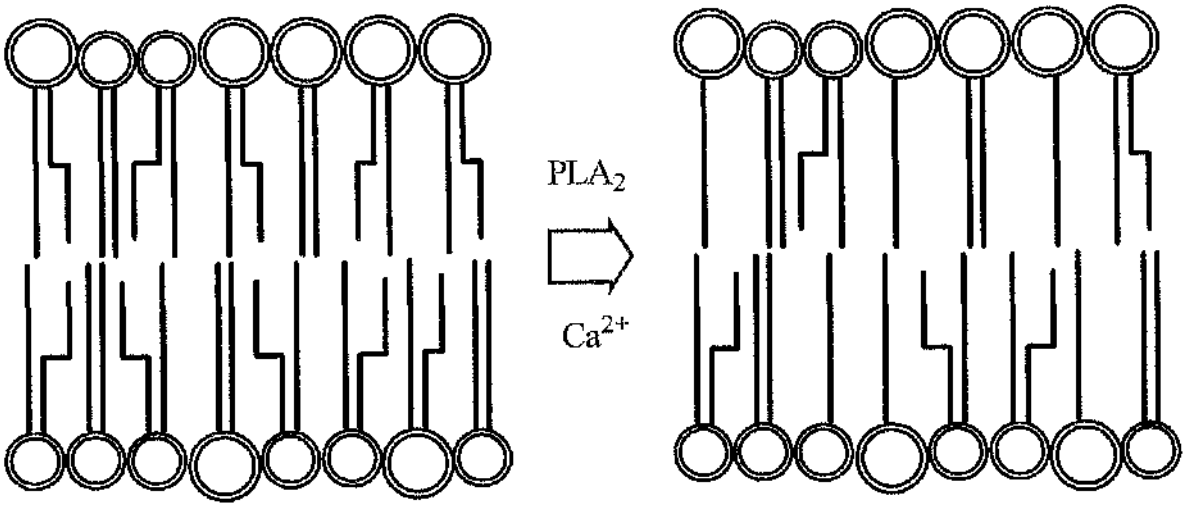

Figure 6.01 Diagramatic representation of the perturbing action of PLA_2 on phospholipid membranes

Hydrolysis of phospholipids by PLA_2 results in the formation of cone-shaped lysolipid molecules which create packing defects and reduce the overall cohesive forces between the acyl chains of the membrane.

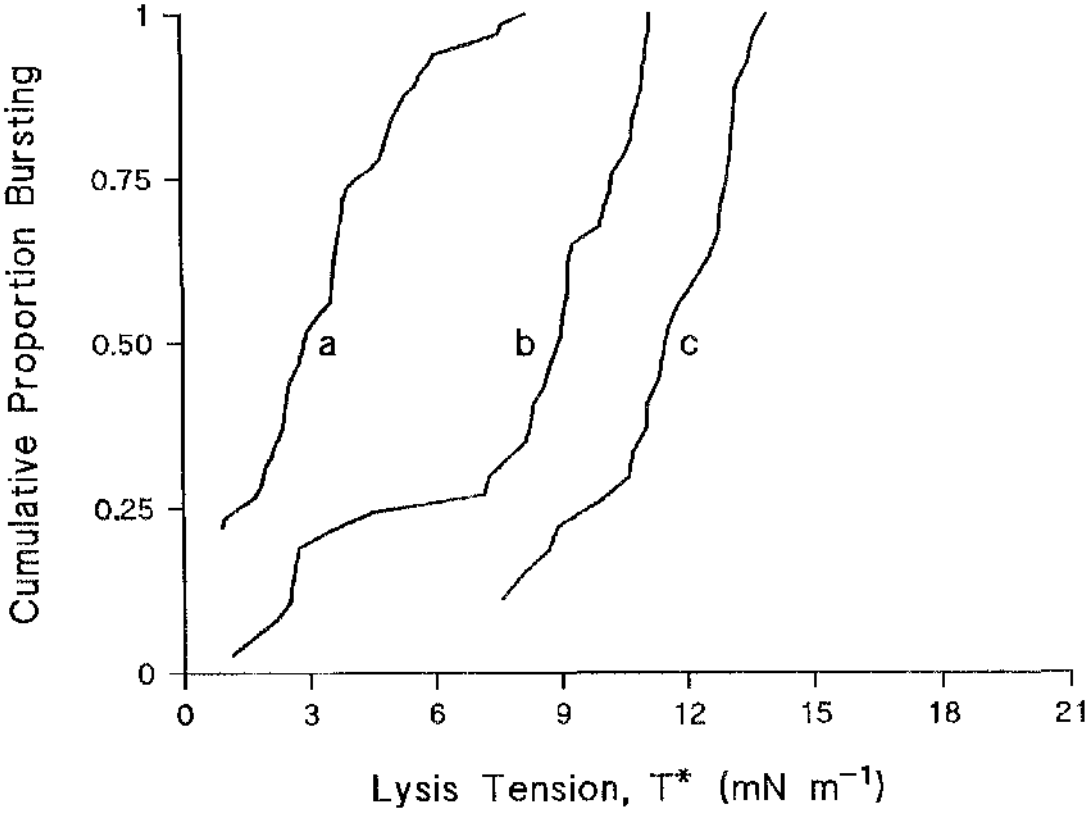

Figure 6.02 Effect of lysophosphatidylglycerol on membrane strength
Cumulative curves of lysis tension for vesicles in 140 mM KCl containing a), 5 μM LPG (n = 64) and b), 2 μM LPG (n = 37); c), untreated controls (n = 27).

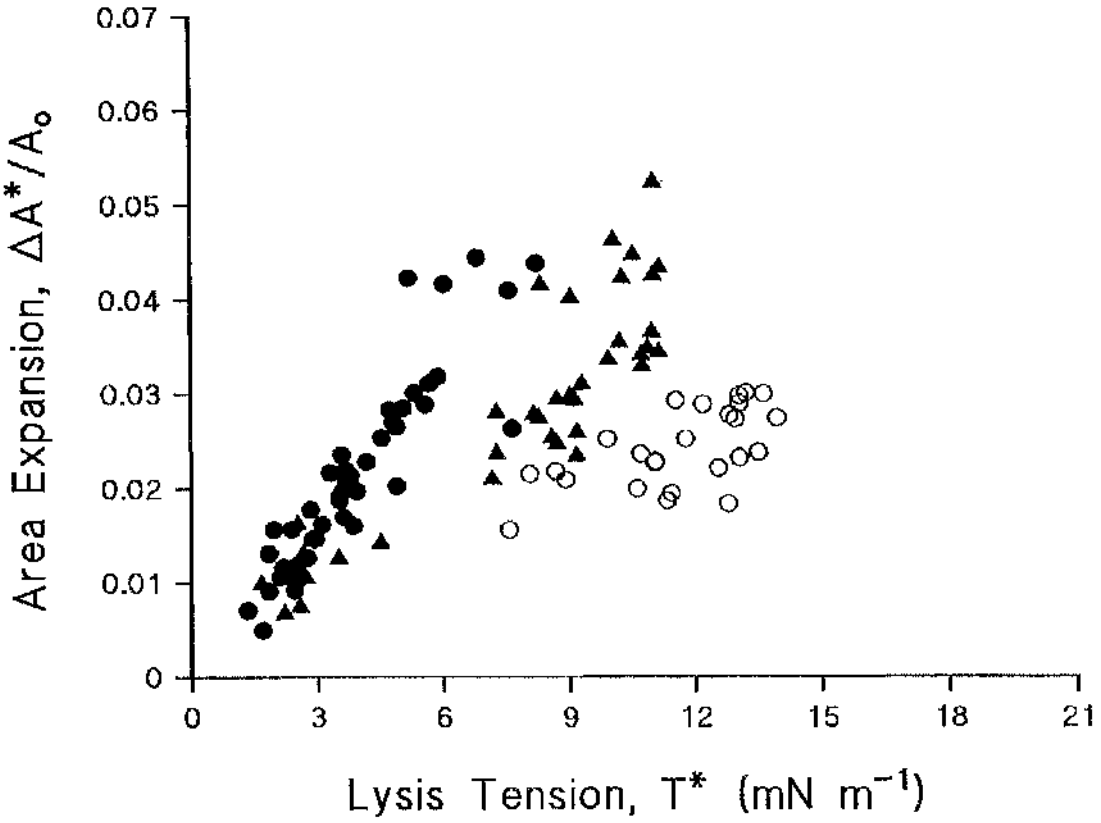

Figure 6.03 Lysis points for vesicles treated with lysophosphatidylglycerol
Lysis tension and corresponding area expansion for vesicles bathed in solutions containing ●, 5 μM (n = 49) and ▲, 2 μM (n = 36) LPG. ○, untreated controls in 140 mM KCl (n = 25).

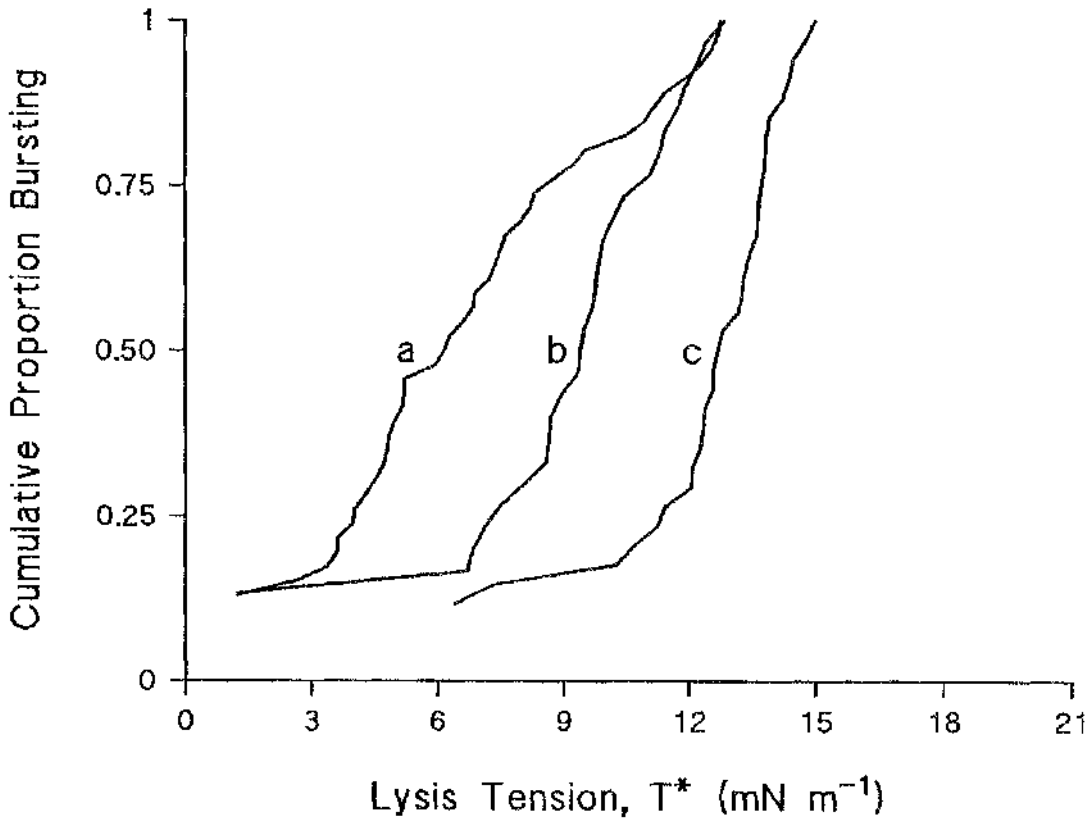

Figure 6.04 Effect of lysophosphatidylcholine on membrane strength
Cumulative curves of lysis tension for vesicles in 140 mM KCl containing a), 25 μM LPC (n = 46) and b), 12.5 μM LPC (n = 30); c), untreated controls (n = 34).

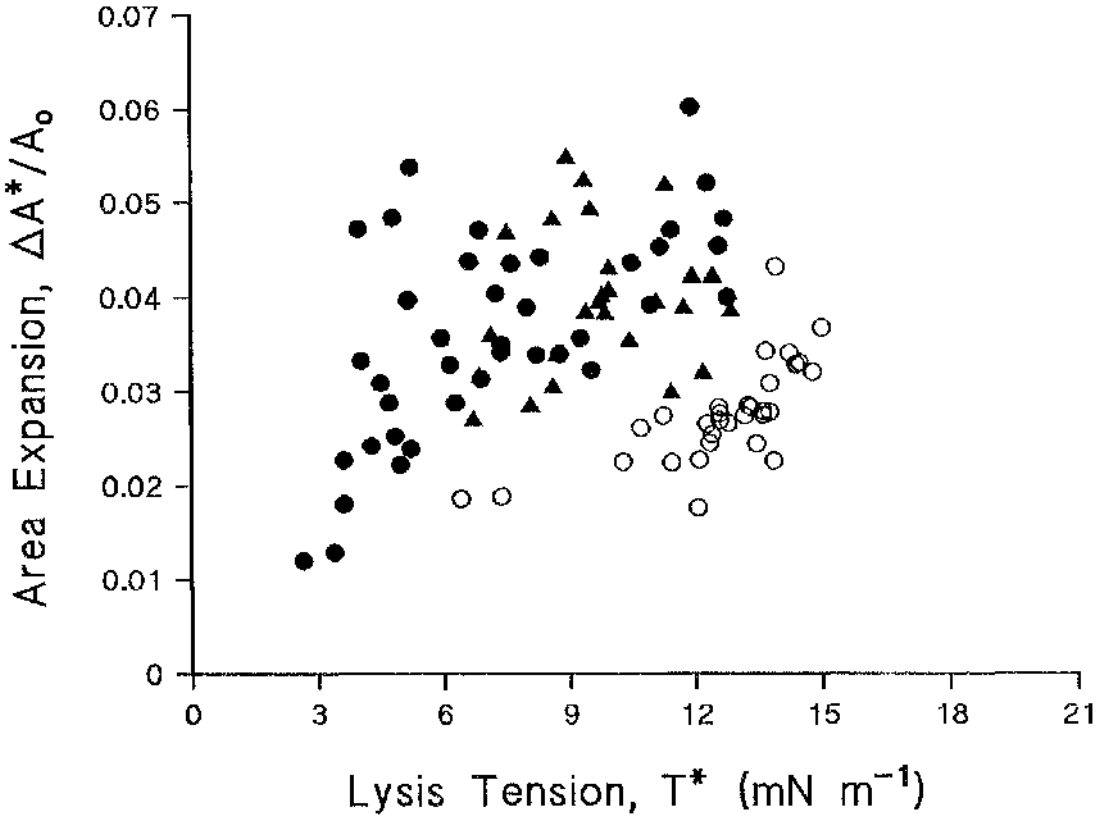

Figure 6.05 Lysis points for vesicles treated with lysophosphatidylcholine
Lysis tension and corresponding area expansion for vesicles bathed in solutions containing ●, 25 μM (n = 40) and ▲, 12.5 μM (n = 26) LPC. ○, untreated controls in 140 mM KCl (n = 31).

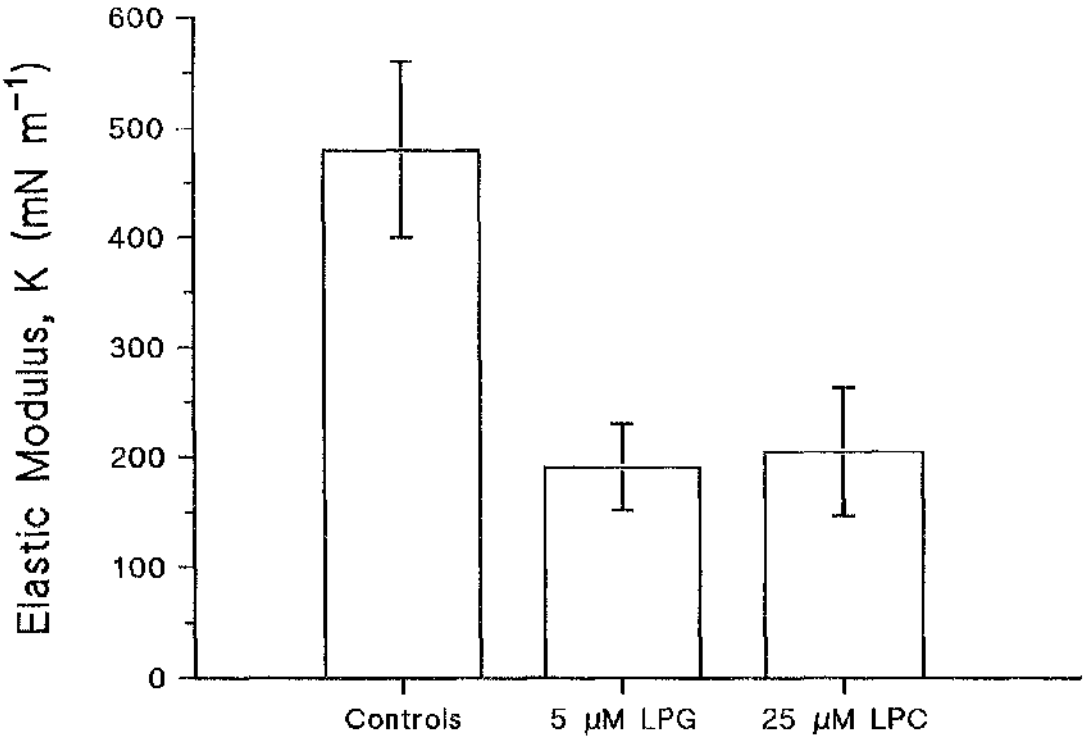

Figure 6.06 Effect on K of exogenous lysolipids

Bar graph showing the values of K (mean ± S.D.) for vesicles exposed to 5 μM LPG (n = 49), 25 μM LPC (n = 40) and untreated controls in 140 mM KCl (n = 56).

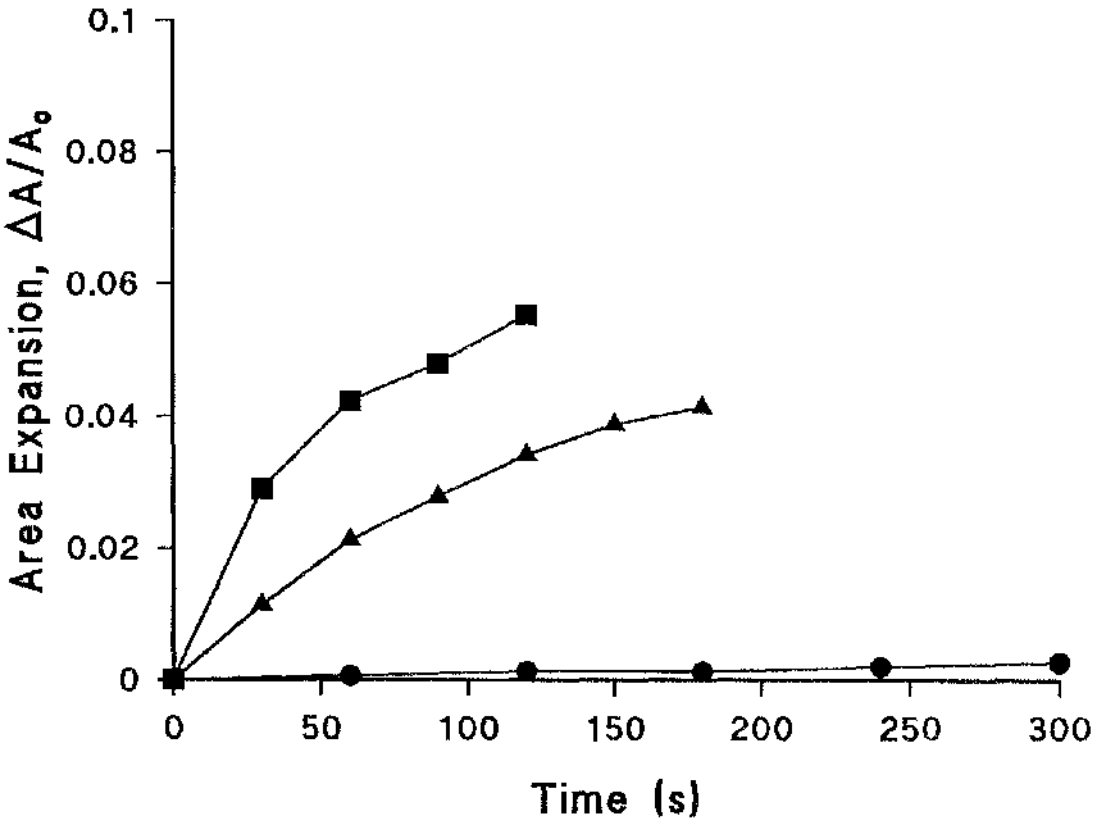

Figure 6.07 Incorporation of LPC into vesicles held at a constant low tension

Fractional increase in membrane area is plotted against time for individual vesicles aspirated with a constant suction pressure of 2 cm H_2O in solutions containing ■, 25 μM (T = 1.23 mM m^{-1}) and ▲, 12.5 μM LPC (T = 1.12 mM m^{-1}).
●, a control vesicle in 140 mM KCl (T = 1.23 mN m^{-1}).

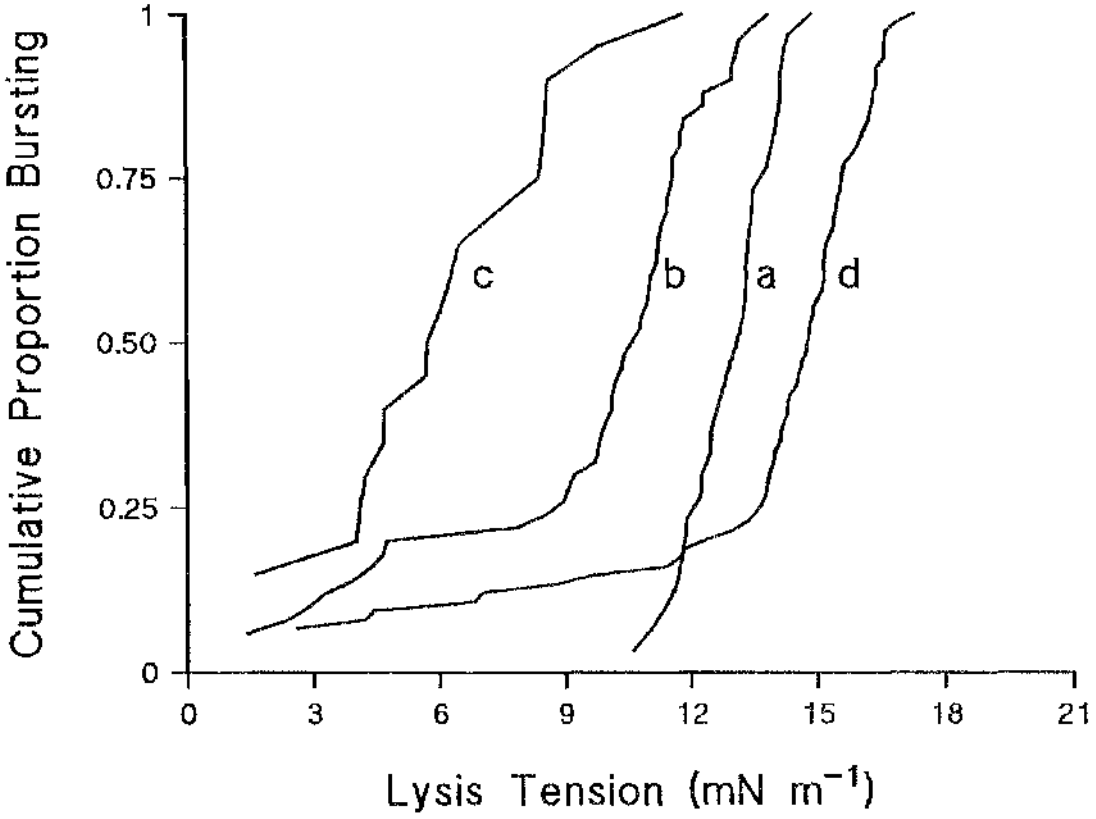

Figure 6.08 Effect of arachidonic acid on membrane strength

Cumulative curves of lysis tension for vesicles treated with: a), 2 μM (n = 30); b), 4 μM (n = 50) and c), 8 μM (n = 20) arachidonic acid; d), untreated controls in 140 mM KCl (n = 74).

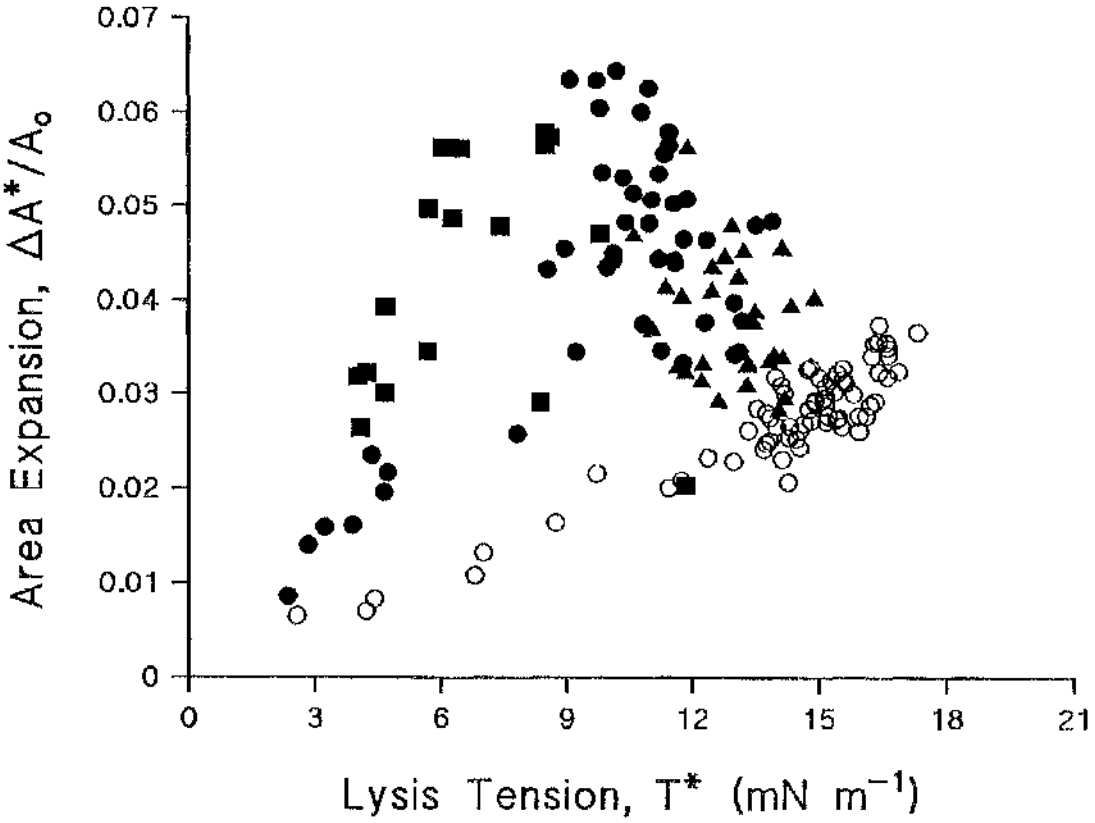

Figure 6.09 Lysis points for vesicles treated with arachidonic acid
Lysis tension and corresponding area expansion for vesicles bathed in solutions containing ▲, 2 μM (n = 30); ●, 4 μM (n = 47) and ■, 8 μM (n = 17) arachidonic acid. ○, untreated controls in 140 mM KCl (n = 70).

PART VII

IS PLA_2 INVOLVED IN CA^{2+} MEDIATED LYSIS OF SARCOLEMMAL VESICLES?

TYPES OF PLA_2

An enzyme releasing fatty acid from phospholipid was first discovered in pancreatic juice. Later, a similar enzyme was found in snake venom, and it was established that the fatty acid removed was the one esterified to the *sn*-2 position of phosphatidylcholine (see van den Bosch, 1980, for a review). With the identification also of other forms of PLA_2, pancreatic and snake venom PLA_2 is now classed as type I. Type II PLA_2 is a membrane bound or secretory enzyme which is found for instance in platelets, synoviocytes, polymorphonucleocytes and macrophages (Vadas & Pruzanski, 1990; Glaser, Mobilo, Chang & Senko, 1993, for reviews). It is extracellularly active (Cockcroft, 1992) and is present in synovial fluid and other exudates. It plays a role in the pathophysiology of inflammation through releasing arachidonic acid, the precursor of eicosanoids. Type II secretory PLA_2 is also known as non-pancreatic s-PLA_2. Although genetically distinct (Johnson, Frank, Vadas, Pruzanski, Lusis & Seilhamer, 1990), Type I and II PLA_2 are molecules of similar size (12-18 kDa) and they share some homologies. They are also similar in both requiring Ca^{2+} in **milli**-molar concentration for optimum activation (Ross, Deems, Jesaitis, Dennis & Ulevitch, 1985; Ulevitch, Sano, Watanabe, Lister, Deems & Dennis, 1988).

In the last few years a novel cytosolic form of PLA_2 with distinct molecular and functional properties has been discovered in a monocyte cell line (Clark, Milona & Knopf, 1990; Kramer, Roberts, Manetta & Putnam, 1991), renal mesangial cells (Gronich, Bonventre & Nemenoff, 1990), rat brain cytosol (Yoshihara & Watanabe,

1990) and platelets (Kim, Suh & Ryu, 1991; Takayama, Kudo, Kim, Nagata, Nozawa & Inoue, 1991). In the presence of **micro**-molar concentrations of Ca^{2+}, $cPLA_2$ is translocated to the membrane where it selectively cleaves arachidonic acid (Channon & Leslie, 1990; Wijkander & Sundler, 1991, 1992). The Ca-dependent phospholipid-binding domain of $cPLA_2$ is homologous to that of PKC (Clark *et al.*, 1991; Sharp *et al.*, 1991), and for full activation at the membrane $cPLA_2$ requires activation by mitogen-activated protein kinase (MAP) (Lin, Wartmann, Lin, Knopf, Seth & Davis, 1993). $cPLA_2$ thus has the characteristics of a regulated intracellular enzyme involved in mediator generation which may be activated by an increase in Ca^{2+}, either as a result of ligand binding to a receptor and consequent release of Ca^{2+} from intracellular stores or by increased influx of Ca^{2+}; or more artificially by use of A23187.

For the sake of completion it should be added that the majority of measurable PLA_2 in mammalian myocardium is a calcium independent cytosolic form of molecular mass 40 kDa which preferentially hydrolyses plasmologen* containing arachidonic acid at the sn-2 position (Gross, 1992).

Some unresolved problems

Most detailed studies on the activation and inhibition of PLA_2 have been carried out on Type I extracellular enzyme from porcine pancreas or cobra venom, because enzyme from these sources has been longest known and is plentiful. Even after the discovery of the related PLA_2 Type II enzymes, the Type I enzyme remained the model for examining the chemical mechanism of PLA_2 action and its possible inhibition. The following salient facts have emerged in this way (i) PLA_2 has little affinity for phospholipid in monomeric form: the enzyme must interact with the substrate in aggregate form i.e. micellar or

* A subclass of choline glycerophospholipids in which the fatty acid at the sn-1 position is linked via a vinyl ether lipid predominating in the myocardial sarcolemma.

bilayer (Hazlett, Deems & Dennis, 1990). It is thought the enzyme possesses both lipid binding and catalytic sites (Dennis, 1987). (ii) The catalytic site requires Ca^{2+} in millimolar concentration, as already mentioned. It follows that hydrolysis of phospholipids may be prevented in various ways (a) by preventing the interaction between the enzyme and its substrate (b) by displacing Ca^{2+} from the membrane (c) by agents that attack the enzymatic site (Flower & Blackwell, 1979). In the first category belong a range of amphiphilic agents typified by the anti-malarial drug mepacrine (see page 101). Cationic local anaesthetics like dibucaine (see page 106) probably also act by interfering with the substrate-enzyme interface, though displacement of Ca^{2+} has also been mooted as a possibility. The best characterized irreversible inhibitor is p-bromophenacylbromine (BPB) which modifies the active histidine residue of PLA_2 I and II (Roberts, Deems, Mincey & Dennis, 1977).

In recent years, the search for anti-inflammatory drugs has yielded a growing number of compounds that inhibit PLA_2 (Wilkerson, 1990). Some are non-hydrolysable phospholipid derivatives which presumably block the enzymes associated with its natural substrate. By contrast the natural marine product manoalide (see page 112) acts irreversibly by modifying lysine residues found in extracellular PLA_2 (Lombardo & Dennis 1985). The compound Ro 31-4493 (see page 109), which inhibits both pancreatic and synovial cell PLA_2, was also designed to interact with the active site of these similar enzymes (Davis, Nixon, Wilkinson & Russell, 1988). However, both manoalide and Ro 31-4493 possess large hydrophilic domains and therefore have the potential also to interfere with the substrate architecture like the non-competitive amphiphilic inhibitors.

No specific inhibitor of $cPLA_2$ has so far been found.

On the strength of its inhibitory action on PLA_2, mepacrine has been used to verify the idea, first canvassed in the early 1970's, that mobilization of arachidonic acid from phospholipase is the first step in the biosynthesis of inflammatory mediators from

perfused lung and spleen slices (Vargaftig & Dao Hai, 1972; Flower & Blackwell, 1976). Mepacrine and other extracelular PLA_2 inhibitors have also been found to prevent aggregation of human platelets which is also initiated by arachidonic acid mobilization (Wallach & Brown, 1981). However, such observations and other similar examples of effective prevention of arachidonic acid mobilization cited later pose a major problem because even in activated cells cytosolic Ca^{2+} is only in the micromolar range. How then is one to explain the effect of inhibitors of $sPLA_2$ when the conditions for activation of that enzyme, which requires Ca^{2+} in the millimolar range, are not met. One possibility, suggested by Feinstein & Sha'afi (1983), is that activators produced by intact cells may be necessary to manifest $sPLA_2$ at low cytosolic $[Ca^{2+}]$. But no evidence for this idea has been advanced. Rather the problem has been mainly overlooked. Evidently, in the face of a positive result a proper explanation of its origin is prone to be neglected, especially when practical considerations like the promotion of new anti-inflammatory drugs is concerned.

Today, it is possible to envisage two further explanations for the above conundrum. First it could be that in some cases when activation of inflammatory cells is brought about by mediators or Ca^{2+} ionophores, the sub-plasmalemmal concentration of Ca^{2+} might be much higher than that in the bulk cytosol. Second, it could be that the recently discovered $cPLA_2$ is involved. This would imply that the non-competitive inhibitors of $sPLA_2$ inhibit also $cPLA_2$. Oddly, the literature seemingly provides no information on this point. But in as much as $cPLA_2$ is also an interfacial enzyme, it would not be surprising if agents altering membrane properties were to interfere with its action.

The lesson to be drawn from the above considerations is that PLA_2 inhibitors have sometimes been used on questionable logic, but nevertheless found to be effective. It was therefore decided to test on empirical grounds whether putative PLA_2 inhibitors, which have been reported to prevent some effects of A23187 mediated influx of Ca^{2+}, can also prevent the effect of elevated $[Ca^{2+}]_i$ on membrane mechanical properties.

EFFECTS OF PLA_2 INHIBITORS

In order to substantiate the idea that elevation of intravesicular Ca^{2+} weakens the membrane by activating PLA_2, it would be necessary to demonstrate (i) the presence in sarcolemmal vesicles of PLA_2 activated by micromolar concentrations of Ca^{2+} (ii) that production of lysolipids and arachidonic acid actually occurs in Ca^{2+} challenged vesicles (iii) that inhibitors of PLA_2 prevent the biochemical degradation of the membrane and that at the same time the mechanical properties of the membrane are maintained normal, or at least in so far as these properties are not disturbed by the inhibitors themselves. In the absence of the necessary biochemical facilities and expertise, the direct tests under (i) and (ii) above were clearly out of reach. However, approach (iii) could be followed, at least in part, with the available technique. In particular, it seemed worthwhile to test PLA_2 inhibitors which have been shown in analogous studies on whole cells to prevent membrane degradation resulting from A23187 mediated Ca^{2+} influx.

Mepacrine (Quinacrine)

Quinine has long been known to inhibit pancreatic lipase and has also been shown to prevent the mobilization of depot fat produced by catecholamines which activate adipose tissue lipase (Mosinger, 1965). Mepacrine and chloroquine, a related antimalarial drug, have similarly been shown to inhibit hormone activated lipolysis (Markus & Ball, 1969). The recognition of mepacrine and chloroquine as inhibitors of PLA_2 came about as result of the intense study of prostaglandin synthesis in the 1970's. Thus mepacrine (50 μM) was found to prevent the release of 'rabbit aorta contracting substance' - a prostaglandin precursor - from perfused guinea pig lung, but not when arachidonic acid was supplied in the perfusate (Varhaftig & Dao Hai, 1972). Subsequently, Flower & Blackwell (1976) showed that the loss of arachidonic acid from the phospholipid fraction of guinea pig spleen cells was blocked by mepacrine (1 mM) and that the release of arachidonate from spleen slices was similarly suppressed. The chemotactic response of leucocytes, which is

controlled by PLA_2 activation, and the associated release of arachidonic acid from leucocytes are also completely blocked by 1 mM mepacrine.

In all the above instances, the cells were stimulated by natural components of the inflammatory system. In other experiments, A23187 was used to induced prostaglandin synthesis. In rat basophilic leukaemia cells, for instance, 1μM A23187, in the presence of physiological $[Ca^{2+}]_o$, stimulated arachidonic acid release; and this response was reduced to one half by 0.1 mM mepacrine (McGivney, Morita, Crews, Hirata, Axelrod & Siraganian, 1981). Similarly, in dispersed renal cortical tubular cells synthesis of PGE_2, induced by A23187 was inhibited by 0.1 mM chloroquine (Wuthrich & Vallotten, 1986).

The effectiveness of mepacrine in preventing activation of PLA_2 has been demonstrated also in the nervous system. The event initiating long term potentiation (LTP) of synaptic transmission is believed to be entry of Ca^{2+} through channels associated with NMDA receptors (Williams & Bliss, 1989). Release of arachidonic acid through activiation of PLA_2 then follows (Dumuis, Sebben, Heynes, Pin & Bockaert, 1988). In accordance with the above, release of arachidonic acid from cultured neurones can be stimulated by ionomycin (10 μM) in the presence of 0.75 mM $CaCl_2$; and this effect is blocked completely by 0.1 mM mepacrine (Dumuis *et al.*, 1988).

Most pertinent from the present point of view, is the work on experimental muscle damage by a group in Liverpool. Using isolated small muscle fibres from the mouse leg, they have shown that A23187 (25 μM) in the presence of physiological $[Ca^{2+}]_o$, causes release from the muscle of cytosolic enzymes e.g. lactate dehydrogenase (LDH) or creatine kinase (CK) (Jones, Jackson, McPhail & Edwards, 1984; Jackson *et al.*, 1984). Poisoning muscle metabolism with DNP similarly caused a Ca^{2+}-dependent release of LDH (Jackson, Wagenmakers & Edwards, 1987). Among some other PLA_2 inhibitors , mepacrine (1 mM) largely suppressed the outflow of LDH. From this, Jackson *et al.*,

(1987) conclude that Ca^{2+} is acting via activation of PLA_2 to induce membrane damage as recognizable by an increase in enzyme efflux.

Results

To test whether mepacrine has a protective effect also on membrane mechanical properties, vesicles were first treated with it and, after an interval, A23187 (5 μM) and $[Ca^{2+}]_o$ (10 μM) were admixed. Vesicles from the same batch exposed to the same concentration of ionophore and Ca^{2+}, but without pre-treatment with mepacrine, served for comparison. And as usual, untreated vesicles served as controls. At 200 μM, mepacrine had no protective effect. But at 1 mM and even more so at 2 mM mepacrine, the cumulative distribution curves were distinctly shifted in downward direction (Fig. 7.01). This was mainly the result of a decrease in the proportion of vesicles so weak as to burst at the lowest aspiration pressure used. With 2 mM mepacrine, this effect was great enough to reduce the proportion of ultra-weak vesicles treated with both 10 μM $[Ca^{2+}]_o$ and 2 mM mepacrine to below even that of the control vesicles. By contrast mepacrine did not restore the strength of the majority of $[Ca^{2+}]_o$ treated vesicles. Thus with 2 mM mepacrine and 10 μM $[Ca^{2+}]_o$ 0.85 of vesicles burst at below 10 mN m^{-1}.

In order better to understand the situation, the effect of 2 mM mepacrine by itself was also studied. Fig. 7.02 shows that mepacrine greatly reduces the tensile strength of the majority of vesicles, but not to the point where they become unmeasurably weak. At the same time, 2 mM mepacrine distinctly increases the distensibility of vesicles (Fig. 7.03, Table 7.1).

In the light of the above findings, one might have expected that the joint effect of mepacrine and elevated $[Ca^{2+}]_i$ would be a more severe reduction in the strength of vesicles than is produced by either agent alone, i.e. that the cumulative curve given by 10 μM $[Ca^{2+}]_o$ plus 2 mM mepacrine would lie to the left of the curve for 10 μM $[Ca^{2+}]_o$ alone. That the opposite is the case suggests that the direct effect of mepacrine on the

membrane masks a protective action of mepacrine against the effect of elevated $[Ca^{2+}]_i$. Thus comparison of Figs. 7.01 & 7.02 shows the leftward shift in the cumulative distribution curve produced by 1 mM mepacrine to be at least as great as that produced by 10 μM Ca^{2+} alone. If it were legitimate to subtract the shift by mepacrine alone from that produced by 10 μM Ca^{2+} plus mepacrine, it would be possible to argue that mepacrine has fully inhibited the effect of raised $[Ca^{2+}]_i$ in membrane tensile strength.

NDGA

Nordihydroguaiaretic acid (NDGA) inhibits both PLA_2 and the lipoxygenase pathway for the production of leucotrines from arachidonic acid (Lanni & Becker, 1985). In studies on the origin of long term potentiation (LTP) in the dentate gyrus of the rat, 200 μM NDGA has been found to block LTP and the release of arachidonic acid normally associated with it (Lynch, Errington & Bliss, 1989). In experiments on isolated mouse soleus muscle treated with A23187 or DNP, similar to those with mepacrine cited above, Jackson *et al.* (1987) found NDGA (5 μM) to decrease CK efflux to about one-third of that found in muscles permeabilized to Ca^{2+} or poisoned, but not exposed to NDGA; and 50 μM NDGA completely inhibited CK efflux. Since the experiments with mepacrine described in the previous sections could arguably be interpreted as consistent with the finding of Jackson *et al.* (1984), it seemed worth testing also NDGA for its effect on vesicle mechanical properties and their modification by elevated $[Ca^{2+}]_i$.

Results

NDGA is not readily soluble and 20 μM was the highest concentration here used. At that level NDGA by itself produced a slight weakening of sarcolemmal vesicles, as evident from the leftward shift of the cumulative lysis tension curve in Fig. 7.04. That it had altered the membrane was also evident from the decrease in K produced by 20 μM NDGA (Table 7.1). In the experiment of Fig. 7.05, 2 μM $[Ca^{2+}]_o$ plus 2.5 μM A23187 caused a greater than usual reduction in membrane strength. Vesicles pre-treated with 20 μM NDGA before exposure to calcium and ionophore as above, were slightly stronger than vesicles treated with Ca^{2+} and ionophore alone, despite the weakening effect produced by NDGA itself. The overall effect of NDGA was thus similar to that produced by mepacrine, but less pronounced.

Dibucaine (Nupercaine)

Local anaesthetics are known to interfere with deacylating lipolytic enzymes including PLA_2 (Kunze, Nahas, Traynor & Wurl, 1976). In experiments on mitochondrial PLA_2 with phosphatidylethanolamine as substrate, 10-50 μM dibucaine stimulated the enzyme, whilst 200-300 μM dibucaine inhibited it (Waite & Sisson, 1972). Using pancreatic PLA_2 and the swelling of mitochondria as index of its activity, Scherphof, Scarpa, & van Toorenenbergen (1972) found 100 μM dibucaine to inhibit the enzyme fully. With cobra venom PLA_2 acting on sonicated C^{14} labelled phospholipids, Kunze *et al.* (1976) found dibucaine to stimulate PLA_2 in concentrations between 100 μM and 1 mM and to inhibit the enzyme at higher concentrations. This diversity in the results obtained may reflect that dibucaine can act both through increasing the susceptibility of the substrate to enzyme hydrolysis and through displacing Ca^{2+} from the enzyme-Ca^{2+} complex (Kunze *et al.*, 1976). In support of the second mechanism, all three groups cited above show that Ca^{2+} antagonizes the inhibitory action of dibucaine on PLA_2 in competitive fashion. Also relevant in this connection is the demonstration that dibucaine in very low concentration can displace Ca^{2+} ions adsorbed on monolayers of acidic phospholipids at the air water interface (Hauser & Dawson, 1968). It should be noted, however, that dibucaine does not simply replace Ca^{2+} adsorbed at the surface of the membrane. Rather dibucaine, like other amphiphiles, becomes intercalated into the membrane matrix (Seelig, 1987); and according to Willman & Hendrickson (1978) it is the positive charge so created which prevents intercalation of enzyme with membrane lipid i.e. a process additional to displacement of Ca^{2+} may also be at work.

Turning now to skeletal muscle, Jackson *et al.* (1984), in experiments similar to those cited above (p 102), found 100 μM dibucaine effective in partly suppressing release of LDH after poisoning muscle metabolism. It seemed interesting therefore to explore whether dibucaine has also a protective effect on the muscle membrane, as judged by measuring membrane mechanical properties.

Two sets of experiments with dibucaine were done at an interval of nearly a year. They are presented separately because the median values and slopes of the cumulative lysis tension curves of the respective control vesicle populations were too different for the results to be pooled.

In the first set of experiments 200 μM dibucaine was tested as to whether it protected against 10 μM $[Ca^{2+}]_o$. Fewer of the vesicles which had been pre-treated with dibucaine were found to burst instantly on aspiration, and the remainder of the dibucaine treated vesicles were slightly stronger than the vesicles exposed directly to 10 μM $[Ca^{2+}]_o$. As a result the cumulative lysis tension distribution curve for the dibucaine pre-treated vesicle lies below and to the right of the curve for vesicles exposed directly to $[Ca^{2+}]_o$ (Fig. 7.06). But in comparison with the control vesicles, the dibucaine pre-treated vesicles remained much weaker. In this set of experiments the effect of 200 μM DBC by itself was not tested. This omission was made good in the second set of experiments (Fig. 7.07) which shows that 200 μM DBC by itself produces a substantial weakening of the membrane. Even if 200 μM dibucaine had completely antagonised the weakening effect of $[Ca^{2+}]_o$ a return of the cumulative curve all the way to the control cumulative curve is therefore not to be expected.

In the second set of experiments, 500 μM dibucaine was used. This higher concentration provided a much greater protection against the effects of $[Ca^{2+}]_o$, as evident from the low proportion of vesicles bursting instantly and the considerable shift to the right in the cumulative distribution curve for the dibucaine treated vesicles as compared with those treated directly with $[Ca^{2+}]_o$ (Fig. 7.08). Moreover, when 500 μM dibucaine by itself was tested (Fig. 7.07), in the absence of $[Ca^{2+}]_o$, it was found to shift the cumulative distribution curve so far to the left that it almost coincided with the cumulative distribution curve for $[Ca^{2+}]_o$ plus dibucaine. It is therefore possible to argue (Discussion p 115) that dibucaine provides almost complete protection against the weakening effect of $[Ca^{2+}]_o$.

Vesicles treated with 500 μM dibucaine were more extensible than untreated vesicles and burst at a higher fractional increase in membrane area than did control vesicle (Fig. 7.09, Table 7.1). Vesicles treated also with 10 μM $[Ca^{2+}]_o$ were still more distensible. But the additional effect of $[Ca^{2+}]_o$ was so small in this case that much of it could have been due to the ionophore and solvent (c.f. Fig. 4.04). At all events, 10 μM $[Ca^{2+}]_i$ did not reduce the effect of dibucaine on extensibility, indicating that Ca^{2+} at that low concentration does not displace dibucaine from the membrane. It may therefore be presumed that the reduction by dibucaine of vesicle tensile strength (Fig. 7.07) was unaffected by the presence of Ca^{2+}.

It has already been mentioned (p 82), that in the presence of LPC, vesicles held at constant tension show a progressive increase in membrane area, as indicated by a creep of the projection up the aspiration pipette. A similar phenomenon was observed with dibucaine (Fig. 7.10). But compared with LPC, or with Ro 31-4493, (see later p 110, 111) the effect of dibucaine was less marked. Thus 500 μM dibucaine produced an increase in membrane area by only 0.021 after 120 sec at 3.57 mN m^{-1}, as compared with an increase by 0.055 for the experiments with 25 μM LPC at a lower tension (Fig. 6.07).

Ro 31-4493

When an isolated heart is perfused with a Ca^{2+} deficient solution, cellular connections are weakened. On readmission of Ca^{2+} in normal physiological concentration, extensive cell damage then occurs. This phenomenon, called the calcium-paradox, has been ascribed to (i) inflow of Na^{+} into cardiac cells through Ca-channels during perfusion with Ca-deficient solution. (ii) On Ca^{2+} repletion, an elevation of $[Ca^{2+}]_i$ to 1-10 μM, owing to the operation of the Na-Ca exchanger (Chapman & Turnstall, 1987; Rodrigo & Chapman, 1991). The cell damage on Ca-repletion has been attributed to (i) membrane damage arising mechanically from a Ca^{2+} provoked contraction, (ii) activiation of Ca-dependent degradative enzymes. In the course of seeking evidence in support of the second possibility Suleiman, Minezaki, Ban, K. & Chapman,(1992)tested the effect of compound Ro 31-4493 on the Ca-paradox. Ro 31-4493 (Fig. 7.11) is an amphiphilic compound whose head group was designed to interact with the active centre of *Bovine* pancreatic PLA_2 as studied with the aid of computer graphics (Davis *et al.*, 1988). In *in vitro* experiments Ro 31-4493 has been shown to inhibit that enzyme as well as human synovial PLA_2 with an IC_{50} of 25 μM. Ro 31-4493 has also been shown to inhibit (IC_{50} = 5.8 μM) the activiation and maintenance of supraoxide (O^{2-}) generation by NADPH oxidase in human neutrophil derived cytoplasts, a process that depends on the availability of arachidonic acid through the operation of PLA_2 (Henderson, Chappell & Jones, 1989). In the experiments of Suleiman *et al.*, (1992) the presence of Ro 31-4493 (50 μM) during Ca depletion/repletion protected the myocardium against damage, as attested by full inhibition of the release of proteins, LDH and CK normally caused by this procedure.

Using a sample of Ro 31-4493 kindly presented by Dr. M-S. Suleiman, Bristol, its effect on membrane mechanical properties was first tested at 50 μM. This concentration proved too high for two reasons. First, the vesicles behaved as if they were sticky and soon clumped together in a fashion not seen with any of the other amphiphilic agents tested*.

* It is in fact possible to study vesicle-vesicle interaction in quantitative manner by an elaboration of the present technique in which two vesicles, each aspirated into a pipette, are brought into contact with one another (Evans & Metcalfe, 1984). However, this was considered outwith the scope of the present thesis, but may be attempted in the future.

Secondly, the majority of vesicles burst instantly on aspiration and the few which could be aspirated were exceedingly weak and distensible, and showed a continuous increase in membrane area between stepwise increases in suction pressure. With 25 μM Ro 31-4493 more vesicles withstood aspiration with low pressures and the usual instant increment in membrane area could be distinguished from the progressive increase in area at steady pressure (see below), so that estimates for T^* and for K comparable with other results could be obtained. Clumping of vesicles took longer to develop, but was still too pronounced to allow an experiment of usual design to be completed. To test whether Ro 31-4493 can protect against the effects of elevated $[Ca^{2+}]_i$ it was therefore used at 12.5 μM, at which level it is still effective in protecting perfused hearts (M-S. Suleiman, personal communication).

At 12.5 μM, Ro 31-4493 still produced a distinct weakening of vesicles and a slight increase in distensibility as compared with control vesicles (Figs. 7.12 & 7.13). And when vesicles pre-exposed to Ro 31-4493 for 20-30 mins were treated also with 10 μM $[Ca^{2+}]_o$ plus ionophore, it was again found that fewer vesicles ruptured instantly on aspiration. But all the vesicles which could be aspirated proved very weak (Fig. 7.14). Even with 12.5 μM Ro 31-4493, pairs of vesicles sticking together were found, but large clumps did not form at this concentration for about 2 hours i.e. until near the end of the experiment. Nevertheless it is clear that the effect of Ro 31-4493 conforms to the pattern already seen with the other PLA_2 inhibitors tested above.

A notable feature of experiments with 25 μM Ro 31-4493 was the greater increase in membrane area which vesicles so treated withstood before bursting. Although this was a feature with all the amphiphilic agents used it was particularly marked with Ro 31-4493 with ΔA^* values around 0.07, i.e. twice the normal limit, were sometimes observed. Some vesicles treated with Ro 31-4493 were also unusually flaccid on initial aspiration, as if an expansion of membrane area had taken place even without applied tension. The striking further increase in membrane area which occurs in the presence of 25 μM Ro 31-

4493 when a low suction pressure is maintained, already mentioned above, is illustrated in Fig. 7.15. Although strict quantitative comparison with the similar phenomenon observable with dibucaine (Fig. 7.10) is not possible because of differences in membrane tension maintained, it is nevertheless obvious that Ro 31-4493 intercalates into the membrane more readily than either of the above agents and is in this respect more similar to LPC (Fig. 6.07).

Manoalide

Manoalide is a sesterterpene (Fig. 7.16) isolated from the marine sponge *Luffariella variabilis*. It has been shown to be an anti-inflammatory compound with a point of action proximal to arachidonic acid release and metabolism (Jacobs, Culver, Langdon, O'Brien, & White, 1985). It inhibits the hydrolysis of phosphatidylcholine by Ca-dependent PLA_2 from bee (DeFreitas, Blankemeier, & Jacobs, 1984) and cobra venom (Lombardo & Dennis, 1985). It also inhibits membrane associated Ca-dependent PLA_2 in mammalian cell lines. For instance, in cultured murine peritoneal macrophages stimulated with 1 μM A23187 (at physiological ($[Ca^{2+}]_o$) manoalide (0.5 μM) causes inhibition of PGE_2 production to 30% of the normal response through limiting the production of arachidonic acid (Mayer, Glaser & Jacobs, 1988). Furthermore, in studies on the macrophage-like cell-line ($P388D_1$) the inhibitory action of manoalide on purified membrane associated Ca-dependent PLA_2 *in vitro* has been correlated with its inhibitory action on A23187 induced PGE_2 release from intact cells (Lister, Sano, Watanabe, Ulevitch, Deems & Dennis, 1987). In the latter experiments manoalide was found to be toxic to cells in concentrations above 0.5 μM at which level it inhibited PGE_2 release by 50%.

In the present experiments, manoalide in concentrations 0.3 and 1.2 μM was found greatly to weaken sarcolemmal vesicles and to reduce K in roughly dose dependent manner (Figs. 7.17, 7.18 & Table 7.1). In order to test whether manoalide protects vesicles against the effect of Ca-loading, still lower concentrations had to be used. At 0.16 μM manoalide by itself seemed to have little direct effect on membrane mechanical properties (Table 7.1). But when vesicles pre-treated with manoalide at this concentration were additionally treated with 1 μM A23187 and 10 μM Ca^{2+} , they became so weak that they all burst immediately. Rather than protecting the membrane, manoalide thus seems to enhance the effect of elevated cytosolic Ca^{2+}.

Compared with lysophosphatidylglycerol (pp 81-82) manoalide is more potent in weakening the membrane and increasing its distensibility. Quite probably manoalide is an even more wedge-shaped compound, its hydrophobic chain being joined to a δ-lactol (B) and γ-hydroxybutenolide ring (A), both of which are potentially reactive (Fig. 7.16) (Potts, Faulkner, de Carvalho & Jacobs, 1992). The potent membrane perturbing action of manoalide here demonstrated probably accounts for its high toxicity on cell cultures mentioned above. It may also account for the inhibition of phosphoinositide specific phospholipase C by manoalide (Bennett, Mong, Wu, Clark, Wheeler & Crooke, 1987), as this membrane associated enzyme is inhibited also by cytotoxic ether-lipid analogues (Powis, Seewald, Gratas, Melder, Riebow & Modest, 1992), another class of compounds with a powerful effect on membrane mechanical properties.

Discussion - PLA_2 Inhibitors

On taking an overall view of the experiments with mepacrine, NDGA, dibucaine and Ro 31-4493 a common pattern can be recognized. By themselves, all these amphiphilic drugs cause a shift in the cumulative tension curve to the left i.e. a reduction in membrane tensile strength. They also cause an increase in distensibility i.e. decrease in K, and an increase in the fractional membrane area increase, ΔA^*, vesicles could sustain (Table 7.1). All these changes and the tendency for progressive area growth on maintained tension, are presumably mechanical expressions of the membrane perturbation and expansion arising from the intercalation of amphiphilic compounds. As regards any modulation of the effect of elevated $[Ca^{2+}]_i$, pre-treatment of vesicles with the above agents resulted in all cases in a greater or lesser, but always clearly recognizable, decrease in the proportion of extremely weak vesicles, i.e. those bursting immediately on aspiration, yet with only a marginal restoration in the strength of those vesicles which could be aspirated. This is a curious result as on first consideration it might be thought that the joint effect of Ca^{2+} and of treatment with an amphiphilic agent would be a greater weakening of vesicles than is produced by each interference alone i.e. seeing that the cumulative curve for vesicles treated with any one of these amphiphilic drugs lies to the left of the control curve, one would expect the cumulative curve for $[Ca^{2+}]_o$ plus amphiphilic drug to lie to the left of the curve applying to vesicles treated with $[Ca^{2+}]_o$ alone, and not to the right of that curve as was in practice found. (Figs. 7.01, 7.05, 7.06, 7.08 & 7.14).

One possible explanation is that the amphiphilic drugs tested possess a true protective action against the effect of elevated Ca^{2+}, but that this effect is masked by their own direct perturbative action on the membrane. On this view, the leftward shift in the cumulative distribution curve produced by the drug alone would need to be subtracted from the cumulative lysis tension distribution curve for vesicles treated with $[Ca^{2+}]_o$ and ionophore plus drug in order to expose the effect of the drug on the action of $[Ca^{2+}]_o$ and ionophore on the membrane. Applying such a procedure to the results obtained, for

instance with dibucaine, yields a curve that overlaps with the control curve. So if such subtraction is valid, it could be argued that dibucaine has fully protected the membrane against elevated $[Ca^{2+}]_i$, the remaining weakening being due to unavoidable effect of dibucaine itself. And similar arguments could also be put forward for mepacrine and in smaller measure for NDGA and Ro 31-4493 in the concentrations found usable.

The attractions for arguing as above is that one arrives at a conclusion that is consistent with expectations based on the work of Jackson *et al.*, (1984, 1987) who, viewing the above drugs as PLA_2 inhibitors, used them to test the hypothesis that Ca^{2+}-induced muscle damage – as measured by the release of cytosolic enzymes – is mediated by activation of PLA_2. But the manipulations involved in arriving at such a satisfactory conclusion have to be viewed with scepticism. It seemed advisable therefore, also to consider an alternative interpretation for the pattern of results obtained.

The experiments with manoalide here come to mind. Manoalide was by far the most powerful agent to weaken the membrane and when treatment with it was superimposed on treatment with $[Ca^{2+}]_o$ and ionophore the population of vesicles was greatly decreased through autolysis. Also after the addition of mepacrine to vesicles exposed to 10 μM $[Ca^{2+}]_o$ plus ionophore, so as to yield 2 mM mepacrine, the impression was gained that the number of vesicles in the dish, especially vesicles large enough for pipette aspiration was lower than before mepacrine was added. If some degree of autolysis generally occurred on addition of $[Ca^{2+}]_o$ and ionophore to vesicles already weakened by amphiphilic drugs, the remaining population might not be strictly comparable to the initial population. Thus if the weakest vesicles were 'weeded out' the cumulative lysis tension distribution based on measurement of the remaining vesicles might conceivably give the false impression that the strength of vesicles had increased.

Table 7.1. Modulus of elasticity, K, and fractional increase in membrane area at lysis, ΔA^* , for n vesicles treated with a selection of putative PLA_2 inhibitors as compared with the batch controls in 140 mM KCl

PLA_2 Inhibitor	concentration (μM)	n	ΔA^*	K (mNm^{-1})
			mean ± S.D.	mean ± S.D.
Mepacrine	2000	41	0.0193 ± 0.0071	245 ± 68
Controls	0	40	0.0218 ± 0.0078	484 ± 73
NDGA	20	22	0.0326 ± 0.0126	280 ± 51
Controls	0	20	0.0229 ± 0.0093	506 ± 98
Dibucaine	500	55	0.0366 ± 0.0173	229 ± 37
	200	43	0.0299 ± 0.0143	307 ± 50
Controls	0	48	0.0264 ± 0.0092	530 ± 62
Ro31-4493	12.5	18	0.0316 ± 0.0042	385 ± 51
Controls	0	18	0.0328 ± 0.0046	455 ± 52
Manoalide	1.25	14	0.0184 ± 0.0040	251 ± 35
	0.31	7	0.0129 ± 0.0025	423 ± 33
Controls	0	28	0.0253 ± 0.0081	546 ± 93

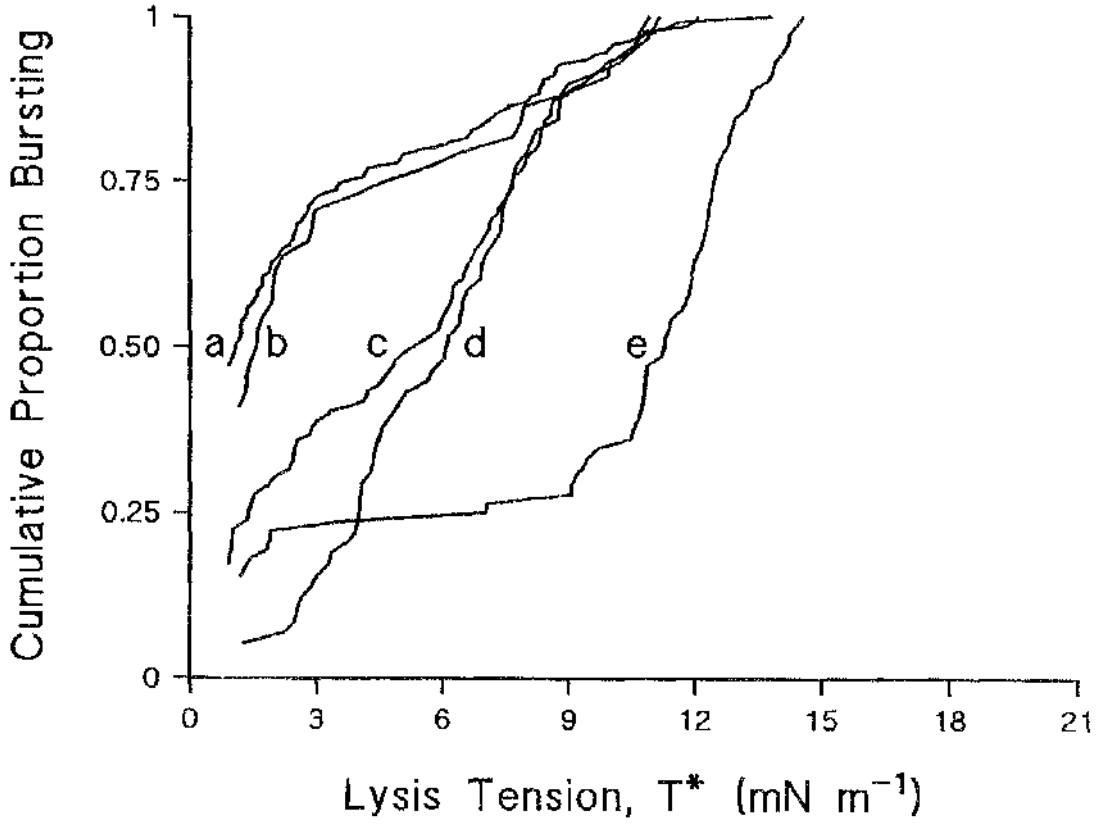

Figure 7.01 Pretreating vesicles with mepacrine reduces Ca^{2+}-induced weakening

Cumulative curves of lysis tension for vesicles in 140 mM KCl: a), loaded in 10 μM Ca^{2+} with 5 μM A23187 (n = 239); b), c) and d) as for vesicles loaded in a) with in addition 200 μM (n = 44), 1 mM (n = 134) and 2 mM (n = 58) mepacrine respectively. e), untreated controls (n = 72).

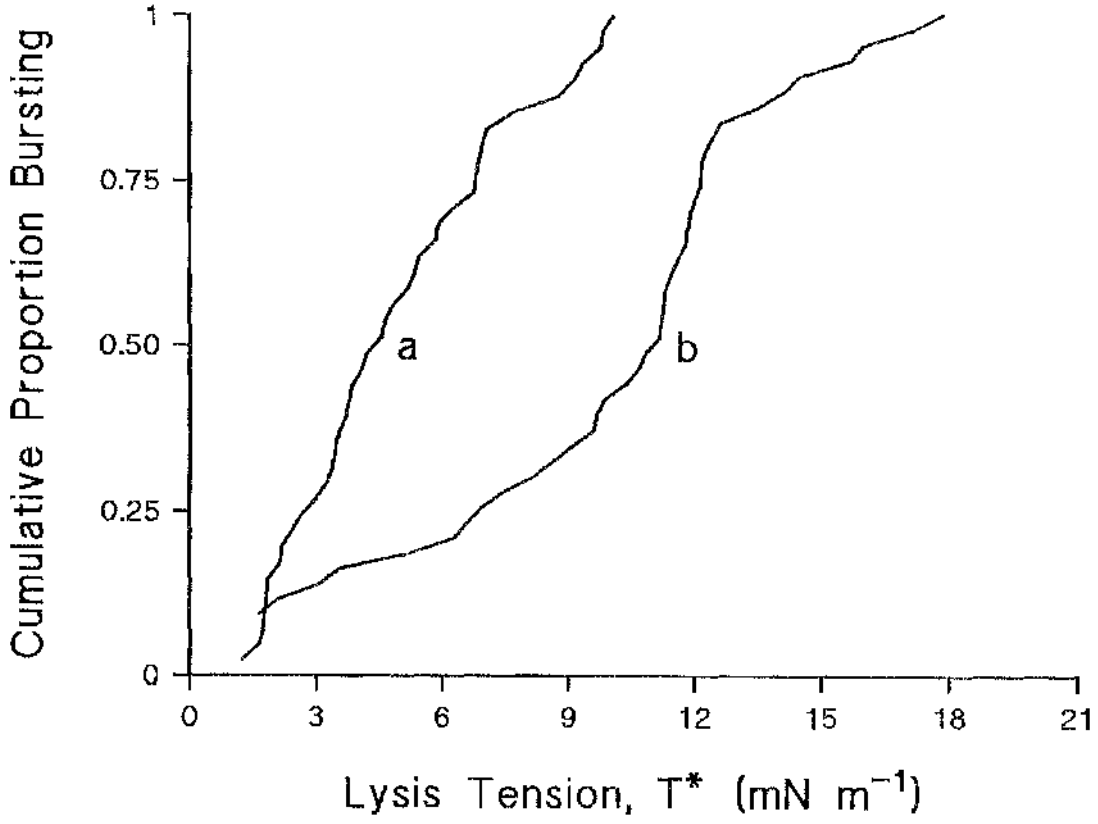

Figure 7.02 Effect of 2 mM mepacrine on membrane strength

Cumulative curves of lysis tension for: a), vesicles exposed to 2 mM mepacrine (n = 41); b), untreated controls in 140 mM KCl (n = 43).

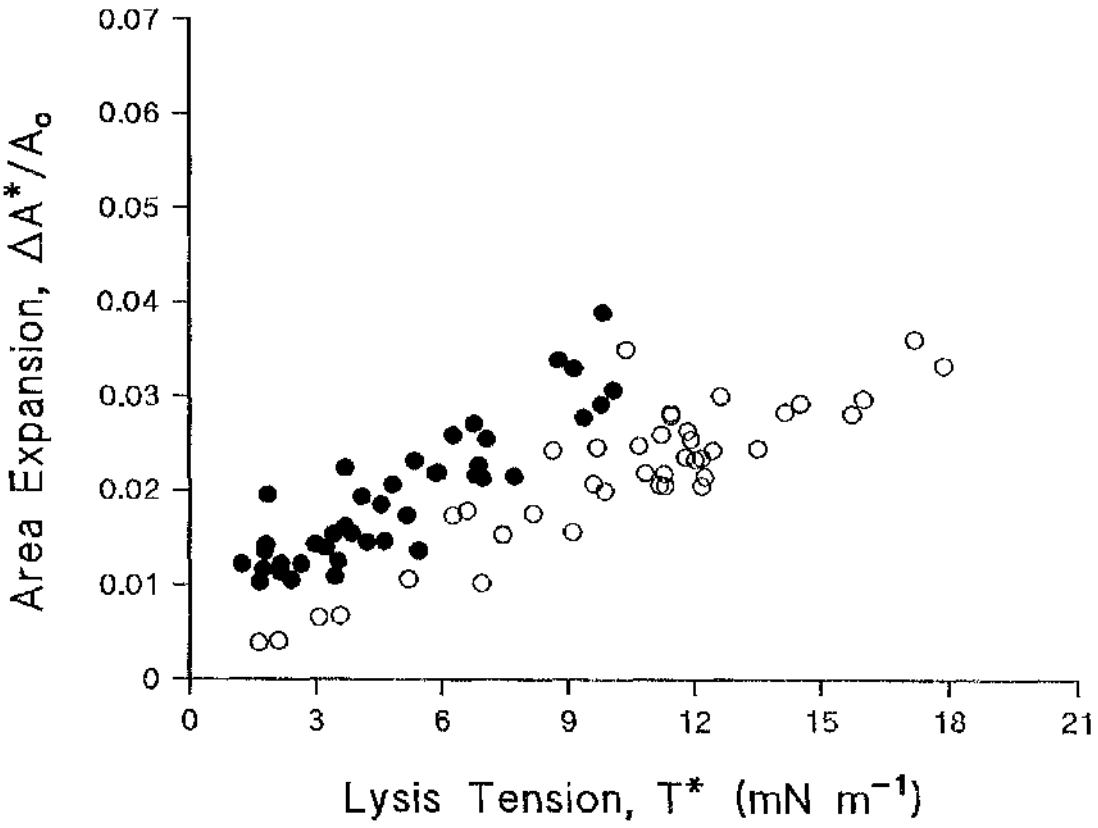

Figure 7.03 Lysis points for vesicles treated with 2 mM MPC

●, lysis tension and corresponding area expansion for vesicles exposed to 2 mM mepacrine (n = 41). ○, untreated vesicles in 140 mM KCl (n = 40).

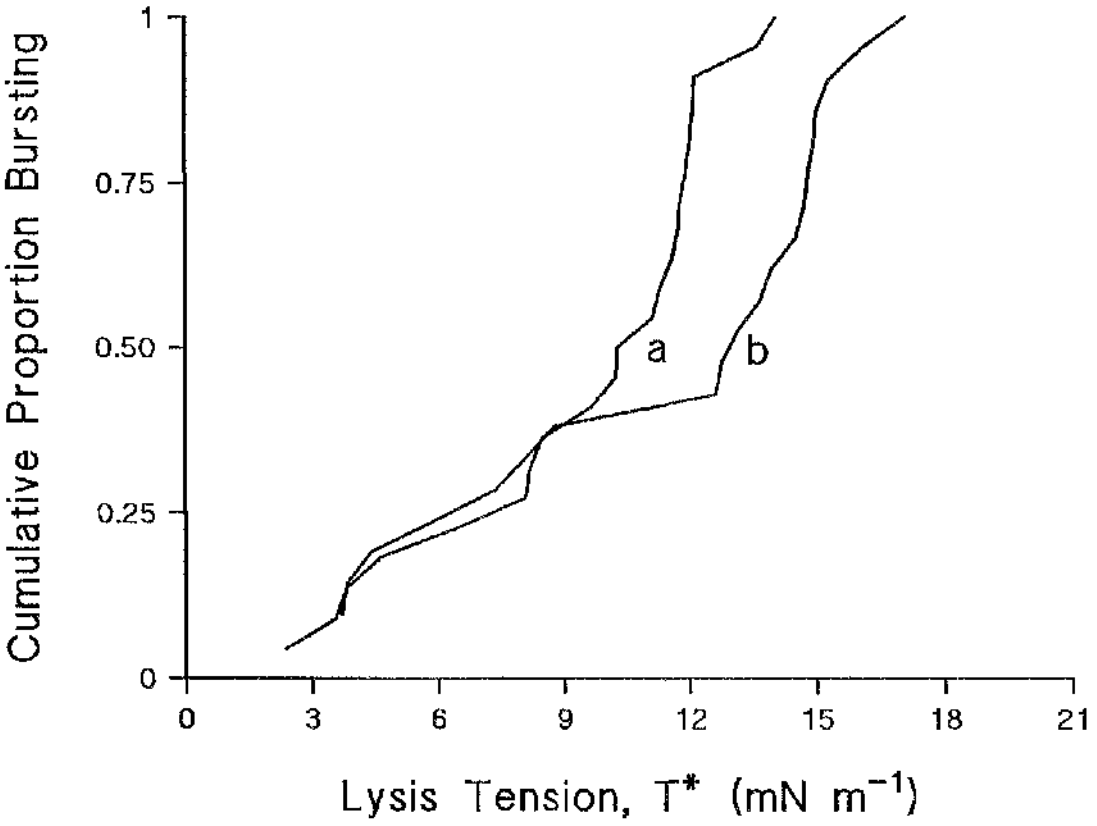

Figure 7.04 Effect of 20 μM NDGA on membrane strength

Cumulative curves of lysis tension for: a), vesicles exposed to 20 μM NDGA (n = 22); b), untreated controls in 140 mM KCl (n = 20).

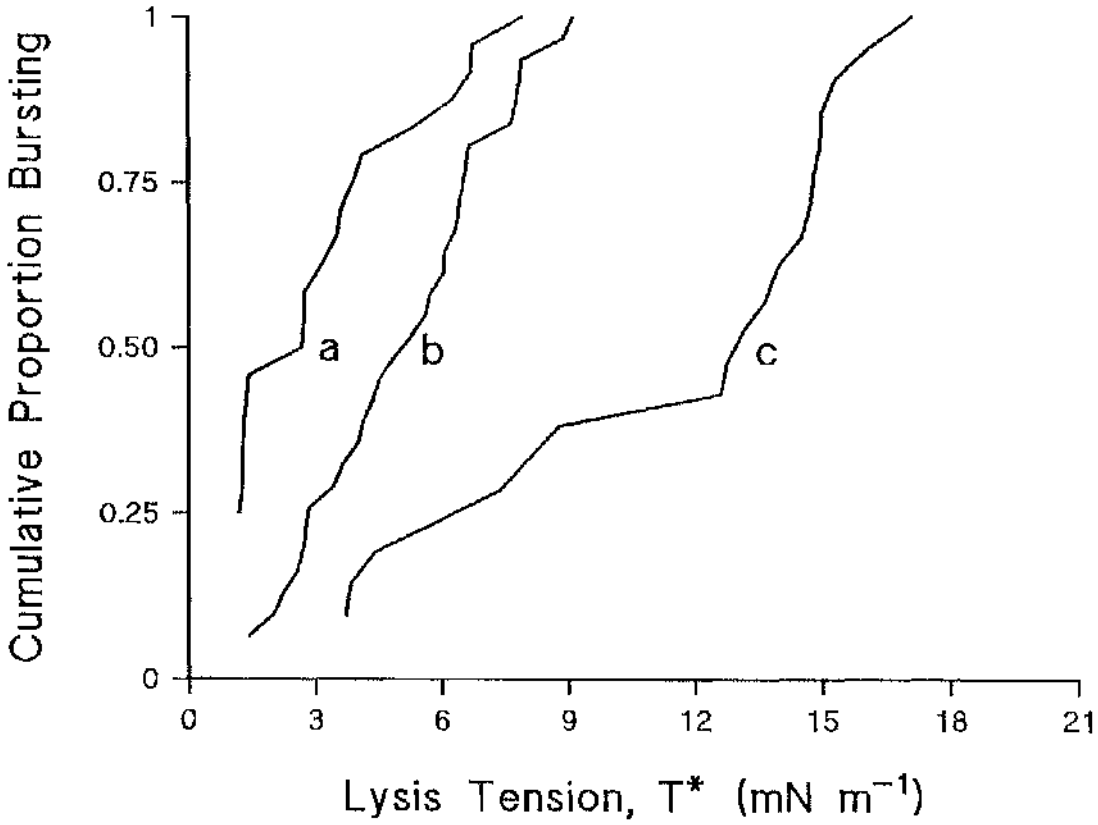

Figure 7.05 Protective effect of 20 μM NDGA on the tensile strength of vesicles loaded with Ca^{2+}

Cumulative curves of lysis tension for: a), vesicles loaded in 2 μM Ca^{2+} and 2.5 μM A23187 (n = 24); b), vesicles pretreated with 20 μM NDGA and then loaded as in a) (n = 31); c), companion controls in 140 mM KCl (n = 20).

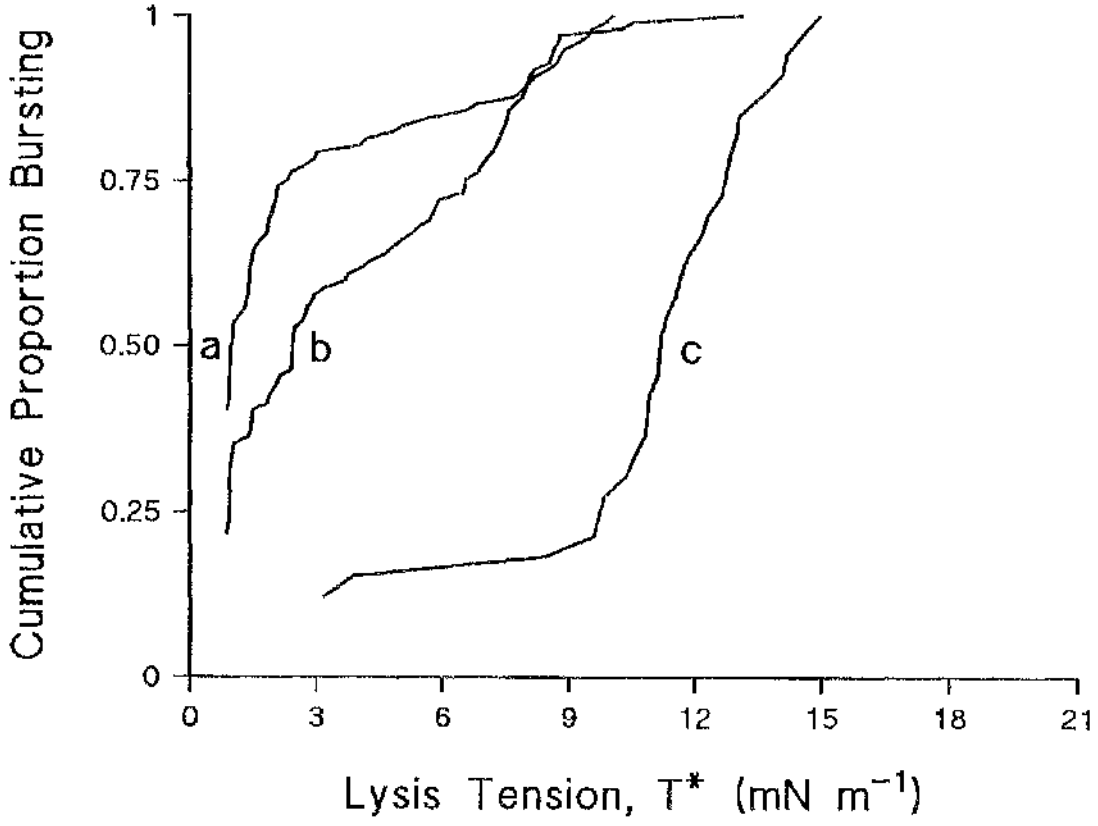

Figure 7.06 Effect of 200 μM dibucaine on the membrane strength of vesicles loaded with Ca^{2+}

Cumulative curves of lysis tension for: a), vesicles loaded in 10 μM Ca^{2+} and 5 μM A23187 (n = 97); b), vesicles pretreated with 200 μM DBC and then loaded as in a) (n = 97); c), companion controls in 140 mM KCl (n = 33).

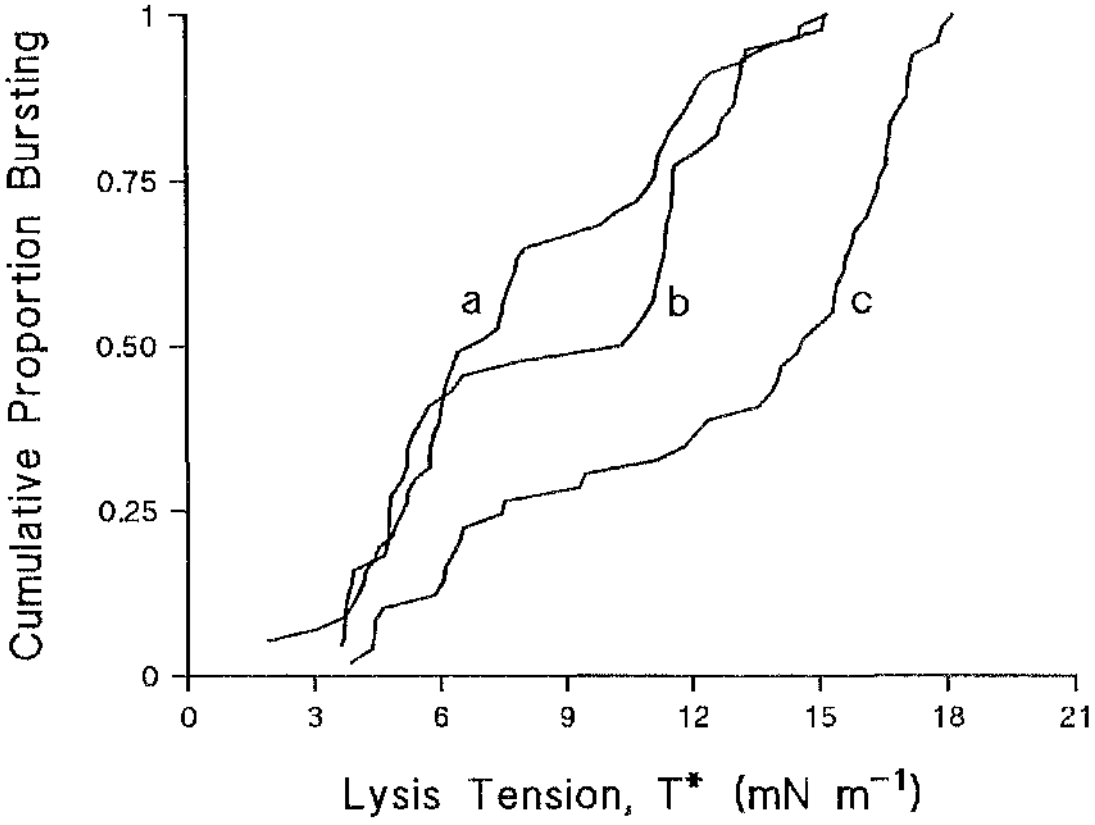

Figure 7.07 Effect of dibucaine on membrane strength

Cumulative curves of lysis tension for vesicles in 140 mM KCl exposed to a), 500 μM DBC (n = 57) and b), 200 μM DBC (n = 44). c), untreated controls (n = 49).

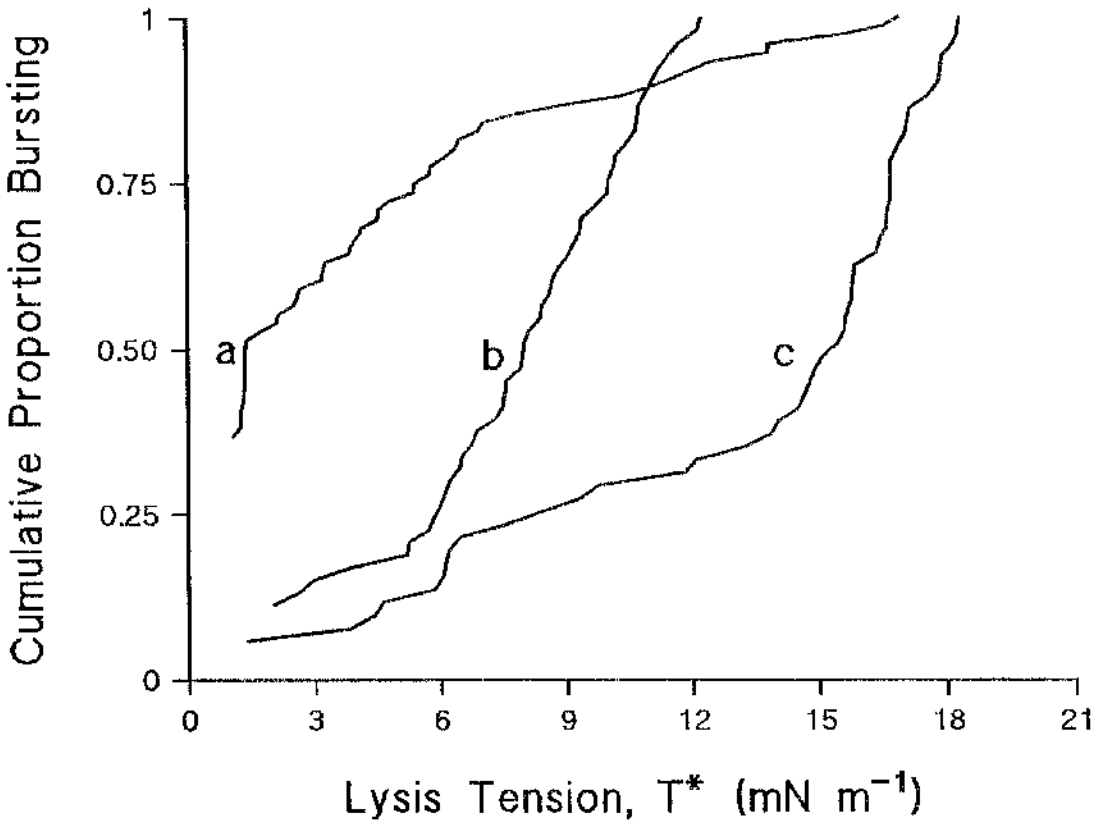

Figure 7.08 Effect of 500 μM dibucaine on the membrane strength of vesicles loaded with Ca^{2+}

Cumulative curves of lysis tension for: a), vesicles loaded in 10 μM Ca^{2+} and 5 μM A23187 (n = 76); b), vesicles pretreated with 500 μM DBC and then loaded as in a) (n = 53); c), companion controls in 140 mM KCl (n = 51).

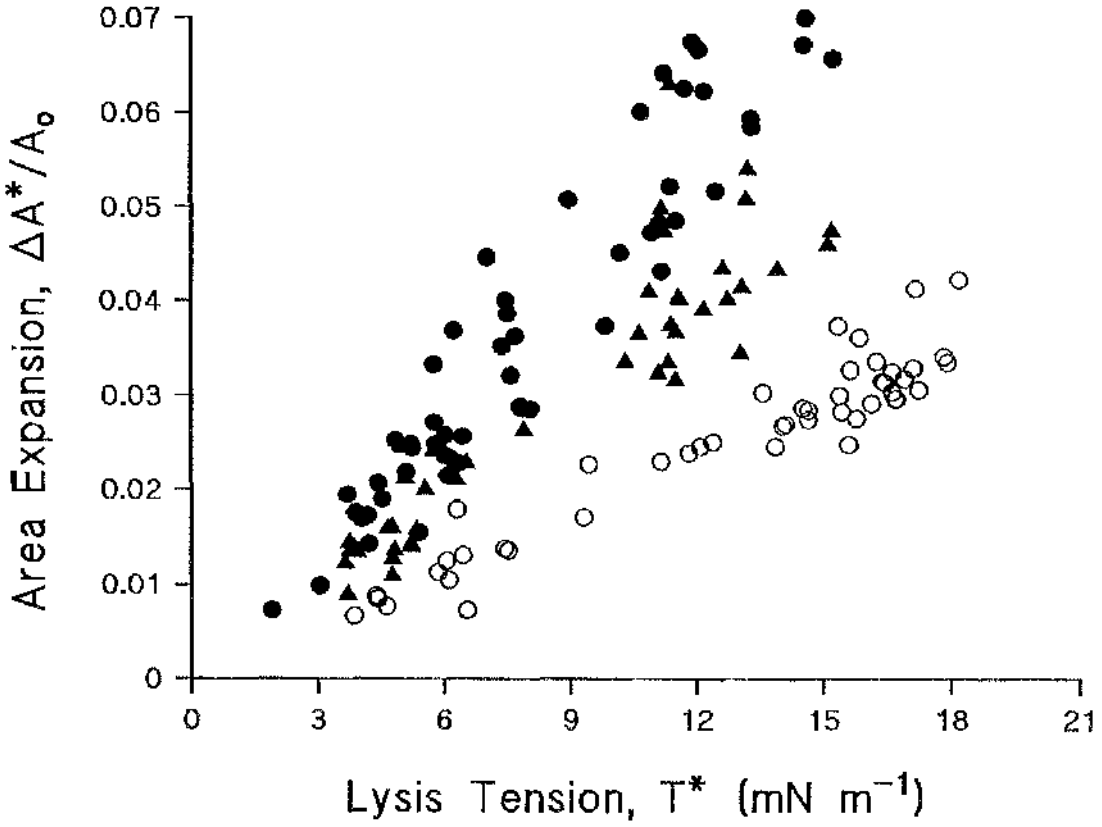

Figure 7.09 Lysis points for vesicles treated with dibucaine

Lysis tension and corresponding area expansion for vesicles bathed in solutions containing ●, 500 μM (n = 55) and ▲, 200 μM (n = 43) DBC. ○, untreated controls in 140 mM KCl (n = 48).

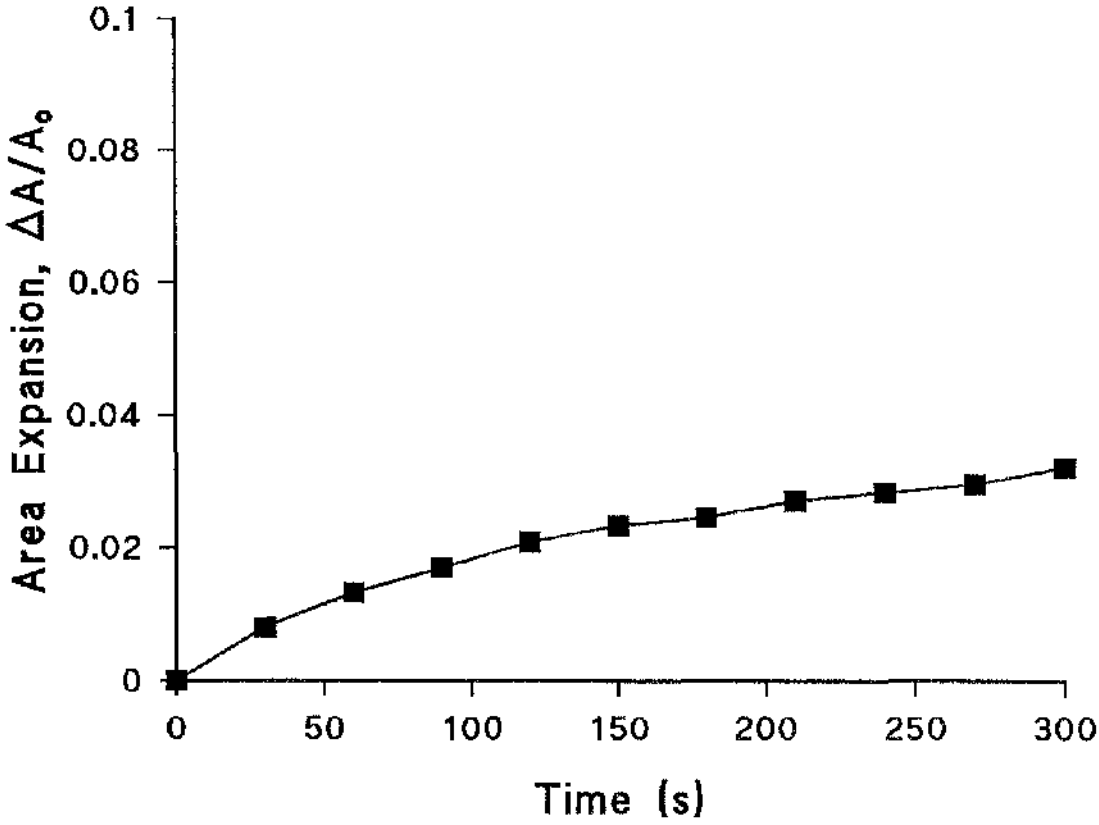

Figure 7.10 Intercalation of dibucaine into a vesicle held at a constant low sub-lytic tension

Fractional increase in membrane area is plotted against time for a vesicle aspirated with a constant suction pressure of 6 cm H_2O in a solution containing 500 μM DBC. Pipette and vesicle dimensions were such that the constant isotropic stress applied to the vesicle was 3.57 mN m^{-1}.

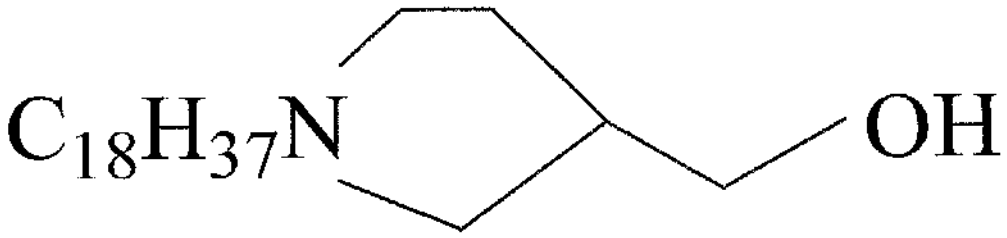

Figure 7.11 Chemical structure of Ro 31-4493

Using molecular graphics, the polar headgroup of Ro 31-4493 was designed to interact specifically with the polar aminoacid side chains contained within the active site of bovine PLA_2

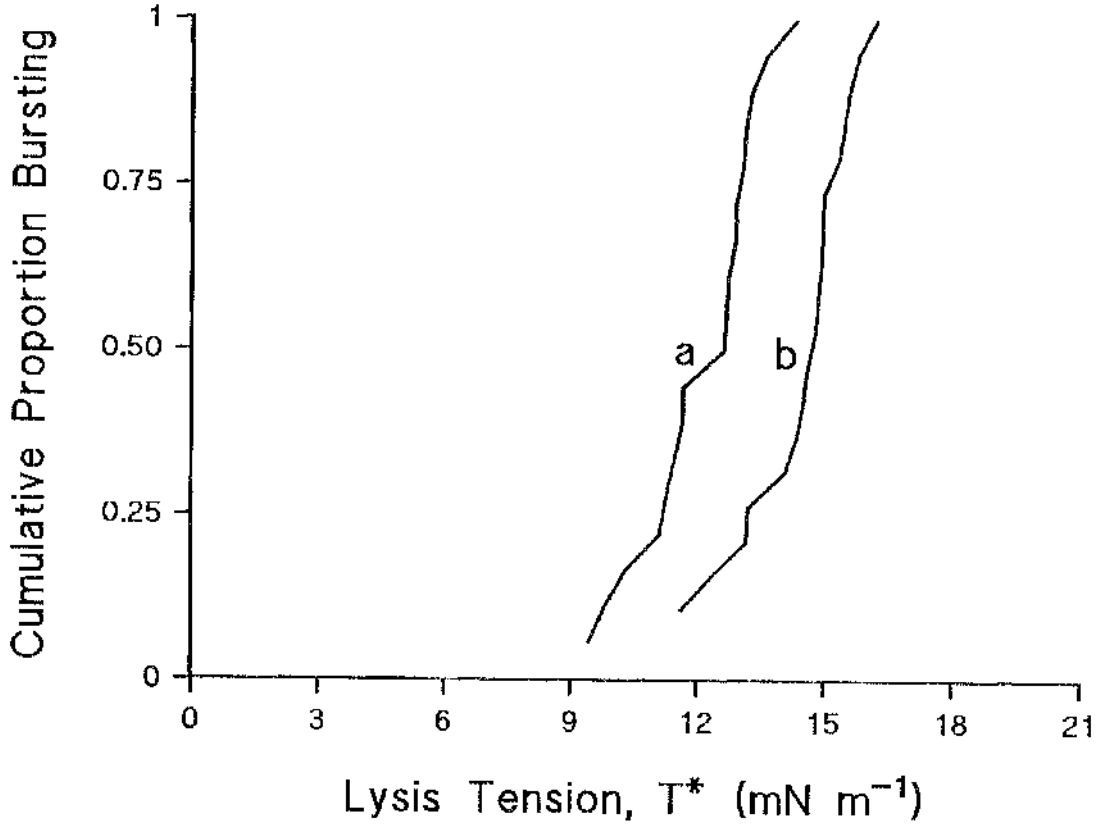

Figure 7.12 Effect of 12.5 μM Ro 31–4493 on membrane strength
Cumulative curves of lysis tension for vesicles in 140 mM KCl exposed to: a), 12.5 μM Ro 31–4493 (n = 18); b), untreated controls (n = 19).

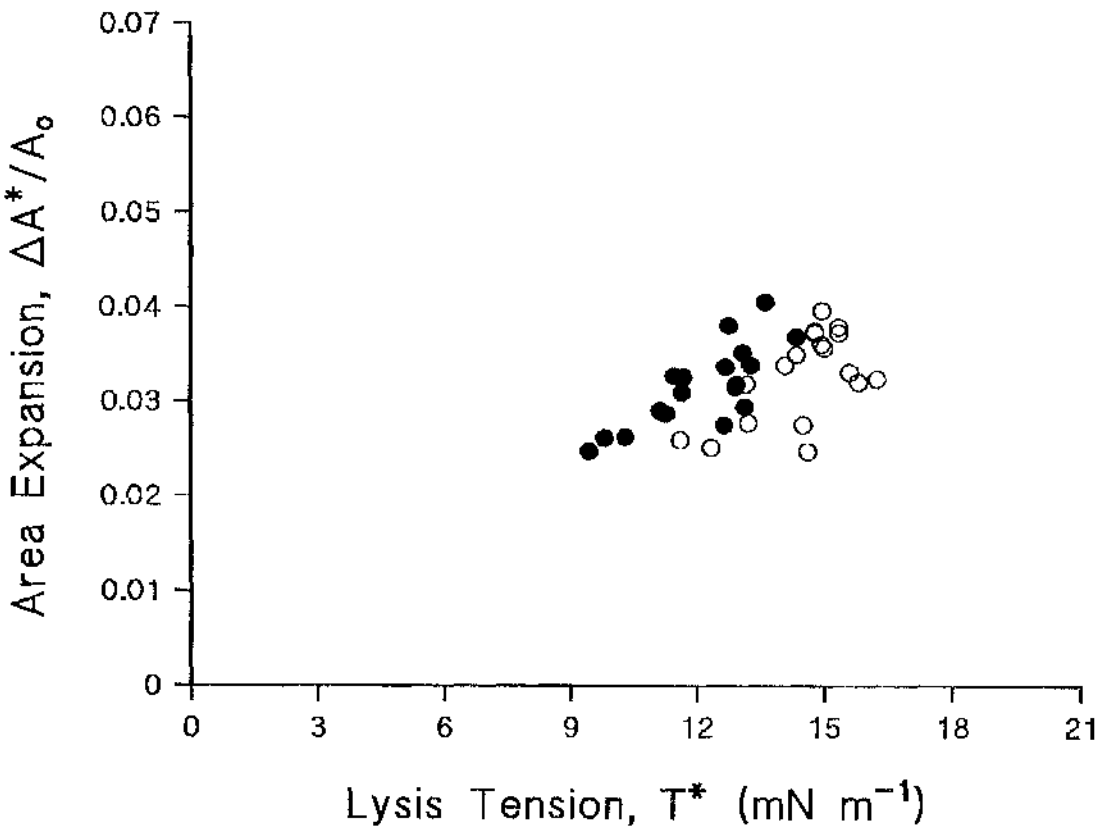

Figure 7.13 Lysis points for vesicles treated with 12.5 μM Ro 31–4493
●, lysis tension and corresponding area expansion for vesicles exposed to 12.5 μM Ro 31–4493 (n = 18). ○, untreated vesicles in 140 mM KCl (n = 18).

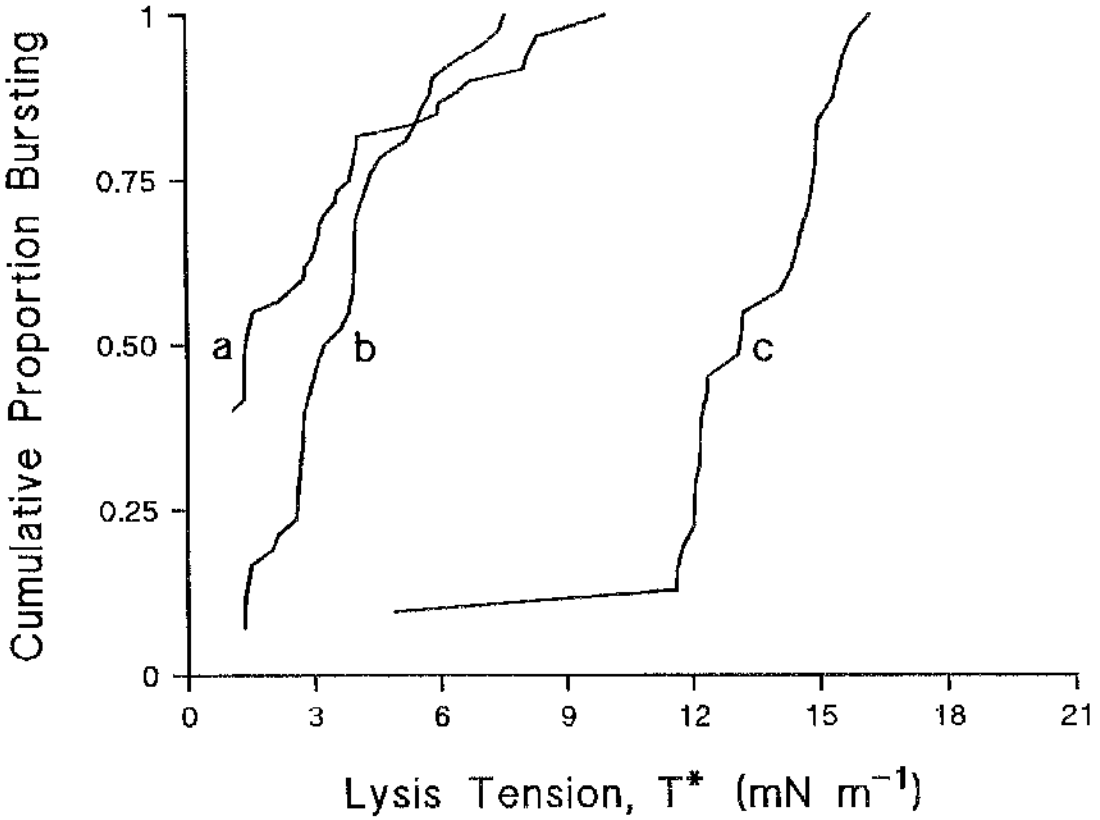

Figure 7.14 Effect of 12.5 μM Ro 31–4493 on membrane strength of vesicles loaded with Ca^{2+}

Cumulative curves of lysis tension for: a), vesicles loaded in 10 μM Ca^{2+} and 2.5 μM A23187 (n = 50); b), vesicles pretreated with 12.5 μM Ro 31–4493 and then loaded as in a) (n = 42); c), companion controls in 140 mM KCl (n = 31).

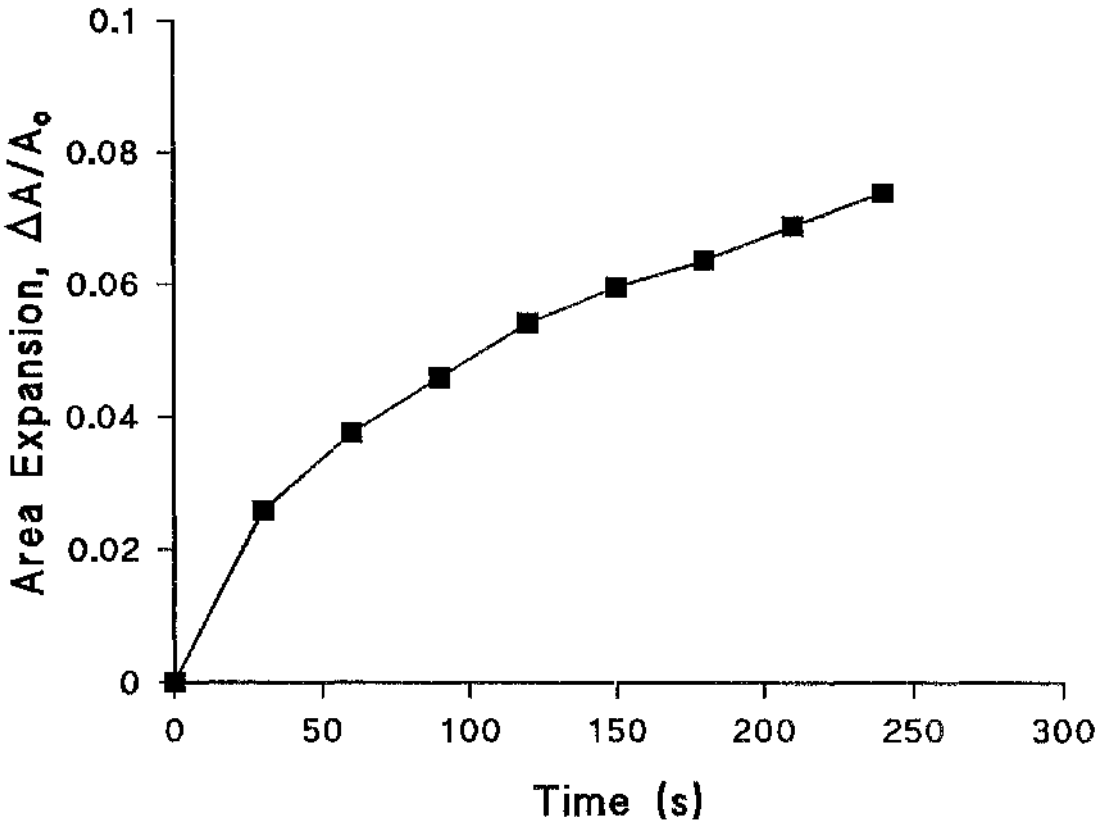

Figure 7.15 Intercalation of Ro 31–4493 into a vesicle held at a constant low tension

Fractional increase in membrane area is plotted against time for a vesicle aspirated with a constant suction pressure of 2 cm H_2O in a solution containing 25 μM Ro 31–4493. Pipette and vesicle dimensions were such that the constant isotropic stress applied to the vesicle was 1.38 mN m^{-1}.

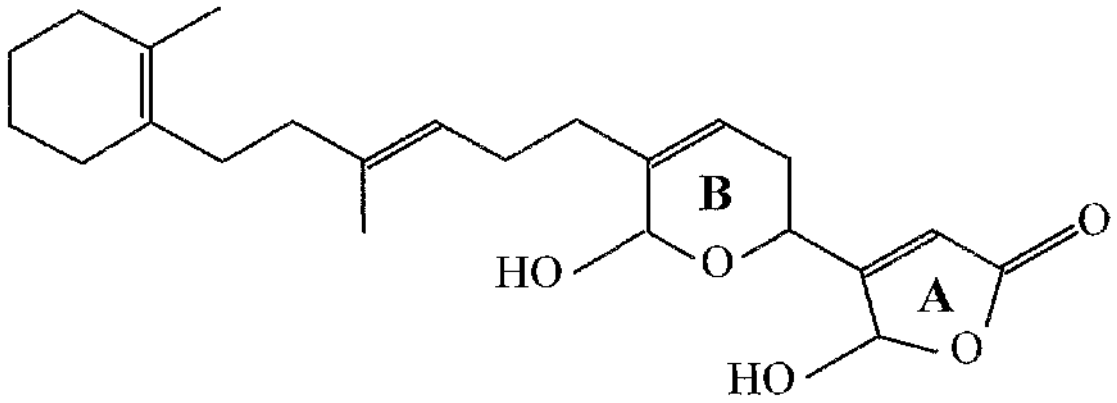

Figure 7.16 Chemical structure of manoalide

Manoalide, a natural sesterterpene, consists of a hydrophobic chain containing two reactive rings, A (γ–hydroxybutenolide) and B (δ–lactol), which together produce irreversible inactivation of bee venom PLA_2.

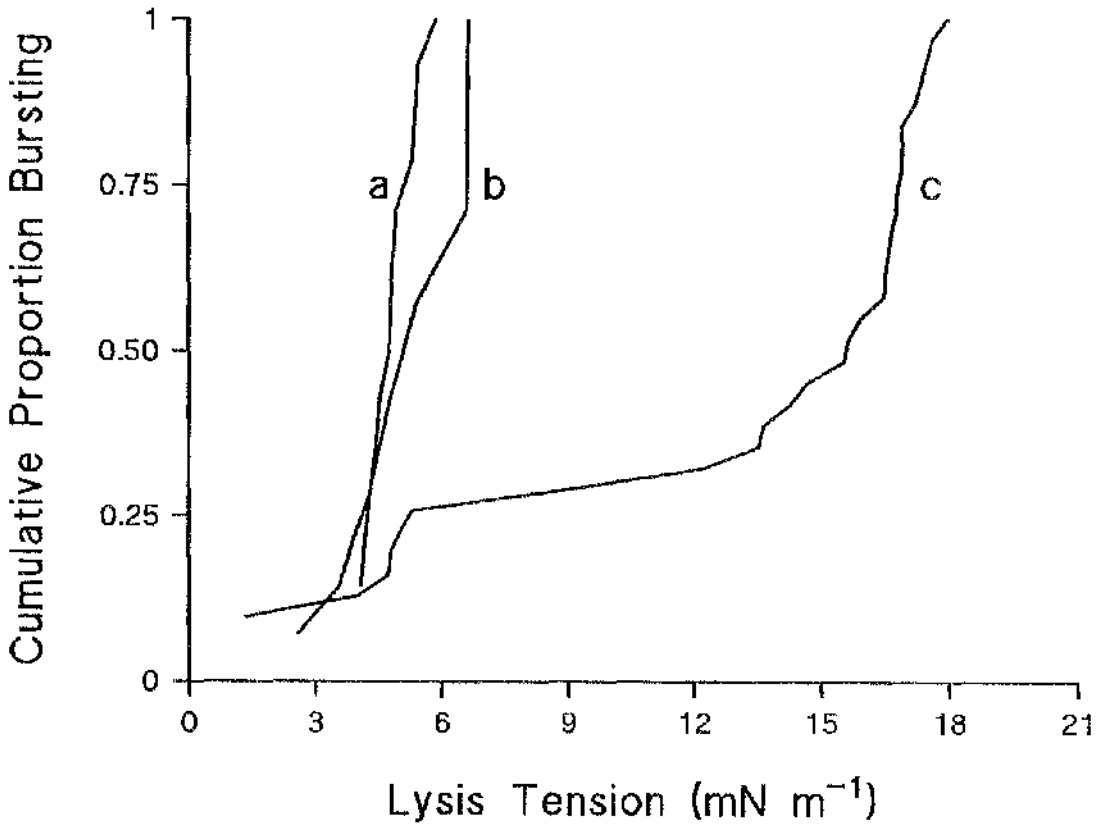

Figure 7.17 Effect of manoalide on membrane strength

Cumulative curves of lysis tension for vesicles treated with: a), 1.25 μM (n = 14) and b), 0.31 μM manoalide (n = 7); c), untreated controls in 140 mM KCl (n = 31).

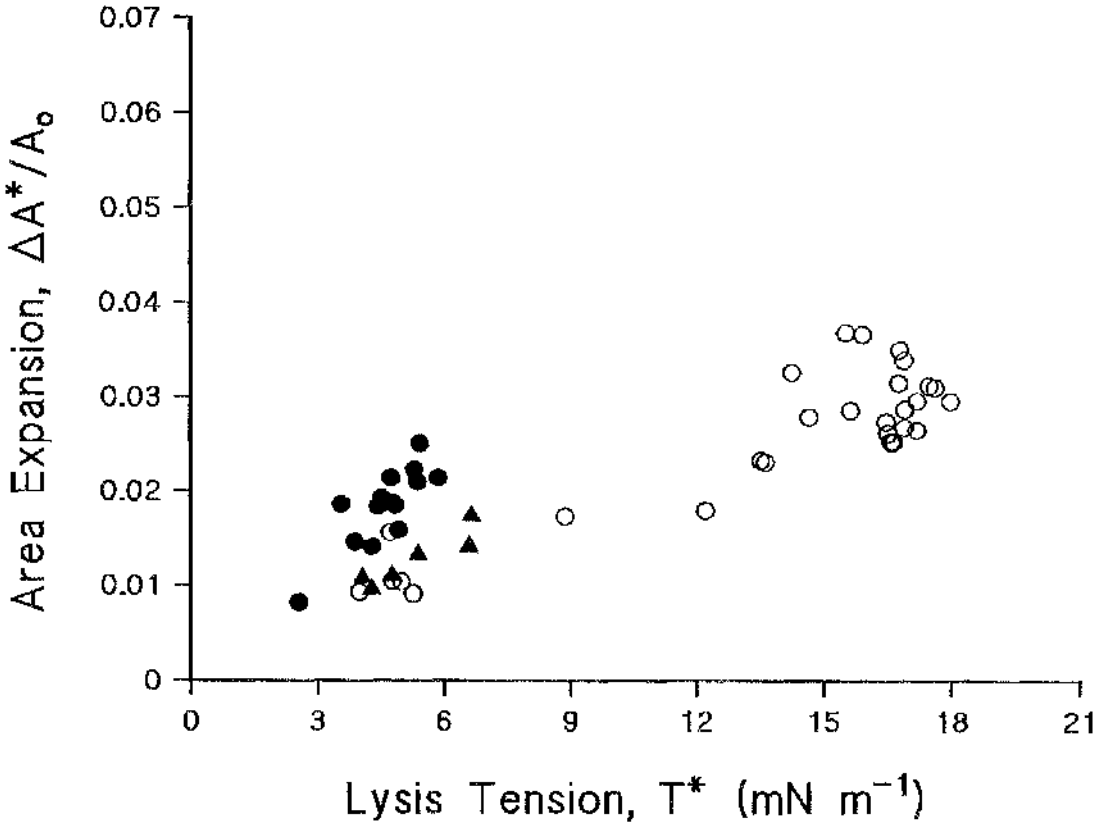

Figure 7.18 Lysis points for vesicles treated with manoalide

Lysis tension and corresponding area expansion for vesicles bathed in solutions containing ●, 1.25 μM (n = 14) and ▲, 0.31 μM (n = 7) manoalide.
○, untreated controls in 140 mM KCl (n = 28).

PART VIII

IS CALPAIN I INVOLVED IN CA^{2+} MEDIATED LYSIS OF SARCOLEMMAL VESICLES?

The best known and most readily available inhibitor of calpain is leupeptin, a tripeptide aldehyde of microbiological source (Fig. 8.01). Leupeptin has been claimed to inhibit intracellular proteolysis in hepatocytes with raised $[Ca^{2+}]_i$ (Nicotera *et al.*, 1986) and of cytoskeletal proteins in platelets under similar conditions (Fox, Austin, Boyles, & Steffen, 1990). However, in other experiments on platelets and red blood cells leupeptin failed to prevent Ca-induced proteolysis and this has been ascribed to its inability to penetrate the membrane, owing to the charged arginine group it contains (Tsujinaka, Kajiwara, Kambayashi, Sakon, Higuchi, Tanaka & Mori, 1988; Medhi, Angelastro, Wiseman & Bey, 1988). To overcome this problem calpain inhibitors composed entirely of nonpolar amino-acids and groupings have been synthesised which are claimed to be membrane permeant. One such compound is MDL 28,170 (Fig. 8.02). Like leupeptin it has a terminal aldehyde group, but this is on an uncharged phenylanaline rather than an arginine residue. Valine replaces the middle leucine and the molecule is terminated by a carbobenzoxy group. According to information kindly supplied by Dr. Bohme, MDL 28,170 in concentrations of 5, 10, 30 μM inhibited A23187 haemolysis in whole red blood by 58, 93, and 91% respectively.

In the first instance, the effect of MDL 28,170 by itself on membrane mechanical properties was tested over a concentration range of 3.5 to 26.0 μM. If anything, it was found to reduce slightly the tensile strength and increase the distensibility of vesicles. But the effect varied from experiment to experiment and showed an irregular dose-dependence. In one experiment in which pre-treatment with 26 μM MDL 28,170 was combined with subsequent exposure of vesicles to 5 μM Ca^{2+} in the presence of 5 μM A23187, the pre-treated vesicles lysed to a much greater extent than did those treated

with Ca^{2+} and ionophore alone. That non-polar peptides which enter the membrane, but do not match the hydrophobic length of the lipid chains, may reduce membrane tensile strength and increase membrane distensibility has already been noted by Evans & Needham (1987).

In the most critically conducted experiments, the effect of 4 or 5 μM $[Ca^{2+}]_o$ in the presence of 1 μM A23187 on vesicles pre-treated with 6.5 μM MDL 28,170 was compared with the effect of the same $[Ca^{2+}]_o$ and ionophore on otherwise untreated vesicles. Under these conditions, no protective effect of MDL 28,170 against the effect of elevated $[Ca^{2+}]_i$ was observable. Whilst a negative result always remains open to questions, the indication is that activation of calpain is not involved in the lytic action of elevated $[Ca^{2+}]_i$.

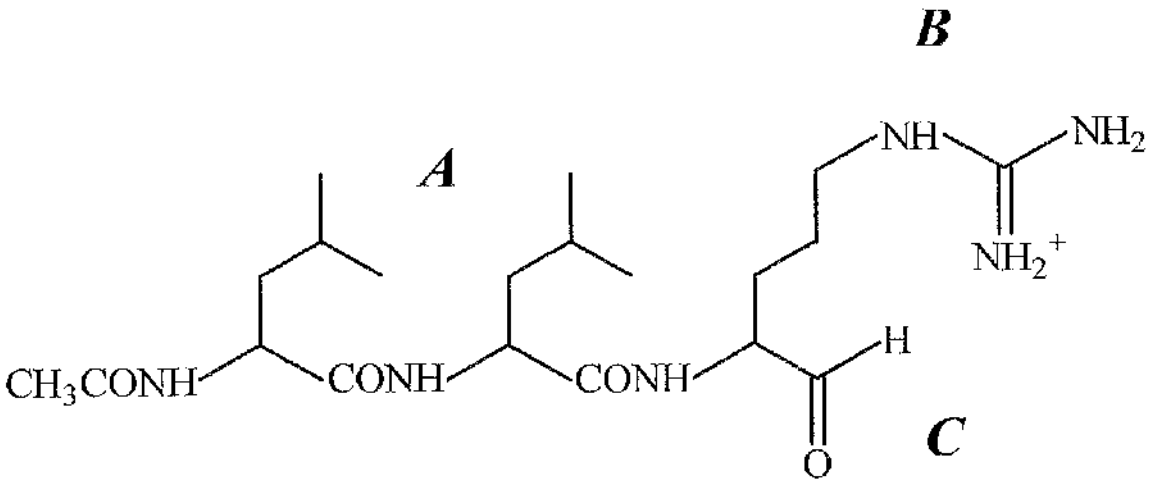

Figure 8.01 Chemical structure of the calpain I inhibitor leupeptin
Leupeptin is a naturally occurring tripeptide aldehyde consisting of 2 leucine (A), arginine (B) and terminal aldehyde (C) groups.

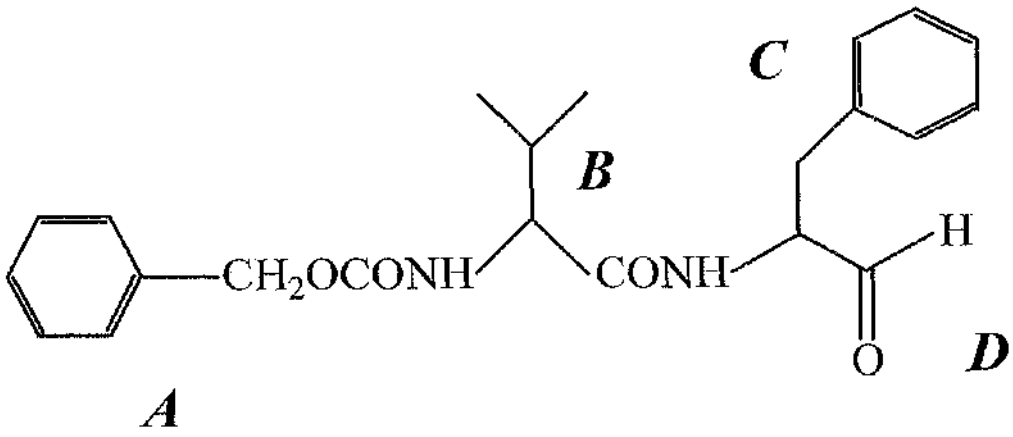

Figure 8.02 Chemical structure of MDL 28,170

MDL 28,170 is a synthetic, uncharged and hence membrane permeable inhibitor of calpain I. The molecule consists of 4 groups; carbobenzoxy (A), valine (B), phenylalanine (C) and aldehyde (D).

PART IX

EXPERIMENTS WITH α-TOXIN

α-toxin from *staphylococcus aureus* has long been known to permeabilize red blood cells and so to lead to their lysis (Bernheimer, 1968). More recently α-toxin has been used to permeabilize also smooth muscle and skeletal muscle fibres (Kitazawa, Kobayashi, Horiuti, Somlyo & Somlyo, 1989; Crichton & Smith, 1990; Török, Patel & Ferenczi, 1991). α-toxin is a monomeric protein of 34 kDa which binds to cell membranes and then undergoes self-association on and in the membrane so as to form an oligomeric annular complex (Füssle, Bhakdi, Sziegoleit, Tranum-Jensen, Kranz, & Wellensiek, 1981). The complex harbours a membrane spanning central pore of 2-3 nm diameter which allows diffusion of molecules up to ~3000 Da, but retains macromolecules within the cell.

The effect of α-toxin on membrane mechanical properties has not been hitherto investigated. The present opportunity was therefore taken to study the effect of α-toxin on sarcolemmal vesicles with the aim, first, to exemplify the mechanical consequences of protein-membrane interaction. Secondly, it was hoped that permeabilization of sarcolemmal vesicles with α-toxin would allow intervesicular Ca^{2+} to be raised without recourse to ionophores.

The α-toxin used was kindly made available by Dr. G.L. Smith of this Department. It was crude α-lysin from staphylococcus aureus (strain Wood 46) and was originally supplied by the Department of Microbiology, Glasgow University in November 1989 as batch 180484 with a haemolytic titre against 1% rabbit erythrocyte of 10,000 H.U. ml^{-1}. Aliquots of this material had been deep frozen on receipt and so stored for 4 years. When reassayed before present use the potency of the batch had dropped to 3200 H.U. ml^{-1}.

As the kinetics of α-toxin incorporation into sarcolemmal vesicles remain unknown, vesicles were exposed to the toxin for at least 15 min before measurements were begun. This seemed a sufficiently long incubation period, as judged from the absence of progressive change in mechanical properties during the subsequent course of the experiments. At a concentration between 3-16 H.U. ml^{-1}, α-toxin caused a graded decrease in the tensile strength of sarcolemmal vesicles. The pooled results from 6 batches of vesicles are summarized in the cumulative lysis tension plots of Fig. 9.01 and the median values are given in Table 9.1*A*. Above 50 H.U. ml^{-1} autolysis supervened as evident from a decrease in the number of vesicles in the dishes, especially of the larger vesicles suitable for pipette aspiration.

α-toxin in above concentration had little effect on the distensibility of vesicles. Such small differences in K as emerged on analysis (Table 9.1*A*) were irregular as regards the concentration of α-toxin used and so small that one remains unsure of their meaning. At all events, compared with the large dose-dependent decrease in K observed with amphiphilic compound e.g. lysolipids, manoalide, Ro 31-4493 etc (cf Figs. 6.03, 6.05, 6.09, 7.13 & 7.18) any effect of α-toxin on membrane distensibility was trivial (Fig. 9.02) In making this comparison, the low molar concentration in which α-toxin is effective needs to be borne in mind. Thus 20 H.U. ml^{-1} is equivalent for the sample of toxin used to 0.18 μg ml^{-1}. Assuming the molecular weight of 3400 and an unrealistic 100% purity this is equivalent to at most 0.006 μM i.e. three orders of magnitude lower than the concentration of amphiphilic compounds studied. When toxin in that concentration is offered to a surplus of erythrocytes less than 5% becomes bound (Bhakdi & Tranum-Jensen, 1985) and the fraction bound by the relatively dilute suspension of vesicles here used is likely to be much less. It seems possible therefore that the weakening of the membrane caused by α-toxin when used in concentrations that can be tolerated by sarcolemmal vesicles is the result of local defects, possibly only a few per vesicle; and even if the lipid membrane is perturbed locally around each defect the modulus of area

elasticity for the whole vesicle, which is a bulk property, may then remain substantially unaltered.

In the above experiments the vesicles were bathed in a solution containing 2 mM NTA with no added calcium, so as to ensure that stray $[Ca^{2+}]_o$ was minimized and that any observed effect of α-toxin was not secondary to an uncontrolled elevation in $[Ca^{2+}]_i$. Having established the direct action of α-toxin on membrane strength, the effect of admixing $CaCl_2$ so as to give 10 μM $[Ca^{2+}]_o$ was next tested. Contrary to expectations based on the supposition that the vesicles had been permeabilized, no further weakening of vesicles was observed. Even 100 μM $[Ca^{2+}]_o$ did not weaken the vesicles further or lead to their autolysis, as would have happened had the vesicles been permeabilized with ionophore. When 1mM $[Ca^{2+}]_o$ was added to vesicles treated with α-toxin a slight increase in strength was observed. These results are summarized in Table 9.1*B* in terms of median tensile strength.

Before interpreting the above results the following additional observations should be noted (i) all the above experiments were done with 16 mM sucrose added to 140 mM KCl so as to render the bathing solution slightly hypertonic and all vesicles lax, as is desirable for pipette aspiration (see methods). Had α-toxin permeabilized the vesicles, sucrose and water would therefore be expected to enter so as to make the vesicles less lax; but this was not observed. (ii) When Trypan Blue (molecular weight 961) was added to vesicles treated with α-toxin as described above, they did not become stained. Taking these observations together with the lack of effect of added $[Ca^{2+}]_o$ on membrane strength, leads one to suspect that α-toxin in the low concentrations which can be tolerated by sarcolemmal vesicles does not permeabilize the membrane. Further considerations presented later (p.145) point in the same direction.

Table 9.1 *A* **Effects of α-toxin on membrane strength and elasticity.**

[α-toxin] (Uml^{-1})	T^* (mNm^{-1})			K (mNm^{-1})	
	n	median	95% C.I.	*n*	mean ± S.D.
0	96	14.8	(13.8, 15.8)	91	557 ± 72
3	52	11.1	(6.4, 12.6)	47	526 ± 97
8	57	4.0	(3.6, 5.1)	45	542 ± 96
16	11	3.3	(1.6, 4.0)	8	640 ± 124

Table 9.1 *B* **Effects of external calcium on on vesicles pretreated with 8 H.U. ml^{-1} α-toxin.**

$[Ca^{2+}]_o$ (μM)	T^* (mNm^{-1})			K (mNm^{-1})	
	n	median	95% C.I.	*n*	mean ± S.D.
0	27	6.5	(4.5, 8.6)	25	518 ± 63
100	26	7.1	(5.0, 7.8)	24	464‡ ± 145
1000	21	7.3†	(6.0, 7.8)	21	503 ± 59

Level of significance in comparison with controls † P=0.54, ‡ P= 0.10

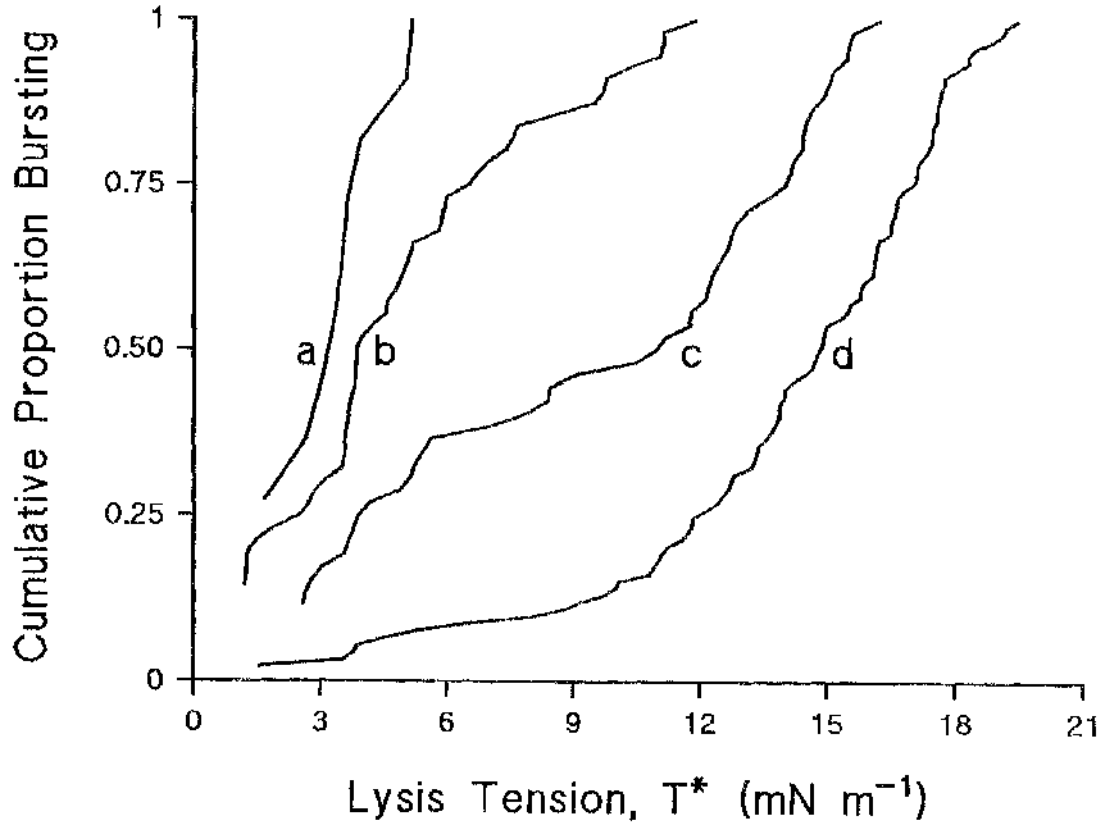

Figure 9.01 Effect of α–toxin on membrane strength

Cumulative curves of lysis tension for vesicles in 140 mM KCl together with: a), 16 H.U. ml^{-1} (n = 11); b), 8 H.U. ml^{-1} (n = 57) and c), 3 H.U. ml^{-1} (n = 52) α–toxin. d), untreated controls (n = 96).

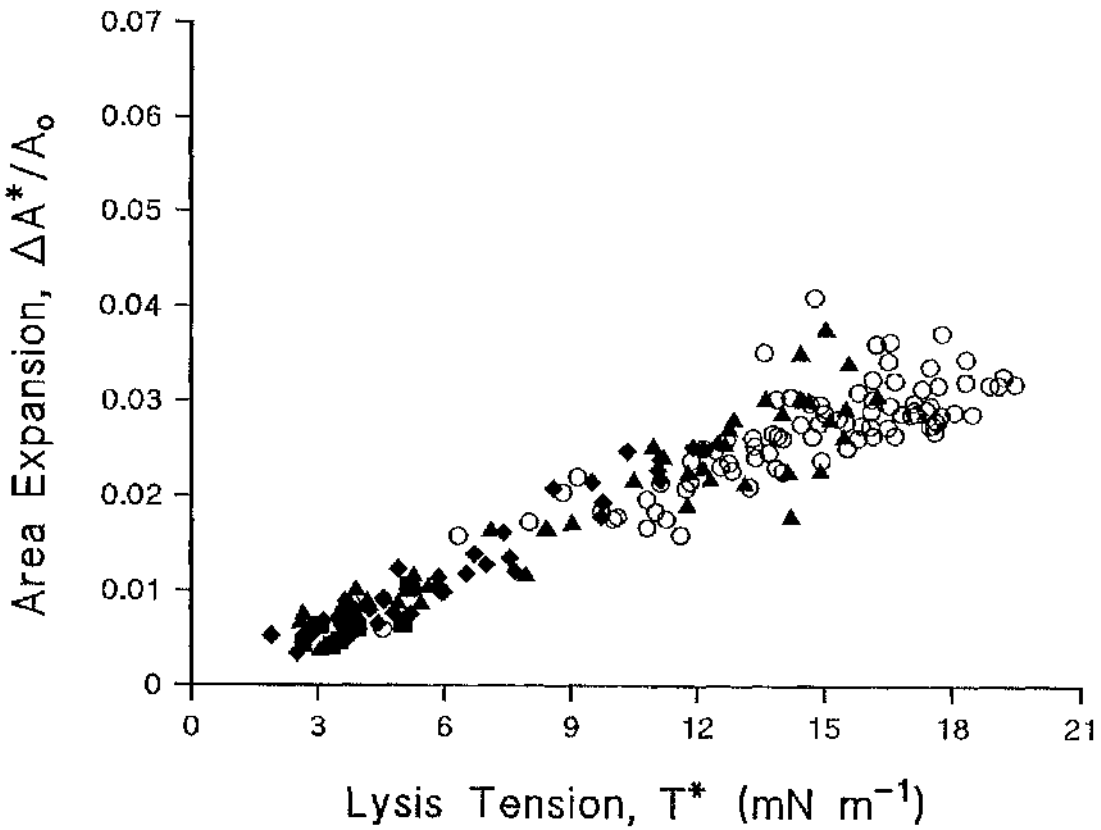

Figure 9.02 Lysis points of vesicles treated with α–toxin

Points show lysis tension and corresponding area expansion for vesicles exposed to α–toxin in concentrations of: ■, 16 H.U. ml^{-1} (n = 8), ◆, 8 H.U. ml^{-1} (n = 45) and ▲, 3 H.U. ml^{-1} (n = 47); ○, companion controls in 140 mM KCl (n = 91).

PART X

CONCLUDING DISCUSSION

The mechanical properties of normal sarcolemmal vesicles and how they compare with the properties of red cell membrane, the only other surface membrane so far studied from a mechanical point of view, have been discussed in Part III (p. 34), and the material subsequently presented has not influenced the views there expressed. However, some other issues deserve final comments in the light of all assembled evidence.

The probity of the effect

As this is the first report of an effect of $[Ca^{2+}]_i$ on membrane tensile strength, it is proper to consider whether we might be dealing with an artefact. Against this eventuality speak the following observations. (i) A23187 and vehicle DMSO by themselves do not reduce the strength of sarcolemmal vesicles and produce only minor decreases in membrane elastic modulus at the highest concentration used (5μM). (ii) Mg^{2+} does not mimic the effect of Ca^{2+}. As the affinity of A23187 for Mg^{2+} is not greatly lower than its affinity towards Ca^{2+}, this result rules out the possibility that controls done with A23187 are misleading, perhaps because the free acid perturbs the membrane less than the metal-acid complex, even though the latter is twice as large. (iii) Ionomycin which forms a smaller metal complex, can substitute for A23187 in carrying Ca^{2+} into the vesicle and in causing a reduction in membrane strength. (iv) With a constant amount of A23187 added to the solution, the strength of the membrane varies with $[Ca^{2+}]_o$ and hence presumably also $[Ca^{2+}]_i$. As regards the attempt to avoid the use of ionophores and to permeabilize vesicles to Ca^{2+} by use of α-toxin, it should be noted that the doses of α-toxin tolerated by vesicles are one order of magnitude lower than are required to render muscle and other types of cells permeable to small molecules, (Kitazawa *et al.*, 1989; Török *et al.*, 1991). Presumably cell membranes supported by a cytoskeleton can carry many annular toxin

complexes. Sarcolemmal vesicles, by contrast, are weakened even by doses of α-toxin so low that most of it probably exists in the membrane in only monomeric form (Bhakdi, Muhly & Füssle, 1984).

The mechanism of action of elevated $[Ca^{2+}]_i$

Of the several possible synergistically reinforcing mechanisms put forward in Part V (pp. 77-80), the hypothesis that activation of PLA_2 by $[Ca^{2+}]_i$ is involved was investigated most actively. Thus it was shown that the products of PLA_2 mediated phospholipid hydrolysis i.e. lysolipids and arachidonic acid (Parts VI A and B pp. 81-96) are each very powerful in decreasing membrane strength. They also produce a striking and regular increase in membrane distensibility. The latter effect was seen only irregularly when $[Ca^{2+}]_i$ was elevated. But this incomplete resemblance might conceivably be due to a difference between the action of extraneously added lysolipid and arachidonic acid and the endogenous release of these products within the membrane. Thus addition of exogenous lysolipid and arachidonic acid is bound to create membrane expansion on a larger scale than the endogenously produced end product of PLA_2 hydrolysis,* especially if the attack of the enzyme on the membrane were patchy, which would suffice to produce weakened points at which lysis under tension is initiated, but not necessarily a striking increase in distensibility (see also discussion p. 82).

Though the above experiments support the plausibility of the activation of PLA_2 by elevated Ca^{2+}, they do, of course, fall short of proving it. To this end it would be necessary to demonstrate (i) the presence in sarcolemmal vesicles of PLA_2 activated by micromolar concentration of Ca^{2+} (ii) that production of lysolipids and arachidonic acid actually occurs in Ca^{2+} challenged vesicles (iii) that inhibitors of PLA_2 here used (Part VII pp. 97-134) actually prevent the biochemical degradation of the membrane. In the absence of the necessary biochemical facilities and expertise, the direct tests were out of reach. They remain to be tested in the future.

* See Fink, K.L. & Gross, R.W. 1984. Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds. *Circulation Research* **55** 585-594.

The other hypothesis pursued was that elevated Ca^{2+} might activate neutral protease (CANP I) which is known to hydrolyse not only cytoskeletal but also integral membrane proteins (see p. 135). To the limited extent to which this hypothesis was tested by use of MDL 28,170, an inhibitor chosen for its reputed ability to reach the inner surface of the membrane, the results did not seem promising. However, the general possibility that membrane proteins may be involved in the mechanical changes produced by elevated $[Ca^{2+}]_i$ should not be discarded on these grounds. Rather it may be reformulated in the light of the results obtained with α-toxin. The reduction in membrane strength which follows the insertion of the pore-forming protein into the membrane serves as an example that the membrane may indeed be weakened by abnormal protein configurations; and in as much as this effect occurred without any increase in membrane distensibility, it corresponds to the response of the majority of vesicles with elevated $[Ca^{2+}]_i$ (Fig. 4.10). In this connection it may be noted that the concentration of cytosolic Ca^{2+} required to activate high conductance K_{Ca} channels in sarcolemma is quite similar to that which produces membrane weakening, so an example of $[Ca^{2+}]_i$ in appropriate concentration having a direct effect on membrane protein configuration is available, though it is not implied that the change in K_{Ca} channel configuration is actually involved in producing the mechanical effect. Of general interest, however, is that the effectiveness of $[Ca^{2+}]_i$ in opening K_{Ca} channels is voltage dependent in the sense that membrane depolarization by an applied outward current which drives Ca^{2+} towards the inner face of the membrane enhances the probability of the open state. Whether the effect of $[Ca^{2+}]_i$ on membrane mechanical strength is similarly voltage dependent is one of the many questions that yet remain to be explored.

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