Ca^{2+} loading reduces the tensile strength of sarcolemmal vesicles shed from rabbit muscle

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1. Sarcolemmal vesicles shed by rabbit muscle were loaded with Ca^{2+} by means of A23187 or ionomycin. $[Ca^{2+}]_o$ was buffered between 0·8 and 20 μM. Membrane strength was measured by pipette aspiration.

2. At 20 μM Ca^{2+} many vesicles underwent autolysis, or were so weak that they burst instantly on aspiration. Between 10 and 2 μM Ca^{2+} a graded decrease in membrane strength was demonstrable. At 0·8 μM Ca^{2+} the mechanical properties of the sarcolemma remained unaltered.

3. Mg^{2+} carried by A23187 does not mimic the effect of Ca^{2+}. The ionophore itself similarly did not cause a decrease in membrane tensile strength.

4. Pre-treatment with BAPTA-AM, so as to buffer internal Ca^{2+}, partly protected vesicles against the decrease in membrane strength produced by Ca^{2+} loading.

5. Membrane strength was not restored by adding excess BAPTA to the bathing solution, so as to reverse the Ca^{2+} gradient. An irreversible degradation of the membrane consequent upon raised $[Ca^{2+}]_i$ seems indicated.

6. These findings are discussed in relation to the mechanisms which have been advanced to account for the role of elevated $[Ca^{2+}]_i$ in cell death.

7. An attempt to use staphylococcal α-toxin as an alternative means to permeabilize the sarcolemma led to the incidental finding that this pore-forming protein itself greatly weakens the membrane in doses lower than required for effective permeabilization.

In the preceding paper (Nichol & Hutter, 1996) we described the cohesive mechanical properties of sarcolemmal vesicles shed by skeletal muscle fibres. Such vesicles contain cytoplasm but no organelles, and from the behaviour of their K_{Ca} channels it may be concluded that the intravesicular concentration of Ca^{2+} ions, $[Ca^{2+}]_i$, is comparable to that in the cytosol of resting fibres (Burton, Dörstelmann & Hutter, 1988). Here we report that elevation of $[Ca^{2+}]_i$, by exposing vesicles to solutions containing Ca^{2+} and ionophore, produces a striking reduction in membrane strength. This finding may be relevant to the mechanism of cell death through Ca^{2+} overload.

A preliminary report has already appeared (Nichol, Burton & Hutter, 1993).

METHODS

The preparation of sarcolemmal vesicles and measurement of membrane mechanical properties by pipette aspiration are described in the preceding paper (Nichol & Hutter, 1996). 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. one-fifth or one-sixth 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 (Nichol & Hutter, 1996), 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.

Solutions

KCl (140 mM) was always buffered with 5 mM Hepes titrated to pH 7·4 with 140 mM KOH at room temperature. When solutions containing < 20 mM Ca^{2+} were required, nitrilotriacetic acid (NTA) was used as metal buffer. The free acid was brought into solution by titration with 1 N 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 1. The table holds for 140 mM KCl, pH 7·4 and is based on a computer program kindly supplied by Dr G. L. Smith (Institute of Physiology, University of Glasgow) using the association constants

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Table 1. Composition of solutions containing 2 mM NTA, 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	—	—
79	4	—	—
40	2	—	—
16	0·8	—	—

for NTA given by Martell & Smith (1974). In practice, a solution containing 4 mM NTA in 140 mM KCl and twice the amount of Ca^{2+} given in Table 1 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 in phials containing 1 mg. Dry DMSO (1·9 ml) 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 1). According to Chandler & Williams (1977), addition of A23187 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 (1977) 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+} and A23187 had been added.

α-Toxin was a crude α-lysin from *Staphylococcus aureus* (stain 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 (HU) ml^{-1}. This would be equivalent to approximately 0·1 mg ml^{-1} of commercially available pure toxin (Calbiochem).

BAPTA-AM (Molecular Probes) was dissolved in dry DMSO to give a 10 mM stock solution. Aliquots (0·2 ml) 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 later.

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–Smirnov test. Skewness in a population was determined from the average value of the third moment about the mean.

RESULTS

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

In experiments designed to elevate $[Ca^{2+}]_i$, the maximum final concentrations of solvent and ionophore used were 0·5% (v/v) DMSO and 5 μM A23187 in 140 mM KCl. DMSO by itself had no significant effect on membrane strength. Similarly with 5 μM A23187 in 0·5% DMSO, no difference in membrane tensile strength was found, provided 2 mM NTA was also present in the solution bathing the vesicles to minimize stray Ca^{2+} (Fig. 1). The effects of treating the membrane with ionophore on membrane distensibility will be described later.

When Ca^{2+} as well as ionophore were admixed to 140 mM KCl solution containing vesicles, striking effects were observed. At above 20 μM Ca_o^{2+} most vesicles became so weak that they burst instantly on aspiration. With continued exposure, their number decreased through autolysis: flocular 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. Figure 2*A* summarizes experiments in which 0·8–10 μM Ca_o^{2+} 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_o^{2+}, 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_o^{2+}, by contrast, only two out of fifty-one 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. 2*B*), 10 μM Ca_o^{2+} produced a similar decrease in membrane strength as it did with the higher dose of ionophore; and with lower concentrations of Ca_o^{2+} the median strength of vesicles increased progressively

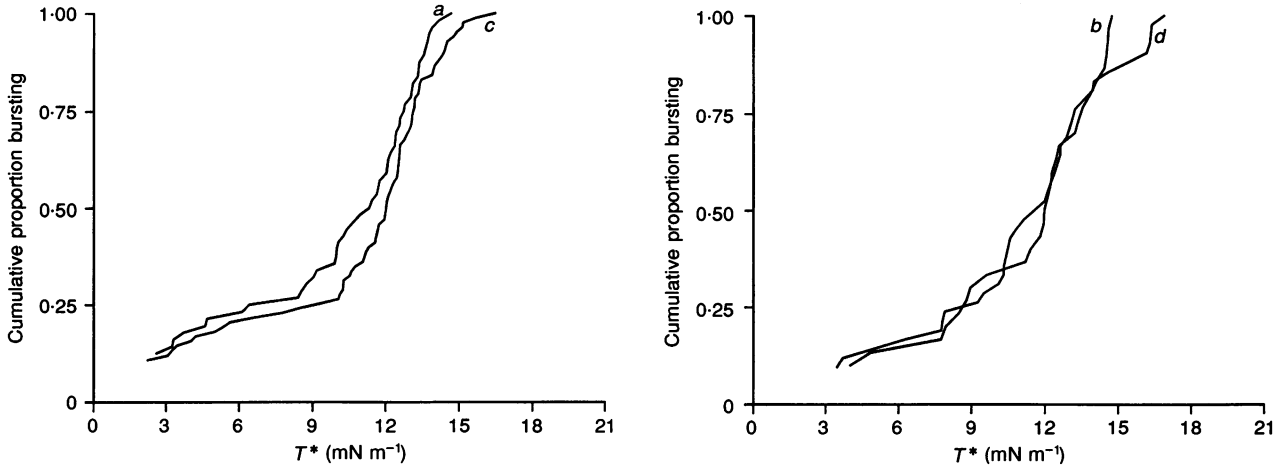

Figure 1. Control experiments on vehicle DMSO and ionophore A23187

Cumulative curves of lysis tension, T^*, of n vesicles in 140 mM KCl plus: *a*, 0·5% DMSO ($n = 52$) and *b*, 0·5% DMSO, 5 μM A23187 and 2 mM NTA to buffer stray Ca^{2+} ($n = 28$); *c* and *d*, cumulative curves for untreated vesicles from the same batches ($n = 76$ and 39, respectively). The differences between the curves in each pair are not statistically significant.

towards that of the control distribution. Interestingly, 2 μM Ca_o^{2+}, 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 polyether 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). Figure 3 shows the cumulative lysis tension for vesicles exposed to 10 or 6 μM Ca_o^{2+} in the presence of 1 or 5 μM ionomycin, together with a cumulative curve for control vesicles from the same batch of muscle. The clear shift in the curves also produced under these conditions rules out the possibility 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, and as it is considerably more expensive, it was used only rarely in later experiments.

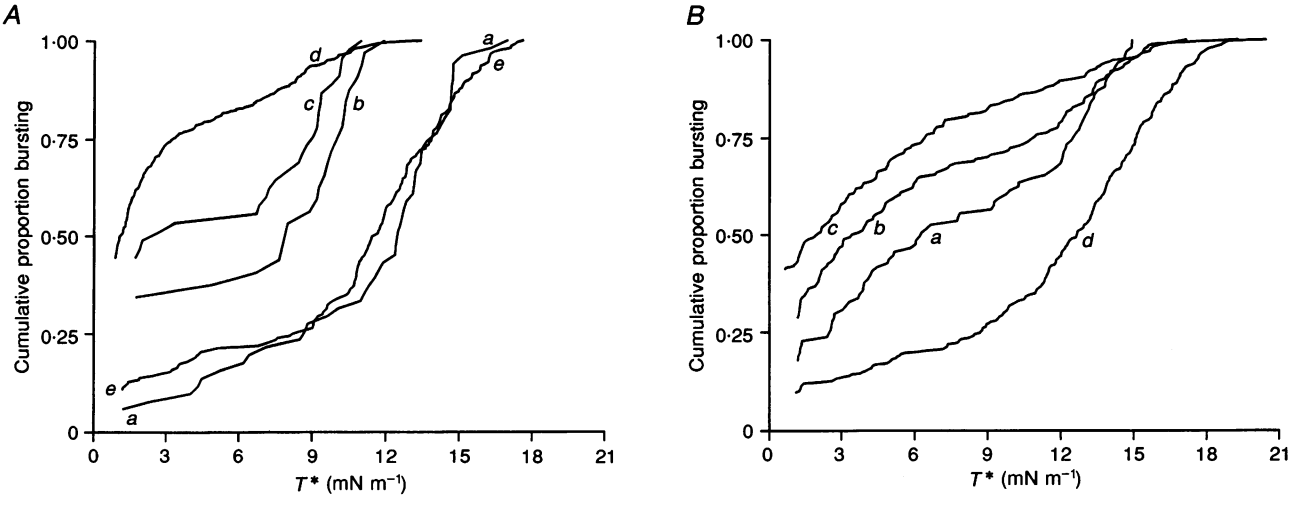

Figure 2. Effect on membrane strength of loading vesicles with Ca^{2+} using A23187

Cumulative curves of lysis tension, T^*. *A*, 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 ($n = 180$). *B*, vesicles treated with 1 μM A23187 and loaded in: *a*, 2 μM ($n = 84$); *b*, 4 μM ($n = 125$); *c*, 10 μM Ca^{2+} ($n = 160$); *d*, untreated vesicles ($n = 285$). The weakening produced by 2 μM Ca^{2+} is significant at $P < 0·01$.

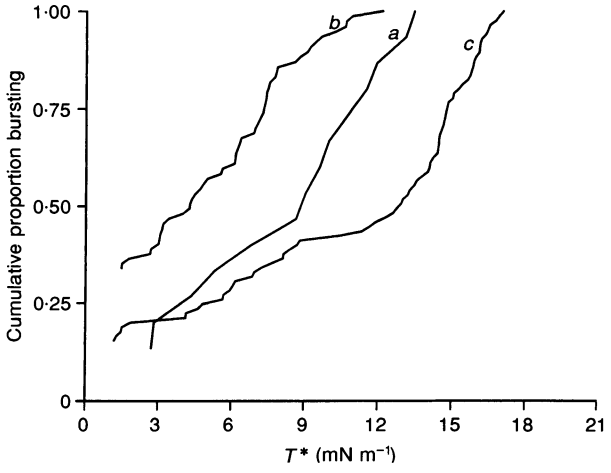

Figure 3. Effect on membrane strength of loading vesicles with Ca^{2+} using ionomycin

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 ($n = 73$).

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).

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+}. 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 treating vesicles with either 0·5% v/v DMSO alone, or with the vehicle plus 5 μM A23187 had no obvious effect on lysis tension (Fig. 1), the fractional increase in membrane area at lysis was slightly higher in both conditions than in untreated vesicles (Fig. 5*A* and *B*). Evidently, membrane distensibility is more sensitive to treatment than is membrane strength. In order to decide whether an increase in $[Ca^{2+}]_i$ has any effect on membrane distensibility, vesicles treated with only 0·1% v/v DMSO and 1 μM A23187 were therefore used; and as control,

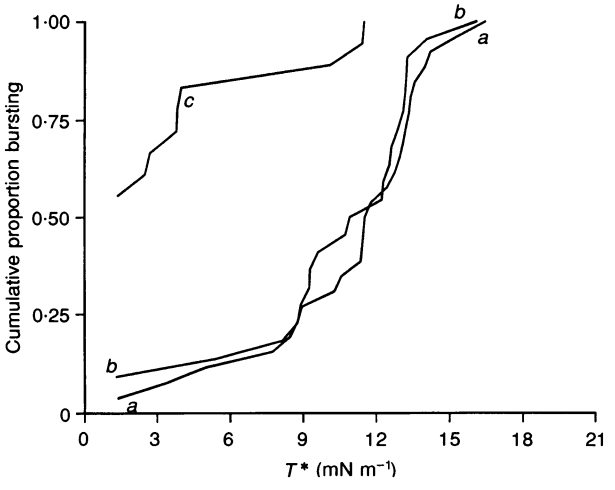

Figure 4. Comparison between the effects on lysis tension of Mg^{2+} and Ca^{2+}

Cumulative curves of lysis tension, T^*, showing: *a*, control vesicles ($n = 29$); *b*, 126 μM Mg^{2+} with 1 μM A23187 ($n = 21$); and *c*, 20 μM Ca^{2+} in combination with 1 μM ionophore A23187 ($n = 9$).

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).

Figure 5*C* is based on the same population as the two right hand curves of Fig. 4. As was to be expected, the lysis points for vesicles treated 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_o^{2+} are depicted in Fig. 5*D*. Noticeable differences are: (i) the large proportion of vesicles lysing at low tension and (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 be offered later.

Table 2 summarizes the data of Fig. 5 in terms of the mean values of the modulus of area expansion, K. Also given are values for vesicles treated with 1 μM A23187 and loaded in 2 or 4 μM Ca_o^{2+}, which were included in Fig. 2*B*, but not in Fig. 5*D* to avoid overcrowding. The experiments of Fig. 5*A* and *B* were done early, those of Fig. 5*C* and *D* late during this work, and the difference in K for the respective control groups reflects the drift illustrated in Fig. 9 of the preceding paper (Nichol & Hutter, 1996). 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_o^{2+} was K significantly ($P = 0{\cdot}0012$) 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 ($P < 0{\cdot}05$) 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. 2*B*).

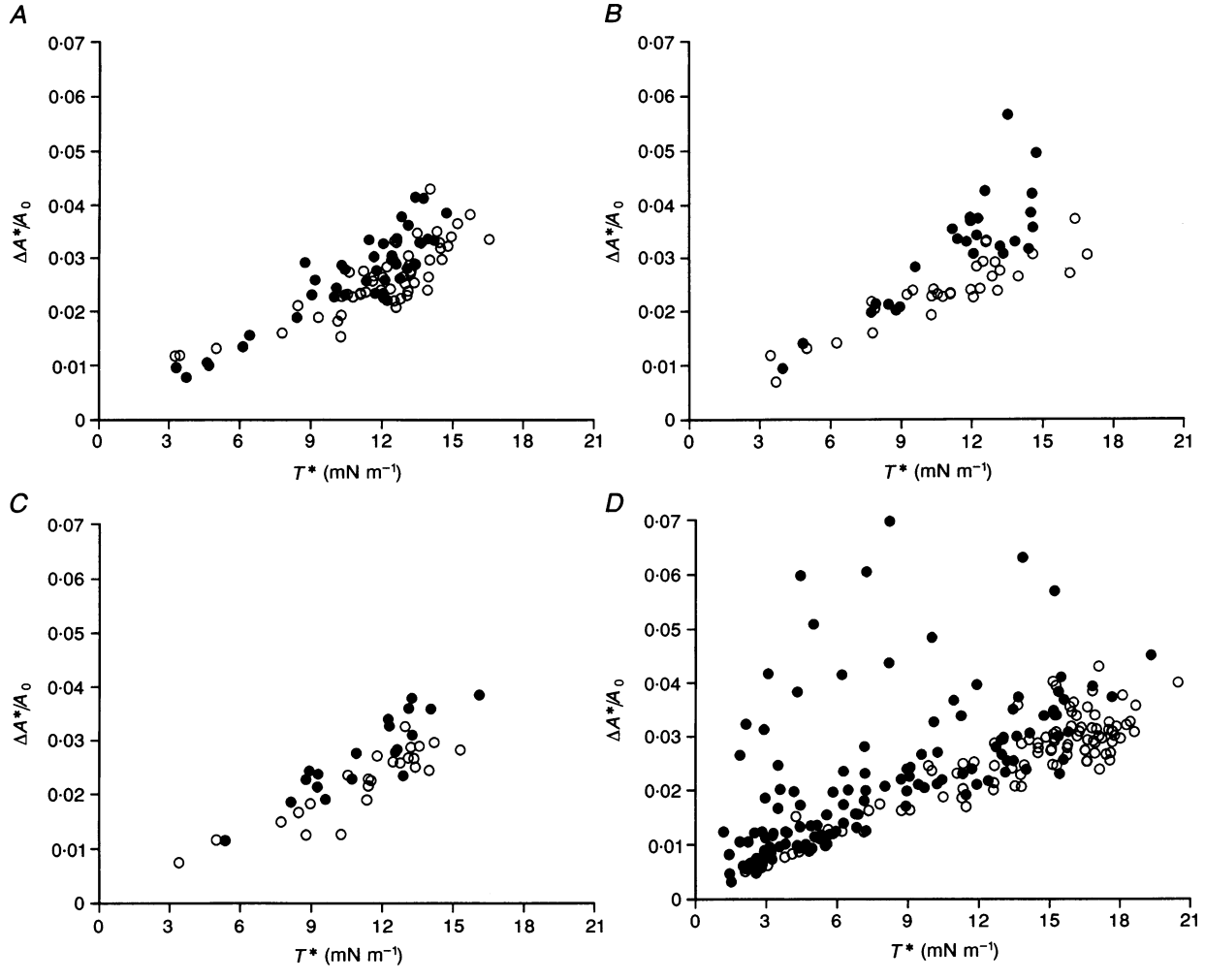

Figure 5. Lysis tension, T^*, and corresponding area expansion for control or Ca^{2+}-treated sarcolemmal vesicles

●, lysis points for vesicles in 140 mM KCl containing: *A*, 0·5% DMSO; *B*, 0·5% DMSO and 5 μM A23187; *C*, 0·5% DMSO, 1 μM A23187 and 126 μM Mg^{2+}; and *D*, 0·5% DMSO, 1 μM A23187 and 10 μM Ca^{2+}; ○, untreated companion vesicles in 140 mM KCl.

Table 2. Modulus of area elasticity for treated sarcolemmal vesicles and respective controls

Treatment	n	Mean ± 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

Protective effect of intracellular Ca^{2+} buffering

A 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+}. In several situations BAPTA so introduced into cells has been shown to delay the effect of A23187-mediated Ca^{2+} influx (Tsien, 1981; Nicotera, Thor & Orrenius, 1988). It seemed interesting therefore to test whether such a protective action of BAPTA can also be demonstrated in sarcolemmal vesicles. Such experiments showed that vesicles pre-treated with BAPTA-AM and then exposed to 10 μM Ca_o^{2+} and 1 μM A23187 were stronger than vesicles so loaded in the ordinary way, but not as strong on average as the control vesicles from the same batches (Fig. 6). Under the conditions of the above experiment BAPTA-AM therefore protected vesicles at least partially against the effect of Ca^{2+} inflow. No time dependence of this effect was observed.

BAPTA-AM is of limited solubility in aqueous solution. It is 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. In the above experiment dilute and fully transparent suspensions of sarcolemmal vesicles were used, and it was noticed that addition of BAPTA-AM in DMSO produced a cloudy solution. Under the microscope formation of crystals was observed. It must therefore be supposed that much of the BAPTA-AM added came out of solution. The addition of pluronic F127 to BAPTA-AM as dispersal agent has been advocated (Haugland, 1992). With a view to improving the experimental conditions by its use, the effect of pluronic F127 (0·05%) by itself 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. Furthermore, 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 sarcolemmal 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 as to whether the effects of elevated $[Ca^{2+}]_i$ are reversible was studied in that way. Several dishes of vesicles were prepared and treated with 10 μM Ca_o^{2+} 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+}, this should reduce $[Ca^{2+}]_o$ to sub-micromolar levels. Half an hour was then allowed for efflux of Ca^{2+} to proceed from the vesicles before further

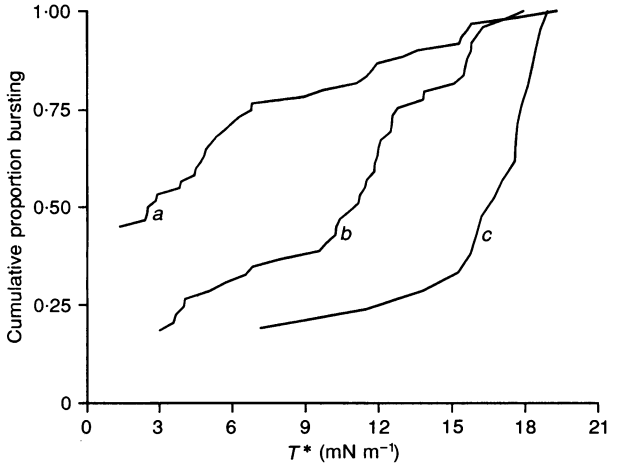

Figure 6. Protective effect of preloading vesicles with BAPTA-AM

Cumulative lysis tension curves for sarcolemmal vesicles loaded with 10 μM Ca^{2+} containing 1 μM A23187 ($n = 34$) (*a*); preloaded with nominally 20 μM BAPTA-AM for 30 min and then loaded as in *a* ($n = 41$) (*b*); companion control vesicles in 140 mM KCl ($n = 18$) (*c*).

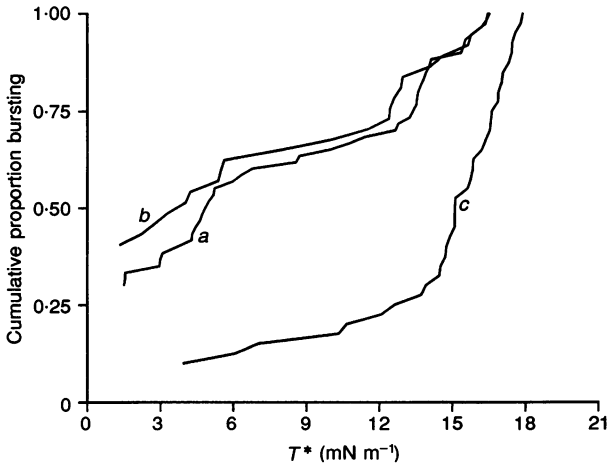

Figure 7. Reduction in membrane tensile strength by Ca^{2+} loading is irreversible

Cumulative lysis tension curves for sarcolemmal vesicles loaded with 10 μM Ca^{2+} containing 1 μM A23187 ($n = 43$) (*a*); loaded as in *a*, and thereafter exposed for at least 30 min to an A23187-containing solution with its Ca^{2+} content mopped up by 200 μM BAPTA sodium salt ($n = 23$) (*b*); and companion controls in 140 mM KCl ($n = 37$) (*c*).

specimens were aspirated. No return of membrane tensile strength was found (Fig. 7). 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.

Experiments with α-toxin

α-Toxin is a monomeric protein which binds to cell membranes and then undergoes self-association to form an oligomeric complex which harbours a membrane-spanning pore of 2–3 nm diameter (Füssle, Bhakdi, Sziegoleit, Tranumjensen, Kranz & Wellensiek, 1981). It has long been known to permeabilize red blood cells and so lead to their lysis. More recently it has been used to permeabilize smooth and skeletal muscle fibres to Ca^{2+} (Kitazawa, Kobayashi, Horiuti, Somlyo & Somlyo, 1989; Török, Patel & Ferenczi, 1991). Ostensibly, therefore, treatment of sarcolemmal vesicles with α-toxin offers opportunity for studying the effects of Ca^{2+} loading without use of a polyether ionophore. In the first instance, however, it was necessary to check on any direct effects of α-toxin on membrane mechanical properties. To this end vesicles in 140 mM KCl with 2 mM NTA, to minimize stray Ca^{2+}, were exposed to α-toxin for at least 15 min before aspiration was begun. It soon became evident that concentrations above 16 HU ml^{-1} caused autolysis of vesicles as shown by a decrease in their number and the appearance of floccular cytoplasmic protein in the dish. In the range 3–16 HU ml^{-1}, α-toxin caused a graded decrease in the strength of sarcolemmal vesicles (Fig. 8*A*). Interestingly, membrane distensibility was not affected by α-toxin in the concentrations tolerated by vesicles (Fig. 8*B*).

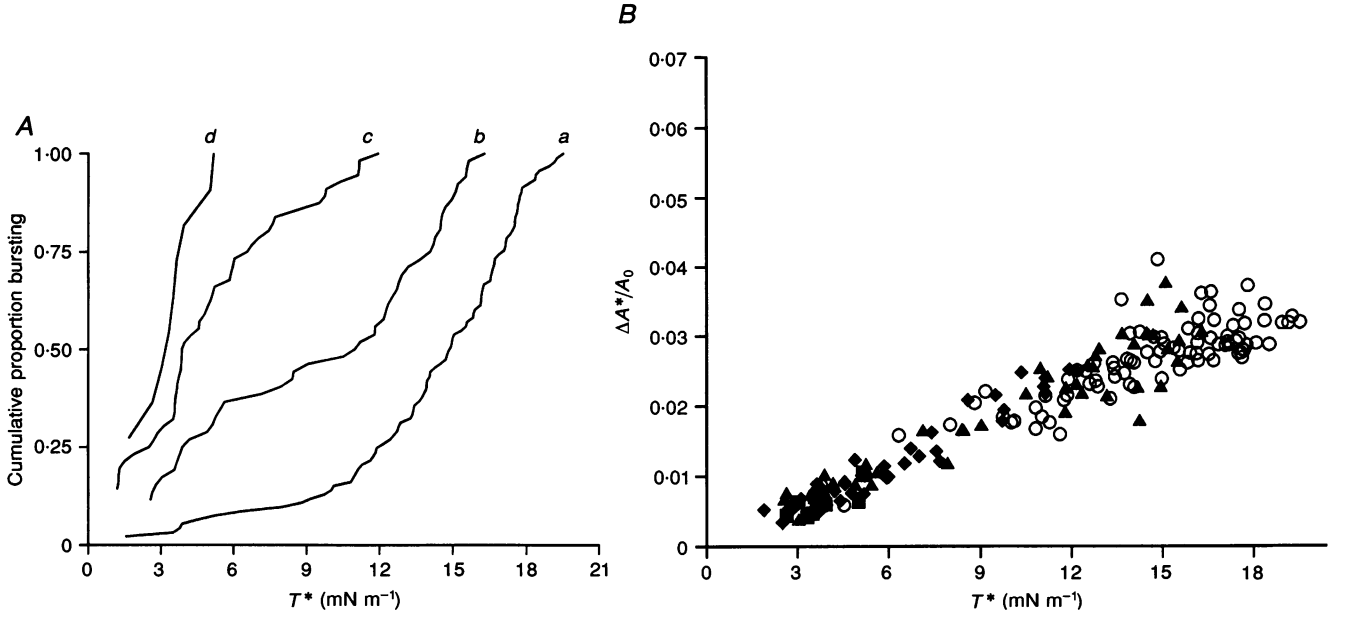

Figure 8. Membrane tensile strength is reduced by α-toxin

A, cumulative tension curves for: *a*, control vesicles in 140 mM KCl ($n = 92$) and sarcolemmal vesicles exposed to α-toxin at 3 HU ml^{-1} ($n = 47$) (*b*), 8 HU ml^{-1} ($n = 45$) (*c*), and 16 HU ml^{-1} ($n = 9$) (*d*). *B*, filled symbols show lysis tension and correponding area expansion $\Delta A^*/A_0$ in vesicles exposed to 3 (▲), 8 (◆) and 16 HU ml^{-1} α-toxin (■), compared with companion controls (○) in 140 mM KCl.

Having established the dose of α-toxin tolerated by vesicles, the effect of admixing $CaCl_2$ so as to give 10 μM Ca_o^{2+} 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_o^{2+} did not weaken the vesicles further or lead to their autolysis, as would have happened had the vesicles been permeabilized with ionophore. In 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 (Nichol & Hutter, 1996). 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 dose which can be tolerated by sarcolemmal vesicles does not effectively permeabilize the membrane, presumably because the concentration of α-toxin in the membrane is too low for the formation of enough annular complexes.

DISCUSSION

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 artifact. 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 (5 μM). (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 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. These include: (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 1 or 5 μM A23187 (Fig. 2*A* and *B*). Regarding the last point, it is relevant that two molecules of A23187 complex with each Ca^{2+} ion. A 5-fold increase in the concentration of A23187 should, therefore, render the ionophore twenty-five 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.

Possible mechanisms involved

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 the 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 from that point of view could be rewarding.

Relevant to the 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 phosphatidylethanolamine 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). Analagous 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 brought about 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).

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

An accumulation of lysolipids in ischaemic Ca^{2+}-loaded tissue is well documented (Man, Slater, Pelletier & Choi, 1983) and points to the involvement of phospholipid-deacylating enzymes. Of particular interest and possible relevance here is the high molecular weight (*ca* 85 000) 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 and so reduce the tensile strength of the membrane (Israelachvili, Marcelja & Horn, 1980); and in adequate proportions endogenous lysolipids, through their detergent effect, would cause autolysis of vesicles such as observed at 20 μM Ca^{2+} and above.

The plausibility of phospholipid deacylation as a causal mechanism is underlined by the finding that extraneous lysophospholipids (5–10 μM) reduce membrane tensile strength. At the same time an increase in membrane distensibility is noted (Nichol & Hutter, 1993). The question whether the decrease in membrane strength produced by intravesicular Ca^{2+} is accompanied by an increase in membrane distensiblilty is therefore of some interest. Vexatiously, no clear-cut results on this point were obtained: only a small proportion of the vesicles weakened by elevated $[Ca^{2+}]_i$ were also demonstrably more distensible than normal (Fig. 5*D*). It should be recalled, however, that elasticity is an overall property of the vesicle membrane, whereas vesicle lysis will occur as soon as a localized packing defect forms in the membrane presumably as a result of stochastic processes. For a vesicle to be abnormally distensible, much of the membrane would need to be perturbed by endogenous lysolipid and such vesicles would probably lyse spontaneously or early on aspiration. A high distensibility together with sufficient membrane strength for the determination of elastic modulus may therefore be a rare combination.

The use of inhibitors as a means of testing whether enzymic phospholipid deacylation is at work in Ca^{2+}-loaded sarcolemmal vesicles brings with it its own problems. Most widely used putative PLA_2 inhibitors, e.g. dibucaine, mepacrine etc. (Jackson *et al.* 1984), are themselves amphiphillic agents which weaken sarcolemmal vesicles. Their use in combination with $[Ca^{2+}]_i$ elevation is possible only within narrow limits. Moreover, such attenuation of the effect of $[Ca^{2+}]_i$ as they may produce seems to be offset by their own direct action, so that the result remains equivocal (J. A. Nichol & O. F. Hutter, unpublished observations). Clearly, before the involvement of PLA_2 in the present situation can be postulated with any confidence, the existence of the enzyme in sarcolemmal vesicles and its activation by Ca^{2+} needs to be directly demonstrated by appropriate immunological and biochemical techniques.

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 diacylglycine 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 (Croall & 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 intramembrane proteins. In red blood cells, for instance, the Ca^{2+}-pump and band-3 proteins are degraded (Salamino *et al.* 1994). Zaidi & Narahara (1989) have demonstrated that calpain is present in skeletal muscle. The possibility therefore exists that by attacking intramembrane 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.

That foreign proteins may indeed have a powerful effect on membrane strength is illustrated by the effect of α-toxin here incidentally reported. Even in concentrations below those required to permeabilize the membrane a decrease in tensile strength is evident, and the indication is that the formation of permeabilizing annular complexes renders vesicles too weak for pipette aspiration. In intact cells permeabilized by higher doses of toxin, the submembrane cytoskeletal network presumably stabilizes the membrane. In view of these findings it would be interesting to test whether the interaction of other pore-forming proteins (Saberwal & Nagaraj, 1994) with membranes is also reflected in altered membrane mechanical properties. Pipette aspiration of sarcolemmal vesicles now offers a method for such investigations.

The reduction in membrane strength by α-toxin occurred without any detectable change in area elastic modulus, presumably because only one or a few flaws in the membrane are sufficient to weaken a vesicle. When more widespread membrane perturbation is produced, as by incorporation into artificial phospholipid–cholesterol vesicles of 1 mole per cent of a peptide whose hydrophobic length is shorter than the thickness of the membrane, the decrease in membrane strength is accompanied by an increase in membrane distensibility. This has been attributed to interference with the lipid–cholesterol interactions (Evans & Needham, 1986; Bloom, Evans & Mouritsen, 1991). Evidently, peptides may cause a detectable increase in membrane distensibility, provided they engender an overall perturbation of the membrane without weakening vesicles so much that pipette aspiration becomes impossible.

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 psoas muscle, 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. In relation to this hypothesis it will be interesting to test in which way extraneously induced lipid peroxidation alters the mechanical properties of sarcolemmal vesicles.

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. The mechanisms we have detailed are open to experimental test, though mostly not with methods at our disposal. Further analysis of the effects here described by other techniques is called for. The loss of membrane strength produced by raised $[Ca^{2+}]_i$ is presumably an expression of the final irreversible events leading to cell death. Any treatment that can prevent it could therefore help minimize cell damage in diverse clinical situations.

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