Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 142-149

A23187 RELEASES BOUND RATHER THAN FREE CALCIUM FROM CALCIUM-LOADED LIPOSOMES

James W. Clack,* Paul S. Brown, Jennifer M. Feldmann,** and Frank A. Witzmann

Department of Biology, Indiana University - Purdue University
Columbus, IN 47203

Received August 5, 1994

Summary: We have investigated sonication-, osmotic shock-, and A23187-induced calcium and horseradish peroxidase release from phosphatidylcholine liposomes. We report that sonication and osmotic shock both cause release of liposome-entrapped horseradish peroxidase activity, thus, completely releasing the entrapped volume into and mixing it with the external medium. However, we find that neither sonication nor osmotic shock releases significant amounts of calcium from liposomes pre-loaded with calcium, while A23187 causes release of significant amounts of calcium from similarly prepared liposomes. We also find that A23187 can release calcium from liposomes after release of entrapment volume by sonication or osmotic shock. Alteration of the net charge of liposomes by substitution of phosphatidylcholine with phosphatidylserine or stearylamine dramatically changes the amount of calcium associated with calcium-loaded liposomes; sonication or addition of A23187 to liposomes containing significant amounts of PS does not appear to release calcium bound to these vesicles. These results suggest that a large fraction (>99%) of the calcium associated with liposomes is bound to the membranes of the liposomes and that the calcium released by A23187 is due to release of bound rather than entrapped calcium.

Numerous investigators have used release of calcium from calcium-loaded liposomes as an assay for cation translocator activity in various cell types. Some studies have employed spectrophotometric assays using extravesicular Arsenazo III to measure calcium efflux from pre-loaded vesicles (1,2,3,4,5,6). Others have used release of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-loaded vesicles to assay for ion channels (7,8,9,10) or Na^{+}-Ca^{2+} exchangers (11). In many of these studies A23187 has been used to release calcium from vesicles and abolish the ability of subsequent test substances to effect calcium release. In these studies, A23187 has been presumed to abolish

* Corresponding author.
Address: 4601 Central Ave.
Columbus, IN 47203.

** Current address: Indiana University School of Medicine, Indianapolis, IN 46202.

Abbreviations: phosphatidylcholine, PC; phosphatidylserine, [PS]; stearylamine, [SA]; horseradish peroxidase, HRP; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), ABTS.

0006-291X/94 $5.00

calcium release by translocating entrapped calcium down its concentration gradient to the external volume. Thus, A23187 abolition of test substance-induced calcium release in the above studies has been interpreted to be due to elimination of the transvesicular calcium gradient.

In attempting to replicate certain of the studies mentioned above, Devenny and Clack (12) discovered that cGMP- and A23187-mediated release of calcium from rod outer segment disk membrane liposomes persisted after calcium-containing liposomes were sonicated or subjected to ionic shock in low calcium media (sonication and ionic shock were sufficient to cause complete exchange of the liposome contents with the external media - measured by release of entrapped ^{3}H-inulin). They concluded that cGMP- and A23187-induced release of calcium from liposomes disrupted in this way was release of bound rather than entrapped calcium. We have performed further A23187-induced calcium release experiments, using both the Arsenazo III and $^{45}Ca^{2+}$ techniques, using liposomes composed of defined lipids and lipid mixtures (phosphatidylcholine [PC] and either phosphatidylserine [PS] or stearylamine [SA]) with different net surface charge. Our results indicate that, as is observed in native rod outer segment vesicles, A23187-mediated release of calcium from liposomes composed of phosphatidylcholine persists after sonication of the liposomes in low calcium medium. Our results also show that micromolar concentrations of A23187 release a fraction of calcium bound to liposomes; percent release of bound calcium is related to the reciprocal of liposome net charge. These results suggest that the overwhelming majority (>99%) of calcium associated with liposomes is bound rather than entrapped calcium (presumably binding to the headgroup of the phospholipids) and that the effect of A23187 is to cause release of calcium from those binding sites.

METHODS

Preparation of Liposomes- Phospholipids were purchased from Sigma (St. Louis, MO) and were purified chromatographically (approx. purity: ≥99%). Phosphatidylcholine (PC) vesicles were prepared by drying 1 ml of a chloroform solution containing 10 mg/ml PC onto the walls of a microcentrifuge tube using a stream of nitrogen, followed by vacuum evaporation overnight. Phosphatidylserine (PS), stearylamine (SA), PC:PS or PC:SA mixtures were similarly dried. $^{40}Ca^{2+}$- or $^{45}Ca^{2+}$-containing liposomes were formed by adding 1 ml of a buffer solution containing 100 mM NaCl, 10 mM Tris, pH 7.4 (Buffer A) and 10 mM $^{40}Ca^{2+}$ or 40 μCi of $^{45}Ca^{2+}$ (specific activity 150 μCi/mmol: ICN, Irvine, CA), respectively, and sonicating with a Fisher model GE50 probe sonicator at approximately 20 watts output for 2 min at 0°C. Lipsomes were then dialyzed against 4 l of Buffer A at 4°C for 48 hr with 2 changes of buffer. Horseradish peroxidase (HRP; Sigma, St. Louis) was entrapped in PC liposomes by sonication of 10 mg/ml HRP dissolved in Buffer A with 10 mg of PC. The HRP-containing liposomes were centrifuged at 150,000 x *g* for 30 min and resuspended in Buffer A five times to eliminate non-entrapped HRP activity.
Measurement of Horseradish Peroxidase Activity- Vesicles containing entrapped HRP were diluted into Buffer A and sonicated for varying amounts of time at 0°C and assayed for HRP activity. The rate of HRP-catalyzed oxidation by H_2O_2 of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was monitored by determing the difference in absorbance between 416 and 480 nm in a custom-built dual wavelength spectrophotometer (Oriel, Stratford, CT). The sample

cuvette was continuously stirred and reagents were injected into the cuvette via a light-shielded port. The distance from cuvette to the photomultiplier tube was very short (~2.3 cm), decreasing or eliminating artifacts resulting from light-scattering (12). Spectrophotometric data were recorded digitally at a sampling rate of 10/sec using an IBM/AT computer equipped with an A/D converter. The data were corrected for baseline drift by subtraction of a linear baseline obtained from the trace before the addition of reagent(s) and/or test substance.

Arsenazo III Assay of Calcium Release- $^{40}Ca^{2+}$ measurements were made by comparing absorbance at 650 nm with that at 730 nm in the spectrophotometer described above (12). Liposomes were diluted into 3.5 ml of Buffer A containing 70 μM Arsenazo III in a stirred cuvette to a lipid concentration of 5 μM. A baseline level of absorbance was determined and, if the liposomes were to be disrupted by sonication, the cuvette was removed from the spectrophotometer, placed in a room temperature water bath, and the liposomes were disrupted using a Fisher GE50 micro-tip probe sonifier at ice temperature. Ionophore A23187 was added (final concentration, 7.5 μM) to measure the releasable Ca^{2+} associated with the membranes. Ca^{2+} (1 μM final concentration) was injected into the cuvette at the end of the experimental run as a calibration standard.

Measurement of $^{45}Ca^{2+}$ Binding and Release From Liposomes. $^{45}Ca^{2+}$-containing liposomes were diluted into a test tube containing 5 ml of Buffer A (5 μM final lipid concentration) in the presence or absence of 7.5 μM A23187. In some cases the liposomes were sonicated at 0°C before subsequent additions or counting. After a 10 min incubation, the liposomes were filtered on 25 mm diameter Pharmacia/LKB Nova PES 100 kdalton filters in a Millipore vacuum manifold and rinsed once with Buffer A. The filters were dried, solubilized with scintillation cocktail and counted in a Packard Scintillation Counter.

RESULTS

The ability of sonication to release the entrapment volume of liposomes was determined by entrapping horseradish peroxidase (HRP) in liposomes and measuring the relative amount of HRP activity released as a function of sonication time. HRP has been established as a molecule that does not cross intact membranes under normal conditions (13), so sonication-induced increases in HRP activity were interpreted to represent ability of sonication to release liposome entrapment volume. Figure 1 shows the effectiveness of sonication in releasing entrapped HRP. Each trace represents the relative concentration of the oxidized form of the indicator dye, ABTS, so that increases in absorbance reflect an increase in oxidized product. The numbers to the right of the traces show the duration of a 20-watt sonication before addition of reactants. The initial slope of each trace was taken to be the velocity of the oxidation reaction. Sonication-induced increases in velocity were due to release of HRP catalytic activity and, thus, due to entrapment volume released. Half-maximal release of entrapment volume occurred at 2 min of sonication. Greater than 95% of the total entrapment volume was released with 4 min or longer sonication. This is in rough agreement with the results of Devenny and Clack (12).

Figure 2 compares A23187-induced Ca^{2+} release (Arsenazo III-assayed) from unsonicated PC liposomes with that from liposomes that were sonicated in Buffer A for 4 min. Sonication of liposomes did not release detectable amounts of Ca^{2+}. Addition of 7.5 μM A23187 released

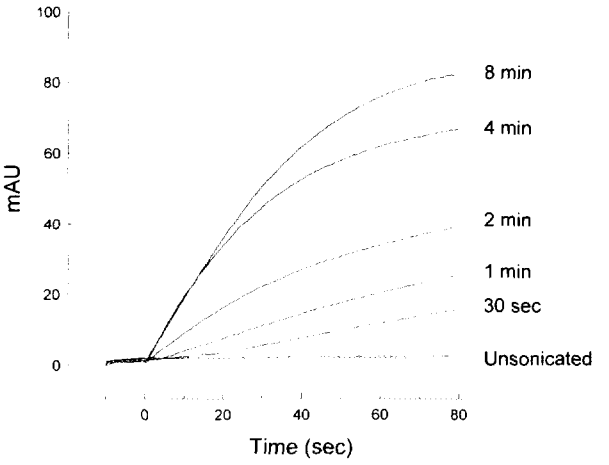

Figure 1. Sonication-induced HRP release from PC Liposomes. HRP-containing liposomes were (30 µl) were diluted into 3.5 ml of Buffer B. Liposomes were then sonicated at 20 watts for the indicated time at 4°C to release entrapped HRP activity. The enzyme reaction was initiated by addition of H_2O_2/2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Increases in absorbance indicate and increase in reduced product. The initial slope of the reactions were interpreted as the rate of the reaction.

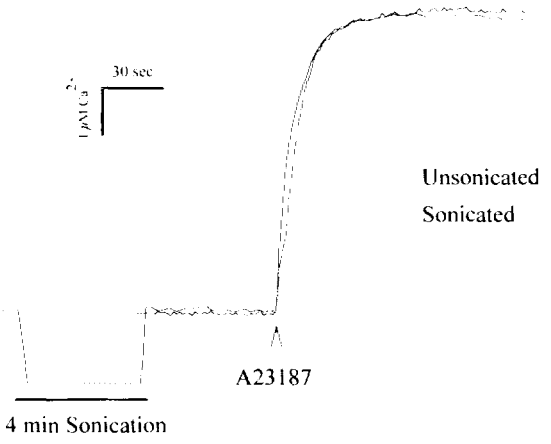

Figure 2. Effect of sonication on release of Ca^{2+} from PC liposomes by A23187. Recording conditions were as describe in the METHODS. Calcium-loaded PC liposomes (50 µg of PC) were diluted into 3.5 ml of Buffer B in a stirred cuvette. Sonicated liposomes (solid trace) were then removed from the recording apparatus, sonicated for 4 min at 4°C and replaced in the recording apparatus. A23187 (7.5 µM final concentration) was injected as indicated. The two traces were fit to the following equation: $f(x\ x_{max})\quad x\ (1 \cdot x\ \tau)$. The data from the sonicated liposomes were well fit to the equation ($r^2 = 0.998$) with $\tau = 2.89$ s. The data from the unsonicated liposomes were slightly less well fit ($r^2 = 0.982$) with $\tau = 5.23$ s. Data from unsonicated liposomes were more readily fit by the following more complicated equation ($r^2 = 0.999$): $f(x\ x_{max})\quad (0.078x \cdot 0.01x^2 - 0.011)\ (1 \cdot 0.023x \cdot 0.01x^2)$
The value of x at which half-maximal release occurs according to this fit is 5.13 s.

approximately 5 μM Ca^{2+} from both the sonicated and unsonicated liposomes. Ca^{2+} release from sonicated liposomes was slightly faster than that from unsonicated liposomes ($\tau_{unsonicated}$ = 5.23 s, $\tau_{sonicated}$ = 2.89 s; cf. Fig. 2 legend). Because this degree of sonication has been shown to release entrapment volume from liposomes under similar conditions, it is very unlikely that a Ca^{2+} concentration gradient existed in the sonicated preparation. Thus, the mechanism of A23187-induced Ca^{2+} release cannot be explained solely in terms of its action as an ionophore.

Measurement of Ca^{2+} release from liposomes using external Arsenazo III allowed us to determine that Ca^{2+} release from PC liposomes is not driven by a concentration gradient, but this assay could not determine the stoichiometry of Ca^{2+} binding to PC liposomes or the amount released from those liposomes by A23187. Experiments using incubation of liposomes with $^{45}Ca^{2+}$ were employed to determine these values. Figure 3 shows the amount of Ca^{2+} bound to phospholipid vesicles as a function of fractional charge (as assayed by filtration, filled circles), and also the amount of Ca^{2+} releasable by A23187 (filled squares, Fig. 3 & inset). The amount of $^{45}Ca^{2+}$ associated with liposomes containing mixtures of PC and PS increased linearly as the

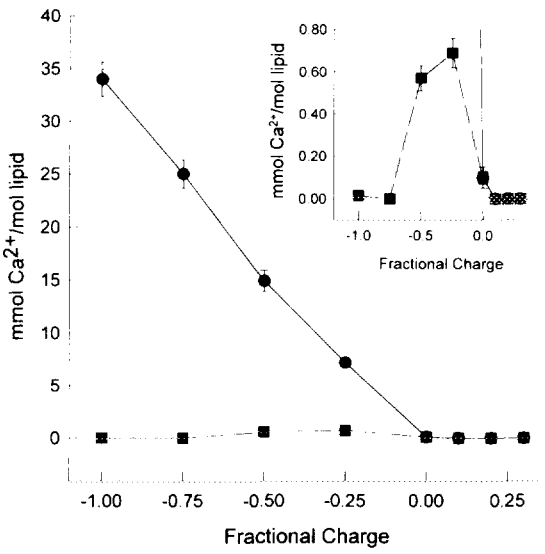

Figure 3. Effect of fractional charge on $^{45}Ca^{2+}$ binding and A23187-releasable $^{45}Ca^{2+}$ from lipid vesicles. Vesicles with varying fractional charge per lipid were prepared as described above. Vesicles were sonicated in Buffer A containing 1 mM $^{45}Ca^{2+}$ (specific activity 150 μCi/mmol) for 4 min at 4° C. They were then dialyzed against Buffer A without added Ca^{2+} for 48 hr (2 changes of buffer). Vesicles (50 μl) were diluted into a test tube containing 5 ml of Buffer A in the presence or absence of 7.5 μM A23187. The vesicles were then filtered on 25 mm diameter Pharmacia/LKB Nova PES 100 kdalton filters in a Millipore vacuum manifold and rinsed once with Buffer A. The filters were dried, solubilized with scintillation cocktail and counted. The filled circles represent the amount of $^{45}Ca^{2+}$ bound to lipid vesicles of varying charge (± SD; n=4). The filled squares (see inset) show the amount of A23187-releasable calcium (± SD; n=3).

fractional charge of the liposomes was decreased. No significant binding/association of $^{45}Ca^{2+}$ was observed in SA/PC liposomes (those with net positive charge). Negative stain electron microscopy showed that unilammelar liposomes were, indeed, formed from low percentage (10-50%) PS and SA liposomes (data not shown). This suggests that $^{45}Ca^{2+}$ dissolved in the liposomes' entrapment volume probably accounts for an extremely small fraction of the $^{45}Ca^{2+}$ found associated with liposomes composed of phospholipid mixtures. The slope of a linear regression fitted to the binding data between fractional charge of 0 and -1 was -0.03. This corresponds to the binding of 33 mmol $^{45}Ca^{2+}$ per mol PS. By contrast, we observed only 100 μmol $^{45}Ca^{2+}$ bound per mol PC in pure (100%) PC liposomes. This binding occurred either in the presence or absence of 7.5 μM A23187 during the incubation period (data not shown), making it unlikely that the diffence in binding of $^{45}Ca^{2+}$ to liposomes with differing phospholipid compositions was due either to differences in phospholipid orientation in the liposome bilayer or formation of multilammelar liposomes. Furthermore, sonication of $^{45}Ca^{2+}$-loaded vesicles for up to 4 min at 0°C did not decrease the $^{45}Ca^{2+}$ associated with the liposomes, confirming that the $^{45}Ca^{2+}$ associated with the liposomes was not contained in the entrapment volume of the liposomes. Figure 3 also illustrates the ability of A23187 to release $^{45}Ca^{2+}$ from liposomes of varying charge (filled squares and inset). A23187 was able to release a large fraction of the $^{45}Ca^{2+}$ bound to PC vesicles; A23187-induced release from liposomes containing an increasing fraction of PS peaked at a fractional charge of -0.25 and then decreased. In fact, A23187 was unable to release a significant fraction of $^{45}Ca^{2+}$ bound to liposomes containing more than 25% PS. Figure 4 plots the A23187 release data from Figure 3 as a fraction of total $^{45}Ca^{2+}$ bound. The increasingly negative environment on the surface of the phospholipid bilayer because of PS incorporation progressively decreased the ability of A23187 to release $^{45}Ca^{2+}$ from the liposomes. Increasing or decreasing the concentration of added A23187 10-fold (range, 750 nM - 75 μM; data not shown) did not alter the percentage of $^{45}Ca^{2+}$ released from liposomes. This suggests that the affinity of PS for calcium is at least 1-2 orders of magnitude greater than the affinity of A23187 for calcium.

DISCUSSION

These results strongly suggest that the overwhelming majority of calcium associated with liposomes after incubation of liposomes in calcium is bound rather than free, entrapped calcium. The presumptive locus of calcium binding is at the head group of phospholipids, which have either a dipole (PC) or acidic region (PS) available for binding. It is very possible that the relatively small amount of calcium found associated with 100% PC liposomes was due to contamination of the PC with an acidic (i.e., phosphatidylserine or phosphatidylethanolamine) phospholipid. We

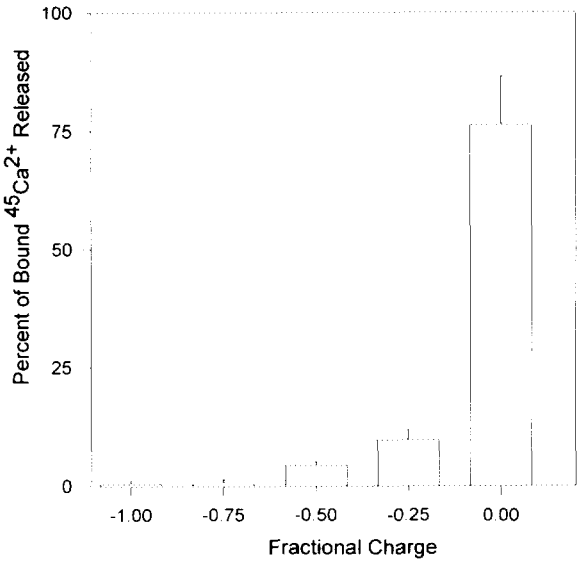

Figure 4. Charge-related percentage of bound calcium releasable by A23187. The release data of Figure 3 are presented as a percentage of total $^{45}Ca^{2+}$ bound for each of 5 different liposomal charge fractions. The data represent the mean ± SD for 3 samples.

calculate that contamination of as little as 0.03% could account for this binding. If calcium binds only to acidic phospholipids, the ability of A23187 to release calcium only at very low percentages of acidic phospholipids may be due to the relatively low charge density on the surfaces of bilayers containing these low fractions of acidic phospholipids. Under our conditions, mixture of fractions of SA, a basic lipid, with PC eliminated sonication-induced association of calcium with liposomes.

These results further suggest that the action of A23187 under these conditions is to release bound calcium from calcium binding sites. This does not mean that A23187 is not a calcium ionophore. Indeed, the ionophore activity of A23187 is well documented (14). The scenario suggested by these results is that A23187 exerts its effects in two phases: first, to compete with phospholipid-calcium binding, causing release of the bound calcium, and, second, to transport released calcium from the inside the liposome to the outside. We could not determine from these experiments whether bound calcium is being released from both the internal and external faces of the liposome membrane. It is possible, owing to the extended dialysis of calcium-loaded liposomes in low-calcium medium, that little, if any, calcium remains bound to the external surface of the bilayer. This possibility is consistent with the increase in velocity of calcium release from sonicated liposomes compared with that from unsonicated liposomes. Sonication of calcium "loaded" liposomes should cause approximately half of the total (previously

internal) PL/Ca^{2+} complexes to redistribute on the outer surface of the liposomes. If one assumes that Ca^{2+} transport is rate-limiting, this should increase the initial velocity of apparent Ca^{2+} release. It is also possible that the increased velocity resulted from decreased size (and volume) of the liposomes as a result of sonication.

The finding that A23187 can act to antagonize binding of calcium to lipid binding sites is an important one. Experiments which use A23187 to transport calcium must employ appropriate controls and must be very carefully evaluated before it can be concluded that effects of A23187 are due to its action as an ionophore. Because A23187 can strip phospholipids of divalent cations such as calcium, it is also probable that micromolar concentrations of A23187 may affect the lipid bilayer (i.e., changing membrane transition temperature or local charge density at the lipid-aqueous interface). Finally, it should be stressed again that the overwhelming majority of calcium associated with "calcium-loaded" liposomes is bound calcium rather than free, entrapped calcium. This suggests that experiments which employ "calcium-loaded" liposomes or cell membrane vesicles to probe mechanisms of calcium transport must be very carefully designed, carried out, and analyzed.

REFERENCES

1. Koch, K.W., and Kaupp, U.B. (1985) J. Biol. Chem. **260**, 6788-6800.
2. Kaupp, U.B., & Koch, K.W. (1984) Vision Res. **24**, 1477-1479.
3. Cook, N.J., Zeilinger, C., Koch, K.-W., & Kaupp, U.B. (1986) J. Biol. Chem. **261**, 17033-17039.
4. Koch, K.W., and Kaupp, U.B. (1987) J. Biol. Chem. **262**, 14415-21.
5. Bauer, P.J. (1988) J. Physiol. (Lond.) **401**, 309-327.
6. Cook, N.J., Molday, L.L., Reid, D., Kaupp, U.B., & Molday, R.S. (1989) J. Biol. Chem. **264**, 6996-6999.
7. Smith, H.G., Jr, Fager, R.S., and Litman, R.J. (1977) Biochemistry **5**, 1399-405.
8. Cavaggioni, A., & Sorbi, R. (1981) Proc. Natl. Acad. Sci. USA **78**, 3964-3968.
9. Puckett, K.L., and Goldin, S.M. (1986) Biochemistry **25**, 1739-1746.
10. Pearce, L.B., Calhoon, R.D., Burns, P.R., Vincent, A., and Goldin, S.M. (1988) Biochemistry **27**, 4396-406.
11. Nicoll, D.A., and M.L. Applebury (1989) J. Biol. Chem. **264**, 16207-16213.
12. Devenny, J.J., and Clack, J.W. (1991) Biochem. Biophys. Res. Comm. **176**, 70-78.
13. Graham, R.C., and Karnovsky, M.J. (1966) J. Histochem. Cytochem. **14**, 291-307.
14. Pohl, P. *et al.* (1990) Biochim. Biophys. Acta **1027**,295-312.