A general method, employing arsenazo III in liposomes, for study of calcium ionophores: Results with A23187 and prostaglandins

(polymeric prostaglandin B₁/endoperoxide analogs/multilamellar vesicles/large unilamellar vesicles/metallochromes)

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ABSTRACT Multilamellar (MLV) and large unilamellar (LUV) lipid vesicles (liposomes) trap the metallochromic dye arsenazo III [2,7-bis(arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid] in their aqueous compartments. When ionophore A23187 was preincorporated into either MLV or LUV above 0.001 mol %, addition of Ca to the outside of liposomes produced spectral shifts characteristic of the Ca-AIII₂ complex. The method permitted detection of two molecules of A23187 per liposome. Liposomes with A23187 were permselective: divalent cations were translocated in the order Mn > Ca > Sr >> Mg ≈ Ba. Because prostaglandins (PGs) may act as Ca ionophores, we have incorporated into MLVs and LUVs stable prostaglandins (PGE₂, PGI₂, PGB₁), endoperoxide analogs, and a water-soluble, polymeric derivative of PGB₁:PGB_x. None acted as ionophore. In contrast, when added to the outside of pre-formed MLV or LUV, PGB_x, at concentrations above 1 μM, provoked permselective uptake of Ca equivalent to that induced by 10 nM A23187. These studies demonstrate not only that liposomes containing arsenazo III may be employed in a sensitive assay for agents that translocate divalent cations, but that a water-soluble derivative of a naturally occurring fatty acid, PGB_x, is a potent ionophore.

Prostaglandins (PGs) such as PGE₁, PGE₂, and PGI₂ exert their biological effects on platelets, smooth muscle, and granulocytes via surface membrane receptors (1–3). Engagement of receptors and their coupling to membrane adenylate cyclase lead to elevation of intracellular cyclic AMP (4–6). It is less clear whether the endoperoxide precursors of prostaglandins, PGG₂ and PGH₂, also act at the cell surface or at intracellular loci. The endoperoxides, which are highly unstable in aqueous media (7), or their stable methanoepoxy analogs release Ca from intracellular membrane preparations; it was suggested that endoperoxides may act as Ca ionophores (1, 4, 8). After earlier suggestions that prostaglandins of the E and F series might themselves function as Ca ionophores (9–11), evidence was adduced that endoperoxide analogs mediate Ca transfer from aqueous to nonpolar solvents (12). More recently, a polymer derived from prostaglandin B₁, PGB_x (13), has been shown to release Ca from intracellular membrane preparations and to mediate Ca transfer from aqueous to organic solvents (14), evidence that the polymer (*M_r* 2400) was a Ca ionophore.

To determine whether prostaglandins, endoperoxide analogs, or PGB_x were, indeed, Ca ionophores, we have devised a sensitive method for the detection of ionophoretic activity, which has distinct advantages over those previously employed. We have prepared multilamellar (MLV) and unilamellar (LUV) liposomes with the metallochromic dye arsenazo III (AIII)

entrapped in their aqueous compartments (15) and quantitated Ca translocation by spectral shifts of the entrapped AIII.*

By this means it was possible to detect one dimer of A23187 per liposome upon preincorporation or 10 nM A23187 when added externally. Permselectivity can be established, because other divalent cations also form complexes with AIII. Moreover, the integrity of MLV and LUV was monitored by adding excess impermeant ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which distinguishes intra- from extra-liposomal dye by dissociating the Ca-AIII₂ complex (15, 18). Having in hand a method that is highly sensitive, tests permselectivity, and reports the integrity of Ca-translocating membranes, we utilized it to study PGE₂, PGI₂, PGB₁, stable endoperoxide analogs, and PGB_x.

MATERIALS AND METHODS

Reagents. A23187 was obtained from Eli Lilly. EDTA, EGTA, Hepes, gramicidin, valinomycin, and Triton X-100 (TX-100) were supplied by Sigma; Statzyme Glucose 50 was supplied by Worthington, and AIII was from Aldrich. MnCl₂, BaCl₂, cholesterol, petroleum ether, and chloroform were from Fisher. MgCl₂ was from Mallinckrodt; SrBr₂, CaCl₂, and dextrose were from Matheson. Sources of lipids have been described (15, 16). Na-Chelex 100 was from Bio-Rad; Sephadex G-50 was obtained from Pharmacia. PGE₂, PGI₂, endoperoxide analogs I and II, and 9,11-azoprostanoid III were the generous gifts of Bengt Samuelsson (Karolinska Institutet, Stockholm). PGB_x was obtained through the courtesy of Edith Polis (Naval Air Development Center, Warminster, PA); another sample was obtained from S. Tsuyoshi Ohnishi and Thomas M. Devlin (Hahnemann Medical College, Philadelphia, PA).

Abbreviations: MLV and LUV denote multilamellar and large unilamellar liposomes, respectively, followed by the molar ratio of constituent lipids as in MLV (PC 7:DCP 2:Chol 1) in parentheses to denote phosphatidyl choline, dicetyl phosphate, and cholesterol in molar ratios as indicated. Entrapped substances are next written in square brackets: [AIII, glucose] designates liposomes after capture of arsenazo III and glucose in aqueous compartments of liposomes. When ionophores were preincorporated their concentration is expressed as mole percentage of liposomal lipid—e.g., 5 mmol % = 5 mol of ionophore per 10⁵ mol of lipid. AIII, arsenazo III [2,7-bis(arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid]; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TX-100, Triton X-100; prostaglandin endoperoxide analog I, (5Z, 9α, 11α, 13E, 15s)-9,11-methanoepoxy-15-hydroxyprosta-5,13-dienoic acid; prostaglandin endoperoxide analog II, (5Z, 9α, 11α, 13E, 15s)-9,11-epoxymethano-15-hydroxyprosta-5,13-dienoic acid; 9,11-azoprostanoid III, (5Z, 9α, 11α, 13E, 15s)-9,11-azo-15-hydroxyprosta-5,13-dienoic acid; PG, prostaglandin; PGB_x, polymeric derivative of PGB₁; X²⁺, divalent cation.

* As previously suggested (16), and agreed (17), a short-hand system of notation for liposomes will be employed.

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Preparation of AIII and Liposomes. AIII, obtained as a mixed, Ca-containing salt, was purified as described (15) by means of K-Chelex 100; aqueous solutions (3.5 mM) were brought to pH 7.5 by KOH. MLV (PC 7:DCP 2:Chol 1) [AIII, glucose] were prepared as described (15) with AIII (3.05 mM), glucose (145 mM), KCl (72 mM), and Hepes (5 mM) at pH 7.5. Swelling time was 2 hr at 23°C. LUV were prepared as described (19). Lipids (PC 7:DCP 2:Chol 1) were injected into a condenser that contained 2.2 ml of the above swelling solution.

Preincorporation of Ionophores into Liposomes. Ionophore A23187, gramicidin, or valinomycin was preincorporated into MLV by addition to lipids in chloroform (at molar ratios described below) before rotary evaporation. A23187 was preincorporated into LUV by addition to lipids in petroleum ether before injection into the condenser chamber (19). PGB_x (free acid), PGB₁, PGE₂, PGI₂, endoperoxide analogs I and II, and 9,11-azoprostanoid were dissolved in either chloroform or petroleum ether for MLV or LUV, respectively, and added to lipids as above. Absorbance spectra of A23187 and PGB_x in lipid solution assured that these had been adequately solubilized. Liposomes were applied to Sephadex G-50 columns (0.75 × 11 inch) prewashed with AIII (3.05 mM). MLV or LUV were eluted in KCl (145 mM)/Hepes (5 mM), pH 7.5; pooled fractions contained 5–6 μmol of lipid per ml. Leakage of D-glucose and solute entrapment were determined either by the solute diffusion method or the described chromatographic procedure (19, 20) by utilizing a Beckman model 25 DB twin-beam spectrophotometer.

Spectrophotometric Analysis of Liposomes with Ionophore Preincorporated. To cuvettes that had been prewashed with EDTA or EGTA were added pooled aliquots of MLV or LUV (1.0 ml). Individual absorbance spectra were obtained (from 750 to 550 nm) against solvent in the reference chamber of liposomes with and without ionophore preincorporated. The two samples were next adjusted to yield equal apparent A_{750} . Difference spectra were next obtained between liposomes with ionophore in the "sample" chamber and liposomes without ionophore in the "reference" chamber. Upon addition of increasing amounts (5 μl) of divalent cations (X^{2+} s) (0.1 M) to both cuvettes at constant time intervals (3.5 min) difference spectra were again recorded. Integrity of liposomes was monitored by addition of EDTA or EGTA in at least 10-fold molar excess of X^{2+} s to both cuvettes. Dissociation of extraliposomal X^{2+} -AIII complexes by impermeant EGTA or EDTA (15) permitted

calculation of X^{2+} uptake by *intact* liposomes after TX-100 (0.06%) had been added to both cuvettes; this was arrived at by subtracting the spectrum obtained after TX-100 lysis from that obtained after EDTA- or EGTA-induced dissociation (15).

Spectrophotometric Analysis of Ionophores Added to Liposomes. Two cuvettes, each containing MLV or LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] were adjusted to yield equal apparent absorbance at 750 nm and placed in "sample" and "reference" chambers. Agents were added to "sample" cuvettes, appropriate solvent (dimethyl sulfoxide and H₂O, or H₂O) was added to the "reference" cuvettes, and difference spectra (750 – 550 nm) were obtained. Sequentially, incremental amounts of X^{2+} , EDTA or EGTA, and TX-100 were added to both cuvettes, and uptake of X^{2+} by intact liposomes was calculated as above.

Translocation of X^{2+} s. To eliminate variables introduced by interactions of X^{2+} with lipids, difference spectra were obtained between two cuvettes containing MLV or LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] to both of which TX-100 (0.06%) had been added. Thereafter, X^{2+} s were added to the "sample" cuvette, and buffer was added to the "reference". Standard curves were generated by adding increasing amounts of X^{2+} to a constant amount of AIII; absorbances were read at the wavelength that yielded the greatest difference between AIII and its X^{2+} -AIII complex (21, 22).

RESULTS

Incorporation of Ionophore A23187 into Liposomes: Entrapment and Diffusion. Preparations of MLV and LUV with varying molar ratios of A23187 were analyzed for entrapment of AIII and glucose and for solute diffusion (19). With no ionophore preincorporated, MLV (PC 7:DCP 2:Chol 1) [AIII, glucose] trapped $4.6 \pm 0.2\%$ ($n = 3$) of AIII or 14 ± 2 mmol of AIII per mol of lipid. Percentage of glucose entrapped was 4.09 ± 0.70 ($n = 13$) or 994 mmol of glucose per mol of lipid. Incorporation of A23187 at 5, 10 or 100 mmol % did not significantly alter entrapment of AIII or glucose. Glucose diffusion (as percentage of total entrapped solute) was 6.01 ± 0.88 at 90 min and 37°C. Incorporation of A23187 at 5, 10, or 100 mmol % did not change this diffusion parameter.

LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] trapped $9.5 \pm 2.0\%$ ($n = 7$) of AIII or 35 ± 2 mmol of AIII per mol of lipid. Entrapment of glucose by LUV was $7.6 \pm 1.4\%$ ($n = 9$) or 2.46 mol of glucose per mol of lipid. Preincorporation of 10 mmol % of A23187 decreased percentage entrapment to 4.7 for AIII and

FIG. 1. Absorbance spectra of LUV with and without A23187 incorporated with AIII and glucose entrapped. (A) Spectra of LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] (bottom) and of LUV (PC 7:DCP 2:Chol 1:A23187 0.001) [AIII, glucose]. (B) Difference spectra of LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] minus LUV (PC 7:DCP 2:Chol 1) [AIII, glucose] plus varying Ca_{out}^{2+} . (C) Difference spectra as in B. (D) Difference spectra of LUV (PC 7:DCP 2:Chol 1:A23187 0.001) [AIII, glucose] minus LUV (PC 7:DCP 2:Chol 1).

FIG. 2. Ca uptake (3.5 min) by MLV [AIII, glucose] with ionophore A23187 preincorporated at varying molar percentages (0.001–0.1%) in the presence of Ca_{out} at concentrations indicated.

to 3.8 for glucose ($n = 2$). However, 5 mmol % of A23187 did not change entrapment ($n = 2$). Glucose diffusion from LUV (PC 7:DCP 2:Chol 1)[AIII, glucose] was $5.79 \pm 1.5\%$ ($n = 7$) at 90 min and was unaffected by addition of up to 10 mmol % of A23187. When MLV and LUV were prepared without cholesterol—i.e., MLV or LUV (PC 8: DCP 2)[AIII, glucose]—entrapment and diffusion data did not differ significantly from controls ($n = 3$).

Ca and Mg Uptake by Liposomes with A23187 Preincorporated. When preincorporated into LUV at 10 mmol %, A23187 provoked Ca uptake (Fig. 1). Absorbances of LUV were unaffected by preincorporated ionophore (Fig. 1A). Difference spectra (Fig. 1B) of LUV $\pm$ ionophore with increasing Ca_{out} yielded absorbance increments characteristic of $\text{Ca}\cdot\text{AIII}_2$ complexes (15, 22). Addition of 10-fold excess EGTA dissociated extraliposomal $\text{Ca}\cdot\text{AIII}_2$ complexes resulting from liposomal fragility (Fig. 1C). Finally, lysis of LUV by TX-100 allowed

Table 1. Divalent cation uptake in MLV induced by A23187 and PGB_x

Ion	mol X^{2+} /mol lipid		
	I*	II†	III‡
Mn^{2+}	0.46	0.46	0.30
Ca^{2+}	0.30	0.38	0.20
Sr^{2+}	0.20	0.38	0.50
Ba^{2+}	0.05	0.19	0.34
Mg^{2+}	0.05	0.10	0.14

* MLV (PC 7:DCP 2:Chol 1:A23187 0.001)[AIII, glucose].

† MLV (PC 7:DCP 2:Chol 1)[AIII, glucose]; 95 nM A23187 (in dimethyl sulfoxide) was added externally.

‡ MLV (PC 7:DCP 2:Chol 1) to which the Na salt of PGB_x (31 μM) had been added externally.

FIG. 3. Permeability (Ca vs. Mg vs. glucose) of MLV or LUV (PC 7:DCP 2:Chol 1: $\pm$ A23187 0.001)[AIII, glucose]. $\bullet$, Ca, $\circ$, Mg; Δ , control; $\blacktriangle$, with A23187. (A) MLV with varying Ca_{out} , Mg_{out} . (B) Glucose diffusion from MLV $\pm$ ionophore with respect to time. (C) LUV with varying Ca_{out} , Mg_{out} . (D) Glucose diffusion from LUV $\pm$ ionophore with respect to time.

access of EGTA to $\text{Ca}\cdot\text{AIII}_2$ within liposomes and permitted calculation of Ca uptake by LUV. To compare uptake of Ca with that of Mg, we performed identical experiments with Mg (Fig. 1D). Addition of Mg (5 mM) produced modest increments in absorbance (maximum at 610 nm); these were reversed upon addition of excess EDTA. No significant translocation of Mg into intact liposomes was demonstrated (see below).

Uptake of Ca proceeded at a constant rate between 3 and 30 min after Ca addition. From standard curves in which AIII (3.05 mM) was titrated against increasing Ca concentration, Ca uptake was quantified by absorbance increments at 656 nm. At various molar percentage of A23187 preincorporated, Ca

Table 2. Calcium uptake by MLVs with 10 mmol % of putative ionophore preincorporated and by MLVs and LUVs with putative ionophores added externally

Agent	MLV (prein- corporated)	Added externally*	
		MLV	LUV
None	0.00	0.00	0.00
A23187	0.20	0.07	1.32
PGB_x	0.02	0.08	1.62
PGB_1	0.00	0.00	0.00
PGE_2	0.00	0.00	0.00
PGI_2	0.00	0.00	0.00
Analog I	0.00	0.00	0.00
Analog II	0.00	0.00	0.00
9,11-Azoprostanoid III	0.00	0.00	0.00

* Concentrations employed: A23187 (22 nM); PGB_x (6.3 μM); PGE_2 and PGB_1 (25 μM); PGI_2 , endoperoxide analogs I and II, and 9,11-azoprostanoid III (7.5 μM).

FIG. 4. Difference spectra of LUV (PC 7:DCP 2:Chol 1)[AIII, glucose] $\pm$ PGB_x (6.3 μ M) added externally. (A) With varying Ca_{out}. (B) With 5 mM Ca. (C) With varying Mg_{out}. (D) With 5 mM Mg_{out}.

uptake by MLV was plotted as a function of Ca_{out} (Fig. 2). After final addition of Ca, excess EGTA was added to dissociate extraliposomal Ca-AIII₂ complexes. Reversal of absorbance increments by EGTA at 656 nm increased from 20% with 0.001 mol % of A23187 preincorporated to 30% at 0.1 mol %. When A23187 was preincorporated into LUV at 1, 5, or 10 mmol % (data not shown), Ca was taken up in amounts equivalent to those shown in Fig. 2. Additional experiments were performed with cholesterol omitted from MLV or LUV—e.g., LUV (PC 8:DCP 2)[AIII, glucose]; Ca uptake in liposomes with 10 mmol % of A23187 was not influenced by omission of cholesterol.

Translocation of Other X²⁺s by A23187: Permselectivity. Various metal X²⁺s produce characteristic spectral shifts of AIII (15, 22). Absorbance changes in AIII, each at the maximum for a given cationic species, were plotted against X²⁺_{out}. Slopes of resultant curves were 100, 40, 40, 40, and 80 for Ca, Sr, Ba, Mn, and Mg, respectively. Ca was taken up by MLV and LUV with A23187 preincorporated far more readily than was Mg (Fig. 3 A and C). Glucose diffusion was unaffected by the presence of A23187 (10 mmol %) in LUV or MLV (Fig. 3 B and D). Translocation of other divalent cations (Mn, Sr, and Ba) was compared with those of Ca and Mg (Table 1). The rank order of translocation mediated by A23187 (either preincorporated or added externally to preformed liposomes) was: Mn > Ca > Sr >> Ba $\approx$ Mg, the order described by Pfeiffer and Lardy (23) for transfer of metal cations by A23187 from aqueous to organic phases. To exclude the possibility that any ionophore incorporated into liposomes might translocate Ca nonspecifically, the channel-forming ionophore, gramicidin, and the K⁺-selective carrier, valinomycin, were preincorporated at 10 mmol %. Neither provoked Ca uptake by MLV.

Prostaglandins, Endoperoxide Analogs, and PGB_x as Ionophores. Prostaglandins, stable endoperoxide analogs, and PGB_x (Table 2) were either preincorporated into LUV and MLV or added externally to preformed liposomes. Under neither condition did any of the prostaglandins, the methanoepoxy analogs, or the azoprostanoid induce significant uptake of Ca. However, PGB_x, when added externally to MLV or LUV, induced uptake of Ca comparable to that induced by A23187, albeit at higher concentration. When either PGB_x or A23187 was added externally, more Ca was taken up by LUV than by MLV. This result is entirely expected; the outermost aqueous compartment of MLV (containing AIII capable of complexing with Ca translocated across the first bilayer) constitutes only 10% of the interior (24). By utilizing spectrophotometric

methods (Fig. 4) analogous to those for A23187, we studied PGB_x with respect to Ca uptake and permselectivity (Table 1). The rank order of translocation of several X²⁺s differs radically from that of A23187; PGB_x induces translocation of Sr > Ba > Mn > Ca > Mg. This order excludes the possibility that the permselectivity found for A23187 is the result of affinity for divalent cation of the metallochrome (AIII) rather than of the ionophore itself. Finally, dose-response comparison (Fig. 5) between A23187 and PGB_x (both added externally) show that on a molar basis PGB_x is 1/100th–1/1000th as active as A23187. Conventional prostaglandins or endoperoxide analogs show no ionophoretic activities.

FIG. 5. Ca uptake as a function of concentration of A23187 (in 1% dimethyl sulfoxide) or PGB_x added externally to MLV (PC 7:DCP 2:Chol 1)[AIII, glucose]. Difference spectra ($\pm$ agent) were obtained in the presence of Ca_{out} (3 mM) at 6 min.

DISCUSSION

The suggestion that prostaglandins and their endoperoxide precursors act as Ca ionophores is based upon two lines of evidence. Firstly, PGE₁, PGB₂, and PGA₁ provoked Ca release from mitochondria (9), PGE₂ released Ca from sarcoplasmic reticulum vesicles (10), and the endoperoxides (PGG₂ and PGH₂) promoted Ca release from a platelet membrane fraction (8). In each instance, millimolar concentrations of prostaglandins were required to produce effects comparable to micromolar concentrations of the carboxylic acid ionophore A23187 (8–10). Secondly, PGB₂, PGA₁, PGE₂, and stable endoperoxide analogs transfer Ca from aqueous into organic solvents in two-phase systems (11, 12). Again, the activity of prostaglandins and endoperoxide analogs, measured at pHs above 8, was found to be 1/100th or 1/1000th that of A23187 (12). The very weak Ca-transporting properties of these compounds differ markedly from those described for PGB_x, a polymeric derivative of PGB₁. This "stable free radical" derivative (13) released Ca from vesicles of sarcoplasmic reticulum or beef heart mitochondria and translocated Ca from aqueous to organic solvents (14). Our results do not support the contention that prostaglandins or their endoperoxide precursors are Ca ionophores at concentrations that can be obtained in cells (10). They do, however, support the suggestion of Ohnishi and Devlin (14) that PGB_x is a potent Ca ionophore.

The sensitivity of the method may be judged by calculating the number of molecules of A23187 per liposome that can be detected when the ionophore is preincorporated. LUVs extruded from petroleum ether have a mean diameter of 2.1×10^3 Å (19). The area of an average lipid in LUV (PC 7:DCP 2:Chol 1) is 80 Å^2 (25). Because the surface area (πD^2) is $1.4 \times 10^7 \text{ Å}^2$, there are 1.7×10^5 lipid molecules in the outer leaflet and 3.4×10^5 in the bilayer. Ca uptake was detectable (see Results) at 1–5 parts of A23187 per 10^5 parts of lipid; we can therefore detect two molecules of A23187 [one dimer: the postulated ionophoretic configuration in lipid bilayers (26)] per liposome. When ionophores were added to preformed liposomes, we were able to detect the activity of 10 nM A23187 or micromolar amounts of PGB_x. Moreover, the method permits study of the rank order of translocation of several X^{2+} s; both A23187 and PGB_x favored movement of Ca over that of Mg across the bilayers. Finally, the integrity of the transporting lipid assembly could be evaluated either by measurements of glucose diffusion or by dissociating extraliposomal Ca-AIII₂ complexes with excess EGTA.

Whereas liposomes containing AIII have the advantages of sensitivity, permselectivity and convenience over other assay systems for ionophoretic activity, no conclusions can be drawn with respect to ionophoretic activity of any agent that would require unusual lipids for its action (e.g., gangliosides, ceramides, etc.) or whose action is dependent upon membrane proteins. The method has, however, permitted two conclusions as to the putative Ca-ionophoretic activity of prostaglandins and their endoperoxide precursors. Firstly, these agents are not Ca-ionophores at micromolar concentration in model lipid bilayers, unlike the reference ionophore A23187 with which

they are usually compared (8–10,14). Secondly, a water-soluble derivative of PGB₁, PGB_x, is a true ionophore for X^{2+} s. Whether polymeric derivatives of PGB₁ or other prostaglandins can be formed by living cells remains to be determined.

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