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Essential adaptation of the calcium influx assay into liposomes with entrapped arsenazo III for studies on the possible calcium translocating properties of acidic phospholipids

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An adapted version of the Ca^{2+}-influx assay of Weissmann et al. (Weissmann, G., Anderson, P., Serhan, C., Samuelson, E. and Goodman, E. (1980) Proc. Natl. Acad. Sci. USA 77, 1506–1510) is presented for studies on the possible ionophoretic properties of acidic phospholipids. This method is based on the use of the metallochromic dye arsenazo III enclosed in liposomal vesicles, to indicate the Ca^{2+} influx. An essential control is introduced to discriminate between Ca^{2+}-arsenazo III complex formation inside the vesicles, as a consequence of Ca^{2+} influx, and outside the vesicles, as a consequence of arsenazo III leakage from the vesicles. Furthermore, some minor improvements are added, like the use of large unilamellar vesicles instead of multilamellar vesicles, and the use of dual wavelength spectrophotometry. Using this method, it was found that dioleoylphosphatidylcholine vesicles, containing 20 mol% dioleoylphosphatidylglycerol, were impermeable to Ca^{2+}. In this system a selective Ca^{2+} permeability could be induced by the addition of the fungal Ca^{2+} ionophore A23187. In contrast, dioleoylphosphatidylcholine vesicles, containing 20 mol% dioleoylphosphatidic acid, incubated in the presence of Ca^{2+} were permeable to both Ca^{2+} and arsenazo III.

From observations on neurotransmitter-excitable membranes it has been speculated that phosphatidic acid may play an active role in calcium translocation over biological membranes [1,2]. Serhan et al. [3,4], using the method of Weissmann et al. [5], demonstrated that phosphatidic acid derived from egg-yolk phosphatidylcholine could act as a calcium ionophore in multilayered liposome systems. Holmes and Yoss [6], however, were not able to reproduce these findings and stated that only oxidized phosphatidic acids could have calcium ionophoretic properties. Recently Chauhan and Brockerhoff [7] confirmed the results of Serhan et al. [4] again using the same method and Nayar et al. [8] came to similar conclusions on basis of $^{45}Ca^{2+}$-influx experiments in vesicles, enclosing different Ca^{2+} chelators. The assay system of Weissmann et al. [5] for determining Ca^{2+} influx into liposomes, is based on a spectroscopic method with the metallochromic dye arsenazo III entrapped inside the liposome structures. Arsenazo III has a high affinity for Ca^{2+} [9], is available in chemically pure form and, when it is bound to Ca^{2+}, has a very intense absorbance at 650 nm [9]. These properties make arsenazo III a useful tool for Ca^{2+} influx studies, notwithstanding the rather complex kinetics of Ca^{2+} binding to arsenazo III [10].

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In this paper an improved version of the Ca^{2+} influx assay with arsenazo III is described. The main adaptation of the assay is the inclusion of a control to see whether the Ca^{2+}-arsenazo III complex formation is a consequence of influx of Ca^{2+} into the vesicles, or efflux of arsenazo III from the vesicles.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized from egg yolk phosphatidylcholine according to standard procedures [11]. 1,2-Dioleoyl-*sn*-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were obtained from DOPC by phospholipase D catalyzed hydrolysis and transesterification [12]. After purification and conversion to their Na^+ salts [13], the products contained no detectable calcium (< 0.5 mol%) as determined by atomic absorbance spectrophotometry. All phospholipids were chemically pure (> 99%) as indicated by two-dimensional high performance thin-layer chromatography. Lipid phosphorus was determined according to Böttcher et al. [14]. Large unilamellar vesicles were prepared in buffer, containing 2.3 mM arsenazo III (Sigma; sodium salt, grade 1), 150 mM KCl and 10 mM Tris/acetate (pH 7.4), by the reverse-phase evaporation method of Szoka and co-workers [15]. To obtain a more homogeneous size distribution, the vesicles were subsequently extruded through a polycarbonate filter (Bio-Rad Uni-Pore; 0.4 μm pore size) [15].

The trapped volume of the vesicles was calculated from K^+ trap measurements [16], and was found to be 5–6 μl/μmol phospholipid. Non-trapped arsenazo III in the vesicle suspension was removed by gel filtration over a 1×10 cm Sephadex G-50 column with 150 mM KCl, 10 mM Tris/acetate (pH 7.4) buffer. The vesicle suspension thus obtained was diluted to a phospholipid concentration of approximately 3 mM and kept on ice prior to the incubations.

The calcium influx experiments were principally based on the method of Weissmann et al. [5]. All experiments were carried out at 20°C. An incubation was started by adding 1.0 ml of the phospholipid vesicle suspension to 4.0 ml of a buffer comprised of 10 mM Tris/acetate (pH 7.4) and 150 mM KCl, containing 10 μmol $CaCl_2$ and, in some test experiments, 40 ng A23187 (Sigma; added as a 10 μg/ml solution in methanol). The presence of Ca^{2+} in the incubation medium prior to the addition of the vesicles prevents temporary occurrence of high local Ca^{2+} concentrations. The incubation was carried out in polyethylene vials (5 ml), which were gently shaken. The amount of Ca^{2+}-arsenazo III complex formed during the incubation was determined with a double beam spectrophotometer (Hitachi Perkin-Elmer; model 356). The absorbance of the samples at 650 nm (maximal extinction coefficient of Ca^{2+}-arsenazo III) was measured with the absorbance at 700 nm as a reference (isosbestic point of the spectra of arsenazo III and Ca^{2+}-arsenazo III). The dual wavelength detection mode was used to correct for variations in light scattering during the incubation instead of the split beam mode with two cuvettes as described by Serhan et al. [4] in which an extra measurement is necessary to correct for the changes in absorbance at 750 nm of the sample. During the experiment the turbidity of the incubation suspension is increased. This is mainly caused by the interaction of Ca^{2+} with the negatively charged phospholipids in the vesicles, resulting in vesicle aggregation and, at higher Ca^{2+} concentrations, in membrane fusion [17]. In typical incubations the change in absorbance (measured at 700 nm) caused by this turbidity increase was in the same order of magnitude as the corrected real absorbance increase at 650 nm caused by the formation of the Ca^{2+}-arsenazo III complex. After 1, 15, 60 and 120 min of incubation, aliquots (1 ml) were taken and the absorbance was measured. To determine whether the increase in absorbance was caused by Ca^{2+}-arsenazo III complex formation inside or outside the vesicles, the following procedure was carried out. To the 1 ml sample in the cuvette 50 μl 0.5 M Na_2EDTA, 10 mM Tris/acetate (pH 7.4) was added. EDTA chelates extravesicular Ca^{2+} and causes dissociation of the Ca^{2+}-arsenazo III complex present outside the vesicles. The immediate decrease in absorbance corresponds to the amount of extravesicular arsenazo III. Subsequently 5 μl 0.1 mg/ml A23187 in methanol was added to the sample. This excess of A23187 makes the membrane highly permeable for Ca^{2+}. In the presence of external EDTA, a fast efflux of Ca^{2+} along with a complete dissociation of intravesicular Ca^{2+}-arsenazo III complex results. This causes a decrease in absorbance which corresponds with

the amount of intravesicular Ca^{2+}-arsenazo III complex. All the presented data are expressed at percentages of the potential maximal absorbance of the incubation suspension, i.e., the absorbance at which arsenazo III is saturated with Ca^{2+}. This maximal absorbance was determined by adding 5 μl 0.1 mg/ml A23187 (in methanol) to 1 ml of the Ca^{2+} containing sample.

Fig. 1 shows the binding of Ca^{2+} to arsenazo III during an incubation of large unilamellar vesicles containing 80 mol% DOPC and 20 mol% DOPG or DOPA. In the case of DOPG-containing vesicles the bilayer is impermeable and Ca^{2+} can only reach the arsenazo III when the fungal Ca^{2+} ionophore A23187 is added. When DOPA-containing vesicles were incubated under the same conditions, a significant amount of Ca^{2+} was bound to arsenazo III. So it seems with these vesicles that Ca^{2+} is translocated to the inside of the vesicles. These findings, with respect to phosphatidic acid containing vesicles, are in agreement with observations of Serhan et al. [3,4] and Chauhan and Brockerhoff [7], who did essentially the same kind of experiments. However, when a discrimination is made between the amount of Ca^{2+}-arsenazo III formed inside and outside the vesicles during the incubation of DOPA-containing vesicles (Fig. 2), it can be concluded that the complex formation of Ca^{2+} and arsenazo III is not only a consequence of Ca^{2+} influx but also of arsenazo III efflux. Fig. 2 shows that EDTA addition after incubation of the vesicles with a Ca^{2+}-containing buffer causes dissociation of a fraction of the Ca^{2+}-arsenazo III complex. Because this fraction is instantly dissociated by the added EDTA, we suppose it to be extravesicular Ca^{2+}-arsenazo III complex. To check that the occurrence of the extravesicular fraction is not caused by the EDTA addition itself as suggested by Serhan et al. [4], control experiments were carried out. The extravesicular Ca^{2+}-arsenazo III complex was removed by gel filtration of samples of the incubation suspension (0.5 ml) over small Sephadex G-75 columns (5 × 60 mm) with an elution buffer containing 2 mM $CaCl_2$, 150 mM KCl and 10 mM Tris/acetate (pH 7.4), essentially according to Serhan et al. [3]. The amount of

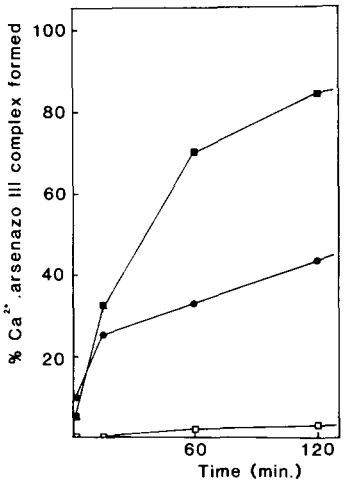

Fig. 1. Ca^{2+}-arsenazo III complex formation during incubation of large unilamellar vesicles with entrapped arsenazo III in a Ca^{2+} containing medium. The vesicles were incubated in a buffer containing 2 mM $CaCl_2$, 150 mM KCl and 10 mM Tris/acetate (pH 7.4) with a phospholipid concentration of approximately 0.6 mM. At various times aliquots were taken and the absorbance at 650 nm was measured with the absorbance at 700 nm as a reference. The data are expressed as percentages of the potential maximal absorbance of the incubation suspension. ●, DOPA/DOPC vesicles (20:80 mol%); □, DOPG/DOPC vesicles (20:80 mol%); ■, DOPG/DOPC vesicles (20:80 mol%), incubated in the presence of 8 ng/ml A23187. For further details, see text.

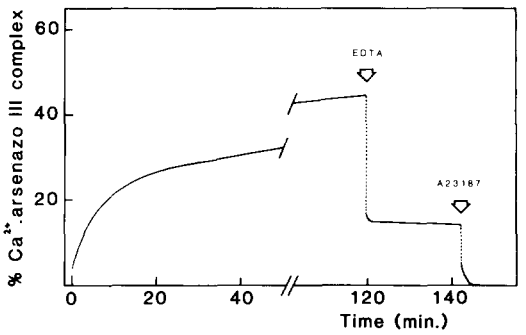

Fig. 2. Incubation of DOPA/DOPC (20:80 mol%) large unilamellar vesicles with entrapped arsenazo III in a Ca^{2+}-containing medium, followed by discrimination between intra- and extravesicular fraction of the formed Ca^{2+}-arsenazo III complex. The experimental conditions are described in the text. The absorbance change of the incubation suspension at 650 nm with the absorbance at 700 nm as a reference was followed continuously. At points indicated with EDTA and A23187, the EDTA-containing buffer and the A23187 solution, respectively, were added as described in the text. The amount of Ca^{2+}-arsenazo III complex formed is expressed as percentage of the potential maximal absorbance of the incubation suspension.

intra- and extravesicular Ca^{2+}-arsenazo III complex, which was determined with this method, was the same as that found with the EDTA addition method. Furthermore, the A23187-induced Ca^{2+} influx did not appear to be accompanied by an efflux of arsenazo III (Fig. 3). These results for DOPA-containing vesicles are in contrast with the findings of Serhan et al. [4], who did not assess higher extraliposomal arsenazo III concentrations than 5% when using multilamellar vesicles containing phosphatidic acid derived from egg-yolk phosphatidylcholine. From the results presented in Fig. 3 it can be concluded that A23187-mediated influx of Ca^{2+} shows the kinetics of a gradient-driven facilitated diffusion. In contrast, phosphatidic acid-mediated Ca^{2+} translocation shows a high initial Ca^{2+} influx which stops gradually within a few minutes, whilst the arsenazo III efflux continues over a larger time period. The arsenazo III efflux is a direct consequence of Ca^{2+}-phosphatidic acid interaction, because incubation of phosphatidic acid containing vesicles in a Ca^{2+}-free buffer does not result in any arsenazo III efflux as detected after Sephadex G-75 gel filtration. The leakage of K^+ from DOPA/DOPC (20 : 80 mol%) large unilamellar vesicles, measured according to Mandersloot et al. [16], was found to be not significantly different in the absence and the presence of 2 mM Ca^{2+}. So, during the incubation of these vesicles with calcium, no lysis or aspecific leakage does occur. This is in accordance with the results of the $^{22}Na^+$ leakage experiments of Nayar et al. [8]. The Ca^{2+}-induced permeability changes in phosphatidic acid-containing membranes seem to be very complicated and further information about the specificity of the permeability change is needed. The understanding of these findings will require a detailed knowledge of the phase behaviour of the phosphatidic acid/phosphatidylcholine bilayers in the presence and the absence of Ca^{2+}.

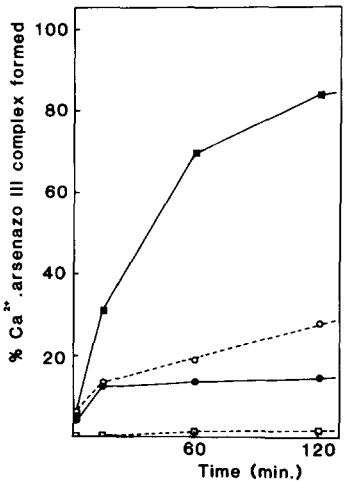

Fig. 3. Discrimination between intra- and extravesicular Ca^{2+}-arsenazo III complex, formed during incubation of large unilamellar vesicles with entrapped arsenazo III in a Ca^{2+} containing medium. The experiments were carried out as described in the text. The data are expressed as percentages of the potential maximal absorbance of the incubation suspension at 650 nm with the absorbance at 700 nm as a reference. ●, ○, DOPA/DOPC vesicles (20 : 80 mol%); ■, □, DOPG/DOPC vesicles (20 : 80 mol%) incubated in the presence of 8 ng/ml A23187. Closed symbols, solid lines: intravesicular Ca^{2+}-arsenazo III; open symbols, broken lines: extravesicular Ca^{2+}-arsenazo III.

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