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Calcium inhibits diacylglycerol uptake by serum albumin

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ABSTRACT

Serum albumin is an abundant protein in blood plasma, that is well-known for its ability to transport hydrophobic biomolecules and drugs. Recent hypotheses propose that serum albumin plays a role in the regulation of lipid metabolism in addition to its lipid transport properties. The present work explores the capacity of bovine serum albumin (BSA) to extract diacylglycerols (DAG) from phospholipid bilayers, and the inhibition of such interaction by divalent cations. Quantitative measurements using radioactive DAG and morphological evidence derived from giant unilamellar vesicles examined by confocal microscopy provide concurrent results. BSA extracts DAG from vesicles consisting of phosphatidylinositol/DAG. Long, saturated DAG species are incorporated more readily than the shorter-chain or unsaturated ones. Divalent cations hinder DAG uptake by BSA. For Ca^{2+}, the concentration causing half-maximal inhibition is ≈10 μM; 90% inhibition is caused by 100 μM Ca^{2+}. Sr^{2+} requires concentrations one order of magnitude higher, while Mg^{2+} has virtually no effect. As an example on how DAG uptake by BSA, and its inhibition by Ca^{2+}, could play a regulating role in lipid metabolism, a PI-specific phospholipase C has been assayed in the presence of BSA and/or Ca^{2+}. BSA activates the enzyme by removing the end-product DAG, but the activation is reverted by Ca^{2+} that inhibits DAG uptake.

1. Introduction

Serum albumin is the major protein present in mammalian plasma. Its physiological roles include maintaining the colloid osmotic pressure, and the transport of apolar substances, such as fatty acids, bilirubin, or drugs [1,2]. Transport of fatty acids, either free or esterified, is particularly important because serum albumin is nowadays considered to play an active role in lipid metabolism beyond mere transport, as a lipid mediator between plasma, lipoprotein and tissue [3]. A recent report [4] on the analysis of esterified fatty acids bound to serum albumin by MALDI mass spectrometry indicated that an abundant species in human albumin lipid extract was diacylglycerol (DAG).

Apart from its role as an intermediate in lipid metabolism, DAG is an important enzyme modulator [5,6] and a potent regulator of cell membrane physiology [7]. DAG plays a major role in cell signalling [8], and in addition it can modify the bilayer properties thus modulating the activities of a number of membrane related enzymes [6,9,10]. DAG can also facilitate bilayer-to-non-bilayer transitions, and subsequent membrane fusion/fission events [6,11]. A major source of DAG in the cell membranes is phospholipase C (PLC), that cleaves phospholipids yielding diacylglycerol plus a water-soluble phosphoryl-base [9,12,13]. At least some PLC are in turn regulated by their end-product DAG [5,14,15].

Serum albumin is known to be effective in extracting DAG from lipid bilayers [16], thus it can influence in this way a number of the DAG effects mentioned above. Indeed, serum albumin has been used as an "activator" in PLC assays, due to its ability to remove the end-product DAG [12]. Serum albumin can bind Ca^{2+} and other cations via electrostatic interactions [17]; co-precipitation of bovine serum albumin (BSA) and Ca^{2+} has also been demonstrated [18]. In addition, Guillaume et al. [19] have shown Ca^{2+}-induced changes in the microenvironment of aromatic residues of BSA, thus confirming the metal–protein interaction. A complex interplay of Ca^{2+} and DAG, among other factors, in the regulation of a protein kinase C, assayed in 1% BSA, has also been described [20].

The present paper reports on the interaction of BSA with DAG, and shows that Ca^{2+} inhibits lipid uptake by this protein. Our study is based on vesicles composed essentially of phosphatidylinositol (PI). This is mainly due to the fact that this work originated from observations in relation to a PI-specific phospholipase C. Actually, an apparent Ca^{2+} inhibition of a PLC assayed in the presence of albumin is presented as an example of a possible biological consequence of our observation.

2. Materials and methods

2.1. Materials

Wheat germ phosphatidylinositol (PI) and egg diacylglycerol (DAG) were purchased from Lipid Products (South Nutfield, UK). Fatty acid

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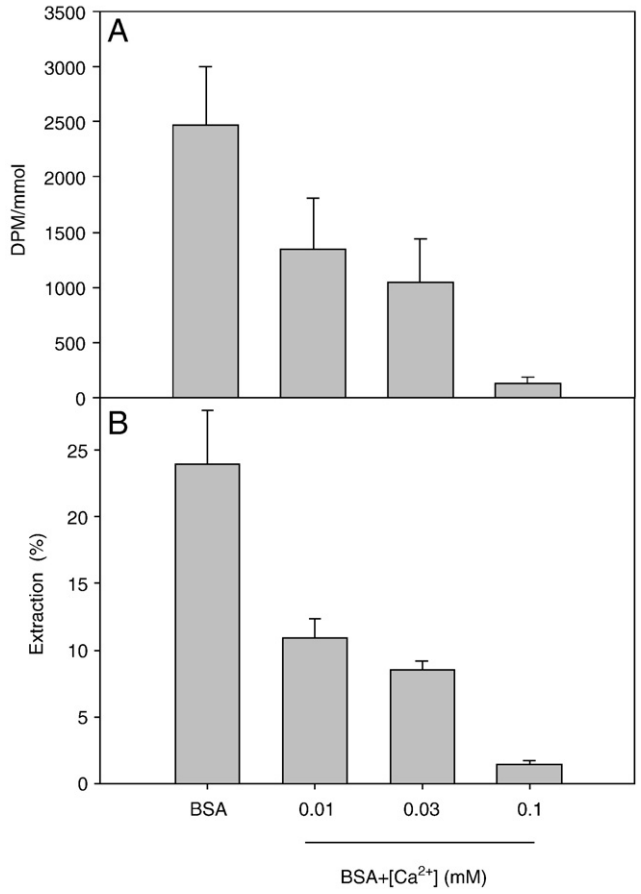

Fig. 1. DAG extraction by BSA from PI/DAG bilayers, and its inhibition by Ca^{2+}. Experiments were performed in the absence (BSA) or in the presence (BSA+Ca^{2+}) of various Ca^{2+} concentrations. Quantification was achieved with 14C-DAG. (A) Radioactive count. (B) Percent BSA extraction. Total concentrations were 1 mMDAG, 15 μM BSA. Average values±SD (n=3–4).

distribution in DAG was: 16:0(32.1%), 16:1(2.1%), 18:0(11.7%), 18:1 (36.2%), 18:2(12.5%), 20:4(5.5%). 1,2-dimyristoyl-*sn*-glycerol (DMG), 1,2-dioleoyl-*sn*-glycerol (DOG), 1,2-dipalmitoyl-*sn*-glycerol (DPG) and 1,2-distearoyl-*sn*-glycerol (DSG) were from Avanti Polar Lipids (Alabaster, AL). PI-PLC from *Bacillus cereus* and *DiI-C18* were purchased from Molecular Probes (Eugene, OR). Dioleoyl-*rac*-glycerol [oleoyl-1-^{14}C] (55 mCi/mmol) was obtained from Larodan (Fine Chemicals AB). The silica gel 60-G plates were from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) (essentially free from fatty acids) was from Sigma Chemical Co (St. Louis, MO). Salts, organics solvents, and other reagents were of analytical grade and were supplied by Merck (Darmstadt, Germany). All buffers were prepared with Milli-Q water (Millipore, Billerica, MA).

2.2. Large unilamellar vesicles

Large unilamellar vesicles (LUV) made either of pure phosphatidylinositol or of phosphatidylinositol:diacylglycerol were prepared by extrusion through polycarbonate filters (Nuclepore, Pleasanton, CA), pore size 0.1 μm. Buffer was 150 mM NaCl, 10 mM HEPES, pH 7.4. The diameter of the resulting vesicles was of 100 nm, according to quasi-elastic light scattering measurements. More details on the preparation of these vesicles can be found in Mayer et al. [21].

2.3. Calcium assay

Ca^{2+} determinations were carried out with an ELAN 9000 ICP-MS (Perkin-Elmer SCIEX, Thornhill, ON, Canada) equipped with a cross-flow nebulizer and Scott spray chamber using the following operating conditions: Nebulizer flow 0.93 L/min, Plasma flow 15 L/min, Auxiliary flow 1.2 L/min, Radiofrequency power 1100 W. External calibration was performed with scandium as internal standard, with calibration and samples containing 1% HNO_3. Standard solutions were prepared out of Specpure stock solutions of 1000 mg/L from Alfa Aesar (Ward Hill, MA) containing each of the elements. $_{43}$Ca and $_{45}$Sr isotopes were measured in peak hopping mode with a dwell time of 100 ms and 3 replicates for each solution.

2.4. Extraction of diglycerides by BSA

2.4.1. TLC assay

After incubation of liposomes (PI/DAG) (1/0.3 mol ratio) in HEPES buffer containing 0.1% BSA (approx. 15 μM), in the presence or absence of different concentrations of divalent cations at 39 °C, the mixture was centrifuged at 510,000 ×g (4 °C, 2 h) in a Beckman TLA 120.2 rotor [22]. After centrifugation, the supernatant was collected and DAG was extracted with chloroform/methanol/HCl (66:33:1, v/v/v). Samples were centrifuged and a known volume of the lower, organic phase recovered and evaporated to dryness. Lipid samples were resuspended in 50 μL chloroform, and applied to thin-layer chromatography (TLC) plates of silica gel 60-G. Plates were run using a solvent containing n-hexane, diethyl ether, and glacial acetic acid in a ratio (7:3:4, v/v/v). The silica plates were then removed, air dried and sprayed with copper sulfate phosphoric acid reagent [23] and heated at 180 °C for 1 min to visualize the lipid spots. The plates were scanned with a GS-800 calibrated densitometer (Bio-Rad) and processed with Quantity-One 4.4 (Bio-Rad). The concentration of DAG was determined by comparing the spots of extracted DAG and the spots of stocks with known DAG concentrations.

2.4.2. Radioactivity assay

Liposomes (PI/DAG) (1/0.3 mol ratio), prepared as described above, were incubated with dioleoyl-*rac*-glycerol [oleoyl-1-^{14}C] (1 μCi/mL) at room temperature for 2 h. To separate the free ^{14}C-DAG, the liposomes were washed three times with HEPES buffer by centrifugation at 510,000 ×g for 2 h and then reincubated for 30 min with 0.1% BSA, in the presence or absence of different concentrations of Ca^{2+} at 39 °C. The mixture was centrifuged at 510,000 ×g (4 °C, 2 h) in a Beckman TLA 120.2 rotor. After centrifugation, the supernatant and pellet were collected. The ^{14}C radioactivity was determined using a Minaxi Tricarb 2900 scintillator.

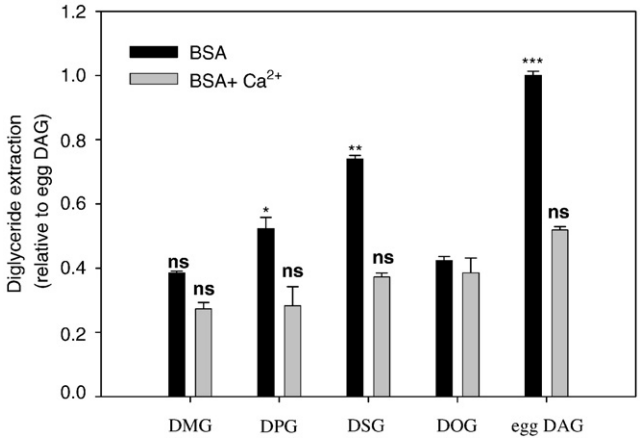

Fig. 2. DAG extraction by BSA as a function of DAG fatty acyl composition in the absence (black) and the presence (grey) of calcium (100 μM). Measurements were performed as in Fig. 1 and normalized to egg DAG extraction in the absence of calcium. DMG, dimyristoylglycerol; DPG, dipalmitoylglycerol; DSG, distearoylglycerol; DOG, dioleoylglycerol. Average values±SD (n=3). Values that are significantly different from DOG data are indicated by asterisks (*p<0.05 Student's t test). ns, not significant.

2.4.3. Fluorescence microscopy study

The electroformation method, as modified by Montes et al. [24], was used to prepare the giant unilamellar vesicles (GUV), using the buffer and AC field conditions described by those authors. GUVs were prepared using the following steps: 3 μL of the lipid stock solution, containing 0.2% DiI-C18, was spread on each of the two sample chamber platinum wires under a stream of dry N_2. GUV lipid composition was sphingomyelin (SM)/PI (1:1 mol ratio)±5 mol% egg DAG. The chamber was then lyophilized for 2 h to remove any remaining trace of organic solvent. The chamber and the buffer were separately equilibrated to temperatures above the lipid mixture phase transition(s) and then 450 μL of buffer was added to cover the wires. Immediately after buffer addition, the platinum wires were connected to an electric wave function generator. The application of the AC field was made in three steps: 1) frequency 500 Hz, amplitude 220 mV for 5 min; 2) frequency 500 Hz, amplitude 1.09 V for 20 min; 3) frequency 500 Hz, amplitude 5.03 V for 90 min. The images were obtained with a confocal microscope (Nikon) at 543 nm excitation.

2.4.4. Hydrolysis of PI by phospholipases

PI concentration was 0.3 mM in all experiments, unless otherwise stated. For optimal catalytic activity, all experiments were performed at 39 °C, in 10 mM HEPES, 150 mM NaCl (pH 7.4) with continuous stirring. According to the type of experiment, the buffer would contain 0.1% bovine serum albumin (BSA) or different concentrations of divalent cations or both. PI-PLC was used at a final concentration of 0.16 U/mL equivalent to 57 ng protein/mL. The total volume in the enzyme assays was 1.0 mL. Enzyme activity was assayed by determining water-soluble phosphorus according to Böttcher et al. [25]. Enzyme activity was assayed as follows: aliquots (50 μL) were removed from the reaction mixture at regular intervals and extracted

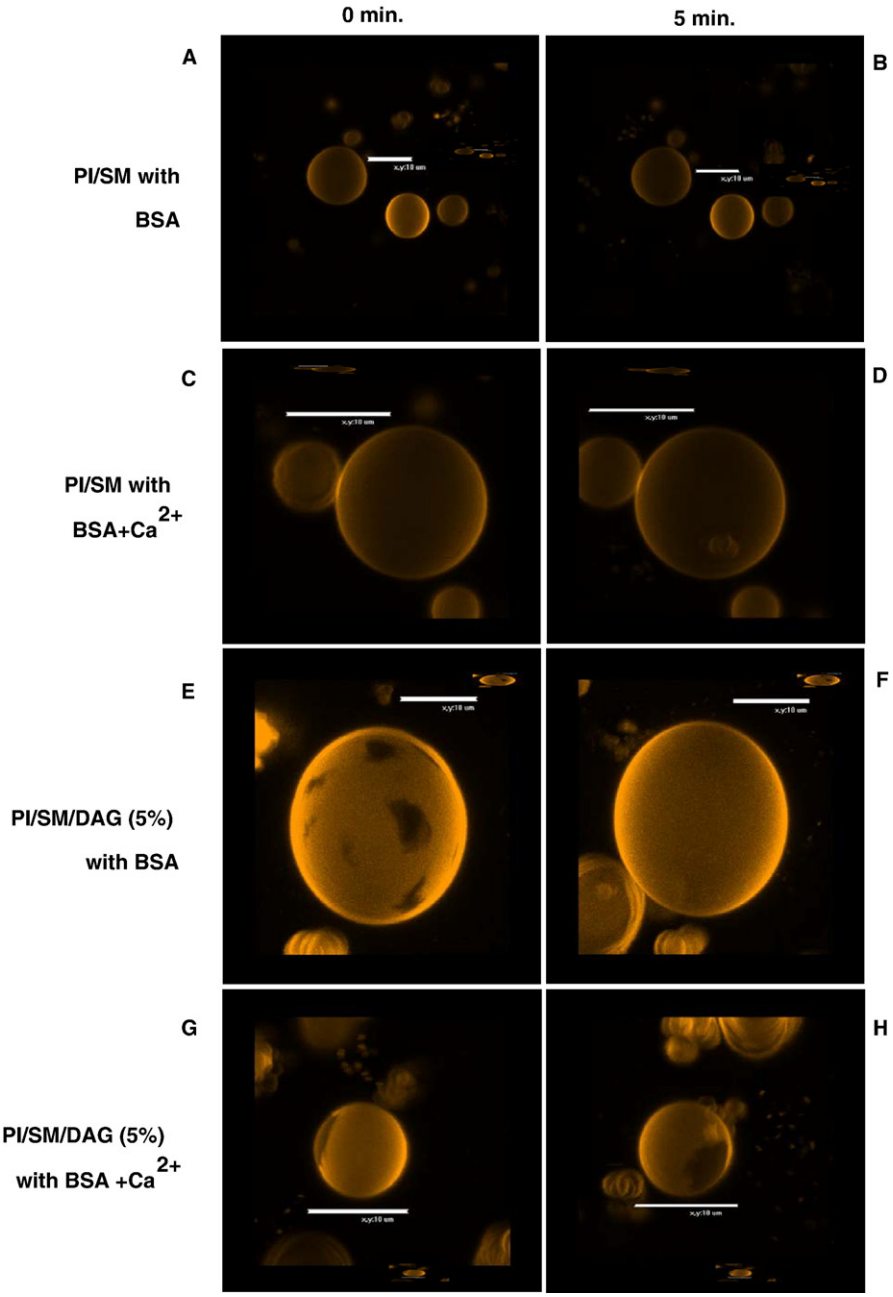

Fig. 3. Confocal microscopy 3D-images of GUV composed of SM/PI (1:1 mol ratio)±5 mol% egg DAG. Images were taken at 0 min (left hand column) and 5 min (right-hand column) after addition of BSA (A, B, E, F) or BSA+10 μM Ca^{2+} (C, D, G, H). Bar: 10 μm.

with 250 μL $CHCl_3$/MeOH/HCl (66:33:1), and the aqueous phase was assayed for phosphorus.

3. Results and discussion

3.1. Diacylglycerol uptake by BSA

One mechanism by which BSA is believed to influence lipid metabolism and membrane function is the binding and extraction of diacylglycerol (DAG) from the lipid bilayers. In order to explore whether Ca^{2+} could interfere with this mechanism, LUV composed of PI:DAG (1:0.3 mol ratio) were incubated with BSA "essentially free of fatty acids", as indicated by the supplier. Total DAG concentration in the system was 1 mM. Calcium concentration varied from 0 to 100 μM. After equilibration, LUV and BSA were separated by ultracentrifugation, and the amount of BSA-bound DAG was measured. The results in Fig. 1, obtained with the radioactive method using ^{14}C-DAG, demonstrate that BSA cause extraction of DAG, and that the phenomenon is hindered by Ca^{2+}. The data in Fig. 1B show that BSA extracts approximately 25% of DAG present in liposomes, however when calcium is added the amount of extracted DAG decreases markedly in a concentration-dependent manner. According to these figures a single BSA molecule binds on average ≈16 DAG molecules. This is in agreement with previous data indicating that 13 fatty acid molecules can be bound to a BSA molecule [26]. Ca^{2+}-inhibition of DAG uptake (Fig. 1) occurs at concentrations such that >90% inhibition is caused at a Ca^{2+}/BSA mol ratio ≈6. Half-maximal inhibition is observed at ca. 10 μM Ca^{2+} (≈0.6 Ca^{2+}/BSA mol ratio).

The fraction of BSA-extracted DAG does not increase significantly when twice the amount of BSA, i.e. 30 μM, is used in the assay (data not shown). This may point to a kind of a stable mixture being formed in our vesicles when ca. 25–30% of DAG is extracted, i.e. at a 1:0.2 PI/DAG mol ratio, so that further DAG uptake by BSA would be prevented. This idea is confirmed by the fact that keeping BSA concentration constant at 15 μM and decreasing total lipid from 4 mM to 2 mM (DAG concentrations 1 mM and 0.5 mM respectively) the proportion of extracted DAG did not vary either (Supplementary Material Fig. S1). The possibility of PI/DAG stabilization at a 1:0.2 mol ratio deserves a separate investigation. A reasonable hypothesis would be that DAG would exist under these conditions under an "umbrella" of inositol, as has been proposed for ganglioside/ceramide and for phospholipid/cholesterol mixtures [27–29].

DAG extraction from PI/DAG vesicles by BSA and its inhibition by Ca^{2+} were also demonstrated semi-quantitatively by a procedure based on TLC separation of the lipids and densitometric estimation of the relative intensities of spots. The results (Supplementary Material Fig. S2) confirm the radioactivity measurements, that Ca^{2+} inhibits DAG uptake by BSA.

The influence of diglyceride fatty-acid composition on the efficiency of extraction of different diacylglycerols by BSA was studied using different liposome compositions: PI/DMG, PI/DPG, PI/DSG and PI/DOG, in addition to PI/egg DAG, in the presence and absence of 10 μM Ca^{2+} (Fig. 2). Egg DAG is the natural diglyceride used throughout this paper (see Materials). The results show that the proportions of diglyceride extracted by BSA from the lipid bilayer vary considerably with DAG fatty acid compositions, in the absence, but not in the presence of Ca^{2+}. In general, extraction in the absence of Ca^{2+} appears to be facilitated by high chain length and by chain saturation (Fig. 2). For a given level of diglyceride extraction, Ca^{2+} inhibition is also more marked for the longer and more saturated chains. Note that egg DAG contains 44% long-chain (C16 and longer) saturated fatty acids. In summary, long, saturated acyl chains favour diglyceride extraction by BSA. Choi et al. [16] had found that the fatty acid binding affinity of albumin depended on the length of the fatty acyl chain, increasing with increasing chain length. This would be in agreement with our observations.

As a further test of DAG uptake by BSA and its inhibition by Ca^{2+}, GUV were prepared composed of PI/SM (1:1 mol ratio). SM facilitated GUV formation, that could not be achieved with pure PI under our conditions. In some cases, these vesicles contained as well 5 mol% egg DAG. The GUV were treated with BSA, in the presence and absence of Ca^{2+}. The results are shown in Fig. 3, for vesicles before and after 5 min BSA treatment. Control vesicles in the absence of DAG (Fig. 3A, B) exhibit a homogeneous appearance, that does not

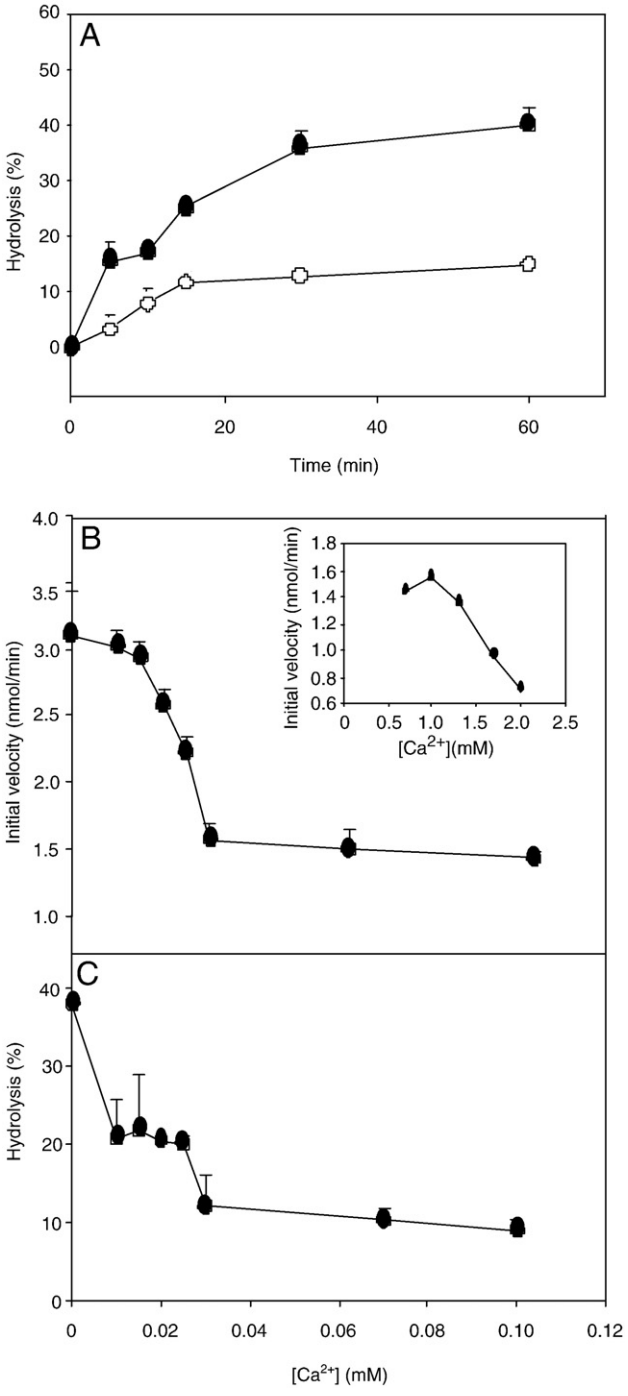

Fig. 4. Hydrolysis of PI by PI-PLC. (A) Hydrolysis time course of PI by PI-PLC in the absence (○) or in the presence (●) of BSA (0.1%). Effect of increasing concentrations of calcium on the initial velocity (B) and percentage (C) of PI hydrolysis in large unilamellar vesicles. PI concentration was 0.3 mM, in the form of LUV, enzyme concentration was 57 ng/mL. In (B) and (C) the experiment was conducted in the presence of BSA (0.1%). The data in (C) were measured after 1 h hydrolysis. Average values±SD (n=3). Inset: Ca^{2+} effect at higher cation concentrations. Average values of two measurements.

change with BSA incubation. The same can be said of GUV incubated with BSA+Ca^{2+} (Fig. 3C, D). However, when DAG is added (Fig. 3E) dark domains become visible. These domains are enriched in the more saturated species of egg DAG and exist probably in a gel-like phase [30]. (Similar images of GUV containing either pure saturated or pure unsaturated DAG species exhibit domain formation only when saturated DAG is present, data not shown). After 5 min treatment with BSA, the domains have disappeared (Fig. 3F). On the contrary, when GUV containing DAG are incubated with BSA+Ca^{2+}, the domains remain visible after 5 min treatment (Fig. 3H). The data in Fig. 3 provide a visual confirmation of the Ca^{2+} property of inhibiting DAG uptake by BSA.

3.2. Effects of BSA and Ca^{2+} on PI-PLC activity

BSA exerts a positive modulation on PI-PLC, as shown in Fig. 4A. The time-course of PI-PLC activity on LUV composed of pure PI shows that, from the initial stages of the process, activity is higher in the presence of BSA. Comparison of initial activity rates with and without BSA shows an increase of about 2-fold in the presence of albumin. As an enzyme end-product, DAG is likely to inhibit PI-PLC activity when accumulating in the bilayer. Nieva et al. [14] observed such an inhibitory effect on a related PLC from *B. cereus*. BSA has a well-known capacity for binding hydrophobic molecules, including DAG [1,4,16].

The possibility that calcium could affect PI hydrolysis by PI-PLC was evaluated in LUV composed of pure PI. For this study, LUVs were incubated with BSA (0.1 wt.%) alone or together with different concentrations of calcium. PI-specific phospholipase C activity on LUV consisting of pure PI at different concentrations of calcium in the presence of BSA is shown in Fig. 4B, C. PI concentration was constant at 0.3 mM. The results (Fig. 4B) show that the initial rate of PI hydrolysis is clearly lowered by calcium. The highest rates of PI hydrolysis were observed in the absence of calcium (3.1 nmol/min). Also in Fig. 4C, the extent of PI hydrolysis was evaluated after 60 min incubation in the presence of Ca^{2+} (10–100 μM). The extent of hydrolysis decreases also very clearly with calcium, Ca^{2+} in a concentration-dependent manner. Initial rates decrease from 3.1 nmol/min to 1.5 nmol/min with Ca^{2+} concentrations increasing from 0 to 30 μM, 50% of the effect being detected at ≈20 μM. The extent of PI hydrolysis decreases from almost 40 mol% PI to about 10 mol% in the 0–30 μM Ca^{2+} concentration range, 50% inhibition being caused by ≈10 μM Ca^{2+}, the same Ca^{2+} concentration causing half-maximal inhibition of DAG uptake. Calcium concentrations above 30 μM had the effect of slowly and gradually decreasing enzyme rate (Fig. 4B, inset) without further decreasing the extent of hydrolysis, that remained at ≈10% with 2 mM Ca^{2+} (data not shown).

An inhibitory effect of Ca^{2+} on PI-PLC had been described, in the absence of BSA, but it was detected only with millimolar concentrations of the cation [31]. Calcium at millimolar concentrations was found to have a stimulatory effect on mammalian PI-PLC [32], but this was observed only in the presence of phosphatidylserine, and was probably related to the interaction of the cation with this negatively-charged phospholipid. Micromolar concentrations of Al^{3+} and La^{3+} inhibit mammalian PI-PLC, particularly when polyphosphoinositide is the substrate [33,34]. These authors explain their

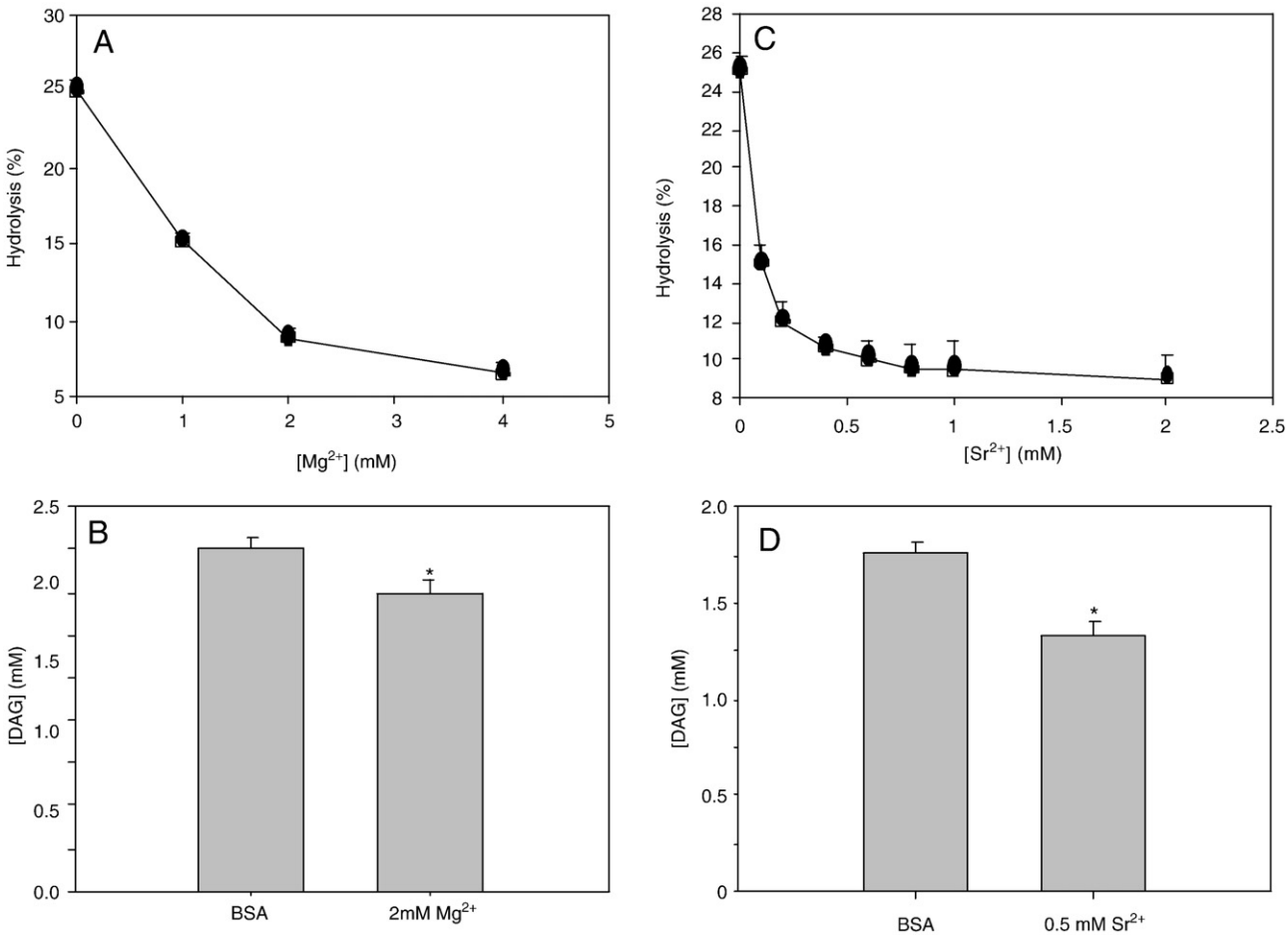

Fig. 5. Effects of magnesium (A, B), and strontium (C, D) on PI hydrolysis after 15 min incubation (A, C). DAG extracted by BSA in the absence and presence of Mg^{2+} or Sr^{2+} (B, D). Average values±SD (n=3). Statistical significance between values in the absence and presence of divalent cations were tested using Student's t-test (*p<0.05).

results on the basis of the metal ions interacting with the lipid bilayer. This reasonable explanation probably does not apply to the observed inhibition of PI-PLC by micromolar Ca^{2+}, mainly because PI as used for vesicle preparation in the present work contains already bound Ca^{2+} (see below).

Importantly, Ca^{2+} had no effect in enzyme assays in the absence of BSA. LUV were incubated in the absence of BSA and in the presence of Ca^{2+} (10–700 μM) (Supplementary Material Fig. S3). Both the initial rate and extent of hydrolysis were, under these conditions, lower than in the presence of BSA (compare with Fig. 3), and Ca^{2+} did not cause any further effect. Thus, the data in Fig. S3 show that PI-PLC activity is not inhibited by Ca^{2+} in the absence of BSA.

Possible internal sources of calcium were explored by determining Ca^{2+} concentration in the various components of the assay medium. Buffers prepared with Milli-Q water did not contain measurable amounts of Ca^{2+}. BSA contained a significant amount of Ca^{2+}. In the concentrations used in our assays, Ca^{2+} arising form commercial BSA preparations amounted to 4.3±0.61 μM. PI contributed with 1.1±0.62 μM Ca^{2+}, and PI-PLC, with 0.2±0.08 μM, final concentrations in the assay. Thus under our conditions, even in the absence of added Ca^{2+}, the concentration of this cation was of about 5–6 μM, in any case well below the concentration range causing enzyme inhibition (10–30 μM).

3.3. Effects of Sr^{2+} and Mg^{2+}

In order to test the specificity of Ca^{2+} effects on BSA and on PI-PLC activity, a series of experiments were performed in which Sr^{2+} or Mg^{2+} was added to the assay buffer. Mg^{2+} effects were far weaker than those of Ca^{2+} (Fig. 5A, B). Mg^{2+} did inhibit PI-PLC activity but only at millimolar concentrations, 50% of the effect being observed at about 1.5 mM, i.e. one hundred times the concentration of Ca^{2+} that produces the same effect (Fig. 5A). Moreover, as shown in Fig. 5B, even 2 mM Mg^{2+} has scarcely any effect on DAG extraction by BSA. Consequently, Mg^{2+} inhibition appears to occur through a different mechanism than that caused by Ca^{2+}. It had been shown by Sundler et al. [31], that both Ca^{2+} and Mg^{2+} could inhibit *B. cereus* PI-PLC at millimolar concentrations, in the absence of BSA. Sr^{2+} in turn has an intermediate effect between those of Mg^{2+} and Ca^{2+} (Fig. 5C, D) decreasing the amount of DAG extracted by BSA although at concentrations about one order of magnitude higher than Ca^{2+}. The fact that millimolar Mg^{2+}, that is known to interact with negatively-charged bilayers [35,36], does not modify DAG extraction by BSA from PI bilayers suggests that the bilayer negative charge is not influencing BSA-DAG interaction.

4. Conclusions

Serum albumin is able to extract diacylglycerols from lipid bilayers, and this effect is inhibited by 10^{-4}–10^{-5} M Ca^{2+}. An example using PI-PLC shows that the serum albumin-DAG-Ca^{2+} interplay may have experimental consequences, and points to a possible regulator role of serum albumin in lipid metabolism, beyond its conventional role as a fatty acid carrier.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2008.11.016.

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