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DEMONSTRATION OF SIMILAR CALCIUM DEPENDENCIES BY MAMMALIAN HIGH AND LOW MOLECULAR MASS PHOSPHOLIPASE A_2

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Abstract—The *in vitro* Ca^{2+} dependencies of arachidonyl (AA)-selective high molecular mass phospholipase A_2 (HMM, 85 kDa-PLA_2) and human low molecular mass (LMM-Type II, 14 kDa)-PLA_2 were compared. When the LMM-PLA_2 and HMM-PLA_2 enzymes were examined for hydrolysis against [^{3}H]AA *Escherichia coli* in an ethyleneglycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA)-free buffer system, neither enzyme demonstrated activity below 10 μM free Ca^{2+}. Beyond 11 μM Ca^{2+} both enzyme activities increased steadily exhibiting 50% of maximal activity at 0.1 and 1.0 mM, respectively. Using EGTA-regulated free Ca^{2+} buffers, both enzymes responded in a biphasic manner, achieving 50% of the maximum response by 0.5 μM Ca^{2+}, stabilizing up to 0.1 mM, then further increasing with exposure to millimolar Ca^{2+} concentrations. Replacement of [^{3}H]AA-labeled phosphatidylethanolamine vesicles for [^{3}H]AA *E. coli* or using Tris–HCl buffer instead of HEPES buffer did not alter these findings significantly. The presence of EGTA had a pronounced concentration-dependent effect on the activity of both the HMM- and LMM-PLA_2 enzymes but only in the range of 0 to 100 μM free Ca^{2+}. EGTA ($EC_{50} \sim 200$ μM) reduced the concentration of Ca^{2+} required by PLA_2 to achieve 50% of maximal acylhydrolysis. In contrast, the Type I bovine pancreatic PLA_2 required millimolar Ca^{2+} concentrations to elicit 50% of the maximal response in both EGTA-free or EGTA-containing systems, which is concordant with its extracellular role as a digestive enzyme. These data suggest that the LMM-Type II PLA_2 and HMM-PLA_2 are both activated at submicromolar, intracellularly relevant, Ca^{2+} concentrations and therefore have the ability to contribute to cellular lipid metabolism.

The family of phospholipases (PLA_2†; EC 3.1.1.4) that hydrolyze the *sn*-2 fatty acyl moiety of phospholipids (PLs) is enlarging. The mammalian low molecular mass (LMM; 13.5–14 kDa) PLA_2 is classified as a non-pancreatic, Type II enzyme having structural and functional features similar to that of various snake venoms [1–5]. This enzyme is often referred to as the "secretory" PLA_2 because it possesses a signal sequence and is released from thrombin-activated platelets [1, 6], glycogen elicited-rabbit peritoneal macrophages [7] and interleukin-1 or tumor necrosis factor-activated smooth muscle cells [8], fibroblasts [9], articular chondrocytes [10, 11] and renal mesangial cells [12]. Because elevated levels of soluble LMM-PLA_2 activity have been measured in serum or exudate fluids in a variety of inflammatory disorders, this enzyme is thought to play a role in inflammation [13–15]. Despite the presence of a signal sequence, the Type II LMM-PLA_2 was found to be cell-associated in human platelets [1] which proved to be identical to enzymes isolated from human placenta [2] and human spleen [5]. Type II LMM-PLA_2 has also been reported to be associated with cytosolic or membrane fractions in a variety of cells (e.g. human neutrophil or gerbil brain) and therefore may be involved in lipid metabolism [16–18]. Indeed, transfection of mouse fibroblasts with human LMM-Type II-PLA_2-cDNA produces a clone where PLA_2 localization in cytosol and microsomes is enhanced in addition to its release into the medium [19]. Transfected clones exhibit an augmented arachidonic acid (AA) release in response to stimuli, supporting a contribution of this enzyme to cellular lipid mediator precursor formation.

A cytosolic high molecular mass enzyme (HMM; 85 kDa) possessing *sn*-2 acylhydrolytic and lysophospholipase activities has been reported in the cytosol of a mouse macrophage cell line, RAW 264.7 [20], resident mouse macrophages [21], rat brain [22], rat kidney [23], mouse mammary gland-derived cells [24], rabbit platelets [25], human platelets [6, 26], and differentiated [27] and undifferentiated [28, 29] human monocytic leukemia U937 cells. In all cases these enzymes exhibited a preference for AA in the *sn*-2 position of substrate PL and were activated *in vitro* by submicromolar concentrations of free Ca^{2+}. In addition, submicromolar levels of Ca^{2+}, associated with cell activation, have been shown to initiate a translocation and/or membrane association of the HMM-PLA_2 in whole cells [22, 27, 30, 31]. These findings led to the hypothesis that the cytosolic HMM-PLA_2 could be an important

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† Abbreviations: AA, arachidonic acid; BP-PLA_2, bovine pancreatic PLA_2 (Type I); CHO, Chinese hamster ovary; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]; THF, tetrahydrofuran; HMM-PLA_2, high molecular mass (85 kDa)-PLA_2; HSF, human synovial fluid; LMM, low molecular mass, Type II (14 kDa)-PLA_2; P_i, inorganic phosphorus; PL, phospholipid; and PLA_2, phospholipase A_2.

regulated enzyme involved in receptor-mediated signal transduction, AA liberation and eicosanoid production.

The Type II-LMM-PLA_2 enzyme is structurally [30] different from the HMM form and biochemically distinct in that it does not show a preference for fatty acid in the *sn*-2 position of PL substrate [1, 3], it is inactivated by sulfhydryl reducing agents such as dithiothreitol [32], and it is preferentially inhibited by a phosphonate-PL transition-state inhibitor [33]. Both the HMM-PLA_2 and LMM-PLA_2 display a requirement for Ca^{2+} for activation, but at different reported concentrations. Both crude [32] and purified [1, 34] synovial fluid LMM-PLA_2 are reported to express full activity at 2–10 mM Ca^{2+} which suggests that this enzyme binds the active site Ca^{2+} at millimolar concentrations similar to the Type I pancreatic PLA_2 enzyme. Since the LMM-PLA_2 has not been thoroughly evaluated at submicromolar Ca^{2+} concentrations, its ability to function intracellularly is not fully understood. To that end, this report describes the re-evaluation and side-by-side comparison of the human HMM and LMM-Type II forms of PLA_2. In our hands, both enzymes responded *in vitro* in a similar manner to Ca^{2+} concentrations found in the activated cell or at higher extracellular levels.

MATERIALS AND METHODS

Reagents. [^{3}H]Arachidonic acid-labeled *Escherichia coli* [20,000 dpm/5 nmol PL inorganic phosphorous (P_i)] was supplied by Dr. Richard Franson, Virginia Commonwealth University (Richmond, VA). 1-Palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylethanolamine (PE) (52 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Unlabeled PE isolated from bovine liver was obtained from Avanti Polar Lipids (Birmingham, AL). *N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) and $CaCl_2$ were purchased from the Sigma Chemical Co. (St. Louis, MO). Reagent grade NaCl was obtained from Mallinckrodt, Inc. (Paris, KY). Enzyme grade Tris[hydroxymethyl]-aminomethane hydrochloride (Tris–HCl) was purchased from BRL, Inc. (Gaithersburg, MD) Amino-propyl columns and tetrahydrofuran (THF) were obtained from Burdick & Jackson (Muskegon, MI). Ready Safe liquid scintillation fluid was obtained from Beckman Instruments (Fullerton, CA).

Enzyme preparation. Since all three enzyme types, bovine pancreatic (BP-PLA_2, Type I), LMM-PLA_2 (Type II) and the HMM-PLA_2, effectively hydrolyzed *sn*-2 [^{3}H]AA *E. coli* PL, enzyme specific activity was determined using this substrate. Bovine pancreatic PLA_2 (specific activity = 39 μmol free fatty acid hydrolyzed/mg/min) was purchased from the Sigma Chemical Co. and solubilized using water purified by a Milli Q system (Millipore, Redford, MA). Human synovial fluid (HSF) from patients with rheumatoid arthritis was collected by Dr. Arthur S. Huppert, Office of Arthritis and Rheumatology (Philadelphia, PA) and was purified as previously described [35]. The active semi-purified G-75 Sephadex fraction of HSF-PLA_2 was used and had a specific activity of 11 μmol free fatty acid hydrolyzed/mg/min. Human recombinant LMM-Type II (rLMM)-PLA_2 was obtained by expression and release from methotrexate-amplified Chinese hamster ovary (CHO) cells.* In brief, a ^{32}P-labeled 47-mer degenerate oligonucleotide probe derived from the published N-terminal amino acid sequence of HSF-PLA_2 [4] was used to screen a human placenta cDNA library constructed in λgt11 (Clonetech, Inc.). DNA sequencing of a 0.8 kb clone (SA2), subcloned into the *Eco*RI site of pUC18, was performed by the method of Sanger *et al.* [36] with double-stranded plasmid DNA primed with universal primers or synthetic oligonucleotides. The sequence is identical to the published cDNA sequence [3] with the exception of a few base changes that do not result in amino acid changes. SA2 cDNA was subcloned into the expression vector RLDN10b, which is a modification of the vector TND [37]. In short, the murine DHFR-SV40 poly(A) was removed from TND and a β-globin promoter-DHFR-bovine growth hormone (bGH) cassette was inserted between the tPA and NEO cassettes. The tPA coding region was excised, replaced with a multiple cloning site linker, and the SA2 cDNA was subcloned (as an *Eco*RI fragment) into an *Eco*RI site in the polylinker. The resulting vector, pSA2-RLDN10b, places the PLA_2 gene under the control of the Rous sarcoma virus LTR and the bGH polyadenylation signal. The DHFR-deficient CHO cells (DG44) [38] were subcultured every 3–4 days. Selections were carried out in MR1.3 containing 400 μg/mL G418 (Geneticin, GIBCO Laboratories) or MT1.3 without nucleotides containing methotrexate. Recombinant LMM-PLA_2 was purified from cell culture medium using the method described for HSF-PLA_2 [35] (95–98% pure; specific activity = 137 μmol free fatty acid hydrolyzed/mg/min).

HMM-PLA_2 was semi-purified from fresh cytosol of the human monocytic cell line U937 and donated by E. Diez, L. Caltabiano and G. Stroup (Cell Sciences, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) or R. Mayer (Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) as previously described [27]. The active fraction obtained from the Sephacryl S-300 column was used (specific activity = 20–89 nmol free fatty acid hydrolyzed/mg/min). In certain preparations, EGTA was omitted from the elution buffer of the S-300 gel filtration column to provide EGTA-free enzyme. HMM-PLA_2 from rat kidney was semi-purified in-house (R. Mayer, Medicinal Chemistry) according to the method of Gronich et al. [23]. The Mono Q fraction used for these studies did not contain EGTA and had a specific activity of 18 nmol free fatty acid hydrolyzed/mg/min.

Phospholipase A_2 assay. Phospholipase A_2 activity was measured by the liberation of *sn*-2 fatty acid

* Stadel JM, Jones C, Livi G, Hoyle K, Kurdyla J, Roshak A, McLaughlin M, Comar S, Strickler J, Bennett CF and Marshall L, Characterization of purified recombinant human secretory phospholipase A_2 expressed in Chinese hamster ovary cells. Manuscript submitted for publication.

from radiolabeled *E. coli* or PE vesicles. Substrate [^{3}H]AA-labeled *E. coli* was stripped of residual Ca^{2+} by washing with a 1 mM EGTA solution which was followed by three Milli Q-filtered water rinses to remove EGTA. PE vesicles were prepared by drying 1-palmitoyl-2-[1-^{14}C]arachidonyl PE and unlabeled PE under a continuous stream of nitrogen, resuspending in assay buffer and sonicating on ice for 5 min in a Bransonic 221 water bath sonicator (50,000 dpm/100 μM PE/assay). The reaction mixture (50 μL final volume for LMM-PLA_2 and BP-PLA_2 and 100 μL final volume for U937 and rat kidney HMM-PLA_2) was buffered with 25 mM HEPES (pH 7.4), unless otherwise stated, and contained 150 mM NaCl, 0–10 mM Ca^{2+}, 100 μM PL substrate and enzyme [35]. Tris buffer contained 100 mM Tris–HCl (pH 7.4) and 0–10 mM Ca^{2+} [39]. Assays were incubated in a shaking water bath at 37° for a time predetermined to be on the linear portion of a time versus hydrolysis plot. In all cases a 10-min time point was used and enzyme was added such that maximal hydrolysis in a given study was no lower than 6% and no greater than 8%. Reactions were terminated by the addition of 1.0 mL THF and liberated fatty acids were extracted over NH_2-propyl solid-phase silica columns with THF:acetic acid (49:1) and quantitated by liquid scintillation counting. Results are calculated as a percentage of radiolabeled free fatty acid hydrolyzed (dpm generated minus background dpm divided by total dpm added).

Preparation of "free" Ca^{2+}/EGTA buffers. Free calcium concentrations were controlled by using Ca^{2+}/EGTA buffers as calculated by the Cation-Ligand Binding Program—IBM PC version 9.0 [40, 41]. The following amount of Ca^{2+} was added to HEPES buffer (pH 7.4) providing the amount of "free" Ca^{2+} in the parentheses, i.e. 0.2 mM EGTA: 0 (0), 145 μM (100 nM), 186 μM (500 nM), 194 μM (1 μM), 209 μM (10 μM); 1 mM EGTA: 0 (0), 722 μM (100 nM), 929 μM (500 nM), 964 μM (1 μM), 1.01 mM (10 μM). The Ca^{2+} concentrations of the assay buffers were determined using the fluorescent calcium indicator, fura-2 (Behring Diagnostics, San Diego, CA), according to the method of Leslie [42] for less than 1 μM and by Inductively Coupled Plasma (ICP) spectrometry using a Jobin–Ynan model 38 spectrometer (393.4 or 317 nm wavelength) or by using an Orion model 90-01 single junction reference Ca^{2+} electrode (Boston, MA) for 1 μM and above. The HEPES and NaCl were contaminated with approximately 800 nM Ca^{2+}; therefore, assays not performed in the presence of a Ca^{2+} chelator were assumed to have up to 1 μM Ca^{2+}. To obtain zero Ca^{2+} in buffers multivalent ion chelator had to be present.

Calculations and statistics. Data are expressed as means ± SD of three determinations within a single experiment. In all cases, figures show one representative experiment which was conducted 2–4 times. Hydrolytic data are expressed as a percentage of the maximum hydrolysis obtained using 10 mM Ca^{2+} concentrations unless otherwise indicated.

RESULTS

Assessment of enzyme Ca^{2+} dependency. Both the native HSF-PLA_2 and the recombinant form (rLMM-PLA_2) were evaluated for activity when exposed to 1 μM to 10 mM final Ca^{2+} concentration. Figure 1A shows that both native and rLMM-PLA_2 enzymes responded identically in EGTA-free buffer, showing no activity between 0 and 11 μM Ca^{2+}, increasing after 11 μM Ca^{2+} and reaching 50% of maximal activity at 60–100 μM. Figure 1B shows the same study performed in the presence of 1 mM EGTA. A small proportion of activity, ~1–5% of the maximal response by both enzymes, was always observed at 100 nM. Both the native HSF-PLA_2 and the rLMM-PLA_2 again displayed similar dependencies for Ca^{2+}, exhibiting 40–60% of their maximal response at 500 nM to 100 μM free Ca^{2+}. Further increases in Ca^{2+} up to 10 mM produced another enhancement in both activities.

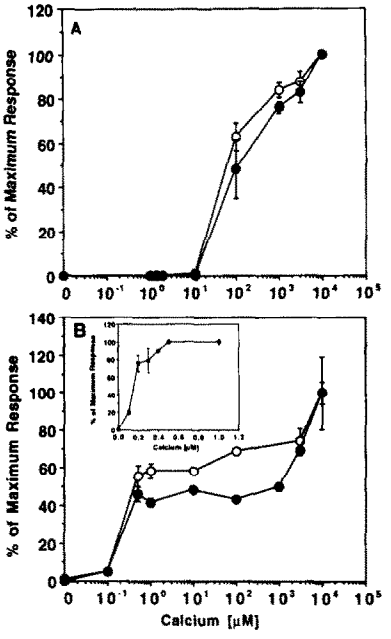

Fig. 1. Effect of different calcium concentrations on HSF or rLMM-PLA_2 hydrolysis of [^{3}H]AA-*E. coli*, assayed with or without EGTA. Panel A shows the activity of the two enzymes analyzed in buffer with no EGTA in the presence of 0 μM to 10 mM free Ca^{2+}. The zero Ca^{2+} point was generated by the addition of 1 mM EGTA and no Ca^{2+} (see Materials and Methods). Panel B shows the activity of rLMM-PLA_2 (●) and HSF-PLA_2 (○) assayed in buffers containing 1 mM EGTA and 0–10 mM Ca^{2+} (see Materials and Methods). The data points represent means ± SD of triplicate determinations of one representative experiment of 2–4 experiments.

The Ca^{2+} dependency of HMM-PLA_2 enzymes was examined in the absence (Fig. 2A) or presence (Fig. 2B) of 1 mM EGTA. Analysis of semi-purified enzyme from differentiated human U937-monocytic cells or rat kidney in EGTA-free buffer revealed that both sources of HMM-PLA_2 exhibited an almost identical Ca^{2+} dependency. No response was noted by either of the enzymes up to 11 μM Ca^{2+}. Above 11 μM Ca^{2+} activity increased continuously up to

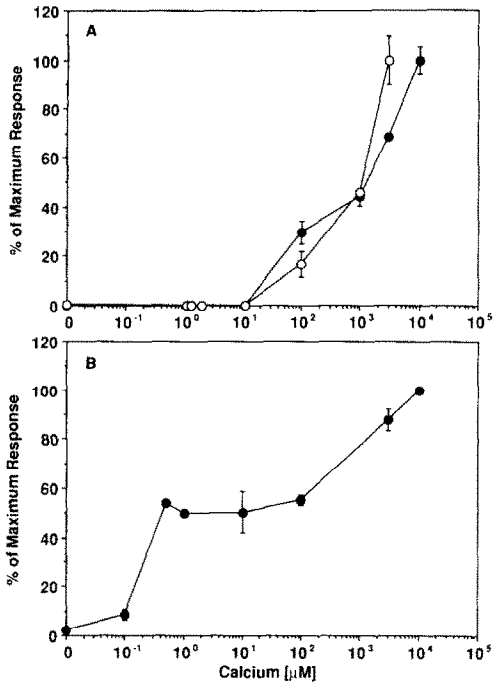

Fig. 2. Effect of calcium concentration on HMM-PLA_2 hydrolysis of [^{3}H]AA-*E. coli* assayed with or without EGTA. Panel A shows the activity of HMM-PLA_2 isolated from U937-monocytes (●) or rat kidney (○) assayed in buffer containing no EGTA and 1 μM–10 mM free Ca^{2+} (see Materials and Methods). The zero Ca^{2+} point was generated by the addition of 1 mM EGTA and no Ca^{2+} (see Materials and Methods). Panel B shows the activity of the U937-HMM-PLA_2 analyzed in buffer with 1.0 mM EGTA in the presence of 0 μM to 10 mM free Ca^{2+}. The data points represent means ± SD of triplicate determinations of one representative experiment of 2–3 experiments.

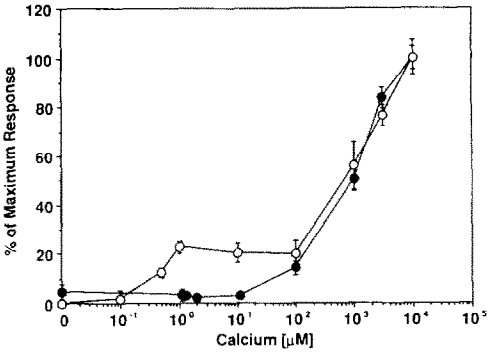

Fig. 3. Effect of calcium concentration on BP-PLA_2 hydrolysis of [^{3}H]AA-*E. coli* assayed with or without EGTA. The activity of BP-PLA_2 (Type I) assayed in buffer containing no EGTA and 1 μM–10 mM free Ca^{2+} (●) (see Materials and Methods) is compared to BP-PLA_2 assayed in buffer containing 1 mM EGTA and 0–10 mM free Ca^{2+} (○) (see Materials and Methods). The zero Ca^{2+} point for assays without EGTA was obtained by addition of 1 mM EGTA and no Ca^{2+}. The data points represent means ± SD of triplicate determinations of one representative experiment of 2–4 experiments.

10 mM Ca^{2+} yielding an EC_{50} of ~1 mM. When U937-derived HMM-PLA_2 was tested in HEPES buffer containing 1 mM EGTA, the enzyme was activated to 50% of maximum by 0.3 to 0.5 μM Ca^{2+} levels. Higher Ca^{2+} concentrations (1–10 mM) produced a further increase in hydrolytic activity.

For comparison, the Type I, 14 kDa bovine pancreatic (BP)-PLA_2 enzyme was evaluated for activity over the same range of Ca^{2+} concentrations. Figure 3 shows that EGTA-free buffers did not elicit hydrolysis until 10–100 μM Ca^{2+} levels were reached. Further increases up to 10 mM Ca^{2+} resulted in still greater activity. In the presence of 1 mM EGTA the BP-PLA_2 exhibited 30% of maximal activity from 0.3 to 100 μM Ca^{2+} which again increased at concentrations above 100 μM.

Effect of varying substrate or buffer on enzyme Ca^{2+} *dependency.* To ensure that our results were not artifacts related to using labeled *E. coli* as substrate, the Ca^{2+} dependencies of U937-derived HMM-PLA_2, rLMM-Type II-PLA_2 and BP-PLA_2 were tested using *sn*-2 [^{14}C]AA PE vesicles. Figure 4A shows that in the absence of EGTA no activity was measured by any enzyme below 11 μM Ca^{2+}. At 11 μM Ca^{2+}, the activity of both the HMM-PLA_2 and the BP-PLA_2 increased, reached 50% of the maximum between 0.3 and 1 mM and displayed no real deviation from the response elicited using [^{3}H]-AA-*E. coli* as PL substrate. The rLMM-PLA_2 tested in this system exhibited a similar trend but reached a maximal hydrolytic response at 1 mM Ca^{2+} which was followed by a rapid decline in activity in the presence of 3 and 10 mM Ca^{2+}. A similar result was reported by Franson and Waite [43] examining the hydrolysis of PE vesicles by an LMM-PLA_2 semi-purified from human neutrophil. The inhibition of activity was attributed to the physical-chemical effect of high concentrations of Ca^{2+} on the surface of the PE vesicles. In a 1 mM EGTA buffer all three enzymes exhibited activity between 100 and 500 nM Ca^{2+}, reached 45–65% of maximum activity by 500 nM Ca^{2+} and remained at this level up to 1 mM (Fig. 4B). Interestingly, in this system the HMM-PLA_2 expressed up to 15% of maximal activity in zero Ca^{2+} which is similar to the findings of others using purified PL as substrate [27, 29]. Hydrolysis of BP-PLA_2 and HMM-PLA_2 against PE vesicles continued to increase as Ca^{2+} increased to 3 and 10 mM. The rLMM-PLA_2 again displayed a sharp decrease in activity at Ca^{2+} levels above 1 mM.

The possibility that buffer type might influence PLA_2 Ca^{2+} dependency was explored. rLMM-PLA_2 and HMM-PLA_2 activities were evaluated in Tris–HCl buffer, pH 7.4 (Fig. 5) using [^{3}H]AA-*E. coli* as substrate. Figure 5A shows the hydrolytic response to the two enzymes in EGTA-free buffer. In these experiments no EGTA was added to the zero Ca^{2+}; therefore, up to 800 nM Ca^{2+} contamination was assumed. The U937 HMM-PLA_2 did not exhibit activity up to 2 μM Ca^{2+} but then increased to 10 and 20% of the maximum response in the presence

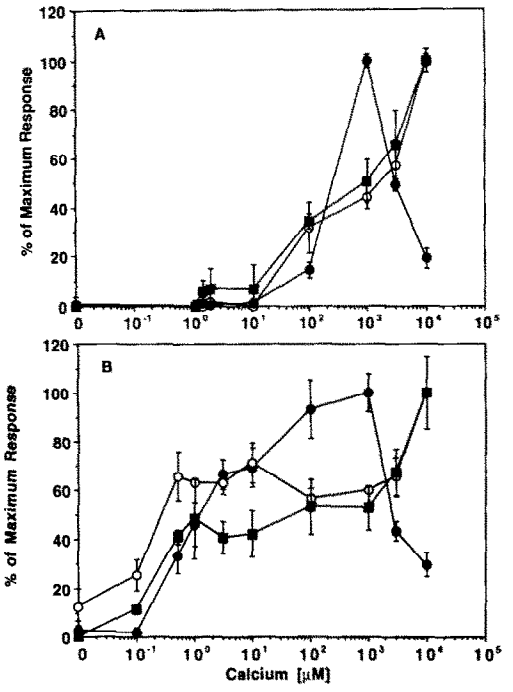

Fig. 4. Ca^{2+} dependency of BP-PLA$_2$, rLMM-PLA$_2$ or HMM-PLA$_2$ activity against [^{14}C]AA-PE vesicles. Panel A shows the Ca^{2+} dependencies of rLMM-PLA$_2$ (●), U937-HMM-PLA$_2$ (○) and BP-PLA$_2$ (■) activities analyzed in EGTA-free buffer containing 1 μM to 10 mM Ca^{2+}. The zero point was generated by the addition of 1 mM EGTA and no Ca^{2+} (see Materials and Methods). Maximal specific activities obtained in a 10-min incubation were 1.2 μmol/min/mg for rLMM-PLA$_2$ and 193.4 nmol/min/mg for BP-PLA$_2$, while 0.21 nmol/min/mg was obtained for HMM-PLA$_2$ in 60 min. Panel B shows the Ca^{2+} dependency of the rLMM-PLA$_2$ (●), U937-HMW-PLA$_2$ (○), and BP-PLA$_2$ (■) activities over 0–10 mM free Ca^{2+} concentrations with 1 mM EGTA (see Materials and Methods). Maximal specific activities obtained in 10-min incubations were 1.4 μmol/min/mg for rLMM-PLA$_2$ and 400 nmol/min/mg for BP-PLA$_2$, while 0.59 nmol/min/mg was obtained for HMM-PLA$_2$ in 60 min. Data points represent means ± SD of triplicate determinations of one representative experiment of 2–3 experiments.

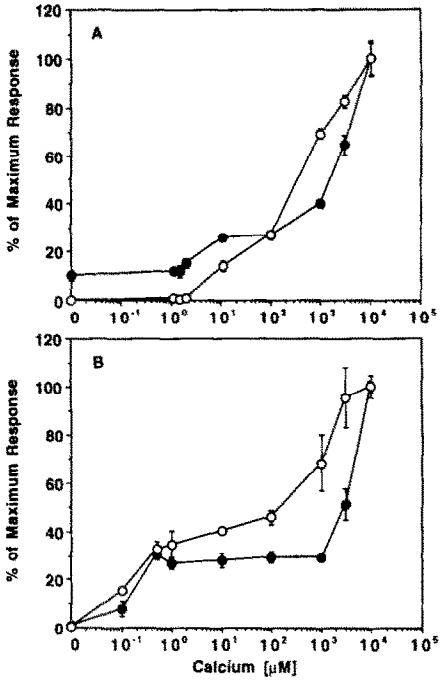

Fig. 5. Effect of Tris buffer on the Ca^{2+} dependency of rLMM-PLA$_2$ or U937-HMM-PLA$_2$ activity against [^{3}H]-AA-*E. coli* with or without EGTA. Panel A represents the activity of rLMM-PLA$_2$ (●) and HMM-PLA$_2$ (○) assayed in Tris buffer containing no EGTA at 1 μM–10 mM Ca^{2+} (see Materials and Methods). The zero Ca^{2+} point represents no Ca^{2+} addition. Panel B represents the activity of rLMM-PLA$_2$ (●) or HMM-PLA$_2$ (○) in Tris buffer containing 1 mM EGTA and 0–10 mM free Ca^{2+} (see Materials and Methods). The data points represent means ± SD of triplicate determinations of 2 experiments.

of 11 and 100 μM Ca^{2+}, respectively. Thereafter, the activity rapidly increased with increasing Ca^{2+} up to 10 mM. The rLMM-PLA$_2$ expressed ~9% of maximal activity at the zero Ca^{2+} point and increased to 25% of maximum with 10 μM Ca^{2+}. This was followed by a plateau of activity up to 1 mM. Calcium from 1 to 10 mM caused both enzyme activities to increase further. Analysis of either HMM-PLA$_2$ or rLMM-PLA$_2$ in Tris buffer with EGTA (Fig. 5B) elicited 24–50% acylhydrolysis at 0.1 to 100 μM Ca^{2+}. Concentrations from 1 to 10 mM produced an additional increase in the activity of both enzymes.

Effect of EGTA on enzyme Ca^{2+} *dependency.* The influence of EGTA on rLMM-PLA$_2$ Ca^{2+} dependence was further examined using buffers containing no EGTA, 200 μM EGTA or 1 mM EGTA prepared and verified as described in Materials and Methods. Figure 6 shows that as the concentration of EGTA increased there was a shift of the hydrolysis curve to the left but only in the 100 nM to 100 μM Ca^{2+} concentration range. No enzymatic activity was observed in EGTA-free buffers up to 1 μM Ca^{2+}. Hydrolytic activity reached 50% of the maximum response between 10 and 100 μM. A 200 μM EGTA buffer supported up to 30% maximal hydrolytic activity at 1 μM Ca^{2+} and reached 50% at approximately 30 μM Ca^{2+}. Up to 60% of the maximum enzymatic response was elicited at 1 μM Ca^{2+} in buffers containing 1 mM EGTA and was sustained through to 100 μM. Further increases in Ca^{2+} levels produced an identical concentration-dependent increase in rLMM-PLA$_2$ activity regardless of the EGTA concentration. The rLMM-PLA$_2$ activity expressed at 10 μM Ca^{2+} was then examined in the presence of 40 μM to 5 mM EGTA. Figure 7 shows that rLMM-PLA$_2$ hydrolytic activity obtained in the presence of 10 μM free Ca^{2+} directly increased in a non-linear fashion as the concentration of EGTA increased. The estimated EC_{50} concentration of EGTA for rLMM-PLA$_2$ hydrolysis in the presence of 10 μM Ca^{2+} was ~200 μM.

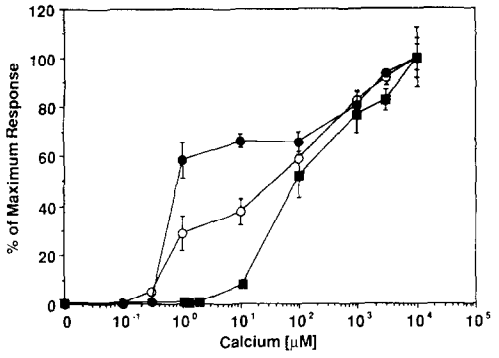

Fig. 6. Effect of different concentrations of EGTA on the Ca^{2+} dependency of rLMM-PLA_2 activity against [^{3}H]AA-*E. coli*. rLMM-PLA_2 was analyzed over 0–10 mM free Ca^{2+} in buffer (see Materials and Methods) containing 1 mM EGTA (●), 200 μM EGTA (○) or no EGTA (■). Data points represent means ± SD of triplicate determinations of 2 experiments.

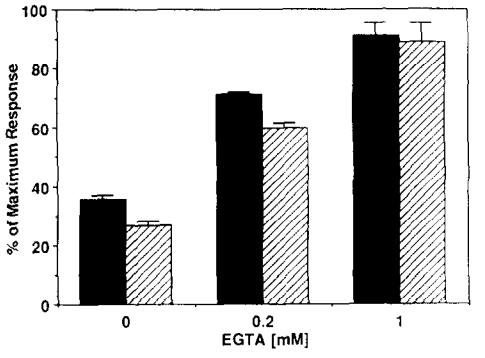

Fig. 8. Effect of phospholipid concentration on EGTA-induced changes in rLMM-PLA_2 Ca^{2+} dependency. rLMM-PLA_2 was assayed in the presence of 2.5 (■) or 8 (▨) nmol PL [^{3}H]AA-*E. coli* in buffer containing 0, 0.2 or 1 mM EGTA and adjusted to 10 μM free Ca^{2+}. The bars represent the means ± SD (N = 3). Data are expressed as a percentage of the maximum response achieved with 10 mM Ca^{2+}.

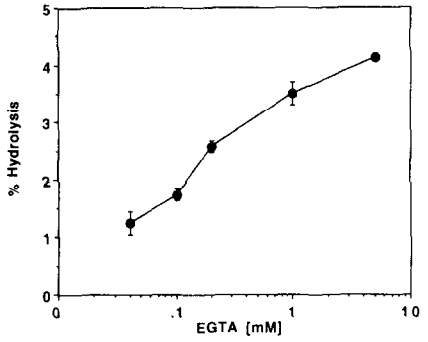

Fig. 7. Effect of increasing EGTA concentration on rLMM-PLA_2 activity in the presence of 10 μM free Ca^{2+}. rLMM-PLA_2 was assayed using [^{3}H]AA-*E. coli* in the presence of 40 μM to 5 mM EGTA at 10 μM free Ca^{2+} (see Materials and Methods). Data represent means ± SD of triplicate determinations of a single experiment.

Phospholipids, particularly those species with anionic polar head groups, are known to interact with Ca^{2+} in *in vitro* systems resulting in physical structural changes in lipid organization. In addition, there are an increasing number of proteins which interact with phospholipids in a calcium-dependent manner [44, 45]. The effect of varying PL substrate concentration on the capability of EGTA to alter Ca^{2+} dependency was investigated. rLMM-PLA_2 hydrolysis was measured in the presence of no EGTA, 200 μM EGTA or 1 mM EGTA-HEPES buffer at two concentrations of *E. coli* PL substrate, i.e. 2.5 nmol P_i, representing the apparent K_m and 8 nmol P_i which is three times the apparent K_m for *E. coli* in this model [35]. *E. coli* membranes are comprised of almost 80% PE. This experiment was performed in the presence of 10 μM free Ca^{2+} where the effect of EGTA was most evident and was compared to the maximum response obtained at 10 mM free Ca^{2+}. A minor but significant decrease ($P < 0.05$; analyzed using ANOVA and Student-Neuman-Keuls multiple comparison test) in percentage of maximal activity occurred in the presence of 8 nmol P_i compared to 2.5 nmol P_i when tested with 0 or 200 μM EGTA buffers (Fig. 8). No difference was observed in hydrolysis between 8 and 2.5 nmol P_i at the highest EGTA concentration.

DISCUSSION

Analysis of the U937-derived HMM-PLA_2 in our assay system, using [^{3}H]AA-*E. coli* as substrate and 1 mM EGTA, duplicated the Ca^{2+} concentration curve previously reported by Diez and Mong [27] using a 50 mM Tris–HCl (pH 8.5) buffered solution and 1-*O*-hexadecyl-2[^{3}H]AA-phosphatidylcholine (PC) vesicles as substrate. This was characterized by a biphasic response beginning with a rise in activity as free Ca^{2+} increased from 0 to 500 nM, a plateau of activity over 1 to 100 μM Ca^{2+} and a further enhancement of activity from 100 μM to 10 mM Ca^{2+}. Side-by-side comparison of the Type II LMM-PLA_2 with the HMM-PLA_2 revealed that the LMM-PLA_2 was activated in much the same way as the HMM-PLA_2 enzyme displaying a submicromolar to micromolar dependence on Ca^{2+}. Neither buffer-type nor substrate used significantly altered this finding. Activities of both the LMM-PLA_2 and HMM-PLA_2 were strongly influenced by the presence or absence of the multivalent ion chelator EGTA which shifted both Ca^{2+} dependency curves to the left, lowering the free Ca^{2+} needed to express up to 50% of maximal activities. The omission of EGTA from buffers compromised our ability to evaluate activity below 1 μM Ca^{2+}. Interestingly, in this system neither LMM-PLA_2 nor HMM-PLA_2 expressed activity below 10 μM Ca^{2+} and expressed greater EC_{50} values of 100 or 1000 μM, respectively.

Submicromolar Ca^{2+} dependencies for LMM-PLA_2-like enzymes have been reported by others in crude enzyme preparations. Acid extracts of human neutrophils [17] or human platelets [46] both displayed an EC_{50} of 50–500 μM Ca^{2+} when assayed in Tris–HCl buffers (pH 7.4 to 9.5) without EGTA assayed against [^{14}C]oleate-labeled *E. coli*. The activity measured was most likely Type II, LMM-PLA_2, activity and not HMM-PLA_2 since the HMM-PLA_2 enzyme is acid labile [26]. Lenting et al. [47] described a $K_{Ca^{2+}}$ of 50 μM for a LMM-Type II-rat liver mitochondrial PLA_2 assayed using a [^{14}C]linoleic acid-labeled mitochondrial membrane system. These data are consistent with our findings that the Type II LMM-PLA_2 was activated, *in vitro*, by submicromolar to micromolar concentrations and not millimolar concentrations of Ca^{2+}.

Additionally, various literature reports support the similarity in Ca^{2+} dependency between the two PLA_2 forms. Baron and Limbird [48] utilized a 10 mM EDTA-HEPES buffer system to prepare 100,000 *g* human platelet supernatant and particulate fractions which were then assessed for PLA_2 Ca^{2+} dependency. Both the Type II-LMM-PLA_2 and HMM-PLA_2 have been purified from platelets and since cellular fractionation was performed in the presence of Ca^{2+} chelator one would expect that the HMM-PLA_2 form was confined predominantly to the soluble fraction [1, 49, 50]. No difference in Ca^{2+} dependency was observed between the two fractions since both responded to the same extent to 0.4 through 4 μM Ca^{2+}. Although one cannot rule out the possibility that both enzymes were equally represented in the two cell fractions, these data may represent the lack of distinction that one would expect using Ca^{2+} dependency to separate the activities of the LMM- and HMM-PLA_2 enzyme. This is further supported by the work of Rordorf *et al.* [18] who found that 50 μM Ca^{2+} similarly activates gerbil brain cytosol PLA_2 activities associated with 60 kDa or 14 kDa molecular mass proteins and microsomal *sn*-2 acylhydrolytic activity associated with 14 kDa proteins. This agrees with our findings that HMM-PLA_2 and LMM-PLA_2 were similarly activated at the same Ca^{2+} concentrations and suggests that they cannot be distinguished on this basis with respect to functional role.

Analysis of the Type I BP-PLA_2 for Ca^{2+} dependency in our system with or without EGTA generated similar respective profiles compared to the LMM- and HMM-PLA_2 enzymes. However, the presence of EGTA supported no more than 30% of maximum activity as opposed to 50–70% between 0.5 and 100 μM Ca^{2+} observed for the other enzymes; 1–2 mM Ca^{2+} was required for expression of 50% or more BP-PLA_2 activity in both EGTA or EGTA-free systems. This is consistent with the reported $K_{Ca^{2+}}$ of 2.8 mM for Ca^{2+} binding to the pancreatic enzyme as determined by ultraviolet absorbance spectroscopy [51]. BP-PLA_2 is a secreted extracellular enzyme possessing both a secretion sequence and heptapeptide which inhibits enzyme activation post secretion until this portion is cleaved by trypsin. The Type I BP-PLA_2 shares only a 37% homology with the Type II-LMM-PLA_2 [1, 3, 6] and no homology with the U937 HMM-PLA_2 [30]. While the catalytic domains of the Type I and Type II-PLA_2 are highly conserved, subtle differences in structure exist [52] and may induce minor differences in the recognition of substrate and/or the binding of Ca^{2+}. This may offer some explanation for the poorer activity response of the BP-PLA_2 at submicromolar Ca^{2+} concentrations compared to that of LMM- or HMM-PLA_2.

To ensure that the results obtained were not artifacts of our *in vitro* assay system, we evaluated Ca^{2+} dependency using a different substrate or a different buffer system. Use of [^{3}H]AA-labeled PE vesicles as substrate resulted in similar Ca^{2+} dependency profiles for both the Type I and Type II LMM-PLA_2 isotypes and the HMM-PLA_2, implying that the form of PL presented did not greatly influence the Ca^{2+} profile observed. This is supported by reports of the nanomolar Ca^{2+} dependency displayed by the HMM-PLA_2 generated against a variety of substrates including 1-*O*-hexadecyl-2-[^{3}H]AA-PC liposomes [27], mixtures of 1-palmitoyl-2[^{14}C]AA-PC and 1,2-dioleoylglycerol [29] or 1,2-diAA-2-[^{14}C]PC liposomes [21]. Likewise, the LMM-Type II-PLA_2 has been evaluated with a variety of substrates including labeled *E. coli* [17], pure PL vesicles [26], Triton-X 100-PL micelles [28] or natural membrane preparations [47]. Historically, Tris–HCl, at alkaline pH, has been the most widely used buffering agent in assessing PLA_2 activity [39]. No significant deviations in Ca^{2+} dependency were observed by either the LMM-PLA_2 or HMM-PLA_2 activity when Tris–HCl was used as the buffer at pH 7.4 compared to HEPES (pH 7.4). This was not surprising since the HMM-PLA_2 submicromolar Ca^{2+} dependency has been demonstrated by others using a variety of buffer systems, e.g. HEPES [23, 29], 80 mM glycine buffer [28] or Tris–HCl [20]. An investigation of pH was not undertaken since similar Ca^{2+} dependency curves for HMM-PLA_2 have been obtained over a range of pHs, e.g. pH 7.1 [27], 7.4 [29], 8.0 [22] and 9.5 [24, 52].

The most profound effects on the Ca^{2+} curves for all three enzyme types were induced by EGTA. First, EGTA-free buffers resulted in a greater Ca^{2+} dependency for all enzymes. Addition of increasing concentrations of EGTA shifted the rLMM-PLA_2 activity versus Ca^{2+} concentration curve to the left but only at Ca^{2+} concentrations below 100 μM. Indeed, as little as 40 μM EGTA significantly enhanced the acylhydrolytic capability of the rLMM-PLA_2 by 12–14% in the presence of 10 μm free Ca^{2+}. The reason for this phenomenon is not clear but one consideration is the possibility that trace metals that are inhibitory at the lower Ca^{2+} concentrations are chelated by the EGTA resulting in the augmentation of enzyme activity. Indeed, such multivalent cations such as Fe^{2+}, Fe^{3+} or Al^{3+} [39] are reported to inhibit HSF-PLA_2 activity while cadmium (Cd^{2+}) was shown to inhibit cytosolic alveolar macrophage PLA_2 [53].

Additionally, the contribution of PL substrate to the EGTA-induced changes in PLA_2 Ca^{2+} dependency is not known nor was it easily studied since fluctuations in PL concentration can induce substrate structural changes which, in themselves, influence enzyme activity and make data unin-

terpretable. Examination of the effect of minor PL concentration changes revealed that significant, albeit, small reductions in hydrolytic activity occurred as *E. coli* PL concentrations increased in the presence of 0 or 200 μM EGTA. The small changes observed could be due to the Ca^{2+}-anionic PL interaction, although compared to the nanomolar Ca^{2+} binding affinity displayed by EGTA they are relatively weak. Indeed, at the highest concentration of EGTA the effects of PL concentration were obviated. It may be more relevant to vary the PL according to its anionicity to study this relationship. This is supported by the work of Leslie and Channon [54] who demonstrated that HMM-PLA_2 enzyme activation is enhanced as PLs with greater anionic capacity are added to the system. This was hypothesized to be due to not only an effect on bilayer packing characteristics but also the charge of the substrate in which case anionic phospholipids could facilitate Ca^{2+}-mediated binding of enzyme to membrane. The role of EGTA in this interaction and whether or not this phenomenon extends to other chelators or Ca^{2+} binding molecules is not clear and requires further investigation. The more intriguing question is whether an EGTA physiological equivalent exists which could regulate PLA_2 activity by altering its Ca^{2+} dependency. Indeed, the lack of activity displayed below 10 μM Ca^{2+} by either the LMM-PLA_2 or the HMM-PLA_2 in the EGTA-free system suggests that such an agent may be required for PLA_2 to exhibit activity at submicromolar Ca^{2+} levels. One example is the receptor-mediated activation of guanine nucleotide binding protein induced reduction in the Ca^{2+} dependencies of either porcine pancreatic PLA_2 or snake venom PLA_2 (in a permeabilized cell system) which invoked greater hydrolysis at intracellularly relevant submicromolar concentrations of Ca^{2+} [55]. The mechanism by which this occurred is unknown but at this time the contribution of a Ca^{2+} binding molecule which may influence activation of PLA_2 hydrolysis cannot be ruled out.

We have shown that the LMM-Type II and HMM-PLA_2 respond similarly to Ca^{2+}, *in vitro*, suggesting that both are capable of functioning at concentrations found intracellularly (0.1 to 2 μM) and extracellularly (1 mM). This is consistent with the occurrence of both cell-associated and extracellular soluble forms of LMM-Type II-PLA_2. The function of the cell-associated LMM-PLA_2 relative to that of the HMM-PLA_2 remains to be established. With the discoveries of new classes of PL-metabolizing enzymes, it appears that cellular arachidonic acid mobilization is much more complex than originally appreciated and possibly regulated through more than one *sn*-2 acylhydrolytic enzyme.

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