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Regulatory Aspects of Rat Liver Mitochondrial Phospholipase A_2: Effects of Calcium Ions and Calmodulin

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A comparative study was made of the metal ion requirement of rat liver mitochondrial phospholipase A_2 in purified and membrane-associated forms. Membrane-bound enzyme was assayed using either exogenous or endogenous phosphatidylethanolamine. Although several divalent metal ions caused increased activity of the membrane-associated enzyme, only Ca^{2+} and Sr^{2+} activated the purified phospholipase A_2. The activity in the presence of Sr^{2+} amounted to about 25% of that found with Ca^{2+}. When the Ca^{2+} concentration was varied two activity plateaus were observed. The corresponding dissociation constants varied from 6 to 20 μM Ca^{2+} and from 1.4 to 12 mM Ca^{2+} for the high- and low-affinity binding sites, respectively, depending on the assay conditions and whether purified or membrane-bound enzyme was used. A $k_{Sr^{2+}}$ of 60 μM was found for the high-affinity binding site. The effect of calmodulin and its antagonist trifluoperazine was also investigated using purified and membrane-associated enzyme. When membrane-bound enzyme was measured with exogenous phosphatidylethanolamine, small stimulations by calmodulin were found. However, these were not believed to indicate a specific rôle for calmodulin in the Ca^{2+} dependency of the phospholipase A_2, since trifluoperazine did not lower the activity of the membrane-bound enzyme to levels below those found in the presence of Ca^{2+} alone. Membrane-bound enzyme in its action toward endogenous phosphatidylethanolamine was neither stimulated by calmodulin nor inhibited by trifluoperazine. Purified enzyme was also not stimulated by calmodulin, while trifluoperazine caused small stimulations, presumably due to interactions at the substrate level. These results indicate that calmodulin involvement in phospholipase A_2 activation should not be generalized. © 1984 Academic Press, Inc.

Phospholipase A_2, catalyzing the release of fatty acids from the *sn*-2 position of phosphoglycerides, is a ubiquitous enzyme (1, 2). Most of the intracellular phospholipases A_2 occur in a membrane-associated form (2, 3). Their presence in conjunction with membrane phosphoglycerides requires a tight regulation of their activity in order to maintain membrane structure. Although several models for this regulation can be put forward ((2, 3) for reviews), detailed knowledge about this process is still lacking.

In recent years membrane-bound phospholipases A_2 have been purified to near homogeneity from rabbit polymorphonuclear leukocytes (4), sheep erythrocytes (5), rabbit (6) and human (7) platelets, rat ascites hepatoma (8), rat liver mitochondria (9), and bovine brain microsomes (10). Like the extracellular soluble phospholipases A_2 from pancreas and venoms (1), these intracellular membrane-bound enzymes require Ca^{2+} ions for enzymatic activity. This knowledge, and the finding that addition of the Ca^{2+} ionophore A23187 to platelets gave rise to the sudden release of arachidonate from platelet phospholipids (11,

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12), led to a simple model in which the activity of the platelet phospholipase A_2 was thought to be regulated by free Ca^{2+} in the cytoplasm (13). In this model the addition of ionophore or thrombin to platelets would result in increased cytoplasmic Ca^{2+} levels and, hence, in increased phospholipase A_2 activity by activation of preexisting phospholipase A_2 molecules. However, a direct correlation of cytoplasmic free Ca^{2+} concentrations and phospholipase A_2 activity could not be made at that time. Recent experiments have shown that the cytoplasmic free Ca^{2+} concentration in platelets varies from about 0.1 μM in the resting state to 2–3 μM after thrombin activation (14, 15). In contrast, *in vitro* studies on platelet phospholipase A_2 activity have been conducted at best in the range 0.2–1.0 mM of Ca^{2+} concentrations (16, 17) with the notable exception of a phosphatidic acid-specific phospholipase A_2 in horse platelet membranes, which was found to be optimally active at 7–10 μM Ca^{2+} (18). In most studies on other intracellular phospholipases A_2, whether in membrane-associated or purified form, Ca^{2+} concentrations up to 10 mM have been used. A detailed study of the Ca^{2+} requirement of cellular phospholipases A_2 has been reported only for the enzyme from sheep erythrocytes. This enzyme was found inactive at Ca^{2+} concentrations below 10 μM, increased sharply in activity above 50 μM, and reached a plateau value at 0.5 mM Ca^{2+} (19). Since this phospholipase appeared to be oriented toward the exterior of the red cell (19) and the plasma concentration of Ca^{2+} was 1.5 mM, these data seem to exclude a regulation of this phospholipase A_2 by availability of Ca^{2+} ions.

Another interesting aspect of membrane-bound phospholipases A_2 is whether their Ca^{2+} requirement is mediated by the ubiquitous Ca^{2+}-binding protein calmodulin. The involvement of calmodulin in phospholipase A_2 activation was first suggested by Wong and Cheung (20), who observed about a 20% increase in the phospholipase A_2 activity of isolated human platelet membranes upon addition of calmodulin. Despite the preliminary nature of these results, several review articles have included phospholipase A_2 in the list of enzymes of which the Ca^{2+} requirement is mediated through calmodulin (21–23).

We have recently purified the membrane-associated phospholipase A_2 from rat liver mitochondria (9). Since between 65 and 80% of the total hepatocyte calcium is mitochondrial (24) and since rat liver mitochondria have been shown to contain calmodulin (25, 26), we have investigated several aspects of the Ca^{2+} requirement of mitochondrial phospholipase A_2. This paper reports on the metal ion requirement, the influence of Ca^{2+} concentration, and the effect of calmodulin on this intracellular phospholipase A_2 in both its membrane-associated and purified forms.

MATERIALS AND METHODS

Materials. [2-^{14}C]Ethan-1-ol-2-amine hydrochloride, [8-^{14}C]adenosine 5'-monophosphate, and 3',5'-cyclic-[8-^{3}H]adenosine monophosphate were obtained from The Radiochemical Centre, Amersham, England. PEI-cellulose plates were obtained from Merck, Darmstadt, FRG. Activator-deficient phosphodiesterase from bovine heart, ionophore A23187 and adenosine 5'-monophosphate were products from Sigma, St. Louis, Missouri. 3',5'-Cyclic adenosine monophosphate was obtained from Boehringer, Mannheim, FRG. 4-Bromophenacyl bromide was bought from Fluka, Buchs, Switzerland. Calmodulin from bovine brain and trifluoperazine were kindly donated by Dr. P. Y.-K. Wong, Valhalla, New York. Rat liver mitochondrial phospholipase A_2 was purified as described previously (9).

Preparation of mitochondria. Unlabeled and [2-^{14}C]ethanolamine-labeled mitochondria were isolated as described earlier (9). Mitochondria were routinely depleted from endogenous Ca^{2+} by incubation for 15 min at 30°C with 3 nmol ionophore A23187/mg protein in a medium containing 0.25 M sucrose, 50 mM Tris (pH 8.0), 5 mM EDTA. After centrifugation for 15 min at 8000 rpm in a Sorvall SS 34 rotor at 4°C, the pellet was resuspended and washed twice in 0.25 M sucrose, 100 mM Tris-HCl (pH 8.0) to remove EDTA. The final pellet was resuspended in the buffer used for the washing procedure.

Assay of mitochondrial phospholipase A_2. When exogenous 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine (sp. radioact, 300 dpm/nmol) was used as substrate the incubations were carried out as described previously (9), except that Ca^{2+} concentrations, as indicated in the respective legends, were used. Prior to use in these assays the substrate was made Ca^{2+}-free by means of an extraction according to Bligh

and Dyer (27) in the presence of 10 mM EDTA. A unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol [1-^{14}C]linoleate from the substrate per minute.

The assay of the enzyme using endogenous substrate was done by incubating [^{14}C]ethanolamine-labeled mitochondria for 30 min at 37°C in 1 ml 0.25 M sucrose, 100 mM Tris-HCl (pH 8.0), and additions as indicated in the legends. The reaction was terminated by extraction of the lipids (27). The chloroform phase was evaporated to dryness, dissolved in a few drops of chloroform/methanol (1/2, v/v), and transferred to silica gel G thin-layer plates. The plates were developed with chloroform/methanol/acetic acid/water (65/50/1/4, v/v). Phospholipid spots were detected by iodine staining. After evaporation of iodine the phosphatidylethanolamine and lysophosphatidylethanolamine plus phosphatidylcholine spots were scraped into scintillation vials. Radioactivity was measured after addition of 14 ml Packard emulsifier liquid scintillation fluid in a Packard 3320 TriCarb liquid scintillation spectrometer. The percentage hydrolysis was calculated from the decrease in radioactivity of the phosphatidylethanolamine spot. In assays with calmodulin, the latter was preincubated with the indicated amounts of Ca^{2+} for 15 min at room temperature prior to addition of enzyme and substrate.

Calmodulin-stimulated phosphodiesterase assay. Cyclic AMP phosphodiesterase was assayed essentially by using the procedure of Klee (28). Under these conditions 0.01 mU of enzyme was stimulated over twofold by addition of 1.2 μM calmodulin.

Analytical procedures. Protein was determined by the method of Bradford (29), as modified by Vianen and van den Bosch (30).

TABLE I

EFFECT OF DIVALENT CATIONS ON MITOCHONDRIAL PHOSPHOLIPASE A_2 ACTIVITY[a]

	Assay conditions		
Metal ion	Purified enzyme	Mitochondria exogenous substrate	Mitochondria endogenous substrate
Ca^{2+}	100	100	100
Ba^{2+}	1	15	17
Cd^{2+}	0	0	0
Co^{2+}	0	0	0
Mg^{2+}	0	26	24
Mn^{2+}	0	4	24
Pb^{2+}	1	7	0
Sr^{2+}	23	33	23
Zn^{2+}	0	1	0

[a] Enzyme assays were done as described under Materials and Methods, and contained either 0.06 μg purified enzyme, 120 μg mitochondrial protein (with 0.2 mM exogenous 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine), or 1.0 mg mitochondrial protein (when hydrolysis of endogenous phosphatidyl[^{14}C]ethanolamine was measured). Metal ions were added to give a final concentration of 8 mM. Mean values of three determinations for membrane-bound enzyme and two determinations for purified enzyme, after correction for blank values in the absence of metal ions, are expressed as the percentage of the activity found in the presence of Ca^{2+}. Data from individual experiments varied by less than 20%.

RESULTS AND DISCUSSION

Table I represents results of studies on the metal ion requirement of mitochondrial phospholipase A_2. The purified enzyme was only active in the presence of Ca^{2+} and, to a much lesser extent, with Sr^{2+}. None of the other divalent cations tested could substitute for Ca^{2+}. When the membrane-associated enzyme was assayed with either endogenous or exogenous substrate, the enzyme appeared to be activated not only by Ca^{2+} and Sr^{2+}, but also consistently by Ba^{2+}, Mg^{2+}, and Mn^{2+}. The reason for this discrepancy is not known at present, but may be caused by a release of Ca^{2+} bound to mitochondrial proteins or phospholipids upon addition of these metal ions.

By analogy to what has been found for pancreatic phospholipase A_2 (31), the mitochondrial enzyme was inhibited by *p*-bromophenacyl bromide, and was protected against this inhibition by the presence of Ca^{2+} ions (Fig. 1). However, Mg^{2+} also exerted a considerable protection. Thus, after 10 min of treatment with the inhibitor in the absence of metal ions, the remaining activity amounts to 11%. In the presence of Mg^{2+} or Ca^{2+} this value is 40 and 65%, respectively. It can be concluded from these data that Mg^{2+}, although not being able to substitute for Ca^{2+} in the catalytic mechanism of the enzyme (Table I, first column), binds to the mitochondrial phospholipase A_2.

When the Ca^{2+} dependency of the purified mitochondrial phospholipase A_2 was determined in more detail, results as depicted in Fig. 2 were obtained. To obtain zero activity at least 4 μM EDTA had to be included in the incubation mixtures.

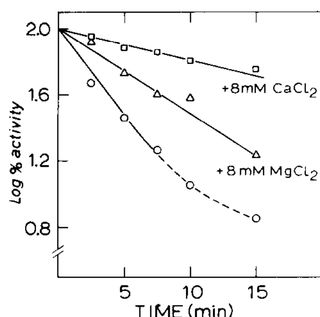

FIG. 1. Inactivation of phospholipase A_2 by *p*-bromophenacylbromide. Enzyme (0.12 μg) was preincubated in 0.13 ml 0.37 M Tris-HCl buffer (pH 8.5) at 30°C for the indicated time intervals in the presence of 0.13 mM *p*-bromophenacylbromide (○) and, when indicated, 8 mM $CaCl_2$ (□) or $MgCl_2$ (△). Enzyme activity was then measured by addition of $CaCl_2$ and substrate to final concentrations of 10 and 0.2 mM, respectively, in a total volume of 0.5 ml. Incubation time was 15 min at 37°C. The percentage remaining activity was calculated relative to enzyme preincubated for 15 min at 30°C without inhibitor but otherwise identical conditions.

Most likely this is due to the presence of trace amounts of Ca^{2+} in buffers used, as atomic absorption measurements indicated a Ca^{2+} content of 3 μM in the distilled water. Interestingly, upon addition of Ca^{2+}, two activity plateaus were clearly found, one in the micromolar and a second one in the millimolar range of Ca^{2+} concentrations. Phospholipase A_2 activity remains rather constant from 50 to 200 μM Ca^{2+} (Fig. 3A), and Fig. 3B shows that this plateau at low Ca^{2+} concentrations can easily be missed when the enzyme is only assayed at millimolar Ca^{2+} concentrations, as is often done for intracellular phospholipases A_2. Double-reciprocal plots (Fig. 3, insets) indicated a value of 14 μM and 2.4 mM for $k_{Ca^{2+}}$ for the high- and low-affinity binding site, respectively. The activity in the first plateau corresponds to about 25% of the maximal activity attained at 10 mM Ca^{2+} (Table II). Similarly, when the influence of Ca^{2+} concentration was determined with the enzyme in its membrane-associated form in mitochondria, and using either exogenous or endogenous phosphatidylethanolamine as substrate, two activity plateaus were found (Fig. 4). The values for $k_{Ca^{2+}}$ and V_{max}^{app} as calculated from Lineweaver-Burk plots are summarized in Table II.

Since experiments with the purified enzyme (Table I) had shown that Sr^{2+} was the only bivalent metal ion that could substitute for Ca^{2+} to produce an active enzyme, the influence of Sr^{2+} concentration on enzyme activity was investigated next. The results were similar to those obtained with Ca^{2+}, except that optimal activity was obtained between 1 and 5 mM Sr^{2+}. At higher concentrations inhibition was found, so that at 10 mM Sr^{2+} 60% of the optimal activity remained (data not shown). For this reason the kinetic parameters for the low-affinity site could not be assessed accurately. From a Lineweaver-Burk plot of the data obtained at Sr^{2+} concentrations below 0.4 mM, a $k_{Sr^{2+}}$ of 60 μM and a V_{max}^{app} of 420 nmol min^{-1} mg^{-1} was calculated. A comparison of these data with those obtained for Ca^{2+} (Table II) shows that both affinity and enzymatic activity were about fourfold lower with Sr^{2+} than with Ca^{2+}. This lower activity found in the micromolar range of metal ion concentrations is also observed at millimolar concentrations of Sr^{2+} and Ca^{2+} (compare data in Table I).

The next question investigated was whether the Ca^{2+} requirement of the mitochondrial phospholipase A_2 was mediated through calmodulin. Figure 5 depicts the effects of calmodulin and trifluoperazine on the membrane-bound

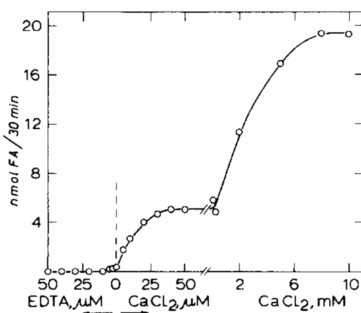

FIG. 2. Ca^{2+} dependency of purified mitochondrial phospholipase A_2. Standard assay mixtures contained 0.26 μg enzyme purified to the stage of hydroxyapatite chromatography (9) and additions as indicated.

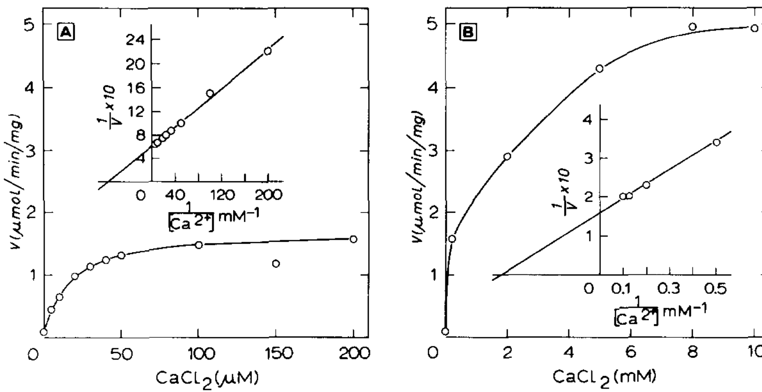

FIG. 3. Effect of Ca^{2+} concentration on purified phospholipase A_2 activity. Standard assay mixtures contained 0.13 μg enzyme purified to the stage of Matrex gel Blue A chromatography (9) and Ca^{2+} concentrations as indicated.

phospholipase A_2 when assayed toward exogenous substrate under isotonic conditions. Phospholipase A_2 activity appeared to be stimulated by 50% at 2 μM calmodulin. To determine whether this relatively small increase had to be ascribed to the presence of residual endogenous calmodulin in the mitochondria, the influence of the calmodulin antagonist trifluoperazine was investigated. The results (Fig. 5B) demonstrate that this drug exerted almost no effect on membrane-bound phospholipase A_2 when the latter was assayed at a Ca^{2+} concentration of 0.1 mM. Further experiments investigated the effect of calmodulin and trifluoperazine over a wider range of Ca^{2+} concentrations, from 0 to 500 μM. In these experiments maximal stimulation by calmodulin amounted to about 20% while trifluoperazine itself caused small stimulations, up to 15% of the phospholipase A_2. To exclude the possibility that these small effects of calmodulin and trifluoperazine were due to the fact that these compounds could only partially reach the membrane-associated phospholipase A_2 in mitochondria under isotonic conditions, the experiment was repeated under hypotonic conditions. Up to 100 μM Ca^{2+}, no significant effects of either calmodulin or trifluoperazine were observed. From 100 to 500 μM Ca^{2+}, the effects were similar to those seen under isotonic conditions, i.e., small stimulation by both compounds, but the magnitude of these effects under hypotonic conditions was even smaller than under isotonic conditions (data not shown). These data suggest that calmodulin is not involved in the regulation of mitochondrial phospholipase A_2. This conclusion is supported by experiments in which the phospholipase A_2 activity against endogenous

TABLE II

KINETIC PARAMETERS FOR PURIFIED AND MEMBRANE-ASSOCIATED MITOCHONDRIAL PHOSPHOLIPASE A_2

	High-affinity site		Low-affinity site	
Phospholipase A_2	$k_{Ca^{2+}}$ (μM)	V_{max}^{app} (mU/mg)	$k_{Ca^{2+}}$ (mM)	V_{max}^{app} (mU/mg)
Purified	14	1700	2.4	6500
Membrane-bound, exogenous substrate	6	0.5	1.4	3.6
Membrane-bound, endogenous substrate	20	0.4	12.0	0.8

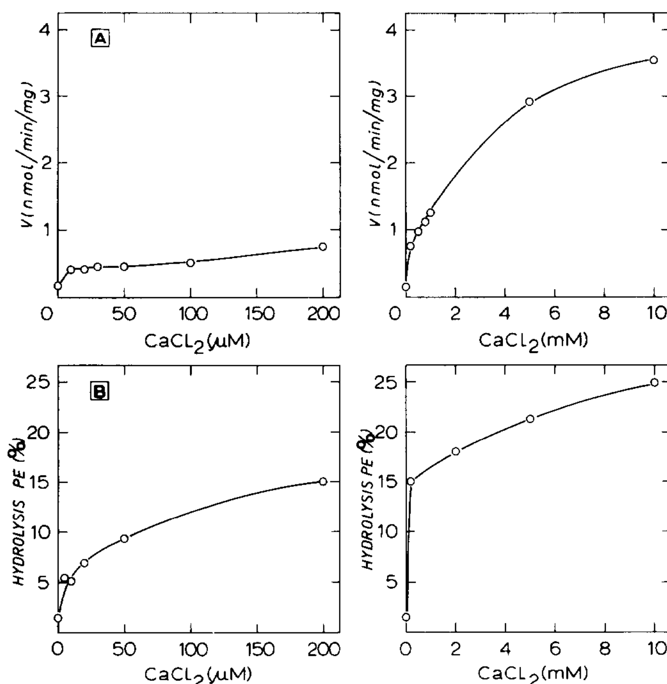

FIG. 4. Effect of Ca^{2+} concentration on membrane-associated phospholipase A_2 activity. Enzyme activity toward exogenous phosphatidylethanolamine was determined with 0.2 mM substrate and ionophore A23187-treated mitochondria (0.25 mg protein) in a medium containing 0.25 M sucrose, 100 mM Tris-HCl (pH 8.5), and the indicated Ca^{2+} concentrations (upper parts). Phospholipase activity toward endogenous phosphatidyl[^{14}C]ethanolamine was measured as described under Materials and Methods, using A23187-treated mitochondria (2.5 mg protein) and Ca^{2+} as indicated (lower parts). Left panels, curves for micromolar Ca^{2+}; Right panels, curves for millimolar Ca^{2+}.

mitochondrial phosphatidylethanolamine was measured (Table III). Hydrolysis of the membranous substrate was stimulated

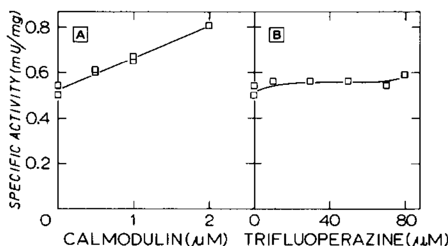

FIG. 5. Effect of calmodulin and trifluoperazine on membrane-bound phospholipase A_2. Ionophore A23187-treated mitochondria (0.25 mg protein) were incubated with 100 nmol 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine in the presence of 0.1 mM $CaCl_2$ under isotonic conditions in 0.25 M sucrose. The indicated amounts of calmodulin (A) or trifluoperazine (B) were added.

almost as effectively by Ca^{2+} alone as by Ca^{2+} plus calmodulin. Again, trifluoperazine has no inhibitory effect and, if anything, caused a slight stimulation. These results are in good agreement with those of Zurini *et al.* (32). These authors also demonstrated a negligible effect of calmodulin on mitochondrial phospholipase A_2 in its action on membranous substrate, and noticed about a 10% stimulation by trifluoperazine. Recently, trifluoperazine has also been shown to stimulate arachidonate release from prelabeled macrophages by about 1.5-fold (33). Since evidence has recently been reported (34) which seems to indicate that trifluoperazine can influence phospholipase A_2 activity by interaction of this hydrophobic compound with phospholipid substrate, further experiments were done with the purified mitochondrial phospholipase A_2.

TABLE III

EFFECT OF CALMODULIN AND TRIFLUOPERAZINE ON MITOCHONDRIAL PHOSPHOLIPASE A_2 ACTING ON ENDOGENOUS PHOSPHATIDYLETHANOLAMINE [a]

Additions	Percentage hydrolysis	
	Experiment 1	Experiment 2
None	4.2	3.9
EDTA, 1 mM	0.1	0.6
$CaCl_2$, 0.5 mM	24.6	26.0
$CaCl_2$, 0.5 mM + calmodulin, 2 μM	28.3	28.3
$CaCl_2$, 0.5 mM + trifluoperazine, 50 μM	26.6	26.5
$CaCl_2$, 0.5 mM + calmodulin, 2 μM + trifluoroperazine, 50 μM	28.6	27.8

[a] Ionophore A23187-treated, [^{14}C]ethanolamine labeled mitochondria (3.9 mg protein) were incubated for 30 min at 37°C in a medium containing 0.25 M sucrose, 100 mM Tris-HCl (pH 8.0) and additions as indicated. Values of duplicate experiments are expressed as the percentage of [^{14}C]phosphatidylethanolamine hydrolyzed after subtraction of a zero-time control.

Figure 6 shows that trifluoperazine indeed stimulates the purified enzyme. These effects of trifluoperazine are fully compatible with those found on the membrane-associated phospholipase A_2, and suggest that they could be caused by a trifluoperazine-induced change in the structural organization of the phospholipid substrate. These stimulations by trifluoperazine of the purified phospholipase A_2 in its action toward phosphatidylethanolamine were noticed at all Ca^{2+} concentrations investigated. Figure 6 also clearly demonstrates that calmodulin does not significantly influence mitochondrial phospholipase A_2, and certainly does not stimulate the enzyme.

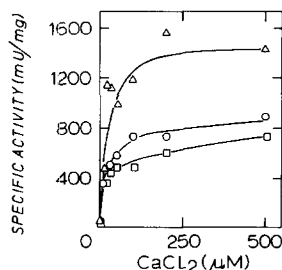

FIG. 6. Influence of Ca^{2+} concentration on purified mitochondrial phospholipase A_2 in the presence of either calmodulin or trifluoperazine. Phospholipase A_2 (0.2 μg) was incubated with phosphatidylethanolamine under standard conditions in the presence of Ca^{2+} concentrations, varied as indicated. Symbols: ○, without additions; □, with 1 μM calmodulin; △, with 50 μM trifluoperazine. Mean values of two experiments are given.

GENERAL DISCUSSION

Rat liver mitochondria are known to contain a phospholipase A_2 which is dependent on Ca^{2+} (35-37), and which has recently been purified to near homogeneity (9, 38). The specificity of the enzyme for Ca^{2+} compared to other divalent cations has not yet been reported. Studies on the metal ion requirement of the enzyme in purified and membrane-associated forms show that different requirements are found depending on the form in which the enzyme is tested (Table I). Although it cannot be ruled out at present that differences in enzyme conformations in soluble and membrane-bound states are responsible for the observed differences in metal ion requirements, these could also be caused by the release of bound Ca^{2+} in the presence of other metal ions which seem to activate the enzyme in membrane-bound form. The combined results presented in this paper suggest that only Sr^{2+} can substitute to some degree for Ca^{2+} in the catalytic mechanism of the mitochondrial phospholipase A_2 (Table I). This result is different from that found for pancreatic phospholipase A_2. This enzyme was completely in-

active with Mg^{2+}, Mn^{2+}, and Co^{2+}, while Ba^{2+} and Sr^{2+} gave activities ranging from only 0.1 to 0.4% of those found in the presence of Ca^{2+} (31). While the first three metal ions had no influence on the enzymatic activity of the pancreatic enzyme, Ba^{2+} and Sr^{2+} behaved as competitive inhibitors, with dissociation constants equal to those for Ca^{2+}. The mitochondrial phospholipase A_2 in the presence of Sr^{2+} reaches an activity of about 25% of the value found with Ca^{2+}. For their interpretation of experiments on Ca^{2+}-stimulated ion losses from mitochondria, Harris and Cooper (39) have used the earlier results with pancreatic phospholipase A_2 to state that Sr^{2+} does not activate the mitochondrial phospholipase A_2 either. The results presented above stress that such extrapolations should be made with caution. Although a rather unspecific assay of free fatty acid release as well as intact mitochondria were also used, Severina and Evtodienko (40) have claimed that Sr^{2+} can activate mitochondrial phospholipase A_2.

Between 65 and 80% of the total liver cell calcium is contained in the mitochondria (24, 41). Liver mitochondria, as normally isolated, contain 5-10 nmol Ca^{2+}/mg protein (24, 42), but the actualCa^{2+} content of liver mitochondria *in situ* is a matter of considerable debate. Recent experiments with isolated liver cells indicated that the mitochondria contained 16 nmol Ca^{2+}/mg protein, corresponding to a free Ca^{2+} concentration in the matrix of about 16 μM (41). It is interesting to note that this value is close to the $k_{Ca^{2+}}$ for the high-affinity Ca^{2+} site of the mitochondrial phospholipase A_2 as reported in this paper (Table II). This suggests that the activity of this enzyme could be influenced by fluctuations in the *in vivo* mitochondrial Ca^{2+} content, at least as far as the enzyme is localized at the inner aspect of the inner mitochondrial membrane. Earlier reports (37, 43) have indicated that about one-third of the phospholipase might be located in the inner membrane. The transverse distribution of this enzyme over both sides of the inner mitochondrial membrane has not been investigated in detail, but Zurini *et al.* (32) obtained indications for a localization on both sides.

At present it remains unknown whether the two activity plateaus observed upon variation of the Ca^{2+} (or Sr^{2+}) concentration (Figs. 2-4) are caused by two Ca-binding sites in a single phospholipase A_2 entity. As mentioned, earlier reports (37, 43) have localized the mitochondrial phospholipase A_2 in both inner and outer membrane. Potentially, the two activity plateaus could thus be caused by the presence of two enzymes, each having a single but different Ca^{2+}-binding site. This point requires investigation. It is interesting to note that porcine pancreatic phospholipase A_2 contains a second, low-affinity, Ca^{2+}-binding site which enables the enzyme to interact with organized lipid-water interfaces at alkaline pH (44).

The first studies on the possible involvement of calmodulin in phospholipase A_2 regulation were reported by Wong and Cheung (20). These investigators found a 15-30% increase in the phospholipase A_2 activity of human platelet membranes upon addition of calmodulin. This relatively small increase was ascribed to the presence of endogenous calmodulin. However, the calmodulin antagonist trifluoperazine did not decrease phospholipase A_2 activity in platelet membranes to below that of basal levels, and thus apparently did not affect the putative endogenous calmodulin. On the other hand, the release of arachidonic acid from phosphatidylcholine in prelabeled human platelets after exposure to thrombin was almost completely inhibited by 100 μM trifluoperazine (45). However, the usefulness of trifluoperazine to unravel calmodulin-mediated processes has been questioned recently in view of the findings that the drug exerts other effects in addition to calmodulin inactivation (46-48). Based on their finding that trifluoperazine inhibits pancreatic phospholipase A_2 in the absence of calmodulin, Withnall and Brown (34) concluded that further work is required before a widespread activation of phospholipase A_2 by calmodulin can be hypothesized. However, these authors also pointed out that their results may differ

from those of Wong and Cheung (20), because they used a soluble rather than a membrane-associated phospholipase A_2.

The availability of a purified mitochondrial phospholipase A_2 has enabled us to study, for the first time, the effect of calmodulin on an intracellular phospholipase A_2 in both its membrane-associated and purified forms. The results with membrane-associated enzyme (Fig. 5 and Table III) confirm and extend earlier data reported by Zurini *et al.* (32), and suggest that calmodulin is not involved in mitochondrial phospholipase A_2 activation. However, lack of stimulation by exogenous calmodulin could potentially be due to saturation of the enzyme with endogenous calmodulin, and in itself does not necessarily indicate that calmodulin is not involved. Subsequent experiments with the purified enzyme (Fig. 6) also indicated that calmodulin does not significantly influence mitochondrial phospholipase A_2. Inasmuch as this enzyme, in the course of its purification (9), eluted from AcA 54 column with an apparent molecular weight of 10,000, the lack of stimulation by added calmodulin cannot be the result of a putative tightly associated calmodulin in our enzyme preparation.

In support of earlier suggestions (20, 45) that phospholipase A_2 stimulation by Ca^{2+} is mediated through calmodulin, Moskowitz and co-workers have recently concluded, from experiments with *Naja naja* snake venom (49) and brain synaptic vesicles (50), that phospholipase A_2 is a calmodulin-regulated enzyme. This conclusion should not be generalized. Rat liver mitochondrial phospholipase A_2 is apparently activated directly by Ca^{2+} ions, and calmodulin does not additionally stimulate its activity. Interestingly, after completion of these experiments, Ballou and Cheung (51) reported that stimulation by calmodulin of phospholipase A_2 associated with platelet membranes was variable, but that neither calmodulin nor trifluoperazine affected a partially purified platelet phospholipase A_2. Similarly, calmodulin did not influence the activity of a purified phospholipase A_2 from rat spleen (52).

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