A Phospholipase A_2 Model System. Calcium Enhancement of the Amine-Catalyzed Methanolysis of Phosphatidylcholine†

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ABSTRACT: In methanolic solutions octylamine catalyzes a random methanolysis of phosphatidylcholine to produce lysophosphatidylcholine. The rate of the reaction is enhanced several hundredfold in the presence of $CaCl_2$. Analysis of the kinetics of the reaction suggests that the reactive intermediate is a calcium–phosphatidylcholine–amine complex. The enhancement is relatively specific for Ca with relative rates Ca > Sr ≫ Ba > Mg. Monovalent ions such as Li and Na and the rare earth ion, cerium, did not enhance the reactions. The reaction proceeds more rapidly in methanol than in ethanol or *n*-propyl alcohol. The reaction in the presence of calcium is inhibited by water. The methanolysis in the absence of $CaCl_2$ is subject to inhibition by CH_3OD. In the presence of $CaCl_2$ there is no deuterium isotope effect. It is suggested that Ca^{2+} lowers the pK of the methanol, thereby facilitating proton abstraction by the amine generating a methoxide ion which attacks the carbonyl group of the phosphatidylcholine. Such a mechanism can also be applied to the hydrolysis of phosphatidylcholine by phospholipase A_2.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acyl ester at the 2 position of phosphoglycerides. The reaction has been shown to proceed by *O*-acyl cleavage without a detectable acyl-enzyme intermediate (Wells, 1971). The enzyme has an absolute and specific requirement for calcium (Wells, 1972). Wells (1973a,b) has presented data which suggest that one role of calcium is to lower the pK of an ϵ-amino group of a lysine which is a catalytically essential group in the enzyme. It was suggested that the amino group may act as a base which abstracts a proton from water to produce a hydroxide ion, which then attacks the ester by a classic hydroxide ion catalyzed ester cleavage. This mechanism would account for *O*-acyl cleavage and the lack of an acyl-enzyme intermediate, as well as the irreversibility of the reaction. Other possible roles of calcium such as participation in substrate binding or in the catalytic step could not be eliminated with the data presently available.

Fujiwara *et al.* (1967) reported that the primary amines, such as dodecylamine, could catalyze the methanolysis of phosphatidylcholine. Some data were presented to suggest that this reaction showed preferential cleavage of the ester at the 2 position of the phosphatidylcholine. Misiorowski and Wells (1973) reported that phosphatidylcholine could bind calcium in methanolic solution. It was therefore of interest to determine what effect calcium and other metal ions might have on the amine-catalyzed methanolysis of phosphatidylcholine, and whether such a model system might produce data which would be useful in interpreting the mechanism of action of phospholipase A_2. This paper describes the results of these studies.

Materials and Methods

Hen's egg yolk phosphatidylcholine was purified as described by Wells and Hanahan (1969). Anhydrous reagent grade salts were obtained from Ventron Corp. (Beverly, Mass.). Perchlorate salts were dried *in vacuo* at 100° for 2 days. Deuterated methanol (CD_3OD, 99.5% D; and CH_3OD, 99% D) was purchased from Bio-Rad Laboratories (Richmond, Calif.). Other chemicals were reagent grade.

Quantitative assessment of reaction rates was by polarimetry. The optical rotation was measured continuously in an OLD-4 (Zeiss) polarimeter (λ 546 nm). The rate constant was evaluated from a plot of ln $[(\alpha_t - \alpha_\infty)/(\alpha_0 - \alpha_\infty)]$ *vs.* time; α_t = optical rotation at time t, α_∞ = equilibrium optical rotation, and α_0 = initial optical rotation.

Analysis of Phosphorus-Containing Compounds. At various times after initiating the reaction, 0.5-ml aliquots of the reaction mixture were neutralized with glacial acetic acid and diluted with 2 ml of chloroform–methanol (9:1, v/v), 1 ml of isobutyl alcohol, and 2 ml of water. After mixing and centrifugation, the upper aqueous phase was removed and the lower phase washed with 1 ml of methanol–water (1:2, v/v). The combined upper phases and the lower phase were analyzed for phosphorus (Dittmer and Wells, 1969). Thin layer chromatography of the lipid products in the lower phase was on silica gel G using the solvent system chloroform–methanol–water (95:35:6, v/v). The products in the upper phase were analyzed on thin layers of cellulose (polygram Cel-300, Brinkman Instruments Inc., Westbury, N. Y.) using the solvent system isopropyl alcohol–concentrated NH_4OH–water (7:1:2, v/v) or on Whatman No. 1 paper using the solvent system butanol–acetic acid–water (5:2:3, v/v).

Analysis of Released Fatty Acids. Aliquots (0.5 ml) of the reaction mixture were neutralized with glacial acetic acid and diluted with 5 ml of chloroform, and the phosphorus-containing compounds were adsorbed onto 100 mg of silicic acid (CC-4, Mallinckrodt Chemical Works, St. Louis, Mo.). The fatty acid products were analyzed by thin layer chromatography on silica gel G using the solvent system hexane–diethyl ether–acetic acid (80:20:1, v/v). Fatty acid methyl esters were analyzed by gas-liquid chromatography using a Hewlett-Packard Model 402 with a flame detector and a Model 3370A integrator. A 6 ft × 0.4 cm (i.d.) column of diethylene glycol succinate (15% on Gas Chromosorb P, 100–120 mesh; Alltech Associates, Arlington Heights, Ill.) maintained at 190° (flash evaporator temperature, 200°; detector tempera-

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TABLE I: Production of Water-Soluble Phosphorus-Containing Compounds during Methanolysis of Phosphatidylcholine.[a]

Time of Incubation (min)	Chloroform-Soluble P (μmol/ml of React. Mixture)	Water-Soluble P (μmol/ml of React. Mixture)
0	25.1	0.0
10	24.3	0.0
20	24.1	0.3
30	23.1	1.3
40	22.1	3.0
50	21.5	3.4
100	19.0	6.5
200	14.0	9.7
400	8.1	17.3
600	5.1	20.1

[a] A sample containing 25 mM phosphatidylcholine, 100 mM $CaCl_2$, and 10 mM octylamine in methanol was incubated for the indicated period of time. After neutralization with acetic acid the phosphorus containing compounds were partitioned between water and chloroform.

ture, 265°; carrier gas helium, 70 ml/min) was used. Fatty acid standards were obtained from the Hormel Institute (Austin, Minn.).

Total fatty acids of the phosphatidylcholine were analyzed after transesterification in 0.5 N methanolic NaOH. Fatty acids released by phospholipase A_2 were isolated (Wells and Hanahan, 1969) and converted to methyl esters (Metcalfe and Schmitz, 1961). Lysophosphatidylcholine produced by phospholipase A_2 was purified (Wells and Hanahan, 1969) and transesterified.

Phosphorus compounds were detected by the method of Dittmer and Lester (1964) for lipid compounds and by the method of Hanes and Isherwood (1949) for water soluble compounds. Neutral lipids were detected on thin layer plates with potassium dichromate in sulfuric acid (Skipski and Barclay, 1969).

Results

General Observations. When a 25 mM solution of phosphatidylcholine in methanol was incubated with 10 mM octylamine at room temperature (22–25°) there was a slow change in optical rotation as shown in Figure 1A. In agreement with Fujiwara *et al.* (1967), thin layer chromatography showed the production of lysophosphatidylcholine. If the reaction was carried out in the presence of 0.1 M $CaCl_2$, there was a marked increase in rate, as shown in Figure 1B. In the presence of $CaCl_2$ the change in optical rotation was biphasic. During the first 30 min, the rapid reaction, there was very little production of water-soluble phosphorus containing compounds (Table I), and complete conversion of phosphatidylcholine to lysophosphatidylcholine. During the slow reaction there was production of a water-soluble phosphorus containing compound (Table I), which was shown by chromatographic analysis to be glycerophosphorylcholine. For purposes of this study only the initial rapid reaction will be considered.

As indicated above, the initial reaction involves the production of lysophosphatidylcholine. The fatty acid was released solely as the methyl ester, as shown by chromatographic analysis. Since there are two fatty acyl esters in phosphatidylcholine, the initial reaction could proceed by either of two

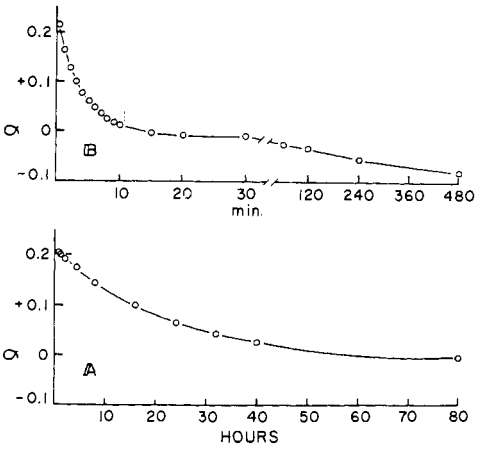

FIGURE 1: The methanolysis of phosphatidylcholine catalyzed by octylamine as reflected in the change of optical rotation (α): (A) 25 mM phosphatidylcholine and 10 mM octylamine; (B) 25 mM phosphatidylcholine, 10 mM octylamine, and 100 mM $CaCl_2$.

steps, as shown in Figure 2. The data of Fujiwara *et al.* (1967) would suggest that $k_2 > k_1$; however, the value of the optical rotation at the end of the rapid reaction, *viz.* $[\alpha]_{546}^{22} = -0.25$, would not be in accord with this suggestion, since $[\alpha]_{546}^{22} = -3.7$ (c 5.0, CH_3OH) for 1-acyllysophosphatidylcholine produced from the starting material by phospholipase A_2. Furthermore, analysis of the fatty acids released, both in the presence and absence of $CaCl_2$, showed that the fatty acids are released from positions 1 and 2 at the same rate (Table II). Based on available data (Hanahan *et al.*, 1952; deHaas and Van Deenen, 1965) one would expect an equimolar mixture of 1- and 2-acyllysophosphatidylcholines to have an optical rotation close to zero. The slow change of the optical rotation to a more negative value during the second phase of the reaction represents the production of glycerophosphorylcholine.

Under the conditions described in this paper the reaction is a random methanolysis of phosphatidylcholine to produce lysophosphatidylcholine. The value of the optical rotation at the end of the rapid reaction is the value of $[\alpha_\infty]$, which was used to evaluate the rate constant. Under the conditions shown in Figure 1A and B, the rate constant in the absence of $CaCl_2$ was 7.4×10^{-4} min^{-1} and in the presence of $CaCl_2$ it was 0.23 min^{-1}.

The effects of concentration of phosphatidylcholine, $CaCl_2$, and octylamine on the apparent first-order rate constant are illustrated in Figures 3 and 4. Figure 3 shows that at constant octylamine the rate constant increases with increasing $CaCl_2$ and decreases with increasing phosphatidylcholine. Figure 4

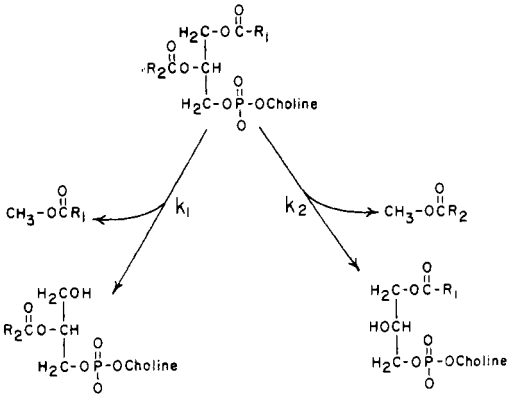

FIGURE 2: Pathways for the conversion of phosphatidylcholine to lysophosphatidylcholine.

TABLE II: Fatty Acid Composition of Phosphatidylcholine and Fatty Acids Released during Methanolysis.[a]

Fatty Acid	Phosphatidylcholine			25 mM Phosphatidylcholine,[e] 100 mM $CaCl_2$, + 10 mM Octylamine incubated for		25 mM Phosphatidylcholine[e] + 10 mM Octylamine incubated for	
	Total[b]	Position 1[c]	Position 2[d]	2 min	10 min	10 hr	36 hr
16:0	36.9	70.2	3.0	37.0	37.5	37.7	37.5
16:1	1.5	2.0	1.0	1.4	1.5	1.6	1.4
18:0	12.8	24.2	1.0	12.6	12.0	13.1	12.6
18:1	31.6	3.6	60.0	32.8	31.4	30.1	32.0
18:2	11.8		23.9	10.9	12.0	12.5	12.2
18:3	0.6		1.1	0.7	0.7	0.6	0.7
20:4	4.8		10.0	4.6	4.9	4.4	3.6

[a] Fatty acids were analyzed as methyl esters. Composition reported as weight percentage. [b] After chemical hydrolysis in methanolic NaOH. [c] Fatty acids of lysophosphatidylcholine after phospholipase A_2 hydrolysis. [d] Fatty acids released by phospholipase A_2 hydrolysis. [e] Released methyl esters.

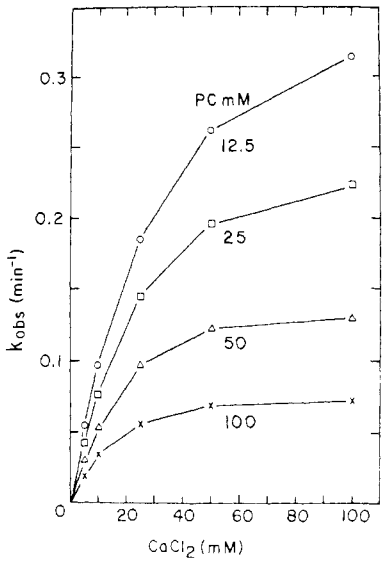

FIGURE 3: The effect of the concentration of phosphatidycholine (PC) and $CaCl_2$ on the apparent first-order rate constant for methanolysis catalyzed by 10 mM octylamine.

shows that at constant phosphatidylcholine the rate constant increases with increasing $CaCl_2$ and octylamine. There was not a linear dependence of the rate constant on the concentration of any of the three components of the reaction. The rate constant was also not linearly dependent on the ratio of any two reactants at a constant concentration of the third component.

Since there was no reaction in the absence of added octylamine, this must be the catalytically active component. In the absence of added $CaCl_2$ the reaction rate was linearly dependent on the concentration of the amine. Previously it was shown that Ca^{2+} can bind to phosphatidylcholine in methanol (Misiorowski and Wells, 1973). Therefore it seemed probable that the reaction could be represented as follows.

$$PC^1 + Ca \rightleftharpoons PC\text{-}Ca$$
$$PC\text{-}Ca + RNH_2 \rightleftharpoons PC\text{-}Ca\text{-}RNH_2$$
$$PC\text{-}Ca\text{-}RNH_2 \longrightarrow \text{products}$$

At a constant concentration of phosphatidylcholine, the concentration of the reactive component, PC–Ca–RNH_2,

[1] Abbreviation used is: PC, phosphatidylcholine.

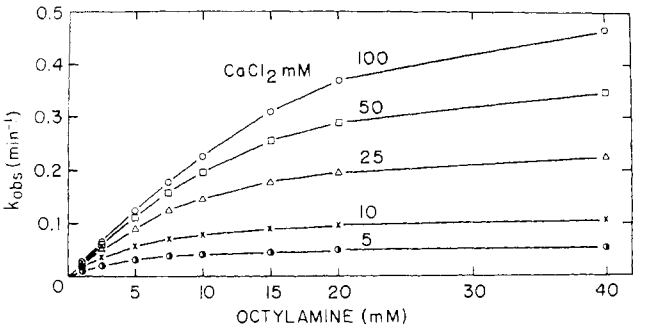

FIGURE 4: The effect of the concentration of octylamine and $CaCl_2$ on the apparent first-order rate constant for the methanolysis of 25 mM phosphatidylcholine.

would increase as the $CaCl_2$ or octylamine concentration increased. However, at constant $CaCl_2$ and octylamine, the fraction of the total phosphatidylcholine present as the reactive intermediate would be reduced as the total phosphatidylcholine concentration increased.

Another mechanism which can also explain the data can be written as follows

$$RNH_2 + Ca \rightleftharpoons Ca\text{-}RNH_2$$
$$Ca\text{-}RNH_2 + PC \rightleftharpoons PC\text{-}Ca\text{-}RNH_2$$
$$PC\text{-}Ca\text{-}RNH_2 \longrightarrow \text{products}$$

There is no *a priori* reason to choose one mechanism over the other. However, since $CaCl_2$ binding to phosphatidylcholine has been studied, the first mechanism has been used to analyze the data.

If one assumes that octylamine does not change the binding of calcium to phosphatidylcholine, then, as shown in the Appendix, one can evaluate the binding of octylamine to the Ca–PC complex and the true rate constant. Conditions were chosen such that ν_1 would be the same (calculated from the data of Misiorowski and Wells, 1973) and the apparent rate constant was plotted *vs.* the octylamine to phosphatidylcholine ratio. Two such plots are shown in Figure 5.

The calculated values of the ν_2 are plotted in Figure 6. The data show 1 mol of amine is present in the active complex and give a value of 0.27 mM^{-1} for K_2. The true first-order rate constant can be evaluated from a plot of $1/k_{obsd}$ *vs.* $1/\nu_1\nu_2$ as shown in Figure 7, which gives $k = 0.77$ min^{-1}. Alternatively, k can be determined from eq 13 (Appendix). These results are presented in Table III.

TABLE III: Conditions Used to Calculate the First-Order Rate Constant (k) and the Equilibrium Constant for Formation of the Ca–PC–RNH_2 Complex.[a]

Conditions	ν_{Ca}[b]	k (min^{-1})
I. A. 12.5 mM PC B. 100 mM PC 50 mM $CaCl_2$ 100 mM $CaCl_2$ Octylamine 1.0, 2.5, 5.0, 7.5, and 10.0 mM	0.55	0.73 ± 0.04 (sd)
II. A. 25 mM PC B. 100 mM PC 50 mM $CaCl_2$ 50 mM $CaCl_2$ Octylamine 1.0, 2.5, 5.0, 7.5, 10.0, 15, and 20.0 mM	0.33	0.78 ± 0.03 (sd)
III. A. 12.5 mM PC B. 100 mM PC 10 mM $CaCl_2$ 25 mM $CaCl_2$ Octylamine 1.0, 2.5, 5.0, 7.5, 10.0, and 15.0 mM	0.18	0.80 ± 0.02 (sd)

[a] Each pair of conditions was chosen such that ν_{Ca} would be the same. The concentrations of octylamine used in the calculations are also indicated. [b] Calculated from the data of Misiorowski and Wells (1973).

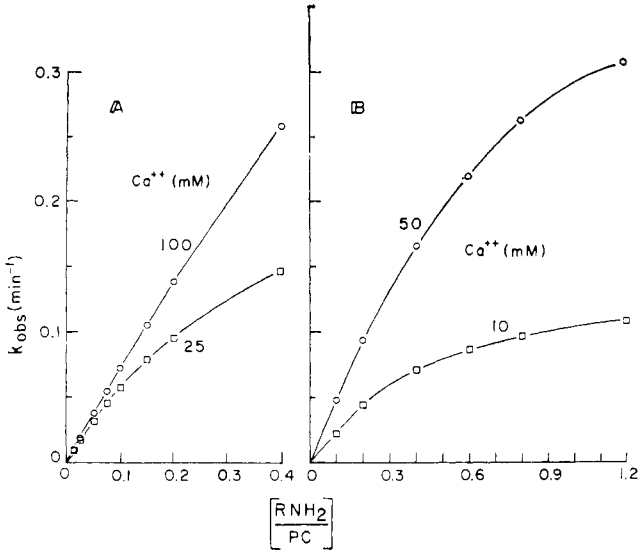

FIGURE 5: The apparent first-order rate constant for the methanolysis of phosphatidylcholine as a function of the octylamine:phosphatidylcholine ratio: (A) the concentration of phosphatidylcholine was held constant at 100 mM; (B) the concentration of phosphatidylcholine was held constant at 12.5 mM. The open circles represent $\nu_{Ca} = 0.55$ and the open squares $\nu_{Ca} = 0.18$.

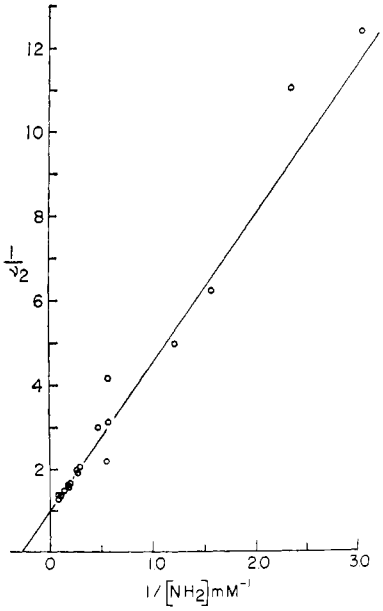

FIGURE 6: Determination of ν_2 (binding of octylamine to the phosphatidylcholine–$CaCl_2$ complex). ν_2 and the concentration of free octylamine were calculated as detailed in the Appendix.

This analysis shows that the reactive complex contains 1 mol each of calcium, octylamine, and phosphatidylcholine and gives a correct value for k. The same results would have been obtained by the use of the alternate mechanism shown above, except that different equilibrium constants would be obtained. For the purposes of this study the important conclusion is that PC–Ca–RNH_2 is the reactive complex, and the pathway for its formation is not critical at this time.

Effect of Deuterated Methanol. Deuterium isotope effects were measured either in CH_3OD or CD_3OD with identical results. As shown in Table IV, in the absence of $CaCl_2$ there was a significant deuterium isotope effect, whereas in the presence of $CaCl_2$ there was no deuterium isotope effect.

Cation Specificity. Table V shows k_{obsd} for several alkaline earth salts. It is apparent that calcium gives the largest rate enhancement. The relative enhancement was Ca > Sr ≫ Ba > Mg (1.0:0.38:0.08:0.04). $BaCl_2$ is insoluble in methanol and could not be tested. At low concentrations there did not seem to be an anion effect since $CaCl_2$ and $Ca(ClO_4)_2$ or $MgCl_2$ and $Mg(ClO_4)_2$ gave comparable results. Precipitation of phosphatidylcholine (15 mM) was observed at 7.5 mM $Ca(ClO_4)_2$ and 30 mM $Mg(ClO_4)$, but no precipitation was observed at 0.1 M $Ba(ClO_4)_2$. In the case of the chloride salts no precipitation was observed at 0.1 M. There was no detectable rate enchancement caused by 100 mM LiCl or NaBr or by 10 mM $CeCl_3$. Previous data showed that NaBr does not complex to phosphatidylcholine, whereas $CeCl_3$ forms a stronger complex than $CaCl_2$ (Misiorowski and Wells, 1973).

Specificity for the Alcohol. The reaction was carried out using 25 mM phosphatidylcholine, 10 mM octylamine, and 100 mM $CaCl_2$ in methanol, ethanol, and *n*-propyl alcohol. k_{obsd} was: methanol, 0.225 min^{-1}; ethanol, 0.008 min^{-1}; propanol, 0.003 min^{-1}. In 0.1 M methanol in acetonitrile, containing 25 mM phosphatidylcholine, 0.1 M $CaCl_2$, and 10 mM octylamine, or in diethyl ether–methanol (95:5 v/v), containing 10 mM $CaCl_2$, 25 mM phosphatidylcholine, and 10 mM octylamine,

TABLE IV: Deuterium Isotope Effect on the Amine-Catalyzed Methanolysis of Phosphatidylcholine in the Presence and Absence of $CaCl_2$.

	$k_{obsd}(CH_3OH)$ (min^{-1})	$k_{obsd}(CH_3OD)$ (min^{-1})	k_H/k_D
I. 25 mM phosphatidylcholine, no $CaCl_2$, and			
10 mM octylamine	7.4×10^{-4}	3.6×10^{-4}	2.06
20 mM octylamine	1.55×10^{-3}	6.8×10^{-4}	2.26
II. 25 mM phosphatidylcholine, 100 mM $CaCl_2$, and			
10 mM octylamine	0.215	0.219	0.98
20 mM octylamine	0.370	0.365	1.01
III. 25 mM phosphatidylcholine, 10 mM $CaCl_2$, and			
10 mM octylamine	0.078	0.075	1.04
20 mM octylamine	0.097	0.101	0.96

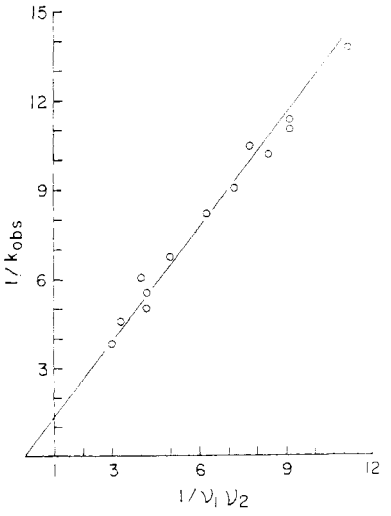

FIGURE 7: The dependence of the apparent first-order rate constant (k_{obsd}) on $\nu_1\nu_2$. $\nu_1\nu_2$ was calculated according to eq 14 of the Appendix.

there was no detectable reaction after 48 hr, as judged by thin layer chromatography.

Specificity for the Amine. As shown in Figure 8 octylamine, butylamine, and diethylamine appear to be equally effective catalysts. Triethylamine was a poor catalyst in spite of a pK very close to octylamine. Ethanolamine was slightly better than triethylamine. No reaction was observed under the conditions described in Figure 8 using 0.1 M imidazole or 0.1 M aniline. There was no correlation of k_{obsd} with the pK of the amine.

Effect of Added Water. Misiorowski and Wells (1973) showed that the binding of calcium to phosphatidylcholine was inhibited by water. This inhibition required four molecules of water per phosphatidylcholine and calcium binding was reduced by one-half at 5.0 M H_2O in methanol. The data in Figure 9A show that the methanolysis of phosphatidylcholine in the presence of calcium was also inhibited by water. However, in this case inhibition occurred with much less water than observed for calcium binding. A plot of $1/k_{obsd}$ *vs.* the water concentration was linear (data not shown) and gave a value of $K_{assn} = 0.83\ M^{-1}$, and showed that only one water molecule was involved. In the absence of calcium there was no inhibition by water (Figure 9B). The addition of water either in the presence or absence of calcium did not lead to hydrolysis, since only methyl esters of fatty acids could be detected by thin layer chromatography of the reaction products.

TABLE V: Effect of Various Cations on the Amine-Catalyzed Methanolysis of Phosphatidylcholine (25 mM Phosphatidylcholine–10 mM Octylamine).

Salt	k_{obsd} (min^{-1}) at Salt Concentration (mM) 25	50	100
$CaCl_2$	0.150	0.200	0.230
$SrCl_2$	0.058	0.075	0.090
$MgCl_2$	0.006	0.008	0.010
$Ba(ClO_4)_2$	0.012	0.017	0.018

Discussion

The data presented are consistent with the hypothesis that a phosphatidylcholine–calcium–amine complex is the reactive intermediate in the methanolysis reaction, although the pathway for the formation of the complex is still ambiguous at the present time. Since the product is a methyl ester of the fatty acid, it is apparent that the nucleophilic attack at the carbonyl carbon must involve a methoxide ion. The following discussion will be concerned primarily with the activating effect of calcium and possible implications for the mechanism of phospholipase A_2.

The mechanism proposed to account for the enhancement of methanolysis by calcium is shown in Figure 10. The conformation shown is taken from the paper by Vanderkooi (1973).[2] The calcium serves to lower the pK of the methanol and thereby facilitate proton transfer to the amine catalyst generating the reactive nucleophile. Calcium also orients the methanol so as to facilitate attack on the carbonyl carbon. In addition calcium may also reduce the electron density developing on the carbonyl group during attack by the methoxide ion.

The loss of a deuterium isotope effect in the presence of calcium supports the conclusion that calcium lowers the pK of methanol. A similar conclusion was reached by Werber and Shalitin (1973) studying metal ion catalysis of the acylation of amino alcohols by active esters.

A rather strict stereochemistry for the reactive complex is supported by the relative effectiveness of various cations in promoting the reaction. Although many of the ions tested form a complex with phosphatidylcholine, only calcium and strontium show effective catalysis with calcium approximately three times more effective. The model shown in Figure 10

[2] An equally probable conformation places the carbonyl at C-1 near the Ca^{2+} and would account for the random methanolysis.

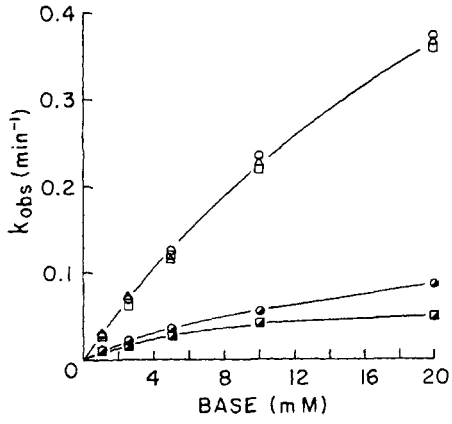

FIGURE 8: The effect of the concentration of various amines on the apparent first-order rate constant for the methanolysis of phosphatidylcholine (25 mM) in the presence of $CaCl_2$ (100 mM): (○) octylamine; (□) butylamine; (△) diethylamine; (◑) ethanolamine; (◪) triethylamine.

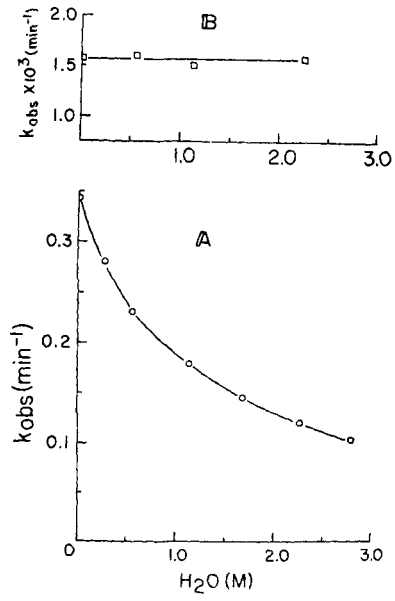

FIGURE 9: The effect of water on the apparent first-order rate constant for the methanolysis of phosphatidylcholine: (A) 25 mM phosphatidylcholine, 100 mM $CaCl_2$, and 10 mM octylamine; (B) 25 mM phosphatidylcholine and 10 mM octylamine.

could explain these effects due to the different sizes of the ions and the resulting different proximity or orientation of the alcohol and the carbonyl group. The low rate seen in ethanol and propanol may be related to steric effects or due to the higher pK values of these alcohols. The effectiveness of various amines also supports the suggestion that the stereochemistry of the complex is a critical factor affecting the reaction rate.

The inhibitory effect of water seems to be related to solvation of calcium, since the reaction in the absence of calcium is not inhibited by water, and the amount of water required for inhibition is well below that concentration of water required to disrupt the calcium–phosphatidylcholine complex. Stockton and Martin (1972) have shown that calcium has a higher affinity for water than for methanol in acetonitrile solutions. The inhibition by water might represent either an improper orientation for reaction or too high a pK of H_2O for proton abstraction by the amine.

Calcium could increase the reactivity of the carbonyl group by polarization of the carbonyl group. Two pieces of data argue against this mechanism: (1) the formation of the calcium–phosphatidylcholine complex does not require the presence of carbonyl groups (Misiorowski and Wells, 1973); (2) infrared spectroscopy shows no observable effect on the carbonyl stretching frequency in methanolic solutions of phosphatidylcholine in the presence of $CaCl_2$.[3]

The results of these experiments suggest a direct role of calcium in the mechanism of action of phospholipase A_2 should be seriously considered. An interaction of Ca^{2+} and phosphatidylcholine similar to that shown in Figure 10 could take place on the enzyme, even though such an interaction does not occur in aqueous solution. The high specificity for calcium in the model system finds a parallel in the absolute specificity of the enzyme for Ca^{2+}. The stereochemistry of the enzyme–Ca^{2+}–phosphatidylcholine complex could account for the positional specificity of the enzyme. Although it has not been demonstrated as yet, it seems probable that the active amino group of the enzyme could abstract a proton from water held in the solvation shell of calcium. The very low pK of this amino group (7.6) certainly suggests that it is in an unusual environment, which may confer reactivity upon it which is similar to that observed in this model system.

[3] K. K. Yabusaki and M. A. Wells, unpublished observations.

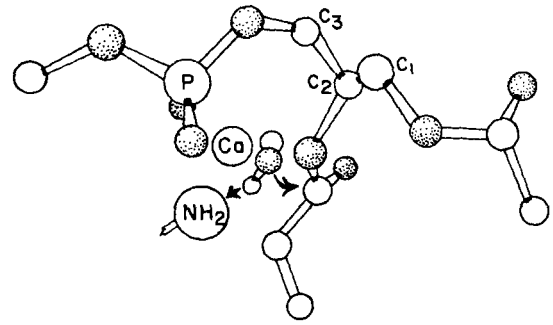

FIGURE 10: Model proposed to account for the enhanced rate of methanolysis of phosphatidylcholine in the presence of $CaCl_2$. C_1, C_2, and C_3 refer to the carbons of the glycerol backbone. The stippled spheres represent oxygen atoms. As drawn, the figure shows attack on the carbonyl of the ester at C_2. Ca is proposed to activate the methanol and facilitate proton transfer to the amine group.

Acknowledgment

Mrs. Norma Hewlett provided expert technical assistance.

Appendix

The formation of the reactive intermediate can be formulated as follows

$$PC + Ca \underset{}{\overset{K_1}{\rightleftharpoons}} PC\text{–}Ca \quad (1)$$

$$PC\text{–}Ca + RNH_2 \overset{K_2}{\rightleftharpoons} PC\text{–}Ca\text{–}RNH_2 \quad (2)$$

$$PC\text{–}Ca\text{–}RNH_2 \xrightarrow{k} \text{products} \quad (3)$$

If we define

$$\nu_1 = \frac{[PC\text{–}Ca] + [PC\text{–}Ca\text{–}RNH_2]}{[PC] + [PC\text{–}Ca] + [PC\text{–}Ca\text{–}RNH_2]} \quad (4)$$

and

$$\nu_2 = \frac{[PC\text{–}Ca\text{–}RNH_2]}{[PC\text{–}Ca] + [PC\text{–}Ca\text{–}RNH_2]} \quad (5)$$

and

$$[PC]_T = [PC] + [PC\text{–}Ca] + [PC\text{–}Ca\text{–}RNH_2] \quad (6)$$

$$[Ca]_T = [Ca] + [PC\text{–}Ca] + [PC\text{–}Ca\text{–}RNH_2] \quad (7)$$

$$[RNH_2]_T = [RNH_2] + [PC\text{–}Ca\text{–}RNH_2] \quad (8)$$

then

$$[Ca] = [PC]_T[([Ca]_T/[PC]_T) - \nu_1] \quad (9)$$

and

$$[RNH_2] = [PC]_T[([RNH_2]_T/[PC]_T) - \nu_1\nu_2] \quad (10)$$

The rate of formation of products (v) is given by

$$v = k[PC\text{–}Ca\text{–}RNH_2] \quad (11)$$

or

$$v = k\nu_1\nu_2[PC]_T \quad (12)$$

The rate constant observed under any set of conditions is k_{obsd} and is given by

$$k_{obsd} = k\nu_1\nu_2 \quad (13)$$

If k_{obsd} is the same under two sets of conditions (a and b), then following Halfman and Nishida (1972), $\nu_1\nu_2$ is given by

$$\nu_1\nu_2 = \frac{\left(\frac{[RNH_2]_T}{[PC]_T}\right)_a - \frac{([PC]_T)_b}{([PC]_T)_a}\left(\frac{[RNH_2]_T}{[PC]_T}\right)_b}{1 - ([PC]_T)_b/([PC]_T)_a} \quad (14)$$

If conditions are chosen such that $(\nu_1)_a = (\nu_1)_b$, then $(\nu_2)_a$ must equal $(\nu_2)_b$ and ν_2 can be evaluated from eq 14. ν_1 is calculated from the data of Misiorowski and Wells (1973). k can then be evaluated from eq 13. Equation 10 is used to determine the free amine concentration in order to evaluate K_2.

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