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Human platelet phospholipase A_2 activity is responsive in vitro to pH and Ca^{2+} variations which parallel those occurring after platelet activation in vivo

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Secretion of human platelet dense granule contents in response to epinephrine and other weak agonists requires the prior liberation of membrane-esterified arachidonic acid by a phospholipase A_2 enzyme species whose activity is regulated by Na^+/H^+ exchange (e.g., Sweatt et al. (1986) J. Biol. Chem. 261, 8660–8673 and Banga et al. (1986) Proc. Natl. Acad. Sci. USA 83, (197–9201). Based on our earlier findings in intact platelets, we postulated that the alkalinization of the platelet interior that accompanies accelerated activity of the Na^+/H^+ antiporter enables the phospholipase A_2 enzyme to function at ambient or low concentrations of intraplatelet Ca^{2+}. To test the hypothesis that the Ca^{2+} dependence of platelet phospholipase A_2 activity is influenced by changes in intraplatelet pH that occur following platelet activation, we characterized the Ca^{2+} dependence of this enzyme as a function of changes in pH (from pH 6.8–8.0), since it is within this range that intraplatelet pH changes occur following platelet activation. Phospholipase A_2 enzymatic activity in platelet particulate preparations was detectable in the presence of micromolar concentrations of Ca^{2+} (EC_{50} 1–2 μM) and plateaued above 10 μM Ca^{2+}. Enzymatic activity measured at 4.8 μM Ca^{2+} was increased by raising the pH from 5.5 to 8.0 (EC_{50} 7.4), was optimal at pH 8.0 and declined at more alkaline values. Furthermore, increases in pH from pH 6.8 to pH 8.0 not only increased maximal enzymatic activity but also enabled detection of enzymatic activity at lower Ca^{2+} concentrations. The interdependent regulation of phospholipase A_2 activity by changes in pH and Ca^{2+} suggests that phospholipase A_2 could serve to integrate changes in intracellular pH and available Ca^{2+} that occur subsequent to activation of human platelets by epinephrine and other weak agonists.

Introduction

Phospholipase A_2 enzymes are Ca^{2+}-dependent esterases which catalyze the removal of an acyl chain from the 2-position of phospholipids to yield the corresponding lysolipid and free fatty acid. Both of these products are rate-limiting precursors for the production of bioactive metabolites in various cell types. Arachidonic acid serves as a precursor for cyclooxygenase and lipoxygenase

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Abbreviations: EDTA, ethylenediaminetetraacetate; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; HETE, hydroxyeicosatetraenoic acid; diHETE, dihydroxyeicosatetraenoic acid; DMSO, dimethyl sulfoxide.

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products in a variety of target tissues. In addition, a particular species of lysolipid serves as a precursor for platelet-activating factor in neutrophils and perhaps other cells. The lysolipid and free fatty acid may also have direct effects such as regulating Ca^{2+} availability [1], initiating membrane fusion [2] and regulating the catalytic activity of protein kinase C [3,4].

Recent observations from our laboratory [5,6] and from others [7] have suggested that epinephrine, ADP and low concentrations of thrombin evoke the secretion of human platelet dense granule contents via the sequential stimulation of phospholipase A_2 and phospholipase C activities. We determined that activation of phospholipase A_2 by epinephrine as well as by ADP involved the functioning of a Na^+/H^+ antiporter, since lysolipid production in response to epinehrine and ADP was blocked by manipulations that bock Na^+/H^+ exchange. Lysolipid production evoked by high concentrations of the strong agonist thrombin, also was attenuated following blockade of Na^+/H^+ exchange, but could be restored by artificially alkalinizing the platelet interior using a membrane-permeant weak base, methylamine [6]. These findings are consistent with the hypothesis that the alkalinization of the platelet interior that occurs as a consequence of Na^+/H^+ exchange may play an important role in agonist stimulation of phospholipase A_2 activity, particularly because the forms of platelet phospholipase A_2 activity that have been characterized to date have pH optima above the resting intraplatelet pH [8–11]. Furthermore, we also observed that alkalinization of the platelet interior with methylamine increased the sensitivity of platelet phospholipase A_2 to the Ca^{2+} ionophore A23187, implying an increased sensitivity of the enzyme to Ca^{2+} when intraplatelet pH was elevated [6].

Because our functional data from intact platelets suggested that the hormone-induced alkalinization and the rise in calcium availability might act in concert to regulate phospholipase A_2 activity [8–11], and since both pH and Ca^{2+} are known to affect phospholipase A_2 activity, we chose to characterize directly the interplay between Ca^{2+} and pH in modulating phospholipase A_2 activity under conditions where calcium concentrations and pH values could be controlled rigorously and independently. Thus, we fractionated lysed human platelets to prepare particulate fractions. We observed a particulate phospholipase A_2 activity that responded to Ca^{2+} in the micromolar concentration range. Except for a recent report [12] of phospholipase A_2 activity in sheep platelets that is sensitive to micromolar Ca^{2+} concentrations, the vast majority of published reports focus on enzymatic activity detected in the presence of millimolar Ca^{2+} concentrations. Furthermore, the present studies demonstrate that increases in pH that have been measured to occur following platelet activation [13–16] increase phospholipase A_2 activity and, more importantly, reduce the threshold for Ca^{2+} activation of the enzyme. Thus it is possible that agonist-evoked changes in pH that occur in vivo, however subtle, may play an important role in modulating the sensitivity of platelet phospholipase A_2 to the Ca^{2+} available following platelet activation.

Experimental procedures

Materials. The radiolabeled phospholipids 1-stearoyl-2-[^{3}H]arachidonylphosphatidylcholine (60–180 Ci/mmol) and 1-stearoyl-2[^{3}H]arachidonylphosphatidylinositol (10 Ci/mmol) were purchased from New England Nuclear research Products. Unlabeled phospholipids were obtained from Avanti Polar Lipids. Arachidonic acid was purchased from Cayman Chemical, Ann Arbor, MI. Silica gel thin-layer plates (LKGDF) were purchased from Whatman. Aspirin, EDTA, dimethylsulfoxide, Hepes, Mes, Tris, soybean lipoxidase type V (EC 1.13.11.12) and bovine gamma globulins Fraction II were from Sigma. The chelator, HEDTA, was from Aldrich. Leupeptin was from the Peptide Institute (Japan). All other organic solvents and inorganic reagents were from Fisher. Ca^{2+} and pH measurements were performed using a Radiometer (Copenhagen) PHM 64 pH meter equipped with an F2112 Ca^{2+} selectrode used in conjunction with a K401 calomel reference electrode (for Ca^{2+} measurements) or a GK2401C pH electrode (for pH measurements).

Human whole blood was obtained from healthy male donors and collected into 1/10 volume of acid/citrate/dextrose (140 mM dextrose/74.8

mM sodium citrate/41.6 mM citric acid) as the anticoagulant. Platelet-rich plasma was obtained following centrifugation (170 × *g* for 15 min) and was aspirated within 2 cm of the buffy coat layer. Platelet-rich plasma was treated with aspirin (1 mM for 20 min at 37°C) to block platelet cyclooxygenase activity. A further 1/10 volume of acid/citrate/dextrose was added and the platelet-rich plasma was chilled on ice. All further manipulations were carried out at 4°C. Platelets were collected by centrifugation (570 × *g* for 15 min) and washed repeatedly by centrifugation, (once using 154 nM NaCl, 50 mM Tris, 20 mM disodium EDTA (pH 7.35) and twice using 154 mM NaCl, 50 mM Tris (pH 6.5)). The final pellet was resuspended quickly on ice in the lysing buffer (1 ml buffer/15 ml platelet rich plasma), 5 mM Hepes-NaOH (pH 7.0) containing 0.5 mg/ml leupeptin and, where specified, 5 mM EDTA. This suspension was subjected to two freeze-thaw cycles and then mechanically disrupted with a Brinkman polytron homogenizer for two 5-s intervals at setting No. 5 separated by 20 s on ice. The preparation obtained following polytron treatment is designated the 'crude lysate'.

The crude lysate was then centrifuged (100000 × *g* for 60 min at 4°C) to yield a particulate and supernatant fraction. The particulate fraction was resuspended in the lysing buffer (1 ml buffer/50 ml platelet-rich plasma) and was stored in aliquots at −70°C until assay for phospholipase A_2 activity.

Phospholipase A_2 activity was recovered after centrifugation in both the supernatant fraction as well as in the pellet. Extensive characterization could reveal no significant difference in the properties of the supernatant versus the particulate enzyme. Furthermore, the supernatant form was progressively lost after longer centrifugation times. Thus, it is possible that the operationally defined 'soluble' phospholipase A_2 (supernatant of a 100000 × *g*, 60 min centrifugation) in fact represents a particulate enzyme associated with smaller, more slowly sedimenting membrane vesicles. Since the properties of the 100000 × *g* supernatant and particulate activities in response to varying Ca^{2+} and pH are indistinguishable, the data described here focus on the particulate enzyme which sediments at 100000 × *g* for 60 min.

Assay of phospholipase A_2 activity. One of two assay procedures was employed, depending upon whether or not the platelets were lysed in the presence of the chelator, EDTA. Similar estimates of phospholipase A_2 activity were obtained with the two procedures.

a. EDTA-lysed platelets. Platelet subcellular fractions were mixed with an equal volume of buffer to give (final concentrations) 2.5 mM EDTA, 2.5 mM HEDTA, 100 mM Hepes, at the pH indicated in the figure legends (pH 6.8–8.0). The solution was then titrated with 0.1 M $CaCl_2$ delivered with a microliter syringe. The free Ca^{2+} concentration was measured with a Ca^{2+}-selective electrode. Duplicate aliquots (100 μl) were withdrawn and placed in polypropylene tubes on ice. Assays were initiated by addition of 0.1 μCi of stearoyl[^{3}H]arachidonylphosphatidylcholine dissolved in 5 μl of DMSO. Tubes were mixed and incubated for 40 min at 37°C. Incubation conditions were chosen to insure that substrate hydrolysis was linear with time and that less than 10% of the labeled substrate was consumed during the incubation. Control incubations indicated that the substrate was stable under our incubation conditions. The labeled substrate chromatographed with authentic unlabeled phosphatidylcholine (chloroform/methanol/acetic acid/water (75:45:12:6, v/v) when incubations were performed in the absence of added platelet preparations (i.e., no enzyme).

b. No added EDTA in lysate. Platelet subcellular fractions (20 μl) were mixed with 80 μl of a buffer previously adjusted to the desired free Ca^{2+} concentration and containing (final concentrations) 10 mM EDTA, 10 mM HEDTA, 100 mM Hepes at the indicated pH. Other conditions are as above.

Isolation of reaction products. At the end of the desired incubation duration, assay tubes were placed on ice and 100 μl of 2 M formic acid/NaOH (pH 3.5) was added. Then, 50 μl of ethyl acetate containing 20 μg unlabeled arachidonic acid and a mixture of HETE and diHETE (approx. 10 μg each, synthesized using soybean lipoxygenase) were added as carriers for isolation of the reaction products. The incubation mixture was then vigorously mixed with 1.05 ml of ethyl acetate, centrifuged to separate the phases, and 1 ml aliquots of the organic phase were removed and evaporated to dryness. Samples were redis-

solved in 30 μl of ethyl acetate, loaded on Whatman LKGDF silica gel thin-layer plates and developed in chloroform/methanol/acetic acid/H_2O (90:8:1:0.8). Radioactivity was located by autoradiography and, in preliminary experiments, was found to be associated exclusively with the iodine-stained carrier compounds. Because platelets were treated in advance with the cyclooxygenase inhibitor aspirin, cyclooxygenase metabolites were not generated. Hence, all liberated [^{3}H]arachidonic acid was recovered either unmetabolized (i.e., as free [^{3}H]arachidonic acid), as HETE or as diHETE. The r_f values of the three products were 0.59, 0.47 and 0.36, respectively. Routinely, arachidonic acid and its lipoxygenase products were localized by I_2 staining. These regions were scraped from the plate and quantified by liquid scintillation counting. Data are generally expressed as cpm [^{3}H]arachidonate recovered, but represent total [^{3}H]arachidonate and [^{3}H]HETEs quantitated as described above.

Effect of unlabeled phospholipids on phospholipase A_2 activity. Unlabeled phospholipids were dissolved in suitable organic solvents (e.g., chloroform/methanol, 9:1) and added to polypropylene tubes in 50 μl of the solvent. The solvent was then dried for 60 min in vacuo using a Savant Speedvac prior to addition of assay buffer (80 μl, as described above) and [^{3}H]phosphatidylcholine (5 μl in DMSO). Tubes were gassed with argon, sealed and the lipid was dispersed by incubation for 30 min in a bath sonicator at 4°C. The tubes were then placed on ice prior to adding enzyme-containing platelet fractions. Incubations were then processed as described above. As noted in the Results, certain unlabeled phospholipids paradoxically increased phospholipase A_2 activity toward the radiolabeled substrate. This increase in enzymatic activity could not be attributed to an effect of the exogenous unlabeled lipid to prevent adsorption of the radiolabeled substrate to the walls of the assay tube.

Other methods. Protein concentrations were determined using the dye-binding method of Bradford [17] using bovine gamma globulin as a standard. Total lipid phosphorus was determined as inorganic phosphate after extraction [18] and ashing as its complex with Malachite green-ammonium molybdate [19].

Ionic equilibria were calculated using a BASIC translation of the computer program of Perrin and Sayce [20] with stability constants given by Sillen and Martell [21], corrected for pH. Ca^{2+} buffer standards were prepared based on these calculations and were used to calibrate the Ca^{2+} electrode.

Results

Phospholipase A_2 activity was assessed in our studies by quantifying the release of radiolabeled arachidonic acid from the synthetic substrate stearoyl[^{3}H]arachidonylphosphatidylcholine. Although several recent papers have described the existence of a phosphatidylcholine-specific phospholipase C activity in mammalian cells [22,23], through which the well-characterized diacylglycerol lipase pathway could also yield arachidonic acid, we could detect no evidence of such activity occurring under our experimental conditions. Thus, we failed to detect any production of radiolabeled diacylglycerol under conditions which gave maximal accumulation of radiolabeled arachidonic acid and its metabolites. In addition, in the presence of an inhibitor of the diacylglycerol lipase enzyme (50 μM RHC 80267), [^{3}H]arachidonic acid production was not significantly reduced nor was there detectable accumulation of [^{3}H]diacylglycerol. Thus, we are reasonably confident that the measurement of [^{3}H]-arachidonic acid release from stearoyl[^{3}H]arachidonylphosphatidylcholine is a valid indicator of phospholipase A_2 activity under our experimental conditions.

Effects of Ca^{2+} on phospholipase A_2 activity

A primary focus of our studies was to characterize phospholipase A_2-catalyzed arachidonate release in the presence of varying H^+ and Ca^{2+} concentrations and bracket the range of concentrations that reasonably could be expected to exist in the intact platelet subsequent to hormone stimulation. Initially, the pH in the incubation was fixed at pH 6.8 or pH 8.0. Resting intraplatelet pH is approx. 7.0–7.1 [6,13–16], whereas pH 8.0 represents the pH value attained following alkalinization of intact platelets by weak bases such as methylamine [6]. Thus, the range of pH

6.8–8.0 should fully bracket all $[H^+]$ that the platelet phospholipase A_2 enzyme would ever encounter in situ.

As indicated under Experimental procedures, two independent protocols were utilized to achieve varying final concentrations of free Ca^{2+} in the incubation. In one procedure, the enzyme preparation was titrated with $CaCl_2$ on ice while free calcium was measured with a calcium-selective electrode. Aliquots were withdrawn and assayed for phospholipase A_2 activity. The second procedure involved the addition of a small volume of concentrated enzyme to a suitable Ca^{2+}-pH buffer system. The capability of the Ca^{2+}-pH buffer system to stabilize Ca^{2+} and pH values was confirmed using a pH as well as a Ca^{2+}-selective electrode. Regardless of the experimental procedure utilized, the findings were indistinguishable. Fig. 1 summarizes the Ca^{2+}-dependence of particulate phospholipase A_2 activities derived from human platelets. At pH 8.0, phospholipase A_2 activity was readily observable at a calcium concentration of 0.26 μM and exhibited an EC_{50} value of 1.0 ± 0.1 μM ($n = 3$), with an apparent plateau in the rate of phosphatidylcholine hydrolysis for

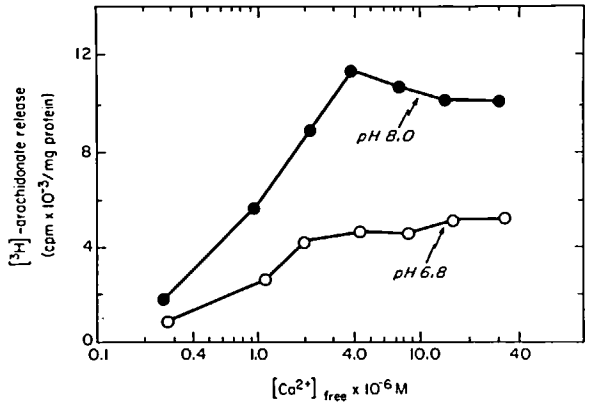

Fig. 1. Calcium and pH dependence of phospholipase A_2 activity in human platelet particulate fractions. Membranes were incubated for 40 min at 37 °C with 0.1 μCi [^{3}H]phosphatidylcholine in 100 μl of 0.1 M Hepes-NaOH buffer at pH 6.8 (open symbols) or pH 8.0 (closed symbols) containing 10 mM EDTA, 10 mM HEDTA and the indicated free calcium concentration. Liberated [^{3}H]arachidonate and arachidonate products were isolated by extraction and thin-layer chromatography as described under Methods. The data shown are from one experiment performed in duplicate and representative of four separate experiments.

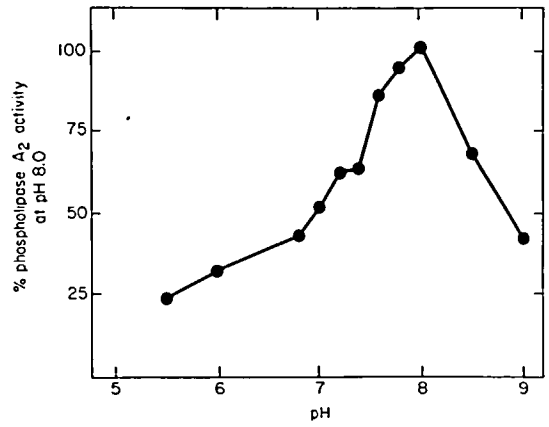

Fig. 2. The pH dependence of Ca^{2+}-dependent phospholipase A_2 activity in human platelet particulate preparations. Calcium-dependent phospholipase A_2 activity was assayed as described in the legend to Fig. 1 except that a single Ca^{2+} concentration was employed (4.8 μM). The pH was varied using the following buffer systems: Mes-NaOH (pH 5.5 and pH 6.0), Hepes-NaOH (pH 6.8–8.0), and Tris-HCl (pH 8.5 and pH 9.0). Shown here are mean values of three experiments performed in duplicate.

calcium concentrations above 10 μM. Raising the pH of the assay buffer from 6.8 to 8.0 increased the rate of hydrolysis at maximal calcium concentrations 2.2 ± 0.3-fold without significantly influencing the concentration of calcium required for half-maximal arachidonate release (i.e., 1–2 μM). However, alkalinization reduced by almost 5-fold the threshold for detection of Ca^{2+}-induced increases in arachidonate release, such that activity was readily measurable at Ca^{2+} concentrations of 0.26 μM at pH 8.0 but was almost undetectable at this Ca^{2+} concentration at pH 6.8.

To obtain more precise information concerning the effect of pH on phospholipase A_2 activity, the rate of [^{3}H]phosphatidylcholine hydrolysis was measured at a constant calcium concentration (4.8 μM) over the pH range of pH 5.5 to 9.0. As shown in Fig. 2, the highest rates of hydrolysis were seen at pH 8.0, with half-maximal rates at pH 7.4.

Since deacylation of several phospholipids has been reported to occur subsequent to hormone stimulation in the intact platelet, we examined the generality of our observations based on phosphatidylcholine hydrolysis to other phospholipid substrates. Initial experiments utilizing [^{3}H]phosphatidylinositol as the labeled substrate gave

qualitatively similar results to those obtained employing [^{3}H]phosphatidylcholine as the substrate. Half maximal hydrolysis of [^{3}H]phosphatidylinositol was observed at 2 μM free calcium, and raising the pH from 6.8 to 8.0 increased the rate of hydrolysis about 2-fold (data not shown). However, we chose to focus on [^{3}H]phosphatidylcholine hydrolysis, since a high rate of Ca^{2+}-independent hydrolysis occurred when [^{3}H]phosphatidylinositol was the substrate, perhaps as a consequence of other phosphatidylinositol-hydrolyzing activities, such as those of the phospholipase C and diacylglycerol lipase enzymes likely to exist in our subcellular fractions. However, it should be noted that phospholipase C activity was not detected using [^{3}H]phosphatidylcholine as substrate (see above).

To obtain further insight into the substrate specificity of the human platelet phospholipase A_2 enzyme, the radiolabeled [^{3}H]phosphatidylcholine substrate was sonicated with various unlabeled lipids prior to initiating the incubation by adding enzyme. Competition at the enzyme-active site by various unlabeled lipids should be reflected by decreases in the rate of hydrolysis of the labeled substrate. As shown in Fig. 3 and Table I, however, three different modes of interaction were observed between the unlabeled lipid, the radioactive substrate and the membrane-bound enzyme.

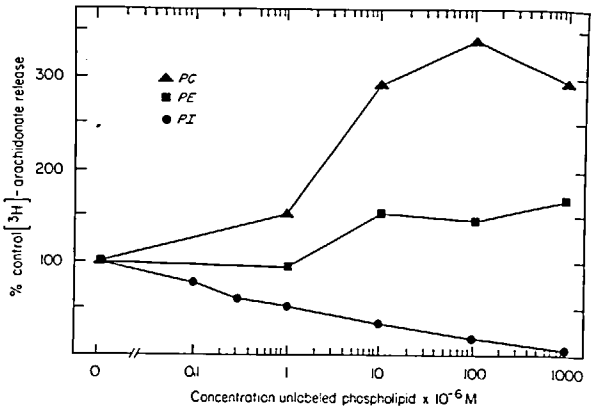

Fig. 3. Effect of unlabeled phospholipids on [^{3}H]phosphatidylcholine hydrolysis by membrane-bound phospholipase A_2 activity. Unlabeled stearoylarachidonylphosphatidylcholine (synthetic), phosphatidylethanolamine (bovine liver), or phosphatidylinositol (bovine liver) were dried in vacuo as a film at the bottom of the assay tubes and dispersed together with the labeled [^{3}H]phosphatidylcholine substrate in the assay buffer (pH 8.0 with no added calcium or containing 4.8 μM free calcium) under argon using a bath sonicator, as described under Methods. Each assay point represents the mean of duplicate data points from 2–5 separate experiments.

TABLE I

STRUCTURAL REQUIREMENTS FOR INFLUENCING HUMAN PLATELET PHOSPHOLIPASE A_2 ACTIVITY

Values are expressed as mean ± S.D. of three independent experiments employing 200 μM unlabeled phospholipid. R_1 and R_2 refer to the acyl chain esterified to the *sn*-1 and *sn*-2 position of the glycerol backbone. Assays contained approx. 40 μg membrane protein in 0.1 ml of 0.1 M Hepes-NaOH (pH 8.0) and the Ca^{2+} buffer: 10 mM EDTA and 10 mM HEDTA. Ca^{2+}-dependent hydrolysis is the difference between that occurring with no added Ca^{2+} and that observed with 4.8 μM free Ca^{2+} and represents approx. 70% or greater of total activity. The data are expressed as percent of control activity, i.e., activity assay in the presence of 0.1 μCi of stearoyl[^{3}H]arachidonylphosphatidylcholine (5.8–16.7 nM final lipid concentration) with no exogenous unlabeled phospholipid present. The 100% control activity varied from 1964 to 4562 cpm [^{3}H]arachidonate released in the three separate experiments summarized here.

Headgroup	R_2	R_1	% control ± S.D.
Phosphatidylcholine	arachidonate	stearate	418 ± 84
Phosphatidylcholine	stearate	stearate	176 ± 46
Phosphatidylcholine	butyrate	butyrate	49 ± 8
Phosphatidylcholine	none	mixed	63 ± 35
Phosphatidylinositol	mixed	mixed	8 ± 9
Phosphatidylinositol	none	mixed	43 ± 8
PO_4	mixed	mixed	22 ± 7
OH	mixed	mixed	82 ± 25
Phosphatidylethanolamine	mixed	mixed	144 ± 18
Phosphatidylethanolamine	arachidonate	stearate	115 ± 4
Phosphatidylserine	mixed	mixed	70 ± 11

Phosphatidylinositol clearly competed for [^{3}H]phosphatidylcholine hydrolysis, and decreased the amount of detectable radiolabeled product with an EC_{50} value of 1 μM. The phospholipase A_2 reaction product, arachidonic acid, also decreased the rate of [^{3}H]phosphatidylcholine hydrolysis, as has been reported previously [34]. In the presence of 200 μM arachidonic acid, phospholipase A_2 activity was reduced to $12 \pm 5\%$ ($n = 3$) of that observed in its absence (data not shown). In contrast, phosphatidylethanolamine and phosphatidylserine had only slight effects on the rate of [^{3}H]phosphatidylcholine hydrolysis suggesting, perhaps, a very low or no affinity of these lipids at the substrate-binding site of the phosphatidylcholine-hydrolyzing phospholipase A_2 enzyme. Paradoxical results were obtained when unlabeled stearoylarachidonylphosphatidylcholine was co-incubated with stearoyl[^{3}H]arachidonylphosphatidylcholine. The apparent rate of hydrolysis of the labeled substrate was increased by as much as 400% over the rate observed in the absence of unlabeled phosphatidylcholine, despite a 20 000-fold decrease in the specific radioactivity of the stearoyl[^{3}H]arachidonylphosphatidylcholine at 200 μM added stearoylarachidonylphosphatidylcholine. As summarized in Table I, this enhancement was mimicked to a lesser extent by distearoylphosphatidylcholine. In contrast, when dibutyryl phosphatidylcholine was incubated in the presence of the stearoyl[^{3}H]arachidonylphosphatidylcholine substrate, only a decrease in the rate of catalysis was observed. A choline-containing head group and arachidonic acid in the *sn*-2 position appear necessary to observe a lipid-induced enhancement of this phospholipase A_2 activity, because stearoylarachidonylphosphatidylethanolamine provided only a slight enhancement of the rate of [^{3}H]phosphatidylcholine hydrolysis, in a manner analogous to the findings with the phosphatidylethanolamine preparation that contained a mixed acyl chain composition. Interestingly, stearoylarachidonylphosphatidylcholine could also activate platelet phospholipase A_2 to a similar extent (281 + 51%, $n = 3$) when [^{3}H]phosphatidylinositol was the substrate. Furthermore, even when phospholipase A_2 activity was amplified dramatically by the addition of 200 μM stearoylarachidonylphosphatidylcholine, the enzyme

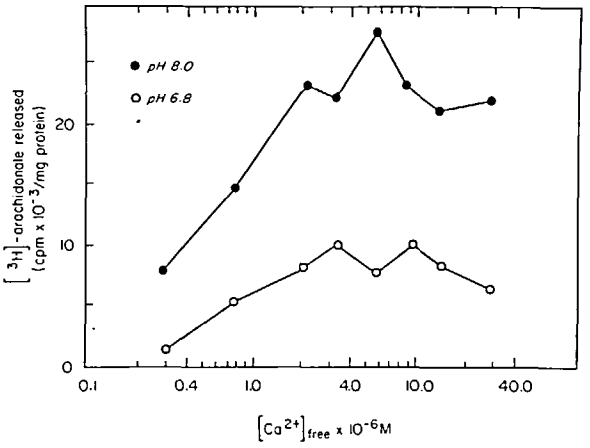

Fig. 4. Calcium and pH dependence of phospholipase A_2 activity of human platelet membranes in the presence of 200 μM stearoyl[^{3}H]arachidonylphosphatidylcholine. Assays were conducted as described in Fig. 1 except that the radiolabeled stearoyl[^{3}H]arachidonylphosphatidylcholine was supplemented with 200 μM unlabeled stearoylarachidonylphosphatidylcholine as described in Fig. 3. The results shown here are from one experiment performed in duplicate and are representative of two separate experiments.

activity still demonstrated its characteristic interdependent regulation by changes in Ca^{2+} and H^+ concentrations (see Fig. 4).

Taken together, the data suggest that stearoylarachidonylphosphatidylcholine not only acts as a substrate for phospholipase A_2 but may also be capable of activating the enzyme by interaction at another site. Thus, it is probable that the lipid composition of the membrane domain on which the phospholipase A_2 enzyme resides may significantly influence enzymatic activity. However, it must be borne in mind that these effects of exogenous lipids are observed despite the presence of considerable endogenous lipid content in both particulate (1.5 mM lipid phosphate) as well as supernatant (18 μM lipid phosphate) preparations of phospholipase A_2 activity. Although the molecular basis for this enhancement of activity by excess exogenous unlabeled phosphatidylcholine is not understood at present, it should be noted that similar enhancement of enzymatic activity by phospholipids has been described for the phospholipase A_2 enzyme isolated from snake venom [24,25].

Discussion

Phospholipase A_2 activity has long been implicated to play an important role in the hormone-induced release of arachidonic acid from membrane phospholipids. However, at least for the human platelet, the question has arisen as to how activation by extracellular agents results in activation of phospholipase A_2 activity. This question arises for several reasons. First, when platelet membranes are fractionated using free-flow electrophoresis, the major portion of phospholipase A_2 activity is recovered with membranes originating from the dense tubular system [26], suggesting a probable physical isolation of phospholipase A_2 from surface membrane receptors that activate this enzyme in the intact platelet. Recent electron microscopic evidence demonstrating that radiolabeled arachidonic acid is incorporated primarily in the dense tubular system complements earlier findings using free-flow electrophoresis [27]. One explanation accounting for the activation of cell surface receptors resulting in the stimulation of a dense tubular system-associated phospholipase A_2 activity is that hormone activation of human platelets may be accompanied by the formation of a diffusible mediator of phospholipase A_2 stimulation. The data presented here suggest that hormone-mediated alterations in intracellular pH and Ca^{2+} availability could provide the means to regulate phospholipase A_2 activity in situ.

Several investigators have reported previously that α_2-adrenergic stimulation of intact human platelets leads to a redistribution of intraplatelet Ca^{2+}. These changes in Ca^{2+} availability probably occur on a local level, since they are detectable using the Ca^{2+} indicators aequorin and chlortetracycline [6,28–30], but are not observed using generalized cytosolic indicators such as quin2 and fura-2 [30,32]. Intraplatelet Ca^{2+}, as monitored by aequorin, can rise following epinephrine stimulation to 5.8 μM [28]. Thus, the Ca^{2+} concentration-response curves observed in the present studies include the range of Ca^{2+} concentrations over which Ca^{2+} changes in vivo. The Ca^{2+} changes measured following epinephrine stimulation of human platelets are not dependent on inositol trisphosphate production, since they are observed in the presence of cyclooxygenase inhibitors, agents which block the epinephrine-stimulated phospholipase C activity that occurs secondary to production of cyclooxygenase metabolites of arachidonic acid [6,28]. Ca^{2+}, however, appears not to be the sole modulator of phospholipase A_2 activity in vivo. Thus, we recently have implicated a role for Na^+/H^+ exchange in mediating phospholipase A_2 activation following epinephrine stimulation of human platelets. The alkalinization resulting from acceleration of Na^+/H^+ exchange can be monitored in the cytoplasm by a variety of techniques. In many cell types, including human platelets, the alkalinization detected amounts to an increase of as much as 0.3 pH units as measured in the total cytoplasmic space [13,33]. It is probable that the changes in intracellular pH (pH_i) that occur in certain microenvironments following receptor stimulation may represent even greater changes in pH_i. Earlier observations that alkalinization of the platelet interior with methylamine increased platelet phospholipase A_2 sensitivity to the Ca^{2+} ionophore A23187 suggest an important interplay between pH and Ca^{2+} in stimulating phospholipase A_2 and evoking, ultimately, phospholipase C activation and human platelet secretion [6].

It should not be overlooked that a large body of literature [8–11,33] has focused on phospholipase A_2 activity at pH values (e.g., pH 9–10) and Ca^{2+} concentrations (e.g., mM) that are unlikely to be obtained physiologically. Nonetheless, a phospholipase A_2 activity in sheep platelets that is measurable at micromolar Ca^{2+} concentrations has been described [12]. The present report extends these findings by demonstrating a similarly-sensitive Ca^{2+}-dependent phospholipase A_2 activity in human platelets and its further modulation by physiologically relevant changes in H^+ concentration (cf. Fig. 2). Thus, the present findings provide a likely explanation for previous reports [6,7] that epinephrine- and ADP-activation of human platelet phospholipase A_2 involves a functional Na^+/H^+ exchange system. Receptor-accelerated Na^+/H^+ exchange would result in a local alkalinization of the platelet interior and thus provide a means for sensitizing the phospholipase A_2 enzyme to ambient Ca^{2+} concentrations.

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