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## PHOSPHOLIPASE C ACTIVITY IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES: PARTIAL CHARACTERIZATION AND EFFECT OF INDOMETHACIN

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Several hormones act at the cellular level to increase diacylglycerol via increased catabolism of phosphatidylinositol by phospholipase C (PLC). Diacylglycerol stimulates protein kinase C, leading to protein phosphorylation and hormone action. Since, PLC activity has not been well studied in man, we have established an assay for PLC in human neutrophils. In this assay sonicates of neutrophils were incubated with L-3-phosphatidyl [ $U^{14}C$ ]-inositol and the incubation mixture extracted with chloroform:methanol. Following the additions of 2M KCl and chloroform, PLC activity was determined by counting [ $U^{14}C$ ] in the aqueous phase. The PLC activity was linear with respect to time and the quantity of added enzyme. Optimum substrate concentration and pH were 2mM and 7.0 respectively. Optimal activity was dependent on $Ca^{2+}$ (2mM) and deoxycholate (2mM). Naloxone, FMLP and PGD <sub>2</sub> , which affect various aspects of leucocyte function, had no significant effects on neutrophil PLC activity. The effects of various compounds with phospholipase A <sub>2</sub> inhibitory activity were also tested on this enzyme. Of these, mepacrine, lidocaine and indomethacin inhibited the enzyme activity. The inhibition by indomethacin was of the noncompetitive type with an apparent $K_m$ of $0.17 \times 10^6 M$ and apparent					
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$K_i$  of  $3.6 \times 10^{-6} M$ . From these data we conclude that indomethacin is capable of inhibiting phospholipase C activity in neutrophils at clinically significant levels and that this may be relevant in the therapeutic action of this drug.

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## INTRODUCTION

Phosphatidylinositol-specific phospholipase C is greatly increased in several tissues in response to various physiological stimuli such as hormones and neurotransmitters (1-5). The increase in phospholipase C activity is associated with changes in phosphatidylinositol turnover and protein kinase C activity (1-5). Phosphatidylinositol-specific phospholipase C is present in several mammalian tissues including brain, kidney, liver, intestinal mucosa, fetal membranes and uterine decidua, macrophages, fibroblasts and platelets (1-3, 6-15). Although phosphatidylinositol turnover has been studied in detail in neutrophils (1-16) phosphatidylinositol-specific phospholipase C has not been well characterized in human neutrophils. In this paper we describe a rapid and sensitive phospholipase C assay to characterize this enzyme in human neutrophils. The effects of various nonsteroidal anti-inflammatory drugs, local anesthetics and antimalarial drugs on neutrophil phospholipase C activity have also been examined. In addition, we have tested the effects of three compounds known to alter leukocyte function (17-20), ie. verapamil, naloxone, and N-formyl-methionyl-leucyl-phenylalanine (FMLP).

## MATERIALS

L-3-phosphatidyl-[U- $^{14}$ C]-inositol (specific activity, 282 mCi/mmol) and L3-phosphatidylinositol-1,2-[1- $^{14}$ C] palmitoyl (specific activity, 60 mCi/mmol) were purchased from Amersham Corporation, Arlington Heights, IL. Bovine, brain, L-phosphatidylinositol, N-formyl-methionyl-leucyl-phenylalanine (FMLP), and sodium deoxycholate were obtained from Calbiochem - Behring, San Diego, CA. MES (2-[N-Morpholino] ethanesulfonic acid), dimethyl sulfoxide (DMSO), fatty acid-poor bovine albumin, (fraction V), 2-mercaptoethanol, myoinositol, myoinositol-2-dipalmitin phosphate, monopalmitin, palmitic acid, phospholipase D (cabbage), phospholipase A<sub>2</sub> (naja naja), lysophosphatidylinositol and naloxone were purchased from Sigma Chemical Company, St. Louis, MO. Precoated thinlayer silica gel G and high resolution plates were obtained from Analtech, Inc., Newark, DE. Whatman No. 1 paper was from Whatman, Inc., Clifton, NJ. Scintillation fluid (Aquasol-2) was purchased from New England Nuclear, Boston, MA. All other chemicals used in this study were of reagent grade. The inositol-1, 2, cyclic-phosphate used in the chromatographic analysis was prepared by the method of Pizer and Ballou (21). Dextran T-500 was obtained from Pharmacia, Uppsala, Sweden. Hanks Balanced Salt Solution (HBSS) and preservative-free heparin were purchased from GIBCO, Grand Island, NY. Lymphocyte separation medium was obtained from Litton Bionetics, Kensington, MD. Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) was from Upjohn, Kalamazoo, MI.



## METHODS

### Isolation of Neutrophils

Whole blood was obtained by venipuncture from healthy medication-free volunteers after obtaining informed consent. Preservative-free heparin (250u/20cc) was used for anticoagulation. Erythrocytes were removed by sedimentation through 2% dextran. The supernatant was centrifuged at 300 x g for 10 min. Residual erythrocytes were removed by hypotonic lysis, after which the neutrophils were suspended at a concentration of  $2 \times 10^6$  cells/cc in HBSS, sonicated and kept on ice until used (20).

### Assay for Phospholipase C Activity

For the standard assay of phospholipase C activity (11), a stock solution of the substrate was prepared as follows: An aliquot (1 ml containing 10 mg) phosphatidylinositol from bovine brain was mixed with 0.1 ml (containing 1uCi) of L-3-phosphatidyl[U- $^{14}$ C]-inositol and taken to dryness at 23°C under nitrogen. Tris buffer (4.5 ml pH 7.0, 50mM) containing 180 mg bovine serum albumin and 2 mM mercaptoethanol was added to the lipid residue and the mixture was sonicated for 1 min at 125 W in a Bransonic 220 bath sonicator. In routine assays of phospholipase C, 0.1 ml of this sonicated substrate was incubated with 0.025 ml of enzyme (50-100 ug protein) in a shaking water bath at 37°C for 30 min. The incubation was terminated by adding 0.5 ml of chloroform:methanol (1:2, vol/vol). After mixing on a Vortex mixer, 0.15 ml of chloroform and 0.15 ml of potassium chloride (2 M) were added and mixed, and the phases separated by centrifugation at 900 x g for 15 min. An aliquot (0.15 ml) of the upper phase was transferred into scintillation vials. Scintillation fluid (10 ml Aquasol-2) was added, and the radioactivity

counted in a scintillation counter. Control tubes without enzyme were included in each experiment, and enzyme was added to these at the end of the incubation period. The counts in the upper phase of the control incubations were subtracted from all samples. Enzyme activity was expressed as units/mg cell protein. One unit of phospholipase C activity was defined as one nmole of myoinositol formed/hr. Protein was determined by the method of Lowry et. al. (22). All points were determined in duplicate.

#### Analysis of Reaction Products

The following procedure (11) was used to analyze the lipid soluble products. Phosphatidylinositol labeled in the fatty acid position was used in place of L3-phosphatidyl-[U<sup>14</sup>C]-inositol and the amount of radioactivity in the incubation mixture was increased by five-fold. After incubation for 30 min, the reaction was stopped by the addition of chloroform: methanol (1:2 v/v) and dried under nitrogen. The dried lipid residue was dissolved in a small amount of chloroform: methanol (2:1 v/v) and the lipid products were chromatographed on silica gel G plates and developed in hexane/diethyl ether/acetic acid (105:45:4.5 by volume). The areas corresponding to diglyceride, monoglyceride and fatty acid were scraped off and, after the addition of scintillation cocktail, were counted in a scintillation counter. The enzyme activity determined by this method was compared with the present method. In addition the diacylglycerols were also analyzed for fatty acid composition by gas chromatography as described previously (11).

In experiments where the water-soluble products were isolated and counted, the assay procedure was modified as follows: The labeled

phosphatidylinositol in the incubation mixture was increased by 5-fold and the KCl (2 M) was replaced by water (11) during extraction. Aliquots of the upper phase were applied on Whatman No. 1 paper and subjected to descending paper chromatography using ethanol/ammonia (3:2, vol/vol) as described by Dawson and Clarke (23) and DiRenzo et al. (11). Since inositol-1,2-cyclic phosphate comigrates with inositol, samples of the upper phase were treated with 1M HCl at 90°C for 3 min before chromatography. This method converts inositol-1,2-cyclic phosphate to inositol-1-phosphate (11) which can easily be separated from inositol. The radioactivity in the areas corresponding to inositol-1,2-cyclic phosphate, inositol-1-phosphate and inositol were counted.

Myoinositol and lysophosphatidylinositol fractions could conceivably appear in the aqueous phase of the extraction mixture as a result of phospholipase D and phospholipase A ( $A_1+A_2$ ) actions respectively. In order to investigate this possibility the following experiments were performed. Phosphatidylinositol, labeled in both fatty acids was incubated with neutrophil sonicates. The incubation mixture was extracted by Folch's procedure (24). After adding a mixture of phosphatidic acid, phosphatidylinositol and lysophosphatidylinositol the lipids were separated and counted as described previously (24). Under the present assay conditions, no radioactivity was detected in the phosphatidic fraction of lysolecithin fractions.

#### Effect of Nonesterified Fatty Acids on Neutrophil Phospholipase C Activity

It has been suggested that free fatty acids might regulate phospholipase C activity. To investigate whether free fatty acids affect neutrophil phospholipase C activity, sonicates of neutrophils were

preincubated with various concentrations (2, 1, 0.5 and 0.1mM) of arachidonic, palmitic, stearic and oleic acids for 15 min at 37°C and following this enzyme assays were performed as described previously.

### Effects of Various Compounds on Neutrophil Phospholipase C Activity

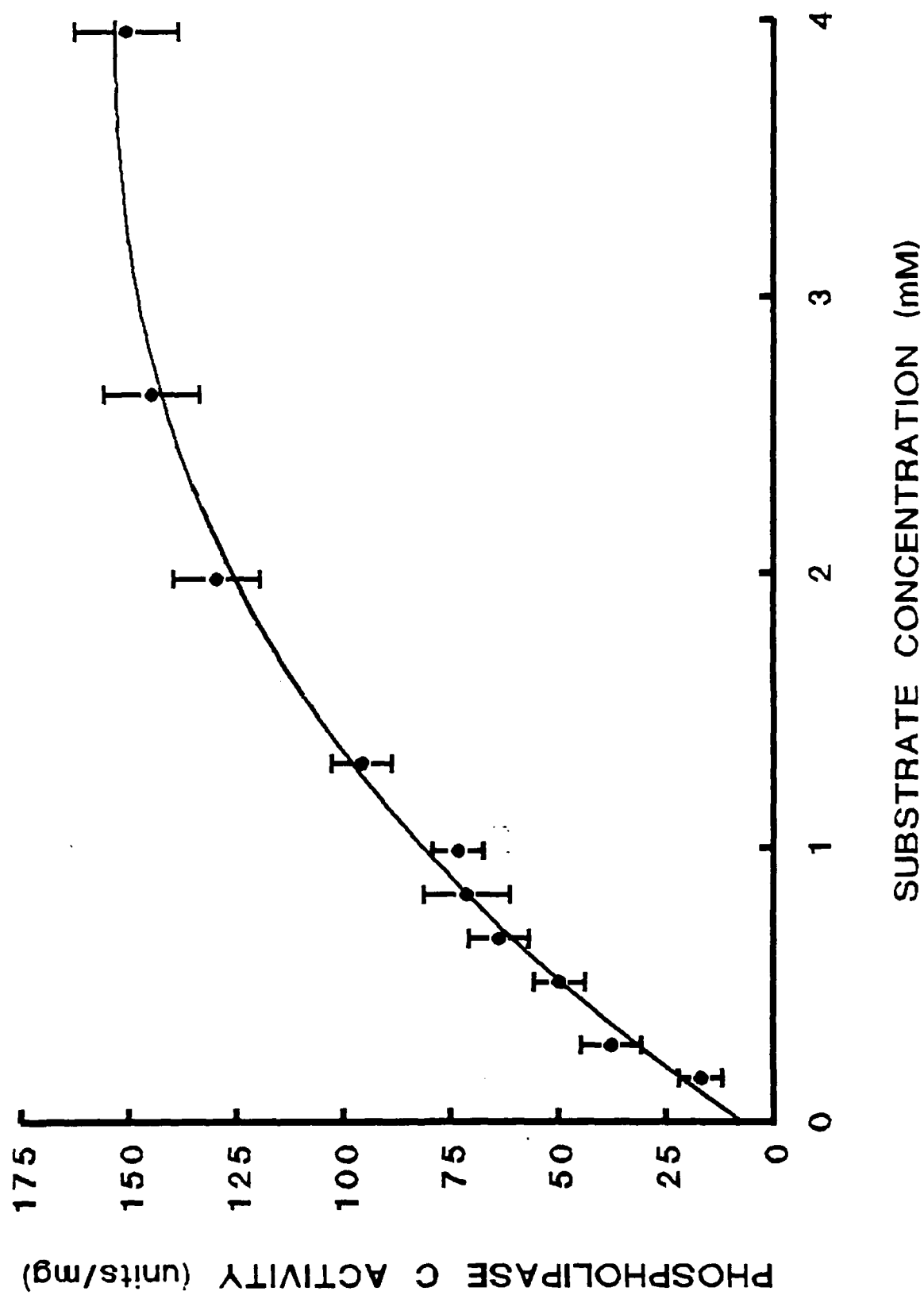
Mepacrine, lidocaine and indomethacin, compounds which inhibit phospholipase A<sub>2</sub> activity (17, 18) were tested for any effect on neutrophil phospholipase C activity. In addition, we have also investigated the effects of naloxone, FMLP, PGD<sub>2</sub> and verapamil on phospholipase C activity. Of these naloxone, FMLP and PGD<sub>2</sub> were selected because of their known effects on neutrophil function (19, 20, 25). Since phospholipase C requires Ca<sup>2+</sup> for optimal activity, the effect of verapamil, a calcium channel blocking drug, was also evaluated (18). For this purpose the various compounds were dissolved in DMSO and incubated with sonicated neutrophils at 37°C. At the end of the incubation period, the sonicates were assayed for enzyme activity.

### RESULTS

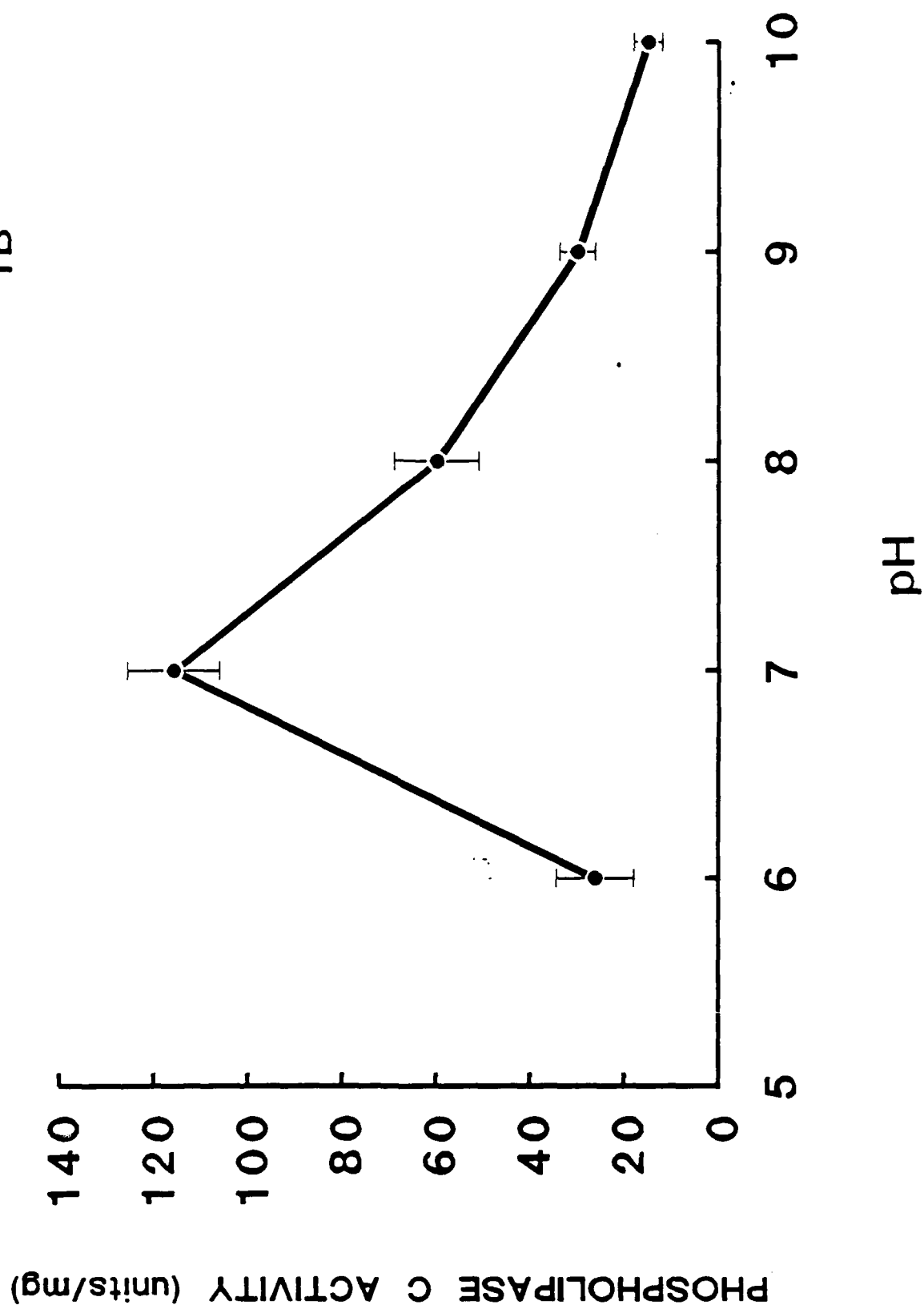
#### Characteristics of Phospholipase C Activity in Sonicates of Neutrophils

Phospholipase C activity was demonstrable in sonicates of neutrophils as shown in Fig. 1; the amount of substrate was not rate-limiting at the concentration (2 mM) used in this assay (Fig. 1A). The enzyme reaction had a pH optimum of 7.0 (Fig. 1B). Enzyme activity was linear over 90 min (Fig. 1C) and directly proportional to the concentration of enzyme (Fig. 1D). The effect of Ca<sup>2+</sup> on the phospholipase C activity is shown in Table 1. Increasing the Ca<sup>2+</sup> concentration from 0.5 to 2mM increased phospholipase C activity by 338%. Additional dependence of phospholipase C on Ca<sup>2+</sup> ions was further documented by the observation that the enzyme activity decreased by 92% in the presence of 2mM EGTA. When the calcium chloride was replaced by magnesium chloride (2 mM) in the assay mixture, the enzyme activity decreased by 87%.

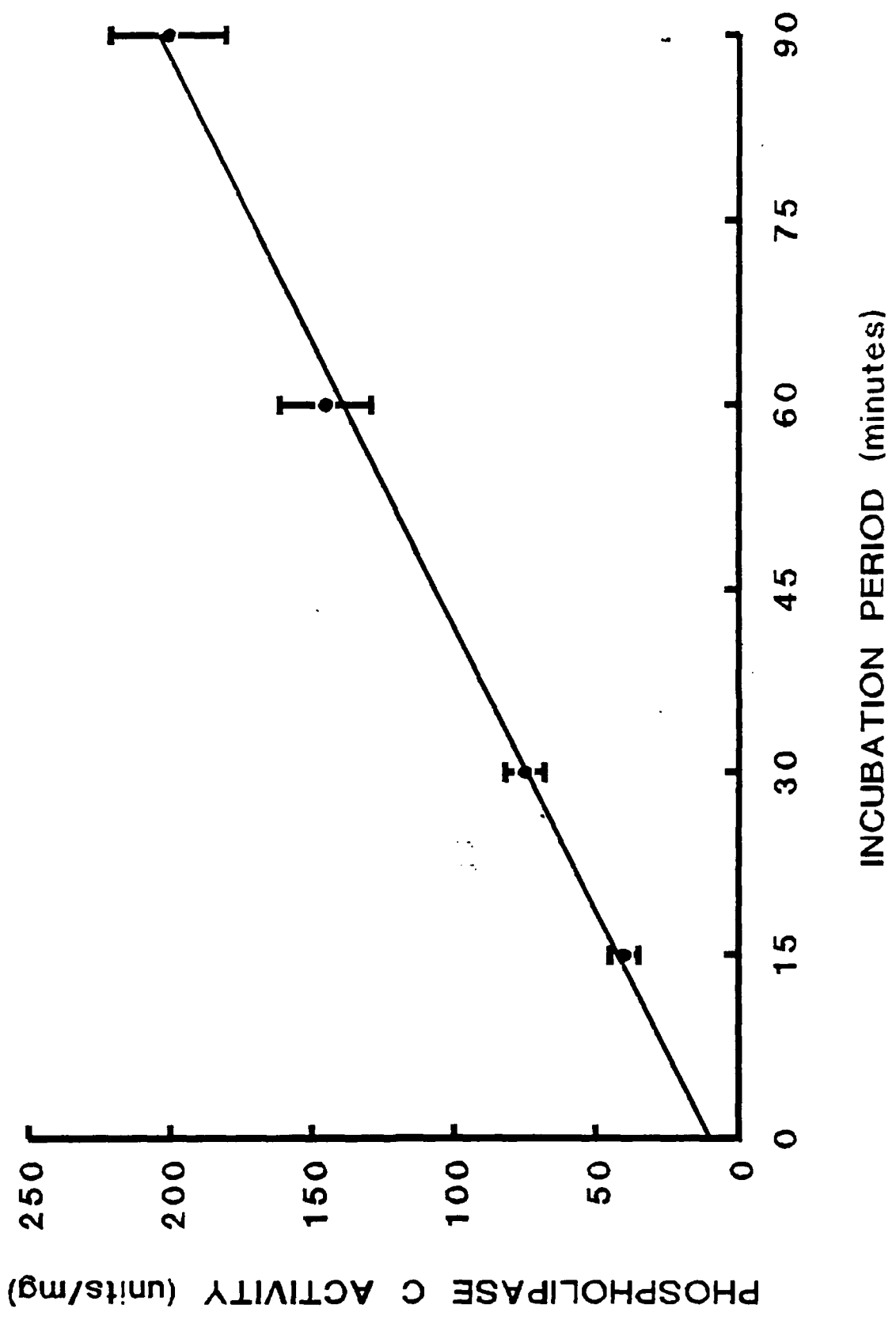
1A



1B



1C





1D

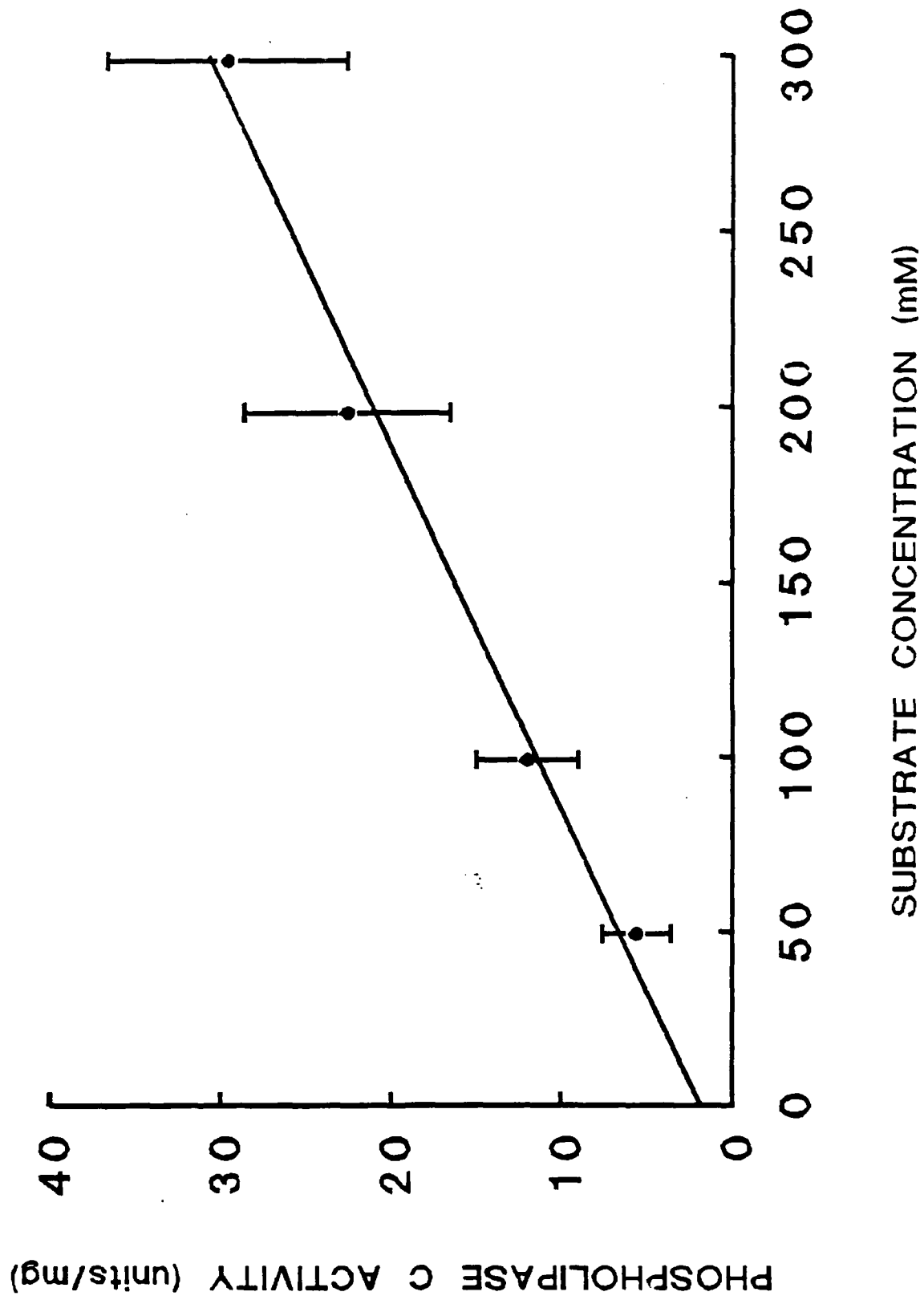


TABLE 1

EFFECT OF  $\text{Ca}^{+2}$  ON NEUTROPHIL PHOSPHOLIPASE C ACTIVITY

Calcium Chloride (mM)	Phospholipase C Activity* Units/mg Protein (Mean + S.E.M.)
0.5	35.6 + 5.5
1.0	94.1 + 13.8
2.0	122.4 + 12.7
3.0	65.8 + 9.6

\*The enzyme assay system contained a mixture of 2 mM 1-3-phosphatidylinositol and L-3-phosphatidyl-[U- $^{14}\text{C}$ ]-inositol (approximately 60,000 CPM) in 0.125 ml of Tris buffer (40 mM, pH 7.0), 2 mM sodium deoxycholate, the indicated concentrations of  $\text{CaCl}_2$  and 50-100 ug protein. The details of the assay are given in Methods. The values represent mean + S.E.M. derived from neutrophils obtained from 3 volunteers.

The requirement by the neutrophil phospholipase C for a bile salt detergent was investigated (Table 2). In the absence of the bile salt, sodium deoxycholate, there was no detectable enzyme activity. Enzyme activity increased with increasing concentrations of sodium deoxycholate, the maximal activity occurring at 2 mM. Increasing the deoxycholate concentration from 2 to 4 mM resulted in a significant decrease in enzyme activity.

Table 3 shows the effects of adding bovine serum albumin to the incubation mixture. A concentration of 4 gm/100 ml resulted in maximal phospholipase C activity and was included in all routine assays.

Sonicated neutrophils retained almost full enzyme activity for at least 4 weeks when stored frozen at  $-23^{\circ}\text{C}$  even when subjected to repeated freeze-thaw cycles. The effect of heat treatment on enzyme activity was also investigated. After 15 minutes at  $50^{\circ}\text{C}$ , the activity decreased by almost 80%; after 15 minutes at  $100^{\circ}\text{C}$  no enzyme activity was detectable.

#### Identification of Phospholipase C Activity

The lipid soluble products from phosphatidylinositol breakdown by neutrophil sonicates were analyzed. After 30 min of incubation of the nonpolar lipids were 1,2-diacylglycerols ( $82.2 \pm 6.8\%$ , Mean  $\pm$  S.E.M.  $n=3$ ) 1,3 diacylglycerols ( $8.4 \pm 1.2\%$ ) monacylglycerols ( $7.3 \pm 0.7\%$ ) and nonesterified fatty acids ( $2.1 \pm 0.08\%$ ).

When the fatty acid composition of diacylglycerols was analyzed by gas chromatography there was 1:1 molar stoichiometry between the total amount of 1,2 and 1,3 diacylglycerols released and the amount of labeled water soluble products released after 30 min of incubation period. In addition, there was good correlation between the fatty acid composition of

TABLE 2

## EFFECT OF SODIUM DEOXYCHOLATE ON NEUTROPHIL PHOSPHOLIPASE C ACTIVITY

Sodium Deoxycholate Concentration (mM)	Phospholipase C Activity* Units/mg Protein (Mean + S.E.M.)
0.5	39.9 + 5.3
1.0	75.2 + 6.3
2.0	127.3 + 13.0
4.0	19.5 + 7.1

\*Conditions of the assay were similar to those described in Table 1.  $\text{CaCl}_2$ , 2mM and the indicated concentrations of sodium deoxycholate were used in the assay mixture. The values are the means + S.E.M. derived from neutrophils obtained from 3 volunteers.

TABLE 3

EFFECT OF BOVINE SERUM ALBUMIN (BSA) ON NEUTROPHIL PHOSPHOLIPASE C  
ACTIVITY

BSA Concentration (gms %)	Phospholipase C Activity* Units/mg Protein Mean + S.E.M.
0.5	3.61 + 8.0
1.0	56.9 + 8.1
2.0	76.8 + 4.7
3.0	99.0 + 5.2
4.0	130.6 + 6.0
5.0	126.9 + 8.8

\*The conditions of the enzyme assay were similar to those described in Table 1. Sodium deoxycholate (2 mM),  $\text{CaCl}_2$  (2 mM) and the indicated concentrations of BSA were used in the assay mixture. The values are the enzyme activities derived from neutrophils of 3 normal volunteers.

the diacylglycerols (Arachidonate  $63.3 \pm 4.1\%$ , stearate  $23.2 \pm 3.2$  oleate  $3.3 \pm 0.3\%$ , linoleate  $3.6 \pm 1.2\%$ , palmitate  $6.1 \pm 1.1\%$ ) and phosphatidylinositol used in the assay (Arachidonate  $60.1 \pm 3.9\%$ , stearate  $25.8 \pm 2.9\%$ , oleate  $3.1 \pm 0.6\%$ , linoleate  $4.1 \pm 1.1\%$ , palmitate  $6.9 \pm 1.3\%$ ).

The water soluble products were analyzed by chromatography (23) and after 30 min of incubation these were inositol-1,2-cyclic phosphate ( $68.3 \pm 8.3\%$  Mean  $\pm$  S.E.M.  $n=3$ ), inositol-1-phosphate ( $27.2 \pm 3.1\%$ ) and inositol ( $4.5 \pm 0.8\%$ ).

#### Comparison of the Phospholipase C Activity Determined by Analysis of the Reaction Products with that of the Present Method

Neutrophil phospholipase C activities from 3 subjects, as determined by chromatographic determination of diglyceride fractions and water soluble products were  $108.6 \pm 9.8$  and  $139.1 \pm 13.2$  units/mg (mean  $\pm$  S.E.M.) respectively. Corresponding values by the present method was  $126.3 \pm 10.3$  units/mg.

#### Effect of Nonesterified Fatty Acids on Neutrophil Phospholipase C Activity

Arachidonic, palmitic, stearic and oleic acids, in various concentrations (2, 1, 0.5, 0.1mM) did not alter neutrophil phospholipase C activity (data not shown).

#### Effects of Various Compounds on Neutrophil Phospholipase C Activity

The various compounds were incubated with sonicated neutrophils for 15 min prior to enzyme assay. Of the various tested compounds which have phospholipase A<sub>2</sub> inhibitory effects, mepacrine, lidocaine and indomethacin had significant inhibitory effects on phospholipase C activity.

Mepacrine ( $10^{-3}$ M) inhibited the enzyme activity by 27%. At  $10^{-6}$ M

concentration this compound inhibited phospholipase C activity by 19%. Lidocaine had inhibitory effect only at  $10^{-3}$ M concentration and  $10^{-5}$ M concentration did not inhibit the enzyme activity. Naloxone, Verapamil, FMLP and  $\text{PGD}_2$  had no significant effects on the enzyme activity (Table 4).

The effect of indomethacin on phospholipase C activity was investigated in detail. The effect of indomethacin was immediate. Inhibition of the phospholipase C activity was dose dependent. Our data in Table 4 show that  $10^{-3}$ M indomethacin decreased phospholipase C activity from 117 to 30 units/mg while  $10^{-5}$ M indomethacin reduced the activity to 76 units/mg. Thus at  $1\mu\text{M}$  concentration there was 35% inhibition and at 0.5 mM, 1mM and 3mM concentration, 54, 74, and 100% inhibition respectively were observed. The extent of inhibition of phospholipase C activity was not dependent on the concentration of substrate over an approximately 32 fold range indicating that indomethacin reacted with the enzyme rather than with the substrate. In Fig. 2, neutrophil phospholipase C activity alone, and in the presence of 0.5 mM and 1 mM indomethacin, is plotted according to Lineweaver and Burke as described previously (26), yielding an apparent  $K_m$  of  $0.17 \times 10^{-6}$ M and apparent  $K_i$  of  $3.6 \times 10^{-6}$ M. Thus, the inhibition of neutrophil phospholipase C by indomethacin appears to be of the non-competitive type.

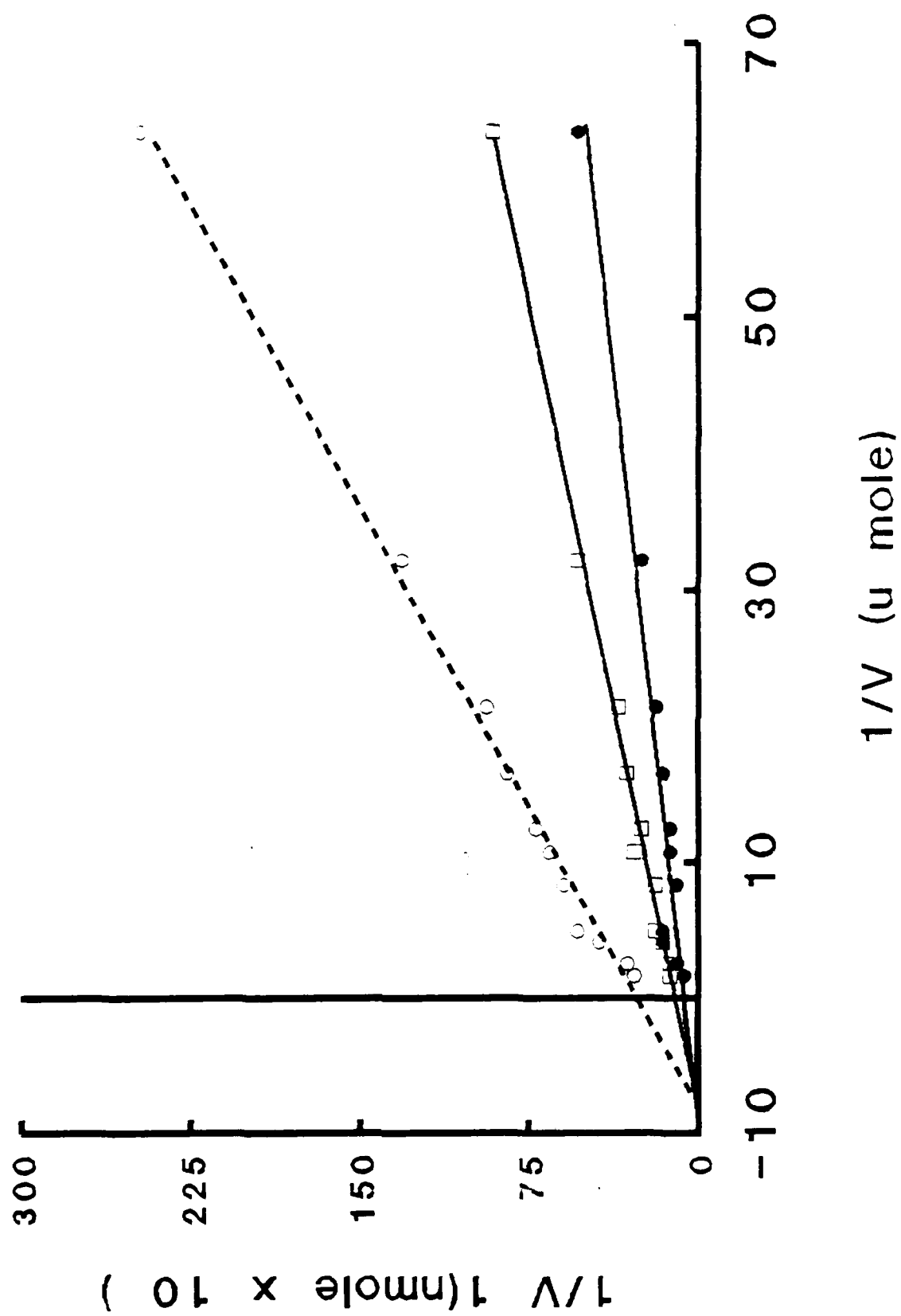
TABLE 4

## EFFECTS OF VARIOUS COMPOUNDS ON NEUTROPHIL PHOSPHOLIPASE C ACTIVITY

<u>Additions</u>	Phopholipase C Activity (Units/mg protein)
None	117.2 + 10.2
Mepacrine	
$10^{-3}M$	85.7 + 7.5*
$10^{-5}M$	95.3 + 10.3
Lidocaine	
$10^{-3}M$	76.9 + 6.8*
$10^{-5}M$	112.7 + 10.3
Indomethacin	
$10^{-3}M$	30.4 + 11.7*
$10^{-5}M$	76.2 + 5.0*
Naloxone	
$10^{-3}M$	125.1 + 3.4
$10^{-5}M$	122.1 + 9.1
Verapamil	
$10^{-3}M$	99.7 + 10.8
$10^{-5}M$	113.2 + 10.0
FMLP	
$10^{-3}M$	133.5 + 8.0
$10^{-5}M$	123.7 + 14.8
PGD <sub>2</sub>	
$10^{-3}$	110.0 + 7.0
$10^{-5}$	120.8 + 5.4

\*The various compounds were dissolved in DMSO and 5 ul of DMSO solution was added to an aliquote of sonicated neutrophils ( $10^6$  cell in 300 ul of Hanks Balanced Solution) and incubated at 23°C for 125 minutes. At the end of the incubation period the sonicated cells (n=3) were assayed for phospholipase C activity as described in Methods. (\*p < 0.04 by Student's t test).





## DISCUSSION

While this is a modification of an earlier method described by DiRenzo et al. (11) several factors contribute to the convenience for analyzing phospholipase C activity in neutrophils by the present method. First, the commercial availability of labeled phosphatidylinositol eliminates the need for the biological or chemical synthesis. Second, the extraction technique used replaces the time-consuming steps of thin-layer chromatography and subsequent extractions. In the present study, we specifically investigated for the presence of phospholipase D in sonicates of neutrophils. It was important to document the absence of this enzyme, since phosphatidic acid can be converted to diglyceride by a phosphatidic acid phosphatase. In this study, phospholipase D activity was absent in neutrophils and this is in agreement with the observations of Water et al (27). Since lysophosphatidylinositol, a product of phospholipase A action, is readily extracted into the upper phase we have investigated for the presence of this compound. Interestingly no radio label was detectable in the lysophosphatidyl fraction and this may be explained by the presence of bovine serum albumin in incubation mixture, assay conditions and certain other compounds present in the neutrophils which inhibit phospholipase A activity (28).

Two classes of phosphatidylinositol-specific phospholipase C have been described in various mammalian tissues. These are: (a) cytosolic or membranebound, active at neutral pH and require  $\text{Ca}^{2+}$  for optimal (1-9, 11, 29, 30) and (b) lysosomal origin, active at acidic pH and does not require  $\text{Ca}^{2+}$  (31-33). The present study confirms that phospholipase C activity assayed under the present conditions in neutrophils belongs to the first

class. It is interesting to note that the specific activity of the enzyme in neutrophils was considerably higher than that observed in rat hepatic tissues and human fetal membranes and uterine decidua (11, 34).

Phospholipase C from platelets, liver, fetal membranes and uterine decidua and skin fibroblasts require  $\text{Ca}^{2+}$  for optimal activity (1-3, 11). In the present study  $\text{Ca}^{2+}$  ions were required for the optimal activity of the enzyme. It has been suggested that  $\text{Ca}^{2+}$  is not necessary for the increase in phosphatidylinositol turnover, although the physiological responses require the ion. In agreement with this latter observation, Tolbert et al. (35) observed that, stimulated turnover of phosphatidylinositol could not be demonstrated under conditions of severe  $\text{Ca}^{2+}$  depletion.

Some mammalian phosphatidylinositol-specific phospholipase C enzymes are activated by the anionic detergent, deoxycholate. Deoxycholate may act as an anionic amphophile, thus regulating activity of the enzyme in vitro. Although it has been suggested that this amphiphilic regulator in vivo may be a free fatty acid (36), in the present study we did not observe any effects of added free fatty acid on enzyme activity. In contrast to our findings, DiRenzo et al. (11) demonstrated that deoxycholate inhibited phosphatidylinositol-specific phospholipase C in fetal membranes and uterine decidua.

Mepacrine (0.1 to 1000  $\mu\text{M}$ ) has a profound inhibitory effect on phospholipase  $\text{A}_2$  activity in platelets, macrophages, polymorpholeukocytes, and lung homogenates (17, 18). In these tissues 50 to 60% inhibition was observed (17, 18) with relatively small concentrations of mepacrine (42 to 200  $\mu\text{M}$ ). The present study shows that mepacrine at 1 mM and 1  $\mu\text{M}$  concentration had significant inhibitory effects on neutrophil

phospholipase C activity. Interestingly, Chan et al. (37) demonstrated a biphasic modulatory role of mepacrine on platelet phospholipase A<sub>2</sub> activity. Although various local anesthetic agents have been shown to inhibit lung phospholipase A<sub>2</sub>, rat liver lysosomal, lymphocyte and platelet phospholipase C activities (17, 18), the results of the present study show that lidocaine had significant effects on neutrophil phospholipase C activity only at mM concentration. Since phospholipase C is a calcium dependent enzyme, we tested the effect of verapamil, a calcium-channel blocking on neutrophil phospholipase C activity. Verapamil had no significant effect on this enzyme. We have also investigated the effects of naloxone, FMLP and PGD<sub>2</sub>, compounds which have effects on various aspects of leukocyte function (19, 20, 38). These compounds had no significant effects on phospholipase C activity. The absence of an effect of FMLP on neutrophil phospholipase C is interesting since Nelson has shown that treatment of human neutrophil with bacterial phospholipase C increased the ability of the cells to bind FMLP (38). Bradford et al. (39) demonstrated FMLP stimulated the breakdown triphosphoinositides probably via phospholipase A<sub>2</sub> and C mediated events.

Of the drugs tested in the present study, only indomethacin demonstrated significant inhibition at relatively low concentrations (1  $\mu$ M). At higher concentrations of indomethacin marked inhibition of phospholipase C activity was observed. Previous studies have shown that indomethacin (10-75  $\mu$ M) inhibited phospholipase A<sub>2</sub> activities by at least 50% in hepatic tissues and platelets, as well as in human and rabbit polymorpholeukocytes (17, 36, 40, 41). In the present study on neutrophil phospholipase C, the apparent K<sub>i</sub> for indomethacin was  $3.6 \times 10^{-6}$ . This

value is similar to the  $K_i$  of indomethacin for cyclooxygenase enzyme (42) and polymorpholeukocyte phospholipase  $A_2$  (41). This suggests that under physiological conditions indomethacin may act on phospholipase C as well as phospholipase  $A_2$  and cyclooxygenase. In the present study, kinetic analysis showed that indomethacin inhibited phospholipase C activity in a noncompetitive manner. The inhibition reported for indomethacin on phospholipase  $A_2$  in polymorpholeukocyte and hepatic tissue was also of the noncompetitive type (40, 41).

From these data we conclude the low concentrations of indomethacin can inhibit neutrophil phospholipase C activity and thus the mechanism of action of indomethacin appears to extend beyond the inhibition of phospholipase  $A_2$  and cyclooxygenase activity.

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#### LEGENDS TO FIGURE

**Figure 1.** Phospholipase C activity as a function of (a) substrate concentration; (b) pH; (c) incubation time and (d) protein concentration. The enzyme assay was performed as described in Methods. Incubations were performed in a shaking water bath at 37°C for 30 min except in (c) where incubation time was altered as indicated. For pH dependence 2 buffers were used; 50mM MES for pH 6.0 and 50mM Tris for pH 7 to 9 (b). The values represent the mean + S.E.M. of values derived from neutrophils of 3 subjects.

**Figure 2.** Neutrophil phospholipase C activity was measured in control conditions (O-----O), in the presence of 0.5mM indomethacin (O-----O) and 1mM indomethacin (O-----O). The data were plotted according to the method of Lineweaver and Burke (26). The values represent the mean + S.E.M. of values derived from neutrophils of 4 subjects.