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SCAVENGING OF SUPEROXIDE ANION BY PHOSPHORYLETHANOLAMINE: STUDIES IN HUMAN NEUTROPHILS AND IN A CELL FREE SYSTEM

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On the basis of previous observations, we attempted to characterize the effects of various products of phospholipid hydrolysis on neutrophil (PMN) respiratory burst activity. We studied the effects of phosphorylcholine (PC) and phosphorylethanoline (PE) on superoxide anion production in PMN and in a cell free system. We found that PE but not PC inhibited measured superoxide anion, but that this was not due to inhibition of cellular superoxide generation but to scavenging of generated superoxide anion. Further, utilizing a system based upon the photo-oxidation of O-dianisidine sensitized by riboflavin, we were able to determine that the scavenging effect of PE was not superoxide dismutase (SOD)-like but rather a general scavenging or glutathione (GSH)-like effect. These data underscore the importance of identifying the mechanism of inhibition of superoxide generation by putative inhibitors as being due to a direct cellular effect or to a scavenging property.

KEY WORDS: Phosphorylethanolamine, phosphorylcholine, scavenging, superoxide anion.

INTRODUCTION

We have recently shown that phospholipase C (PLC), a membrane bound enzyme responsible for the hydrolysis of membrane phospholipids to diacylglycerol (DAG) and phosphoryl derivatives phosphorylinositol (PI), phosphorylcholine (PC), phosphorylethanolamine (PE), or phosphorylserine (PS) (depending on the substrate preference of the phospholipase) can inhibit certain aspects of neutrophil (PMN) oxidative function while stimulating other PMN functions.[1] In our experiments we used an enzyme which perferentially hydrolyzed phosphatidylcholine, not phosphatidylinositol. In an attempt to better characterize the effects of various products of phospholipid hydrolysis on PMN respiratory burst activity, we studied the effects of PC and PE on superoxide anion production in PMN and in a cell free system. We found that PE, but not PC, inhibited measured superoxide generation in a dose dependent manner. However, we found that this lowering of measured superoxide was not due to inhibition of cellular superoxide generation but rather to scavenging of generated superoxide anion by this phospholipid hydrolysis product, and that the PE acted as a general free radical scavenger rather than as a specific superoxide dismutase type scavenger.

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MATERIALS AND METHODS

Reagents

Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Corp., Forrester, NY, and stored at − 4° C at a stock concentration of 10 mg/ml in dimethylsufoxide (DMSO), and used in a final concentration of 10–100 ng/ml. N-formylmethionyl-phenylalanine (fMLP) was purchased from Sigma Chemicals, St. Louis, Mo., stored at a stock concentration of 10^{-6} M in DMSO and used at a final concentration of 10^{-7} M. Dianisidine and superoxide dismutase (SOD) were obtained from Sigma Chemicals and riboflavin from Eastman Organic Chemicals. PE and PC were all obtained from Sigma and stored dessicated at 0° C. Preparations at the appropriate concentrations were made fresh each day in Hanks Balanced Salt Solution (HBSS). Xanthine and xanthine oxidase were purchased from Sigma. Xanthine (purity 99–100 %) was stored at room temperature. Xanthine oxidase was stored at 4° C and had a specific activity of 1.2 U/mg protein.

Isolation of Polymorphonuclear Leukocytes

PMN were isolated as previously described[2]. Briefly, 30–50 cc of blood were drawn into a syringe containing 1 u/ml of heparin and were allowed to sediment with one half volume of 6.5 % dextran and 0.98 % sodium chloride. The supernatant was collected, the remaining red cells lysed by hypotonic lysis and the pellet was resuspended and layered onto a Ficoll-Hypaque gradient and centrifuged at 450 × g for 30 min. at 20° C. The pellet contained approximately 95 % PMN and was suspended in HBSS with 0.2 % human serum albumin (HBSS-HSA) or HBSS alone.

Superoxide Generation

Superoxide anion generation was determined by two methods. Both relied on the superoxide dismutase (SOD) inhibitable reduction of cytochrome C. In PMN the assay was performed as described by Goldstein *et al.*[3] PMN were incubated with varying concentrations of PE and PC, then stimulated with agonists PMA (10 ng/ml) or fMLP (10^{-7} M). Reduction of cytochrome C at 550 nm (using a molar extinction coefficient for this change in absorption of 21,000) was determined at 15 min, and in some experiments was measured in a continuous assay using a Varians spectrophotometer. Superoxide generation was also measured in a cell free system with xanthine-xanthine oxidase (X:XO) as the source of free radical (.1 U XO) with and without added phosphoryl derivative.

Biochemical Augmentation Assay

The photo-oxidation of O-dianisidine sensitized by riboflavin was measured as described by Misra and Fridovich[4]. Illumination for the photochemical reactions was provided by two parallel 20-W Sylvania Gro-Lux fluorescent bulbs mounted in the center of an aluminum foil lined box. Reaction mixtures containing riboflavin (stock solution 1.3 × 10^{-5} M in 0.01 M potassium phosphate buffer at pH 7.5) and o-dianisidine (10^{-2} M) in ethanol were added to quartz cuvettes with and without SOD. Absorbance was measured at 460 mm on a Varians spectrophotometer before and after 4 minutes illumination and the difference in absorption was calculated. This

assay can be used to classify scavenging compounds as SOD-like or glutathione (GSH)-like, since SOD-like compounds produce an augmentation and GSH-like compounds an inhibition of absorption.[5]

RESULTS

Effects of Phosphoryl Derivatives on Superoxide Generation

We found that phosphorylethanolamine (PE) but not phosphorylcholine (PC) (data not shown) inhibited PMA and fMLP induced production of O_2^- when pre-incubated with PMN (Table I). This effect was dose dependent and was seen at concentrations as low as 2 mM PE when fMLP was the agonist and 4 mM PE when PMA was the agonist. The inhibition occurred whether PMN were pre-incubated with PE (Table I) or when agonist and PE were added simultaneously (Figure 1a, b) and superoxide generation measured in a continuous assay system. The high concentrations required suggested that the effects were mediated by scavenging or toxicity.

Effects of Phosphoryl Derivatives in a Cell Free System (X:XO)

In order to help determine whether the inhibitory effect of PE on measured O_2^- generation was due to scavenging or inhibition of production of free radical, we repeated the experiments with varying concentrations of PE in a cell free system, measuring the production of O_2^- by a mixture of xanthine and xanthine oxidase. We found that there was maximum inhibition of O_2^- generation after approximately 1 min of incubation, but that over time the SOD inhibitable reduction of cytochrome C in presence of PE returned to close to normal levels (Figure 2), suggesting consumption or inactivation of the PE.

Photo-oxidation of Dianisidine

To determine whether the observed scavenging of O_2^- by PE was due to a specific SOD-like effect or was due to a general free radical scavenging activity, we measured

TABLE I[1]

Addition to PMN	*Agonist*	O_2^{-2}
0	cyt B + FMLP[3]	24.8 ± 2.5
PE 20 mM[4]		2.6 ± 1.9
PE 15 mM		2.8 ± 0
PE 10 mM		7.4 ± 2.8
PE 4 mM		12.8 ± 2.6
PE 2 mM		14.5 ± 0.9
0	PMA[3]	48.8 ± 0.95
PE 20 mM		0
PE 15 mM		3.7 ± 0.28
PE 10 mM		8.5 ± 0.57
PE 4 mM		21.9 ± 2.0

[1]Results are expressed as mean ± S.E. of a representative experiment in duplicate. Similar data were found in two additional experiments for each agonist.

[2]O_2^- is expressed as nanomoles of cytochrome C reduced/10^6 PMN

[3]Cytochalasin B 5 μg/ml and FMLP 10^{-6} M. PMA 10 ng/ml without cytochalasin B.

[4]PMN are pre-incubated with PE for 10 min. prior to activation.

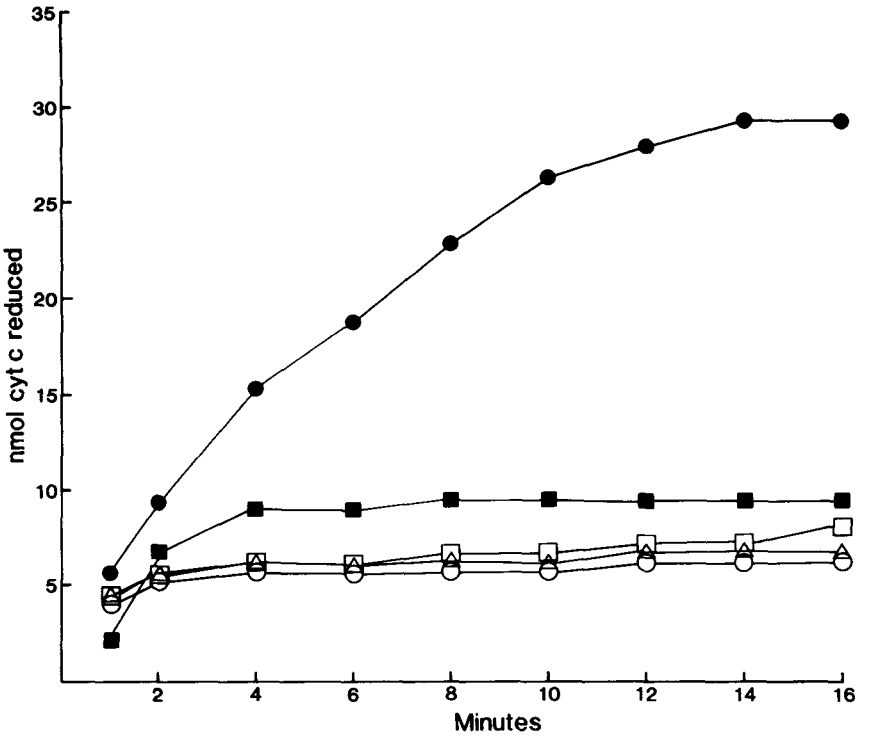

FIGURE 1 Human polymorphonuclear leukocytes (PMN) were incubated with varying concentrations of phosphorylethanolamine (PE) (control ●—●, 20 mM PE ○—○, 15 mM PE △—△, 10 mM PE □—□, 5 mM ■—■ PE) then simultaneously stimulated with cytochalasin B 5 μg/ml and FMLP 10^{-6} M. Superoxide generation, measured as SOD inhibitable reduction of cytochrome C was assayed at 1-minute time intervals on a Varians spectrophotometer. There was dose dependent inhibition when PE incubated PMN were compared to controls.

the effects of PE on the photo-oxidation of dianisidine. We found that when PE was added to the reaction mixture (described in Materials and Methods), there was inhibition of the photo-oxidation of dianisidine (Table II). This effect is similar to that produced by glutathione (GSH) and leukotrienes,[5] and is opposite of the augmentation that is seen with SOD alone (Table II).[4,5]

TABLE II[1,2]

Addition	*Absorption after 8 min*	*% inhib*
0	0.0877 ± 0.001	
PE 5 mM	0.0107 ± 0.0011	85.4
PE 2.5 mM	0.0355 ± 0.0032	52
SOD 10 μg/ml[3]	0.3453 ± 0.0275	

[1]The effects of PE on the photo-oxidation of O-dianisidine were measured as described in Materials and Methods.

[2]Data were expressed as mean ± S.E. absorption of a representative experiment in duplicate. Similar data were found in 2 additional experiments.

[3]When superoxide dismutase (SOD) was added, there was expected[4,5] augmentation of photo-oxidation of O-dianisidine.

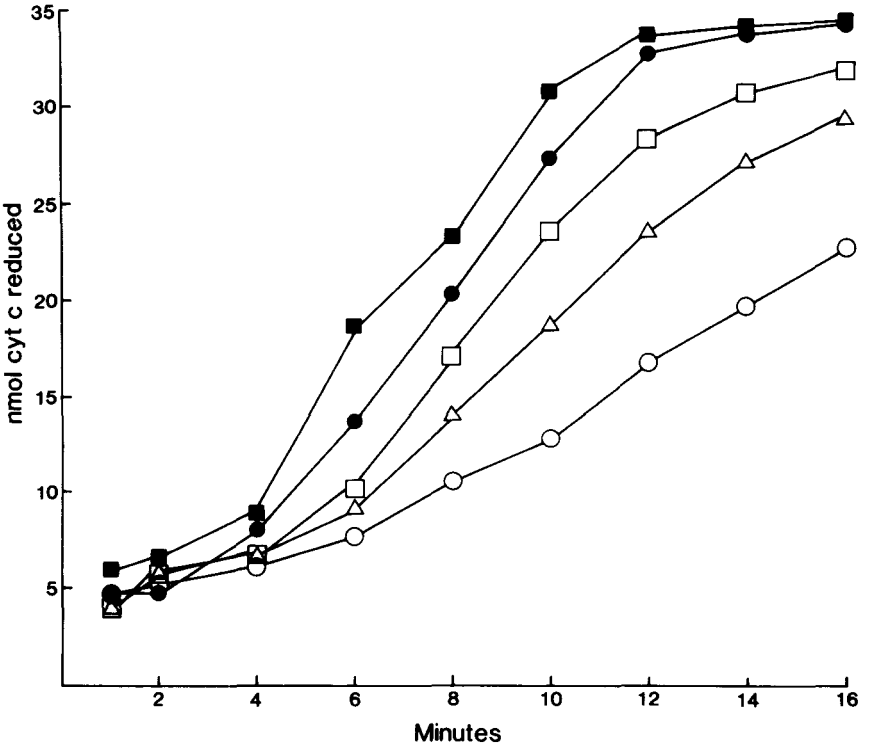

FIGURE 2 Control PMN (●—●) treated as above, but the stimulus is PMA (10 mg/ml). 20 mM PE ○—○, 15 mM PE △—△, 10 MM PE □—□, 5 mM ■—■ PE.

DISCUSSION

We have demonstrated that phosphorylethanolamine but not phosphorylcholine diminishes measured O_2^- generation in human PMN, but that this effect is due to scavenging of generated O_2^-. Moreover, using a unique assay which relies upon the photo-oxidation of dianisidine to a colored compound that can be spectrophotometrically measured at 450 mm, we found that the effect of PE was due to a scavenging effect and was not the result of a specific SOD-like catalytic effect.

The study of superoxide production by human PMN and the subsequent characterization of the biochemical pathways which regulate this production have been the focus of intense interest in biology over the past several years.[6] Several recent reviews highlight the importance of the respiratory burst system in human PMN,[7,8] and we,[9] and others[10,11] have shown that regulation of PMN respiratory burst activity in PMN has important consequences in a variety of biological systems. A number of compounds which have been found to inhibit various components of the respiratory burst system have now been identified,[12] and clarification of the site of action and mechanisms of these putative inhibitors is warranted.

We were interested in further characterizing the effects of hydrolysis products of

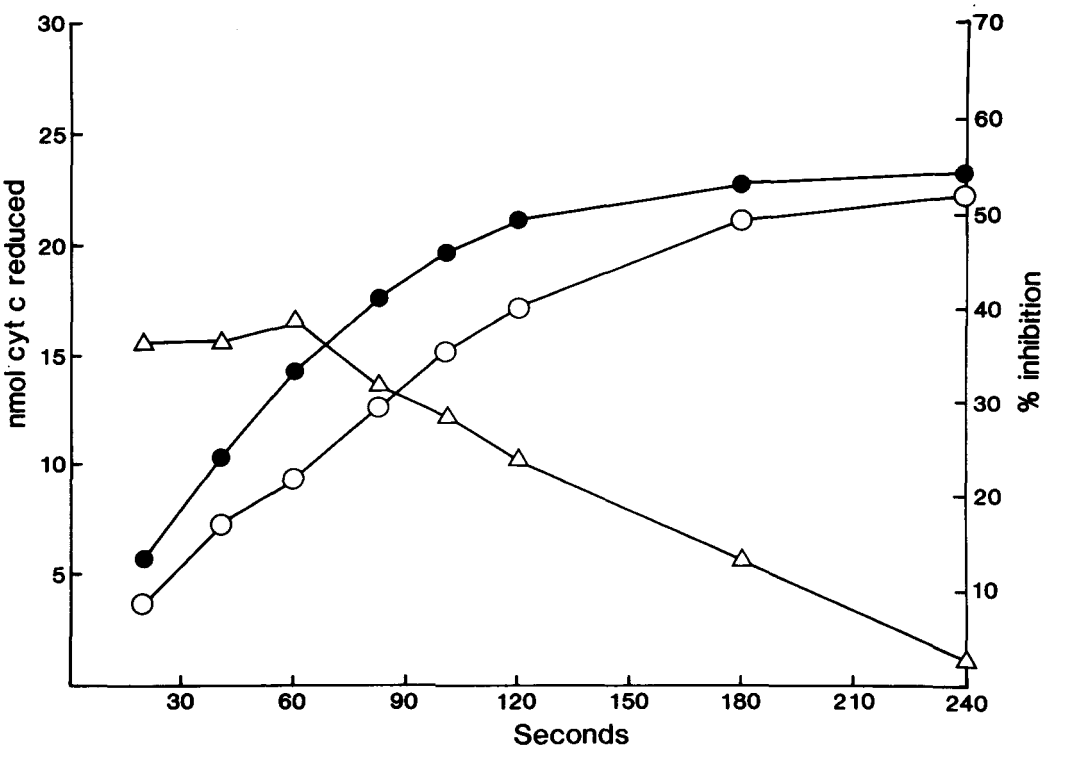

FIGURE 3 Superoxide generation is measured in a cell free system using a mixture of xanthine and xanthine oxidase (specific activity 1.2 U/mg protein) as described in materials and methods, in the absence (●—●) and presence (○—○) of 20 mM PE. Percent inhibition with time (△—△) is shown on the ordinate on the right.

phospholipases, since we have recently shown that phosphatidylcholine-preferring phospholipase C can inhibit PMN respiratory burst activity while stimulating other PMN functions.[1] This apparently paradoxical effect led us to investigate the effects of various phospholipid hydrolysis products on PMN function. We found that phosphorylethanolamine, but not phosphorylcholine, inhibits measured O_2^- generation, and that this effect is due to scavenging rather than to a direct cellular effect on the oxidase. These experiments did not, however, elucidate the mechanism of the effect of phosphatidylcholine preferring phospholipase C on PMN O_2^- generation but instead appeared to identify unexpected scavenging properties of phosphorylethanolamine.

Finally, we sought to investigate whether the scavenging effect of PE was SOD-like, that is, specific for the dismutation of superoxide anion, or was a more general scavenger of free radicals. To answer this question we utilized a system which is based upon the observations by Misra and Fridovich that the photo-oxidation of O-dianisidine involves a complex series of free radical chain reactions which utilizes the superoxide anion as a chain propagating species.[4] Thus:

a) Riboflavin (Rf) + light (hv) → Rf^*
b) $Rf + DH_2 \rightarrow RfH^0 + DH^0$

c) $RfH^0 + O_2 \rightarrow Rf + O_2^- + H^+$
d) $DH^0 + O_2^- + H^+ \rightarrow DH_2 + O_2$
e) $DH^0 + DH^0 \rightarrow D + DH_2$
f) $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

In a) the riboflavin is excited by light, and in b) it oxidizes dianisidine, giving a flavin semiquinone and a dianisidine radical which would spontaneously dismute as in e) to yield the colored product D, which absorbs at 460 mm. However, the flavin semiquinone produced in b) can reduce O_2 to O_2^- (reaction c) which will reduce the dianisidine radical (reaction d). SOD, by scavenging O_2^- (reaction f) prevents the reduction of the dianisidine radical, allowing more of the colored D to accumulate. On the other hand, any radical scavenging species acting on reaction b would diminish the formation of D, and a decrease in absorbtion would be observed. In our system, PE caused an inhibition of absorbance, thus indicating that it was acting on reaction b as a general free radical scavenger. This is exactly as has been described for GSH, and different from what would be expected[5] and what we observed (Table II), for SOD.

Thus, we have shown that PE scavenges generated free radicals, and that this effect is not SOD-like. Further description of putative inhibitors of PMN respiratory burst activity should include evaluation of their ability to scavenge generated free radicals, as this information may have pharmacologic implications as systems to modulate free radical production are developed. In addition, an understanding of the scavenging properties of physiologic compounds may provide insight into important biological phenomena.

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