Phospholipase A$_2$ Is Involved in the Mechanism of Activation of Neutrophils by Polychlorinated Biphenyls

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Aroclor 1242, a mixture of polychlorinated biphenyls (PCBs), activates neutrophils to produce superoxide anion ($O_2^-$) by a mechanism that involves phospholipase C-dependent hydrolysis of membrane phosphoinositides; however, subsequent signal transduction mechanisms are unknown. We undertook this study to determine whether phospholipase A$_2$-dependent release of arachidonic acid is involved in PCB-induced $O_2^-$ production. We measured $O_2^-$ production in vitro in glycogen-elicited, rat neutrophils in the presence and absence of the inhibitors of phospholipase A$_2$: quinacrine, 4-bromophenacyl bromide (BPB), and mannoalide. All three agents significantly decreased the amount of $O_2^-$ detected during stimulation of neutrophils with Aroclor 1242. Similar inhibition occurred when neutrophils were activated with the classical stimuli, formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate. The effects of BPB and mannoalide were not a result of cytotoxicity or other nonspecific effects, although data suggest that quinacrine is an $O_2^-$ scavenger. Significant release of $^3$H-arachidonic acid preceded $O_2^-$ production in neutrophils stimulated with Aroclor 1242 or fMLP. Mannoalide, at a concentration that abolished $O_2^-$ production, also inhibited the release of $^3$H-arachidone. Aspirin, zileuton, or WEB 2086 did not affect Aroclor 1242-induced $O_2^-$ production, suggesting that eicosanoids and platelet-activating factors are not needed for neutrophil activation by PCBs. Activation of phospholipase A$_2$ and $O_2^-$ production do not appear to involve the Ah receptor because a congener with low affinity, but not with one high affinity for this receptor, stimulated the release of arachidonic acid and $O_2^-$. These data suggest that Aroclor 1242 stimulates neutrophils to produce $O_2^-$ by a mechanism that involves phospholipase A$_2$-dependent release of arachidonic acid. Key words: arachidonic acid, 4-bromophenacyl bromide, mannoalide, PCBs, quinacrine, signal transduction, superoxide anion. Environ Health Perspect 104:52-58 (1996)

Polychlorinated biphenyls (PCBs) are a class of industrial chemicals that persist in the environment. In laboratory animals PCBs cause a variety of biological effects including cancer, immunotoxicity, neurotoxicity, and hepatotoxicity as well as dermatisis, edema, induction of cytochrome P450, and increased mixed-function oxidase activities. Although many of these effects have been related to activity of the Ah receptor, recent studies suggest that mechanisms independent of this receptor may also be important (1-3). For example, investigations concerning the neurotoxicity of PCBs suggest that ortho-substituted congeners, which have little affinity for the Ah receptor, cause greater decreases in neurotransmitter levels (4) and perturbations of Ca$^{2+}$ homeostasis in neural cells (3,4) than planar congeners, which bind the Ah receptor with high affinity.

Studies in our laboratory indicate similar results in neutrophils. When exposed in vitro to Aroclor 1242, a PCB mixture, neutrophils are activated to undergo degranulation and to produce superoxide anion ($O_2^-$). These effects do not appear to involve Ah receptor activity since similar results are observed with an ortho-substituted congener, 2,2',4,4'-tetrachlorobiphenyl (2,2',4,4'-TCB), whereas a planar congener, 3,3',4,4'-tetrachlorobiphenyl (3,3',4,4'-TCB), does not alter neutrophil function (5). Furthermore, the activation of neutrophils by PCBs appears to involve mechanisms similar to those reported by Kodavanti et al. (4,5) for neural cells, namely, the activation of phospholipase C, the production of inositol 1,4,5-triphosphate (IP$_3$), and the subsequent mobilization of intracellular calcium (6). Activation of phospholipase C appears to be important in PCB-stimulated stimulation of neutrophils because the concentration-response relationship is similar for production of inositol phosphates and neutrophil activation, and IP$_3$ accumulation precedes the onset of $O_2^-$ production in neutrophils exposed to PCBs. Furthermore, activity of this enzyme parallels production of O$_2^-$; i.e., 2,2',4,4'-TCB causes the accumulation of inositol phosphates but 3,3',4,4'-TCB does not (6). Finally, 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoic acid, an inhibitor of mobilization of intracellular calcium, significantly attenuates PCB-induced neutrophil activation, suggesting that IP$_3$-induced mobilization of intracellular calcium plays an important role in the activation of neutrophils by PCBs (7). These results are consistent with the hypothesis that PCBs activate neutrophils by a mechanism that involves phospholipase C. Little is known, however, about subsequent signaling pathways involved in the activation of neutrophils by PCBs.

Phospholipase C appears to play an important role in neutrophil activation in response to several physiologic activators of neutrophil function, including the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (8,9), opsonized zymosan, and antigen-antibody complexes (10). Several enzymes are activated subsequent to the increase in phospholipase C activity, including phospholipases A$_2$ and D, as well as protein kinase C (11). Activation of these enzymes is important in regulation, initiation, and/or maintenance of neutrophil activation. For example, inhibitors of phospholipase A$_2$ such as quinacrine and 4-bromophenacyl bromide (BPB) abolish $O_2^-$ production in response to fMLP and opsonized zymosan, as well as to phorbol myristate acetate (PMA) (12-14), suggesting that activity of phospholipase A$_2$ is required for activation of the NADPH oxidase responsible for reduction of $O_2^-$ to $O_2^-$. Because early signal transduction events are similar in neutrophils exposed to PCBs and other stimuli such as fMLP, it is possible that subsequent signal transduction mechanisms are also similar. Therefore, the purpose of the present study was to determine whether PCBs activate neutrophils by a mechanism that involves phospholipase A$_2$ and consequent release of arachidonic acid.

Materials and Methods

Chemicals. Aroclor 1242, 2,2',4,4'-TCB, 3,3',4,4'-TCB, and 3,3',4,4',5-pentachlorobiphenyl (PeCB) were obtained from ChemService (West Chester, Pennsylvania). The congeners were reported by the manufacturer to be greater than 99% pure. fMLP, PMA, cytochalasin B, cytochrome C, superoxide dismutase (SOD), quinacrine, BPB, xanthine, and xanthine oxidase were...
obtained from Sigma Chemical Company (St. Louis, Missouri). WEB 2086 was obtained from Boehringer Ingelheim (Ridgefield, Connecticut) and zileuton from Abbott Laboratories (Chicago, Illinois). Manoalide was purchased from Biomol (Plymouth Meeting, Pennsylvania), and 3H-arachidonate was obtained from DuPont (Boston, Massachusetts). For all experiments Aroclor 1242 was dissolved in methanol, and the congeners were dissolved in dimethylformamide at a concentration of 10 mg/ml. Neutrophils received 1 μl/ml of this stock solution, and control cells received 1 μl of the appropriate vehicle. fMLP, PMA, and cytochalasin B were dissolved in DMSO and diluted so that the final concentration of DMSO was less than 1%. Manoalide and BPB were suspended in ethanol so that the final concentration of ethanol to which neutrophils were exposed was less than 1%. Quinacrine was suspended in Hanks’ balanced salt solution (HBSS).

**Neutrophil isolation.** We obtained glycerol-elicted neutrophils from the peritoneal cavities of one to two male, Sprague-Dawley, retired breeder rats per experiment (Charles River Laboratories, Portage, Michigan) as described previously (5). Briefly, rats were anesthetized with diethyl ether and 30 ml 1% glycerol was injected intraperitoneally. After 4 hr the rats were anesthetized again and killed by decapitation. The peritoneum was washed with 30 ml of heparinized (1 U/ml), 0.1 M phosphate-buffered saline (PBS). The solution obtained from the peritoneum was collected, filtered through gauze, and centrifuged at 500 g for 7 min. Contaminating red blood cells were lysed with 15 ml of 0.15 M NH₄Cl, and neutrophils were suspended to a final volume of 50 ml with PBS and centrifuged for 7 min at 300 g. Cells were washed once with PBS and resuspended in HBSS.

**Generation of O₂⁻.** We measured O₂⁻ production by neutrophils (2 × 10⁶ cells/ml) in response to Aroclor 1242, 2,2′,4,4′-TCB, 3,3′,4,4′-TCB, 3,3′,4,4′,5-PCB, PMA, and fMLP. In these and all experiments with fMLP, neutrophils were pretreated with cytochalasin B (4 μM) for 5 min because fMLP does not cause significant O₂⁻ production in rat neutrophils in the absence of cytochalasin B (6,15).

We measured O₂⁻ production in the presence and absence of the inhibitors of phospholipase A₂, BPB (1–100 μM), quinacrine (1–200 μM), and manoalide (0.1–10 μM). Neutrophils were preincubated with BPB or quinacrine for 5 min or with manoalide for 20 min before addition of stimuli. To test the role of metabolites of arachidonic acid in neutrophil activation, O₂⁻ production was measured in stimulat-
ed neutrophils in the presence and absence of aspirin or zileuton, inhibitors of cyclooxygenase or 5-lipoxygenase, respectively. We used the receptor antagonist for platelet-activating factor (PAF), WEB 2086, to examine the potential involvement of PAF. Neutrophils were incubated with aspirin or WEB 2086 for 30 min or with zileuton for 15 min before addition of stimuli. In all experiments, cumulative O₂⁻ production in response to stimulation was measured over 20 min.

**Detection of O₂⁻.** We measured O₂⁻ generated by monitoring the reduction of cytochrome C (10 mg/ml) in the presence and absence of SOD (16). For every sample, two tubes were incubated, one to which SOD (840 U/ml) was added before stimulation and one to which SOD (840 U/ml) was added at the end of incubation. The amount of cytochrome C reduced was estimated from the difference in absorbance (550 nm) between the cell-free supernatant in the two tubes using an extinction coefficient of 18.5 cm⁻¹ mM⁻¹.

Phospholipase A₂ inhibitors could decrease the amount of O₂⁻ detected by mechanisms unrelated to inhibition of production of O₂⁻ by neutrophils. To test whether any apparent decrease in O₂⁻ production by inhibitors of phospholipase A₂ was due to scavenging of oxygen radicals, we measured O₂⁻ generated during oxidation of xanthine by xanthine oxidase in a cell-free system in the presence and absence of the compounds. Cytochrome C was incubated for 5 min at 37°C with xanthine (9 mg/ml) and xanthine oxidase (2.5 × 10⁻⁴ U/ml) in the presence or absence of quinacrine, BPB, and manoalide. We determined the amount of O₂⁻ generated by xanthine and xanthine oxidase by the difference in absorbance (550 nm) between tubes containing both xanthine and xanthine oxidase and tubes containing xanthine alone.

In addition, absorbance of reduced cytochrome C measured in the presence or absence of phospholipase A₂ inhibitors to ensure that apparent inhibition of neutrophil-generated O₂⁻ was not a result of quenching of absorbance by these compounds. Cytochrome C was reduced by O₂⁻ generated from neutrophils exposed to Aroclor 1242 as described above, and absorbance at 550 nm was measured in the cell-free supernatant fluids after the addition of quinacrine, manoalide, BPB, or the appropriate vehicle. There were no differences in absorbance values in the presence and absence of inhibitors of phospholipase A₂.

**Determination of cytotoxicity.** Cytotoxicity was determined by release of the cytotoxic enzyme lactate dehydrogenase (LDH). Neutrophils were incubated with manoalide, BPB, or quinacrine as described above, and LDH activity was determined in the cell-free supernatant fluids as described by Bergmeyer and Bernt (17). A separate aliquot of neutrophils was lysed with Triton-X and sonication, and total LDH activity was determined in the cell-free supernatant fluid of this lysate so that cytotoxicity could be expressed as the percentage of total LDH released.

**Labeling of neutrophils with 3H-arachidonic acid.** Neutrophils (10⁷/ml) were suspended in Mg²⁺-free and Ca²⁺-free HBSS containing 0.1% bovine serum albumin and incubated in the presence of 0.5 μCi/ml 3H-arachidonic acid (specific activity = 180–240 Ci/mM) for 2 hr gently shaking at 37°C. At the end of the labeling period, neutrophils were washed 2 times with Mg²⁺-free and Ca²⁺-free HBSS and resuspended in HBSS containing albumin, Ca²⁺ and Mg²⁺. The cell count was adjusted so that the final concentration of neutrophils was 2 × 10⁶/ml. The incorporation of 3H-arachidonic acid was routinely greater than 80% of the total radioactivity added to the cells.

**Stimulated release of 3H-arachidonic acid.** Neutrophils labeled as described above were stimulated with either fMLP, PMA, Aroclor 1242, 2,2′,4,4′-TCB, 3,3′,4,4′-TCB, 3,3′,4,4′,5-PeCB, or the appropriate vehicle, and release of 3H-arachidonic acid was measured at various times. In some experiments, we measured 3H-arachidonic acid release in the presence and absence of manoalide (10 μM). At the end of each incubation, neutrophils were placed on ice and centrifuged at 0°C for 10 min. The cell-free supernatant fluids were transferred to vials containing scintillation cocktail (14 ml), and the total radioactivity in each sample was determined by liquid scintillation counting. To determine the total cellular uptake of 3H-arachidonic acid, for each experiment total cellular radioactivity was measured in a 1-m1 aliquot of washed cells (2 × 10⁶ cells/ml).

**Statistical analysis.** Results are expressed as means ± standard error of the mean (SEM). Results for 3H-arachidonic acid are expressed as percentage of total cellular radioactivity released into the medium. Data were analyzed by analysis of variance, and group means were compared using the Student-Newman-Keuls’ test. We performed appropriate transformations on all data that did not follow a normal distribution (e.g., percent data). Neutrophils taken from a single rat or pooled from two rats were used to obtain data for a single replicate or n. For all studies, the criterion for statistical significance was p ≤ 0.05.


**Results**

No $O_2^-$ was produced by unstimulated neutrophils either in the presence or absence of the phospholipase A$_2$ inhibitors BPB, manoalide, or quinacrine (data not shown). The effect of BPB on $O_2^-$ production in neutrophils stimulated with Aroclor 1242, fMLP, and PMA is shown in Figure 1A. Cumulative $O_2^-$ release in response to Aroclor 1242 was $25.6 \pm 5.8$ nmol/10$^6$ cells/20 min. Responses to PMA and fMLP were included so that effects of PCBs could be compared to those of other neutrophil stimuli with well-studied mechanisms of action. PMA and fMLP produced $45.8 \pm 14.5$ and $19.6 \pm 4.6$ nmol/10$^6$ cells/20 min, respectively. $O_2^-$ produced by all three agents was significantly different from unstimulated neutrophils. $O_2^-$ generated in the presence of Aroclor 1242 was significantly inhibited by 1 $\mu$M BPB and abolished by 10 $\mu$M BPB (Fig. 1A). Similar inhibition of fMLP- and PMA-induced $O_2^-$ production was observed.

Manoalide also inhibited $O_2^-$ production in neutrophils exposed to Aroclor 1242, fMLP, and PMA; however, the concentration–response relation differed slightly in neutrophils exposed to fMLP and PMA compared to Aroclor 1242 (Fig. 1B). Inhibition of fMLP- and PMA-induced $O_2^-$ production was complete in the presence of 1 $\mu$M manoalide, i.e., maximal, inhibition of $O_2^-$ generation in the presence of Aroclor 1242 was achieved with 10 $\mu$M manoalide (Fig. 1B). Neither BPB nor manoalide was cytotoxic to neutrophils in the presence of stimuli (Fig. 2). In addition, neither of these phospholipase A$_2$ inhibitors scavenged $O_2^-$ radicals at concentrations that inhibited neutrophil $O_2^-$ production as evidenced by the inability of these agents to reduce the amount of $O_2^-$ detected in the presence of xanthine and xanthine oxidase (Table 1). The quantity of $O_2^-$ measured in neutrophils exposed to Aroclor 1242, fMLP, or PMA was also decreased in a concentration-dependent manner by quinacrine (Fig. 3); however, this compound inhibited the amount of $O_2^-$ detected in the presence of xanthine and xanthine oxidase in a cell-free system (Table 1), suggesting that quinacrine scavenges $O_2^-$ radicals.

The effect of BPB and manoalide to inhibit $O_2^-$ production by Aroclor 1242-stimulated neutrophils suggested that Aroclor 1242 activates phospholipase A$_2$. A consequence of activation of this enzyme is liberation of arachidonic acid from membrane phospholipids. Accordingly, we examined the kinetics of $^3$H-arachidonate release in response to Aroclor 1242. Experiments were performed in the presence of albumin to inhibit the metabolism and reacylation of arachidonic acid; therefore, the data represent the cumulative release of arachidonic acid. Little radiolabeled arachidonate was released from neutrophils in the absence of activation (Fig. 4). Significant release of $^3$H-arachidonate was observed within 5 min from neutrophils exposed to Aroclor 1242, and no further release was observed after this time (Fig. 4A). $^3$H-arachidonate release in response to fMLP occurred within 2 min, with no further release thereafter (Fig. 4B). The magnitude of release of $^3$H-arachidonate was greater in neutrophils exposed to Aroclor 1242 when compared to fMLP (Fig. 4A, B). In neutrophils exposed to PMA, a small but statistically significant release of $^3$H-arachidonate was observed, but not until 20 min after stimulation (Fig. 4C).

Because release of arachidonic acid can occur by mechanisms independent of phospholipase A$_2$ (18), we investigated the effect of manoalide on PCB-, PMA-, and

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**Figure 1.** Percent inhibition of superoxide anion ($O_2^-$) generation by (A) 4-bromophenacyl bromide (BPB) and (B) manoalide. $O_2^-$ production was measured in neutrophils as described in Materials and Methods. Neutrophils ($2 \times 10^6$ cells/ml) were incubated at 37°C for 3 min in the presence of BPB or vehicle or for 20 min in the presence of manoalide or vehicle and then stimulated for 20 min with Aroclor 1242 (10 $\mu$g/ml), phorbol myristate acetate (PMA; 20 ng/ml) or formyl-methionyl-leucyl-phenylalanine (fMLP; 1 $\mu$M). Cytochalasin B (4 $\mu$M) was added 5 min before stimulation with fMLP. $O_2^-$ production in response to Aroclor 1242, PMA, and fMLP was (A) $25.6 \pm 5.8, 45.8 \pm 14.5$, and $19.6 \pm 4.6$ nmol/10$^6$ cells/20 min and (B) $13.8 \pm 5.1, 21.3 \pm 2.4$, and $14.9 \pm 3.9$ nmol/10$^6$ cells/20 min. *Significantly different from respective value obtained in the absence of inhibitor; $n = 4$–5.

**Figure 2.** Lack of cytotoxicity of manoalide and 4-bromophenacyl bromide (BPB). Neutrophils were exposed to Aroclor 1242, phorbol myristate acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence or absence of manoalide (10 $\mu$M) or BPB (100 $\mu$M) as described in the legend to Figure 1, and cytotoxicity was assessed from release of the cytosolic enzyme lactate dehydrogenase (LDH) as described in Materials and Methods. Percent release of LDH from unstimulated neutrophils was 7.5 ± 1.8%; $n = 3$. There were no significant differences between groups.
fMLP-induced $^3$H-arachidionate release. As can be seen in Figure 5A, manoalide, at a concentration which abolished $O_2^-$ production by all three agents (Fig. 2B), significantly inhibited the release of $^3$H-arachidonate in neutrophils stimulated with Aroclor 1242. Similar results were observed in neutrophils stimulated with fMLP (Fig. 5B). PMA induced a small increase in arachidonic acid release that was significant in the absence, but not the presence of manoalide, and these values were not significantly different from each other (Fig. 5C). Similar results were obtained with BPB (data not shown).

The release of $^3$H-arachidonate and the production of $O_2^-$ by neutrophils in response to stimulation with Aroclor 1242 were concentration dependent (Fig. 6A). Likewise, exposure to 2,2',4,4'-TCB caused generation of $O_2^-$ and release of $^3$H-arachidonate in a concentration-dependent manner (Fig. 6B), whereas 3,3',4,4'-TCB failed to elicit either response in neutrophils (Fig. 6C). A small but significant release of $^3$H-arachidonate was observed in neutrophils exposed to 3,3',4,4',5-PeCB; however, this congener did not cause significant production of $O_2^-$ (Fig. 6D).

Arachidonic acid is metabolized in the neutrophil by cyclooxygenase and 5-lipoxygenase to products that can alter neutrophil function (19). To test whether arachidonic acid metabolites were involved in activation of the NADPH oxidase, we measured $O_2^-$ production in stimulated neutrophils in the presence and absence of the inhibitor of cyclooxygenase, aspirin, or the inhibitor of 5-lipoxygenase, zileuton (Table 2). $O_2^-$ production in neutrophils exposed to Aroclor 1242 was not affected by these inhibitors at concentrations that have been shown to attenuate eicosanoid production (20). Similarly, no effect of these inhibitors on fMLP- or PMA-induced $O_2^-$ production was observed. PAF is a product of phospholipase A$_2$, which has been suggested to be involved in the generation of $O_2^-$ in rabbit neutrophils (21); therefore, experiments with the PAF receptor antagonist WEB 2086 were performed to test for any involvement of this product. WEB 2086, at concentrations which significantly attenuate PAF-induced responses (22), did not alter the generation of $O_2^-$ by Aroclor 1242, PMA, or fMLP (Table 2).

**Discussion**

We have demonstrated previously that the PCB mixture Aroclor 1242 activates isolated rat neutrophils to produce $O_2^-$ (5). In the present study, Aroclor 1242 stimulated the release of arachidonic acid by a mechanism involving phospholipase A$_2$. The release of arachidonic acid and the production of $O_2^-$ in neutrophils exposed to Aroclor 1242 occurred with similar kinetics and concentration–response relations. Two inhibitors of phospholipase A$_2$, BPB and manoalide, caused concentration–dependent inhibition of $O_2^-$ production upon exposure of rat neutrophils to Aroclor

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**Table 1. Detection of $O_2^-$ generated from xanthine and xanthine oxidase***

| Phospholipase A$_2$ inhibitor | $O_2^-$ detected (nmol/ml) |
|------------------------------|---------------------------|
| Vehicle                      | 33 ± 6.2                  |
| Quinacrine                   | 4.7 ± 2.7*                |
| 100                          | 6.0 ± 3.6*                |
| Manoalide                    | 27 ± 0.1                  |
| 4-Bromophenacyl bromide      | 29 ± 4.8                  |

*The effect of inhibitors of phospholipase A$_2$ on $O_2^-$ generated from a mixture of xanthine (9 mg/ml) and xanthine oxidase (2.5 × 10$^4$ U/ml) was measured in a cell-free system. $O_2^-$ generated was assessed as described in Materials and Methods. The amount of $O_2^-$ detected was significantly decreased in the presence of quinacrine, but not manoalide or 4-bromophenacyl bromide. *Significantly different from value obtained in the presence of vehicle; n = 3.

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**Figure 3. Quinacrine induced-inhibition of $O_2^-$ generation.** Experiments were performed as described in the legend to Figure 1, except that neutrophils were incubated at 37°C for 3 min in the presence or absence of quinacrine prior to stimulation for 20 min. *Significantly different from respective value obtained in the absence of quinacrine; n = 4–5.

**Figure 4. Time course of cumulative release of $^3$H-arachidonic acid from neutrophils.** Neutrophils were labeled as described in Materials and Methods and incubated in the presence and absence of (A) Aroclor 1242, (B) formyl-methionyl-leucyl-phenylalanine (fMLP), or (C) phorbol myristate acetate (PMA) as described in the legend to Figure 1. Release of $^3$H-arachidonic acid into the extracellular medium was determined as described in Materials and Methods. Data are expressed as percent of total cellular radioactivity. *Statistically different from respective value obtained in the absence of quinacrine; n = 3–4.

**Figure 5. The inhibitory effect of manoalide on release of $^3$H-arachidonic acid in neutrophils stimulated with (A) Aroclor 1242, (B) formyl-methionyl-leucyl-phenylalanine (fMLP), or (C) phorbol myristate acetate (PMA).** Neutrophils were labeled with $^3$H-arachidonic acid, preincubated with manoalide (10 µM) or vehicle for 20 min, then stimulated as described in Figure 1. Cumulative release of $^3$H-arachidonate was measured over 20 min. Data are expressed as percent of total cellular radioactivity. *Statistically different from respective value obtained in the absence of stimulus; **Statistically different from respective value obtained in the absence of manoalide; n = 4.
1242. These inhibitors also attenuated the release of $^3$H-arachidionate in prelabeled neutrophils, suggesting that the inhibitory effect of BPB and manaoalide on $O_2^-$ production was a result of inhibition of phospholipase A₂. Release of LDH by neutrophils was not altered in the presence of these compounds (Fig. 2), indicating that the inhibition of $O_2^-$ generation was not related to cyto toxicity. In addition, these agents did not act by scavenging oxygen radicals because reduction of cytochrome C by xanthine and xanthine oxidase was not affected by either agent (Table 1).

Although other nonspecific effects of these compounds cannot be ruled out entirely, the argument that the effects on $O_2^-$ production are due to inhibition of phospholipase A₂ is strengthened by the use of compounds that inhibit phospholipase A₂ by different mechanisms. Manaoalide inhibits the enzyme by covalently modifying selective lysine residues within the protein (21), whereas BPB causes irreversible inhibition by binding covalently to a histidyl residue at the active site (21). Furthermore, quinacrine, which inhibits phospholipase A₂ by perturbing the enzyme–substrate interface (21), also decreased $O_2^-$ measured in Aroclor 1242-treated neutrophils. The results with quinacrine, however, are difficult to interpret because concentrations that inhibited $O_2^-$ production by neutrophils also inhibited the reduction of cytochrome C by $O_2^-$ generated by xanthine and xanthine oxidase (Table 1), suggesting that quinacrine is capable of scavenging $O_2^-$: Thus, it is not possible to know from these data what contribution inhibition of phospholipase A₂ made to the decrease in $O_2^-$ detected from activated neutrophils in the presence of quinacrine. These results suggest that studies using quinacrine to relate phospholipase A₂ activity to the production of oxygen radicals should be interpreted with caution.

Quinacrine is not the only inhibitor of phospholipase A₂ that has pleiotropic effects. In general, all inhibitors of phospholipase A₂ available at this time have been shown to have effects unrelated to the inhibition of phospholipase A₂ (20,23-26). Because of the lack of availability of an inhibitor specific for phospholipase A₂, it is difficult to make definitive conclusions concerning the role of this enzyme in cellular activation. Despite considerable efforts to minimize the probability that nonspecific effects of the inhibitors were responsible for our results, the possibility remains that inhibition of $O_2^-$ production by manaoalide and BPB occurred via other means. For example, the inhibition of $O_2^-$ production in the presence of BPB and manaoalide may have resulted from the ability of these agents to inhibit phosphoinositide-specific phospholipase C (23,24), as this enzyme has been shown to be involved in the activation of neutrophils by fMLP and Aroclor 1242 (6). However, this possibility seems unlikely because inhibition of phospholipase C occurs at higher concentrations of BPB [IC$_{50}$ = 100 μM (23)] and manaoalide [IC$_{50}$ = 80 μM (27)] than the concentrations found to be effective in this study. Furthermore, BPB and manaoalide inhibited the response to PMA, an agent that does not involve activation of phospholipase C (28), thus suggesting that the inhibitory effect of these compounds on $O_2^-$ was unrelated to their ability to inhibit phospholipase C.

If activation of phospholipase A₂ is important in initiation of production of $O_2^-$ in response to stimuli, release of $^3$H-arachidonic acid from prelabeled neutrophils should precede generation of $O_2^-$. Significant release of $^3$H-arachidionate was seen within 5 min in neutrophils exposed to Aroclor 1242 (Fig. 3A), and $O_2^-$ production was observed between 5 and 15 min (6). Thus, the results are consistent with the hypothesis that phospholipase A₂ is involved in initiation of NADPH oxidase activity in neutrophils activated by Aroclor 1242.

Similar results were observed with fMLP: release of $^3$H-arachidionate was seen within 2 min (Fig. 3B) and $O_2^-$ was detected within 5 min (6). The concentration—

![Figure 6. Concentration-dependent release of $O_2^-$ and $^3$H-arachidionate in neutrophils stimulated with (A) Aroclor 1242, (B) 2,2',4,4'-tetraclorobiphenyl (TCB), (C) 3,3',4,4'-TCB, or (D) 3,3',4,4',5-pentachlorobiphenyl. Cumulative generation of $O_2^-$ and release of $^3$H-arachidionate were measured in neutrophils stimulated for 20 min with the indicated concentrations of PCBs, as described in Materials and Methods. *Statistically different from respective value obtained in the presence of vehicle; n = 4.](image-url)
response relations for $^3$H-arachidonate release and $O_2^*$ production in neutrophils exposed to Aroclor 1242 (Fig. 5A) are also supportive of this hypothesis, as are most of the results from studies examining the effects of specific PCB congeners. For example, 2,2',4,4'-TCB caused production of $O_2^*$ and release of $^3$H-arachidonate from neutrophils at a concentration of 10 pg/ml but not at 1 pg/ml. Conversely, 3,3',4,4'-TCB failed to elicit either response. The exception was 3,3',4,4',5-PeCB, which like 3,3',4,4'-TCB has high affinity for the Ah receptor. 3,3',4,4',5-PeCB caused small, but significant release of arachidonic acid in the absence of production of $O_2^*$. These results suggest that $O_2^*$ generation by neutrophils exposed to PCBs is accompanied by the release of arachidonic acid, but this release is not sufficient to cause production of $O_2^*$. Alternatively, 3,3',4,4',5-PeCB may stimulate the release of arachidonic acid from a phospholipid pool that is different from that released in response to Aroclor 1242 and 2,2',4,4'-TCB, and this pool may be unavailable for activation of the NADPH oxidase.

In rat neutrophils stimulated with PMA, it does not appear that activation of phospholipase $A_2$ is required for initiation of $O_2^*$ generation. Release of $^3$H-arachidonate was significant only after 20 min of exposure to the stimulus, while $O_2^*$ production occurred much earlier (29). In addition, the magnitude of release of $^3$H-arachidonate was considerably lower in neutrophils stimulated with PMA when compared to the results obtained with fMLP or Aroclor 1242, whereas the amount of $O_2^*$ produced was comparable. One explanation for these data is that, although phospholipase $A_2$ is required for PMA-induced production of $O_2^*$, its role is not to initiate activation of NADPH oxidase. An alternative explanation for these results is that PMA activates phospholipase $A_2$ to release low concentrations of arachidonic acid that were not detectable by the methods employed in this study but that are necessary and sufficient in concert with other signal transduction pathways activated by PMA (e.g., protein kinase C) to stimulate production of $O_2^*$. This latter explanation is consistent with previous studies using human peripheral blood neutrophils in which $O_2^*$ production in response to PMA was inhibited by BPB and quinacrine and was also abolished by staurosporine, an inhibitor of protein kinase $C$ (14). The effect of staurosporine was likely due to inhibition of protein kinase $C$-mediated phosphorylation and translocation of the 47-kD cytosolic factor p47, an event that has been proposed to be required for assembly and activation of the NADPH oxidase (30,31). Thus, it appears that activation of the NADPH oxidase by PMA in human neutrophils requires at least two events: protein kinase $C$-mediated phosphorylation of p47 and phospholipase $A_2$-induced release of arachidonic acid. The same events may be required in rat neutrophils.

Although $O_2^*$ production in response to Aroclor 1242 was abolished completely by 10 $\mu$M manoline (Fig. 2B), release of $^3$H-arachidonate was inhibited only partially (Fig. 4). There are at least three possible explanations for these results. One is that arachidonate release must reach a threshold to cause activation of the NADPH oxidase, and manoline reduces arachidonic acid to levels below that threshold. The second is that, although the mechanism of release of arachidonic acid involves a phospholipase $A_2$ that can be inhibited by manoline and BPB, other phospholipases $A_2$ may contribute to overall release of arachidonic acid. Several isoforms of phospholipase $A_2$ exist in neutrophils, all of which may not be inhibited by the compounds used in this study. For example, it is known that neutrophils contain a cytosolic phospholipase $A_2$ that is not a target for either manoline or BPB (20).

A third possible explanation for the partial inhibition of arachidonate release by manoline is that mechanisms independent of phospholipase $A_2$ are responsible, in part, for the release of arachidonic acid in stimulated neutrophils. One potential mechanism involves phospholipase $D$-dependent conversion of phosphatidylcholine or phosphatidylethanolamine to phosphatidic acid and subsequent release of arachidonic acid from diglyceride and monoglyceride by the actions of diglyceride lipase and monoglyceride lipase, respectively (20). Another potential source of arachidonic acid in activated neutrophils is diacylglycerol lipase-dependent release from diacylglycerol, which arises from membrane phosphinositides as a consequence of phospholipase C activation (32). Although these pathways contribute less total arachidonic acid than phospholipase $A_2$ (32), they may be responsible for the arachidonate released in Aroclor 1242-activated neutrophils that was insensitive to manoline. Indeed, in rat neutrophils exposed to PCBs, phospholipase C activity is increased (6), and diacylglycerol levels are likely to be elevated. Further studies are needed to address the role of these pathways in the release of arachidonic acid in neutrophils exposed to PCBs.

It is likely that arachidonic acid, rather than its metabolites, is responsible for stimulation of NADPH oxidase activity in neutrophils activated with Aroclor 1242. Inhibitors of cyclooxygenase and of 5-lipoxigenase as well as the PAF receptor antagonist WEB 2086 failed to alter $O_2^*$ production in response to Aroclor 1242, fMLP, or PMA. These results are consistent with those obtained using other agents that stimulate neutrophils. Exposure of human neutrophils to exogenous arachidonic acid activates the NADPH oxidase (33,34), and this response is unaffected by inhibitors of the lipoxygenase or cyclooxygenase pathways. In addition, several other unsaturated fatty acids that are not substrates for metabolism via these pathways (e.g., linoleic, oleic, docosahexanoic, and eicosapentaenoic acid) also stimulate respiratory burst activity in intact neutrophils (34,35). Thus, it appears that phospholipase $A_2$-mediated release of arachidonic acid (or other unsaturated fatty acids) is an important event in activation of rat neutrophils by PCBs.
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