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Protein Tyrosine Kinase Involvement in the Production of Superoxide Anion by Neutrophils Exposed to Aroclor 1242, a Mixture of Polychlorinated Biphenyls

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ABSTRACT. Neutrophils produce superoxide anion (O_2^-) when exposed *in vitro* to Aroclor 1242, a mixture of polychlorinated biphenyls (PCBs). The mechanism for this effect shares some similarities with the mechanism by which the physiologic agonist f-Met-Leu-Phe (fMLP) activates neutrophils. Since production of O_2^- in response to fMLP involves GTP-binding proteins and protein tyrosine kinases (PTKs), the current study was undertaken to determine whether these signalling pathways are involved in PCB-induced neutrophil activation. Neutrophils exposed to Aroclor 1242 or fMLP produced significant O_2^-. Pretreatment of intact neutrophils with pertussis toxin or cholera toxin or exposure of permeabilized cells to GDPβS significantly inhibited O_2^- production in fMLP-treated neutrophils but did not alter the response to Aroclor 1242. Pretreatment with genistein, an inhibitor of PTKs, significantly inhibited O_2^- production in both Aroclor 1242- and fMLP-treated neutrophils; however, daidzein, a structural analogue of genistein which lacks activity against PTKs, was without effect. Exposure of neutrophils to Aroclor 1242 resulted in an increase within 1 min in tyrosine phosphorylation of proteins in the 40 and 60 kDa molecular mass ranges which persisted for up to 10 min. Similar results were obtained with 2,2′,4,4′-tetrachlorobiphenyl (2,2′,4,4′-TCB), a PCB congener that stimulates O_2^- production. In contrast, 3,3′,4,4′,5-pentachlorobiphenyl (3,3′,4,4′,5-PeCB), a congener that does not generate O_2^-, caused only a transient increase in tyrosine phosphorylation of proteins in the 40 kDa range with no effect on 60 kDa proteins. These data suggest that Aroclor 1242 activates neutrophils to produce O_2^- by a mechanism that requires tyrosine kinase activity; however, heterotrimeric G-proteins are not likely to be involved. BIOCHEM PHARMACOL **53**;12:1833–1842, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. G-protein; PCBs; fMLP; phosphorylation; cholera toxin; pertussis toxin

PCBs§ are persistent environmental contaminants that cause a wide range of biological effects in laboratory animals including tumor promotion [1] and alterations in neural function [2], endocrine function [3], and immunity [4–7]. In addition to the effects of PCBs on cell-mediated and humoral immunity [4–7], PCBs have been shown to alter the function of neutrophils, a cell type involved in nonspecific immune responses [8]. Neutrophils are circulating leukocytes that provide the first line of defense against invading pathogens. Upon exposure *in vitro* to Aroclor 1242, a mixture of PCBs, neutrophils are activated to undergo degranulation and to produce O_2^-. Moreover, exposure to PCBs alters the response to subsequent stimuli. The mechanisms of these effects are not understood fully [8].

Although many toxic effects of PCBs have been related to the activity of the Ah receptor, neutrophil activation by Aroclor 1242 appears to occur independently of this mechanism [8–11]. Congeners with low affinity for the Ah receptor, such as 2,2′,4,4′-TCB, cause the same effects as Aroclor 1242, but congeners with high affinity for the Ah receptor, like 3,3′,4,4′-TCB and 3,3′,4,4′,5-PeCB, do not [9, 10]. These data suggest that mechanisms unrelated to Ah receptor activity are involved in PCB-induced neutrophil activation.

Several agents, including fMLP, stimulate neutrophils to produce O_2^- and to degranulate by interacting with G-protein-linked receptors on the cell surface [12–15]. These receptors are linked to a variety of enzymes within the cell that play obligatory roles in neutrophil activation including PTKs [16–18], phosphatidylinositol 3-kinase [19, 20], and

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§ *Abbreviations:* fMLP, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage-colony stimulating factor; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; MAP kinase, mitogen-activated protein kinase; 3,3′,4,4′,5-PeCB, 3,3′,4,4′,5-pentachlorobiphenyl; PCB, polychlorinated biphenyl; PTK, protein tyrosine kinase; O_2^-, superoxide anion; SOD, superoxide dismutase; 2,2′,4,4′-TCB, 2,2′,4,4′-tetrachlorobiphenyl; 3,3′,4,4′-TCB, 3,3′,4,4′-tetrachlorobiphenyl; and TNF-α, tumor necrosis factor-α

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phospholipases C [21, 22], D [23, 24], and A_2 [10, 25]. Recent studies in our laboratory indicate that PCBs stimulate O_2^- production by a mechanism that requires the activity of phospholipases C and A_2 [9, 10], suggesting that the signalling mechanisms involved in PCB-induced neutrophil activation are similar to the pathways activated by physiologic agonists such as fMLP. Given these similarities, the purpose of the present study was to test the hypothesis that Aroclor 1242 activates neutrophils by a mechanism that involves G-proteins and/or PTKs.

MATERIALS AND METHODS

All animals were used in accordance with the guidelines of the Michigan State All University Committee for Animal Use and Care.

Materials

Aroclor 1242 was obtained from ChemService (West Chester, PA). Chick egg albumin, cytochrome *c*, SOD, xanthine, xanthine oxidase, fMLP, digitonin, genistein, pertussis toxin, and cholera toxin were obtained from the Sigma Chemical Co. (St. Louis, MO). GDPβS was purchased from Calbiochem (La Jolla, CA). Daidzein was obtained from Biomol (Plymouth Meeting, PA). Mouse antiphosphotyrosine antibody was obtained from Upstate Biochemical (Lake Placid, NY), and anti-mouse antibody and rainbow molecular weight markers were purchased from Amersham (Arlington Heights, IL). For all experiments, Aroclor 1242 was dissolved in methanol, and 2,2′,4,4′-TCB and 3,3′,4,4′,5-PeCB were dissolved in dimethyl formamide. Neutrophils received 1 μL/mL of Aroclor 1242, 2,2′,4,4′-TCB, or 3,3′,4,4′,5-PeCB and control cells received 1 μL of the appropriate vehicle. fMLP, genistein, daidzein, and cholera toxin were dissolved in DMSO and diluted so that the final concentration of DMSO was less than 1%. Pertussis toxin was dissolved in glycerol, and GDPβS was dissolved in HBSS.

Neutrophil Isolation

Neutrophils were obtained from the peritoneal cavities of male, Sprague–Dawley, retired breeder rats (Charles River Laboratories, Portage, MI). Rats were anesthetized with diethyl ether, and 30 mL of 1% glycogen was injected intraperitoneally. Four hours later, the rats were anesthetized again, killed by decapitation, and the peritoneal cavity was washed with 30 mL of heparinized (1 U/mL) 0.1 M PBS. The peritoneal fluid was collected, filtered, and centrifuged at 500 *g* for 7 min. Contaminating red cells were lysed with 15 mL of 0.15 M NH_4Cl, and neutrophils were washed one time with 0.1 M PBS. The percentage of neutrophils in this preparation is routinely greater than 95% [26].

Permeabilization of Neutrophils

In experiments with GDPβS, it was necessary to use permeabilized neutrophils because this agent cannot gain entry into cells in the absence of permeabilization. Neutrophils (10^7/mL) were incubated at 37° for 20 min in Ca^{2+}-free medium containing 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES, 5.6 mM glucose, 15 μM EGTA, and 1 mg/mL BSA. At the end of this incubation, cytochalasin B (5 μg/mL), MgATP (5 mM), and EGTA (0.6 mM) were added. After 5 min, the neutrophils were permeabilized by the addition of 0.75 μM digitonin for 8 min. The reaction was stopped by the addition of excess, ice-cold buffer. Neutrophils were washed two times at 4° with buffer in the absence of EGTA and albumin and then resuspended in HBSS of the following composition: 4.5 mM KCl, 0.6 mM Na_2HPO_4, 0.62 mM KH_2PO_4, 120 mM NaCl, 23 mM Tris, 1.6 mM $CaCl_2$, 0.68 mM $MgCl_2$, 10 mM glucose, and 14 mM $NaHCO_3$. The neutrophils were kept on ice until used. Under these conditions, the plasma membrane was selectively permeabilized. The percentage of cells permeabilized was routinely 60–70%, as assessed by uptake of trypan blue.

Generation of O_2^-

To determine the role of pertussis toxin-sensitive and/or cholera toxin-sensitive G-proteins in PCB-induced neutrophil activation, generation of O_2^- was determined in the presence and absence of pertussis toxin or cholera toxin. Neutrophils (2×10^6/mL) were incubated at 37° for 90 min with pertussis toxin (50 ng/mL) or for 60 min with cholera toxin (10 μg/mL) before stimulation with fMLP (1 μM) or Aroclor 1242 (10 μg/mL) for 20 min, and O_2^- production was determined after 0, 5, 10, and 20 min. In these and all experiments with fMLP, cells were pretreated with cytochalasin B (4 μM) for 5 min because rat neutrophils do not produce significant amounts of O_2^- in the absence of cytochalasin B [9]. To determine the role of GTP-binding proteins that are not substrates for either pertussis toxin or cholera toxin, O_2^- production was measured in permeabilized neutrophils (10^7/mL) in the presence or absence of GDPβS, a nonhydrolyzable analogue of GDP which binds irreversibly to and inactivates GTP-binding proteins [27]. Permeabilized neutrophils were incubated at 37° for 2 min with GDPβS (10^{-3} M) or its vehicle and stimulated with fMLP or Aroclor 1242 for 20 min.

To determine the role of protein tyrosine kinases in PCB-induced O_2^- production by rat neutrophils, experiments were performed in neutrophils pretreated with the tyrosine kinase inhibitor genistein. O_2^- production was measured in neutrophils that were incubated at 37° for 15 min with genistein or vehicle and stimulated for 20 min with Aroclor 1242 or fMLP. The same experimental protocol was followed using daidzein, a structural analogue to genistein which is inactive against tyrosine kinases [28].

Detection of O_2^-

Cumulative O_2^- generation was measured as the reduction of cytochrome *c* in the presence or absence of SOD [29]. Neutrophils were suspended in Ca^{2+}- and Mg^{2+}-containing HBSS in the presence of cytochrome *c* (10 mg/mL). For every sample, two tubes were incubated, one to which SOD (840 U/mL) was added before stimulation and one to which SOD was added at the end of the incubation period. The amount of O_2^- produced was estimated from the amount of cytochrome *c* reduced as determined from the difference in absorbance between the cell-free supernatant fluids of the two tubes, using an extinction coefficient of 18.5 cm^{-1} mM^{-1} as described previously [29].

Tyrosine Phosphorylation

Neutrophils (3×10^7/mL) were suspended in HBSS and incubated for 5 min at 37° before stimulation with 10 μg/mL Aroclor 1242, 10 μg/mL 2,2′,4,4′-TCB, 10 μg/mL 3,3′,4,4′,5-PeCB, or vehicle. Neutrophils were exposed for 1, 5, or 10 min to PCBs or vehicle. At the end of the incubation time, the samples were placed on ice and centrifuged at 0° for 1 min at 15,000 *g*. The pellets were resuspended in 400 μL of ice-cold PBS, sonicated, and added to 400 μL of sample buffer [Tris–HCl (pH 6.8), 2 mM sodium orthovanadate, 10 mM nitrophosphate, 10 mM NaF, 10 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 20% SDS, 10% mercaptoethanol, 17.5% glycerol, and 0.1% bromophenol blue]. The samples were denatured by boiling for 5 min at 100° and separated on a 10% denaturing SDS–polyacrylamide gel (constant current at 11 mA for approximately 16 hr, 4°). Approximately 500 μg of protein was added to each lane. Proteins were transferred electrically (100 V, constant voltage for 1 hr, 4°) to prepared Immobilon-P membranes. Transfer of rainbow molecular weight standards run alongside lysate samples indicated the success of transfer. Immobilon-P membranes were blocked for 3–4 hr in Tris-buffered saline + Tween-20 (0.1%; TBS-T) containing 4% chick ovalbumin and 0.25% sodium azide. Mouse antiphosphotyrosine antibody was incubated with blots overnight in blocker (1:7500, 4°) with constant rocking. Blots were washed three times with TBS-T (30, 5, and 5 min) and once with TBS (5 min). Anti-mouse antibody linked to horseradish peroxidase (1:7500) in TBS was added for 1 hr and incubated with blots at 4° with constant rocking. Blots were washed using the same protocol as described above. Enhanced chemiluminescence using Amersham reagents was performed on blots to visualize antiphosphotyrosine antibody-labeled proteins.

Determination of Cytotoxicity

To test whether inhibition of O_2^- production by genistein resulted from injury to the cells, cytotoxicity was determined in neutrophils exposed to genistein. Neutrophils were incubated with genistein as described above, and activity of the cytosolic enzyme, LDH, was determined in the cell-free supernatant fluids as described by Bergmeyer and Bernt [30]. A separate aliquot of neutrophils was lysed with Triton X-100 and sonication, and total LDH activity was determined in the cell-free supernatant fluid of this lysate. Cytotoxicity was expressed as the percent of total cellular LDH released into the medium.

Statistical Analysis

Data are expressed as means ± SEM. Data were analyzed by ANOVA and group means compared using the Student–Newman–Keuls' test. Appropriate transformations were performed on all data that did not follow a normal distribution. For all studies, the criterion for statistical significance was $P \leq 0.05$.

RESULTS

Neutrophils stimulated with Aroclor 1242 or fMLP produced significant amounts of O_2^-, however, little O_2^- was produced by unstimulated neutrophils (Fig. 1, A and B). Pertussis toxin did not alter significantly O_2^- generation by Aroclor 1242-treated neutrophils at 20 min (Fig. 1A). As expected, O_2^- production in response to fMLP was abolished in the presence of pertussis toxin (Fig. 1B). Similar results were obtained with cholera toxin: at concentrations that significantly attenuated O_2^- production by fMLP-stimulated cells (Fig. 2B), cholera toxin did not alter the response to Aroclor 1242 (Fig. 2A). Neither pertussis toxin (Fig. 3A) nor cholera toxin (Fig. 3B) affected the rate of production of O_2^- in response to Aroclor 1242.

To examine the role of pertussis toxin- and cholera toxin-insensitive GTP-binding proteins in PCB-induced O_2^- production, permeabilized neutrophils were pretreated with the nonhydrolyzable analogue of GDP, GDPβS. Little O_2^- was produced by permeabilized neutrophils in the absence of stimulation; however, significant amounts of O_2^- were generated by permeabilized neutrophils stimulated with Aroclor 1242 (Fig. 4A) or fMLP (Fig. 4B). Pretreatment with GDPβS had no effect on the response to Aroclor 1242 but abolished O_2^- production by fMLP-stimulated cells.

To evaluate the role of PTKs in Aroclor 1242-induced neutrophil activation, O_2^- production was measured in neutrophils after treatment with the PTK inhibitor genistein. Genistein inhibited Aroclor 1242-elicited O_2^- production in a concentration-dependent manner (Fig. 5A). The concentration–response relation was similar for O_2^- generation in neutrophils stimulated with Aroclor 1242 or with fMLP (Fig. 5A). Release of LDH was not significantly different in control and stimulated neutrophils in the presence or absence of genistein, indicating that genistein-induced inhibition of O_2^- production was unrelated to cytotoxicity (Fig. 5B). To determine whether the reduction of O_2^- was related to nonspecific effects of genistein, O_2^-

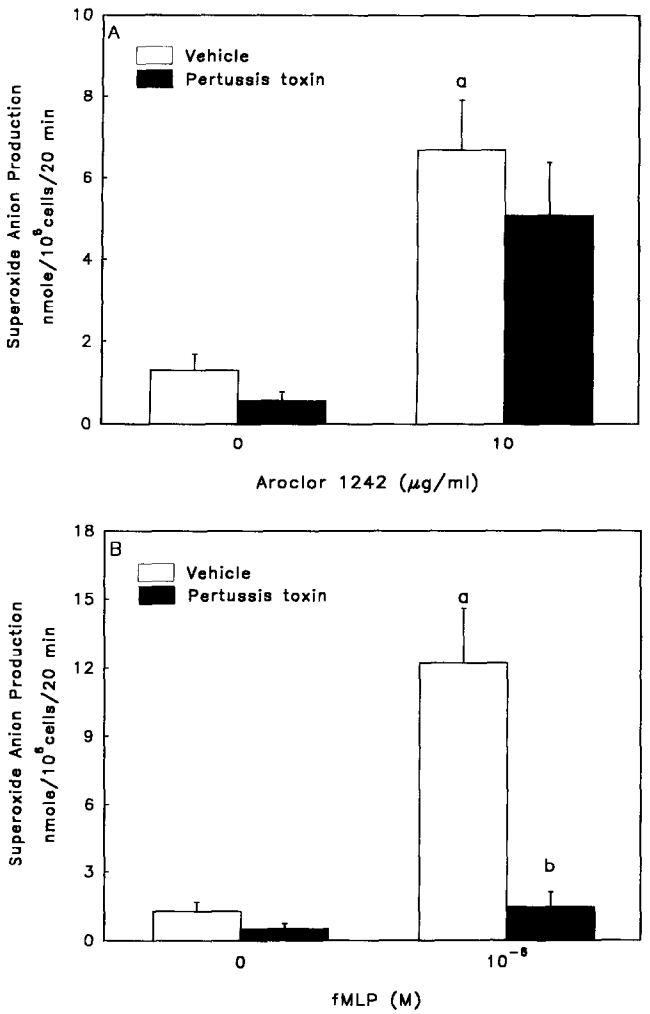

FIG. 1. Effect of pertussis toxin on superoxide anion (O_2^-) production by neutrophils. Glycogen-elicited neutrophils were incubated at 37° for 90 min with 50 ng/mL pertussis toxin or vehicle and then were stimulated for 20 min with 10 μg/mL Aroclor 1242 (A), 1 μM fMLP (B), or the appropriate vehicle. Cytochalasin B (4 μM) was added 5 min before stimulation with fMLP. Cumulative production of O_2^- was measured as the SOD-sensitive reduction of cytochrome c. Values are means ± SEM, N = 5–6. Key: (a) significantly different from the respective value obtained in the absence of stimulus; and (b) significantly different from the respective value obtained in the absence of pertussis toxin ($P \leq 0.05$).

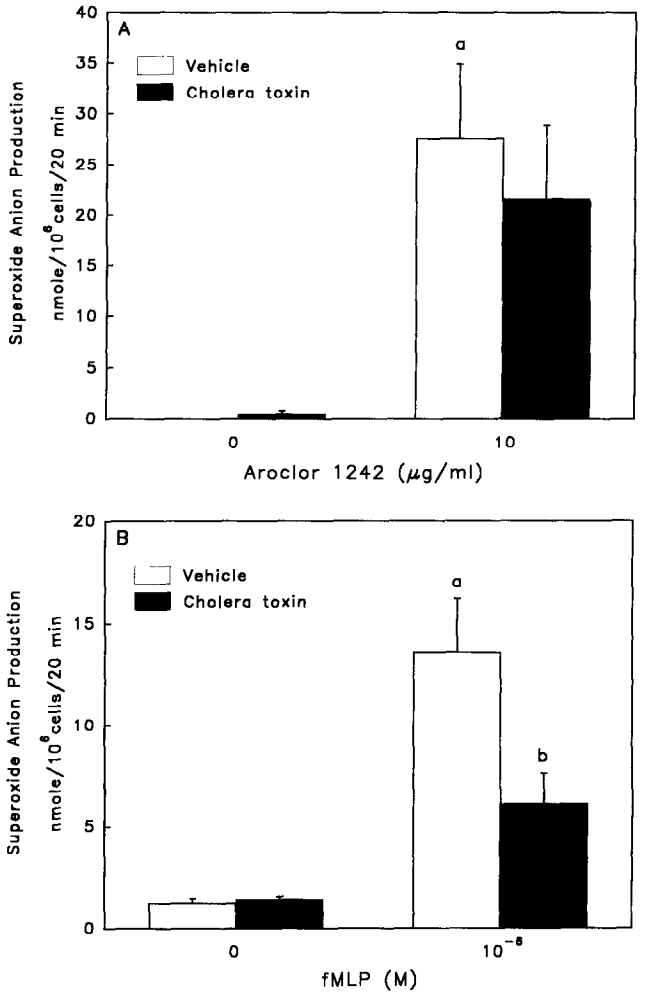

FIG. 2. Effect of cholera toxin on O_2^- production by neutrophils. Neutrophils were incubated at 37° for 60 min with 10 μg/mL cholera toxin or vehicle and then were stimulated for 20 min with 10 μg/mL Aroclor 1242 (A), 1 μM fMLP (B), or the appropriate vehicle. O_2^- production was measured as described in the legend to Fig. 1. Values are means ± SEM, N = 4. Key: (a) significantly different from the respective value obtained in the absence of stimulus; and (b) significantly different from the respective value obtained in the absence of cholera toxin ($P \leq 0.05$).

generation was measured in the presence and absence of daidzein, a structural analogue of genistein that lacks activity against PTKs. Daidzein at concentrations up to 10 μM did not alter significantly O_2^- produced in response to either fMLP or Aroclor 1242 (Fig. 6).

Inhibition of PCB-induced O_2^- production by genistein suggested the involvement of PTKs in the mechanism of O_2^- production by Aroclor 1242. A consequence of PTK activation is phosphorylation of proteins on tyrosine residues. Accordingly, tyrosine phosphorylation in response to Aroclor 1242 was investigated. Tyrosine phosphorylation was determined in response to 2,2',4,4'-TCB, a PCB congener that stimulates O_2^- production, and 3,3',4,4',5-PeCB, a congener that does not. Under the conditions of the experiment, several proteins were phosphorylated on tyrosine residues in a constitutive manner including proteins in the 20, 50–60, and 70–80 kDa range (Fig. 7). The level of tyrosine phosphorylation of these proteins did not change in cells stimulated with Aroclor 1242, 2,2',4,4'-TCB, or 3,3',4,4',5-PeCB. A protein of approximately 60 kDa, which was visible in control cells, was phosphorylated further in neutrophils stimulated with Aroclor 1242 or 2,2',4,4'-TCB, but not 3,3',4,4',5-PeCB. This increase in phosphorylation was seen at all time points. The most prominent change in tyrosine phosphorylation was seen in the 40 kDa molecular mass range. In this range, there was slight constitutive tyrosine phosphorylation that was enhanced markedly at 1, 5, and 10 min in the presence of

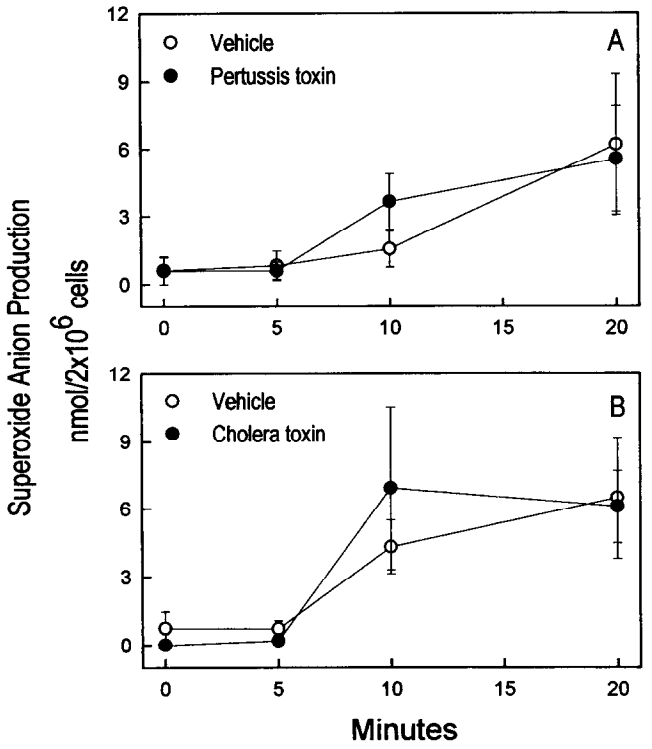

FIG. 3. Lack of effect of pertussis toxin (A) or cholera toxin (B) on the time–course of production of O_2^- in response to Aroclor 1242. Neutrophils were preincubated with (A) pertussis toxin (50 ng/mL for 90 min) or its vehicle or (B) cholera toxin (10 μg/mL for 60 min) or its vehicle followed by stimulation with 10 μg/mL Aroclor 1242. O_2^- production was determined at the times indicated. Values are means ± SEM, N = 3. No statistical differences were observed between values for the toxins and their respective controls at any time.

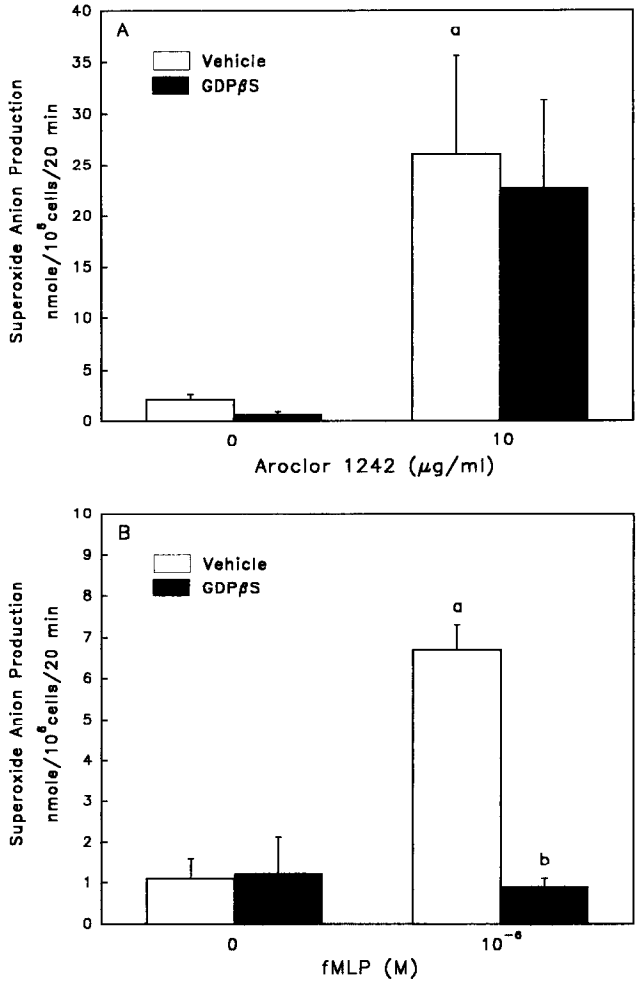

FIG. 4. Effect of GDPβS on O_2^- production by neutrophils. Neutrophils were permeabilized with digitonin as described in Materials and Methods and incubated at 37° for 2 min with 1 mM GDPβS or vehicle. O_2^- production was measured as described in the legend to Fig. 1 in neutrophils stimulated for 20 min with 10 μg/mL Aroclor 1242 (A), 1 μM fMLP (B), or the appropriate vehicle. Values are means ± SEM, N = 5. Key: (a) significantly different from the respective value obtained in the absence of stimulus; and (b) significantly different from the respective value obtained in the absence of GDPβS ($P \leq 0.05$).

Aroclor 1242 or 2,2',4,4'-TCB. A small increase in phosphorylation in the 40 kDa molecular mass range was seen in neutrophils exposed to 3,3',4,4',5-PeCB; however, this increase was not evident at 5 min (Fig. 7).

DISCUSSION

We have demonstrated previously that Aroclor 1242, a mixture of PCBs, stimulates neutrophils to produce O_2^- by activating phospholipases C and A_2, a mechanism that is similar to the process by which physiologic agonists like fMLP stimulate neutrophils [9, 10]. Since activation of phospholipases by fMLP involves GTP-binding proteins and PTKs, the present study was undertaken to examine the involvement of these cellular messengers in PCB-induced neutrophil activation.

Role of GTP-Binding Proteins in PCB-Induced Production of O_2^- by Neutrophils

Pertussis toxin ADP-ribosylates and inactivates G-proteins belonging to the G_i and G_o families, whereas cholera toxin modifies the activity of members of the G_i, G_s, and transducin families of G-proteins. As expected, fMLP-elicited O_2^- production was abolished in neutrophils pretreated with pertussis toxin (Fig. 1B), cholera toxin (Fig. 2B), or GDPβS (Fig. 4B), indicating efficacious treatment with these agents. These results with rat neutrophils are consistent with studies in human neutrophils and suggest that both pertussis toxin-sensitive and cholera toxin-sensitive G-proteins are involved in the mechanism of fMLP-induced generation of O_2^-.

Heterotrimeric GTP-binding proteins are involved at multiple sites in the signalling pathway in human neutrophils activated by fMLP [14, 27]. In human peripheral blood neutrophils, pertussis toxin-sensitive G-proteins link the receptor for fMLP to phospholipases C and A_2, enzymes that are important in fMLP-stimulated O_2^- production [31]. Although the site of action of pertussis toxin and cholera

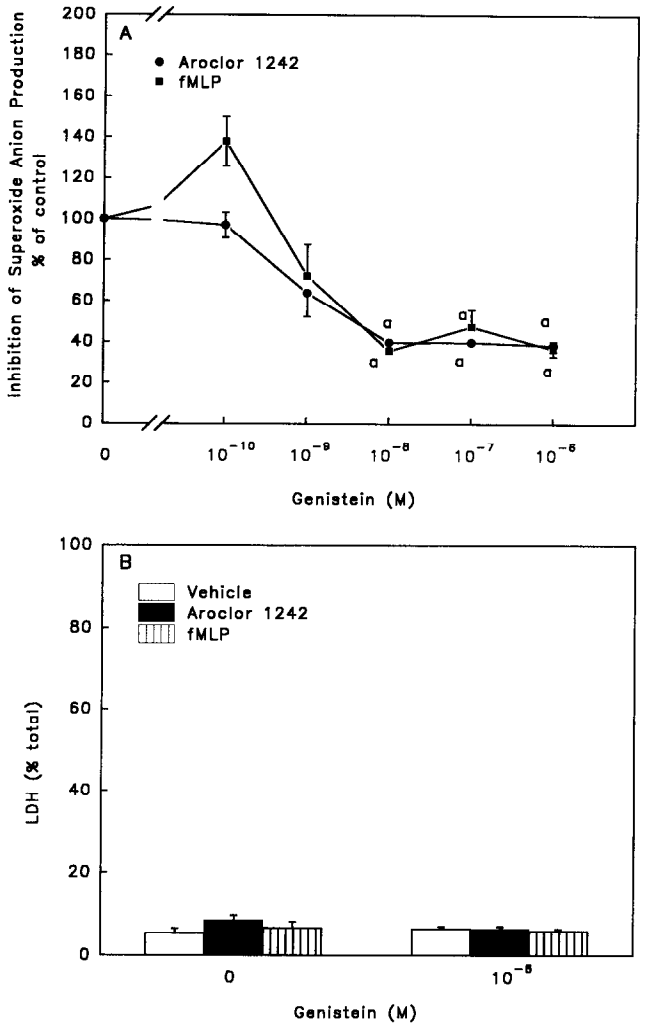

FIG. 5. Attenuation of O_2^- production and lack of cytotoxicity of genistein. Neutrophils were incubated with genistein for 15 min, and stimulated for 20 min with 10 μg/mL Aroclor 1242 or 1 μM fMLP. The production of O_2^- (A) and the release of LDH (B) were measured as described in Materials and Methods. Values are means ± SEM; (A) N = 7–8, and (B) N = 3. Panel A: (a) significantly different from the respective value obtained in the absence of genistein ($P \leq 0.05$). Neutrophils stimulated with Aroclor 1242 or fMLP produced 31.9 ± 6 or 29.2 ± 3.3 nmol O_2^-/20 min/10^6 cells, respectively. (B) there were no statistical differences among groups.

toxin was not addressed by the present study, these agents likely inhibit the production of O_2^- in rat neutrophils by a mechanism similar to the effect on human neutrophils, i.e. by uncoupling the fMLP receptor from intracellular enzymes such as phospholipases C and A_2.

In contrast to results in fMLP-activated neutrophils, neither pertussis toxin nor cholera toxin inhibited significantly the rate of generation of O_2^- or the maximal production of O_2^- in neutrophils exposed to Aroclor 1242. These results suggest that, although generation of O_2^- by PCBs requires phospholipases C and A_2 [9, 10], the mechanism is different from the activation by fMLP and does not involve heterotrimeric GTP-binding proteins. The observation that interference with G-protein function does not affect PCB-induced O_2^- production provides evidence that PCBs, unlike fMLP, do not activate phospholipases by interacting with G-protein-linked receptors on the cell surface or by directly stimulating activity of pertussis toxin- or cholera toxin-sensitive heterotrimeric G-proteins. Accordingly, an alternative mechanism must be invoked, such as direct stimulation of phospholipase activity or other signalling mechanisms that regulate phospholipase activity.

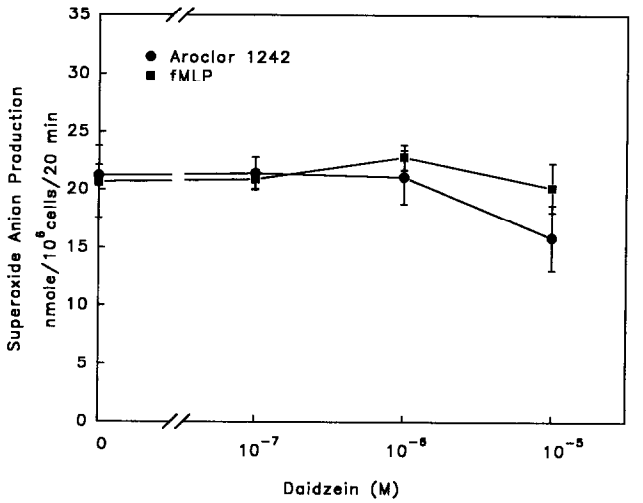

FIG. 6. Lack of inhibition of O_2^- by daidzein. Neutrophils were incubated at 37° with daidzein for 15 min and stimulated for 20 min with 10 μg/mL Aroclor 1242 or 1 μM fMLP. The production of O_2^- was measured as described in the legend to Fig. 1. Values are means ± SEM, N = 4. No statistical differences were observed.

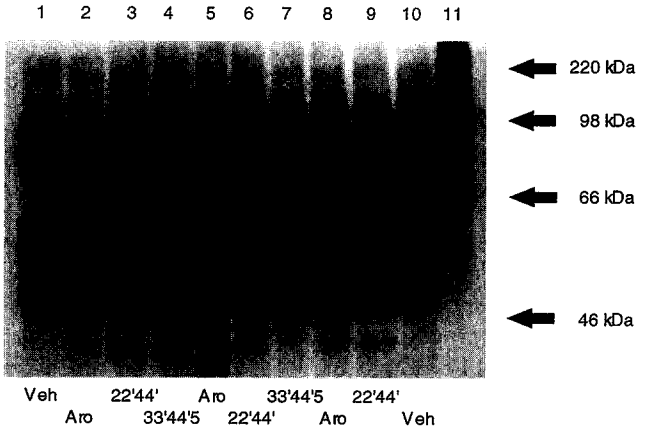

FIG. 7. Time-course of tyrosine phosphorylation in neutrophils stimulated with PCBs. Neutrophils (30 × 10^6/mL) were stimulated for 1 min (lanes 1–4), 5 min (lanes 5–7), or 10 min (lanes 8–10) with vehicle (Veh; lanes 1 and 10), 10 μg/mL Aroclor 1242 (Aro; lanes 2, 5, and 8), 10 μg/mL 2,2',4,4'-TCB (22'44'; lanes 3, 6, and 9) or 10 μg/mL 3,3',4,4',5-PeCB (33'44'5; lanes 4 and 7). Neutrophils were lysed and proteins were separated by SDS–PAGE, and bands corresponding to proteins containing phosphorylated tyrosine residues were visualized as described in Materials and Methods. The autoradiograph is representative of two separate experiments. Lane 11, molecular weight markers.

In addition to heterotrimeric G-proteins, neutrophils contain at least 25 low-molecular-weight G-proteins including Ras, Rac, and Rap1A [32–36]. These three G-proteins regulate effects such as activation of MAP kinase [36], exocytosis [37], and NADPH oxidase assembly and subsequent O_2^- production [33, 34]. Although Ras can be inhibited by pertussis toxin [36], the other low-molecular-weight G-proteins are not affected by either pertussis or cholera toxins [32, 38]. All GTP-binding proteins cycle between an inactive state to which GDP is bound and an active state to which GTP is bound. GDPβS is a nonhydrolyzable analogue of GDP which binds to G-proteins irreversibly and prevents subsequent binding of GTP, a process required for activation. Thus, studies with GDPβS allow the potential to examine effects mediated by G-proteins that are not sensitive to pertussis toxin or cholera toxin. Generation of O_2^- in the presence of Aroclor 1242 was unaffected by GDPβS. These results suggest that GTP-binding proteins, including those insensitive to pertussis or cholera toxins, are not involved in the mechanism of activation of neutrophils by PCBs; however, we cannot rule out the possibility that GTP-binding proteins involved in PCB-induced neutrophil activation are located in sites within the cell that were not reached by GDPβS under the conditions used. For example, both Rap 1 and Rap 2 are present within gelatinase-containing granules of neutrophils [39] and may be inaccessible to GDPβS because it does not permeate cell membranes. Thus, these data suggest that neither heterotrimeric nor low molecular weight G-proteins are involved in PCB-induced generation of O_2^- by rat neutrophils; however, a possible role for G-proteins inaccessible to GDPβS cannot be excluded.

Role of PTKs in PCB-Induced Generation of O_2^- by Neutrophils

Activation of PTKs is important in response to several agents that stimulate and/or prime neutrophils including fMLP [40], monosodium urate crystals [41, 42], lipopeptides [43], TNF-α [44], and GM-CSF [45]. The importance of PTKs in neutrophil activation is substantiated by the results of studies with PTK inhibitors that significantly attenuate neutrophil responses such as O_2^- production [46, 47], degranulation [48], chemotaxis and adherence [49]. The results presented here suggest that tyrosine kinase activity is involved in activation of neutrophils by Aroclor 1242 as well. An increase in protein tyrosine phosphorylation was seen within 1 min after exposure to Aroclor 1242 (Fig. 7), whereas the onset of production of O_2^- was not seen until after 5 min of exposure to PCBs (Fig. 3), indicating that tyrosine kinase activation precedes the production of O_2^-. In addition, exposure to PCBs resulted in increased tyrosine phosphorylation of proteins in the 40 kDa molecular mass range, a change that has been linked causally to the production of O_2^- in human neutrophils exposed to chemoattractants [50–53]. Stimulation of tyrosine phosphorylation by PCB congeners correlated well with their ability to stimulate O_2^- production. 2,2′,4,4′-TCB, a PCB congener that causes O_2^- production [8–10], stimulated a marked and prolonged increase in tyrosine phosphorylation of both 40 and 60 kDa proteins; however, 3,3′,4,4′,5-PeCB, which does not stimulate the production of O_2^- [10], caused only a slight and transient increase in tyrosine phosphorylation of proteins in the 40 kDa range, with no change in phosphorylation of proteins in the 60 kDa range. Finally, inhibition of PTK activity with genistein resulted in significant attenuation of O_2^- generation in PCB-treated neutrophils. The action of genistein to inhibit phosphorylation of tyrosine residues has been demonstrated repeatedly in numerous systems including neutrophils [50, 54–57] and monocytes [58]. In neutrophils, fMLP-stimulated O_2^- production and tyrosine phosphorylation were inhibited by concentrations of genistein in the range of those used in this study [50]. Inhibition of tyrosine phosphorylation was most evident for proteins in the 40 kDa range, the range in which protein phosphorylation was observed upon exposure to Aroclor 1242. This observation is consistent with our results and supports the interpretation that Aroclor 1242 activates neutrophils to produce O_2^- by a mechanism that involves activity of PTKs.

The specific proteins phosphorylated on tyrosine residues in PCB-treated cells were not identified so it is not possible to discern which proteins are important in the mechanism by which PCBs stimulate neutrophils. The tyrosine kinase substrates present in the 40 kDa molecular mass range are likely to be MAP kinases, a family of serine/threonine protein kinases with molecular masses ranging from 40 to 45 kDa that are regulated by both tyrosine and threonine phosphorylation [51, 53, 59]. This possibility is supported by previous studies with human neutrophils, which have shown that the mechanism of O_2^- production in response to fMLP involves tyrosine phosphorylation and subsequent activation of MAP kinases [52]. The results of our study suggest that activation of this pathway, although necessary, is not sufficient in the absence of other signalling mechanisms to activate O_2^- production. This conclusion is based on results with 3,3′,4,4′,5-PeCB, which increased tyrosine phosphorylation of proteins in the 40 kDa range, but did not elicit the production of O_2^- [10]. Similar results have been obtained with GM-CSF, a hematopoietic growth factor that causes rapid phosphorylation on tyrosine residues of several proteins, including MAP kinase. GM-CSF potentiated the production of O_2^- in response to other agents, but did not elicit the generation of O_2^- when added alone to neutrophils [53].

Another interpretation of the profile of tyrosine phosphorylation depicted in Fig. 7 is that phosphoproteins in the 60 kDa range play an important role in O_2^- production by rat neutrophils. Aroclor 1242 and 2,2′,4,4′-TCB, which cause the production of O_2^-, increased phosphorylation on tyrosine residues of proteins in this mass range, whereas 3,3′,4,4′,5-PeCB, which does not cause O_2^- generation, did not. Phosphorylation on tyrosine residues of a protein with a molecular mass of 62 kDa was seen in rabbit peritoneal

neutrophils stimulated with fMLP [40]. This protein belongs to the src family of tyrosine kinases, and it was suggested that this protein is important in activation of NADPH oxidase activity, which leads to generation of O_2^- [40]. A similar mechanism may be in effect in PCB-stimulated neutrophils.

The mechanisms of activation of PTKs are complex and poorly understood. Neutrophils contain both particulate and soluble PTKs that can be activated by several pathways that may include receptor autophosphorylation, G-protein activation, FcγRI receptor cross-linking [60], β_2 integrin-dependent adhesion [44, 61], or calcium-dependent activation [42]. In addition, recent studies suggest that activation of the cytosolic Ah receptor in the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is associated with an acute increase (within 10 min) in PTK activity in guinea pig adipose tissue [62]. Any or all of these mechanisms may be involved in activation of PTKs by PCBs. The involvement of calcium in PCB-induced activation of PTKs seems likely since PCBs cause an increase in IP_3 formation within 5 sec in neutrophils [9] and also stimulate the accumulation of intracellular calcium in neural cells [63]. Furthermore, inhibition of intracellular calcium mobilization depresses activation of neutrophils by PCBs [11]. Since neutrophils possess a cytosolic Ah receptor [8], PCB binding to this receptor may be involved in PCB-induced activation of PTKs. Ah receptor binding, however, may be less important in activation of PTKs than other mechanisms in view of the fact that 2,2′,4,4′-TCB, which binds the Ah receptor with much less affinity than 3,3′,4,4′,5-PeCB, was a stronger activator of tyrosine phosphorylation. It is impossible to exclude the possibility that PCBs increase tyrosine phosphorylation by directly interacting with PTKs or by modulating the activity of other proteins that affect tyrosine phosphorylation. For example, PCBs may exert a negative influence on the activity of phosphotyrosine phosphatases within the cell. Further experiments are necessary to determine the mechanism(s) by which PCBs increase tyrosine phosphorylation in neutrophils.

In summary, the results of this study are consistent with the hypothesis that PCBs activate neutrophils by a mechanism that involves tyrosine kinase activity; however, heterotrimeric G-proteins are not likely to be involved. Stimulation of PTK activity by PCBs may be important in the mechanism of toxicity of these compounds. Enhanced activity of tyrosine kinases has been implicated in proliferative diseases including many types of cancer [64], as well as in nonmalignant proliferative diseases such as atherosclerosis [65] and psoriasis [66]. Therefore, it is interesting to speculate that activation of PTKs in cells exposed to PCBs may be important in the mechanism by which these compounds exert some of their toxic effects.

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