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Participation of Ca^{2+}/calmodulin during activation of rat neutrophils by polychlorinated biphenyls

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Abstract

The effects of Ca^{2+} and Ca^{2+}/calmodulin on the polychlorinated biphenyl (PCB)-induced activation of phospholipase A_2 (PLA_2) in rat neutrophils were examined. The commercial PCB mixture Aroclor 1242 induced activation of PLA_2 and promoted an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$). Bromoenol lactone (BEL), an inhibitor of the Ca^{2+}-independent PLA_2 isoform ($iPLA_2$) activated by PCBs, did not abrogate the increase in $[Ca^{2+}]_i$, suggesting that this change in Ca^{2+} concentration is not downstream from the activation of $iPLA_2$. TMB-8 [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate], a blocker of the release of intracellular Ca^{2+}, decreased Aroclor 1242-induced stimulation of PLA_2 with a maximal inhibition of 17% at 50 μM. These two results suggest little direct dependence between the PCB-induced activation of $iPLA_2$ and increase in $[Ca^{2+}]_i$. Calmidazolium and W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], two chemically distinct calmodulin inhibitors, inhibited Aroclor 1242-induced PLA_2 activity, whereas trifluoperazine (TFP), another inhibitor of calmodulin, had no effect at noncytotoxic concentrations. Thus, activation of PLA_2 is dependent, in part, on calmodulin. Furthermore, both TFP and Aroclor 1242 inhibited neutrophil degranulation stimulated by the bacterial peptide formyl-methionyl-leucyl-phenylalanine. These results raise the possibility that some of the effects of PCBs on neutrophil function can be explained by effects on Ca^{2+}/calmodulin-dependent processes.

Keywords: Arachidonic acid; Phospholipase A_2; Trifluoperazine; Polychlorinated biphenyls; Calmodulin

1. Introduction

The chemical stability of PCBs makes this group of organochlorine compounds one of the most ubiquitous and persistent chemicals in the environment. These chemicals have been detected around the world in remote areas [1], wildlife refuges [2], dairy products [3], and human breast milk [4,5]. In environmental matrices, PCBs are found as complex mixtures resembling those found in commercial mixtures known as Aroclors that, in general, are termed according to their percentage of chlorine content by weight [6]. For example, Aroclors 1221, 1242, and 1248 have 21, 42, and 48% chlorine content by weight, respectively. The multiple cellular mechanisms of action, widespread environmental distribution, physicochemical properties, and differences in activity among congeners have made the toxicological profile of PCBs both extensive and complex. The physiological effects of PCBs in mammals include behavioral impairment [7], stimulation of oscillatory uterine contraction [8], changes in immunoglobulins M and G [9], and enzyme induction [10]. Some evidence has suggested a relationship between PCB exposure and effects on motor functioning [11], neurotoxicity [12], cancer [13], and cancer-derived mortality [14].

The mechanisms of action of PCBs are multiple and structure-dependent. Many of the PCBs can bind with relatively high affinity to the Ah receptor and elicit toxic responses similar to those observed for TCDD. Another group of PCBs has low affinity for the Ah receptor and exhibits different biological activities such as neurotoxicity

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Abbreviations: PCB, polychlorinated biphenyl; PLA_2, phospholipase A_2; $iPLA_2$, calcium-independent PLA_2; BEL, (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; TFP, trifluoperazine; Ah, aryl hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; fMLP, formyl-methionyl-leucyl-phenylalanine; TKs, tyrosine kinases; PLC, phospholipase C; AA, arachidonic acid; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide CMDZ, calmidazolium, HBSS, Hanks' balanced salt solution; DMF, *N*,*N*-dimethylformamide; and MPO, myeloperoxidase.

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[15,16], stimulation of insulin release [17], and effects on neutrophil function [18,19]. With respect to neutrophils, PCBs activate these cells and alter their response to other stimuli. For example, phorbol ester-stimulated superoxide anion production is increased in cells treated with PCBs [20]. On the other hand, neutrophil degranulation induced by the peptide fMLP is inhibited by PCBs [20].

In addition to alterations in cellular function, several intracellular signals are activated by PCBs in neutrophils including PLA_2, TKs, and PLC [21–23]. PLA_2 hydrolyzes phospholipids at the *sn*-2 position to release the second messenger AA. This enzyme regulates neutrophil function by modulating superoxide anion production [24,25] and degranulation [26]. Indeed, inhibition of PLA_2 prevents PCB-induced stimulation of these cells [22]. In neutrophils, both Ca^{2+}-dependent and -independent isoforms of PLA_2 have been identified, and most of the PCB-induced activity is attributed to activation of $iPLA_2$ [22]. The biochemical mechanisms underlying the regulation of $iPLA_2$ are not known. It has been shown that $iPLA_2$ can be regulated by ATP [27,28], nitric oxide [29], and peptides such as [Arg^8]vasopressin [30] and parathyroid hormone [31]. An intriguing observation is that $iPLA_2$ can be modulated by direct protein–protein interactions with biomolecules such as phosphofructokinase [32] and calmodulin [33].

Given that $iPLA_2$ plays an important role in neutrophil stimulation by PCBs, it was of interest to determine whether activation of this enzyme depends on other intracellular signals. The objective of this study was to examine the interaction among several signaling pathways involved in activation of neutrophils by PCBs. In particular, the roles of intracellular calcium and calmodulin in the activation of PLA_2 were explored.

2. Materials and methods

2.1. Chemicals

Aroclor 1242 (Lot No. 187–56B; 99% pure) was purchased from ChemService; BEL was purchased from Biomol. [^{3}H-5,6,8,9,11,12,14,15]AA ([^{3}H]AA; 180–240 Ci/mmol; radiochemical purity $>$ 98%) was purchased from DuPont NEN. Cytochalasin B, fMLP, TFP, TMB-8, W7, and CMDZ were obtained from the Sigma Chemical Co. Fura 2/AM was obtained from Molecular Probes.

2.2. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague–Dawley, retired breeder rats by glycogen elicitation as described [34]. Isolated neutrophils were resuspended in HBSS, pH 7.35, containing 1.6 mM $CaCl_2$. The percentage of neutrophils in the cell preparations was $>$95%, and the viability was $>$ 95% determined by the ability to exclude trypan blue. The isolation procedure was performed at room temperature.

2.3. Exposure to PCBs

The PCB mixture Aroclor 1242 was used to study the effects of PCBs in the activation of rat neutrophils because in environmental samples PCB congeners are found in complex mixtures similar to those present in Aroclor 1242. In addition, toxicological information from these mixtures has been proven to be effective in risk assessment processes [35]. Aroclor 1242 stock solutions were prepared by dissolution in DMF. Neutrophils were suspended in HBSS in borosilicate glass test tubes, 12 $\times$ 75 mm (VWR), and 1 μL of a stock solution of Aroclor 1242 was added to the cells to achieve the desired concentration. Control neutrophils received 1 μL DMF.

2.4. Determination of PLA_2 activity

Neutrophils (10^7/mL) were suspended in Mg^{2+}- and Ca^{2+}-free HBSS containing 0.1% bovine serum albumin and incubated in the presence of 0.5 μCi/mL of [^{3}H]AA for 2 hr, gently shaking at 37°. Neutrophils were then washed twice with Mg^{2+}- and Ca^{2+}-free HBSS and resuspended in HBSS containing Mg^{2+}, Ca^{2+}, and bovine serum albumin (0.1%). The cell count was adjusted so that the final concentration of neutrophils was 2 $\times$ 10^6/mL. Total cellular uptake of [^{3}H]AA was measured in a 1-mL aliquot of suspended cells: the incorporation of [^{3}H]AA was between 80 and 88%. Release of [^{3}H]AA from labeled neutrophils was measured in cells incubated with inhibitors for 20 min and then exposed to Aroclor 1242 at 37° for 30 min. At the end of each incubation, neutrophils were placed on ice and centrifuged at 4000 *g* at 0° for 10 min. The cell-free supernatant fluids were transferred to vials containing scintillation fluid (14 mL), and the total radioactivity in each sample was determined by liquid scintillation counting. Control neutrophils (DMF) released about 3–5% of the total incorporated radioactivity.

2.5. Neutrophil degranulation

Degranulation was measured by the release from neutrophils of the enzyme MPO. Neutrophils (2 $\times$ 10^6) were suspended in HBSS and pretreated with 5 μg/mL of cytochalasin B for 5 min at room temperature. Then the quiescent neutrophils were exposed to PCBs or vehicle for 10 min at 37° followed by incubation for an additional 10 min at 37° with 100 nM fMLP. Cells were centrifuged at 4000 *g* at 4° for 10 min, and the cell-free supernatant fluids were collected. Activity of MPO in the medium was determined using the method of Henson *et al.* [36]. Total cellular MPO activity was determined in 2 $\times$ 10^6 neutrophils lysed with Triton X-100 and sonicated. Degranulation was ex-

pressed as a percent of total cellular MPO released into the medium.

2.6. Intracellular free CA^{2+} measurements

Cells were incubated with 2 μM Fura-2/AM for 20 min at 37° in HBSS and then washed with HBSS. Neutrophils (2.5×10^6/mL) were loaded in a spectrofluorometer cell, and data collection was started 50 sec before adding the PCBs or TFP. Experiments with BEL were done after Fura-2/AM labeling by incubating the cells with the inhibitor for 20 min at 37°. Fluorescence emission at 505 nm was monitored at room temperature using a dual wavelength spectrofluorometer system with excitation at 340 and 380 nm. An increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) was detected by a change in the fluorescence ratio 340/380 [37]. Maximal fluorescence was obtained by adding 0.01% Triton X-100 to the cells, whereas the minimal fluorescence ratio was determined after adding 30 μL EGTA (10 mM). These two parameters did not change significantly in the presence of Aroclor 1242. Fluorescence at 360 nm was monitored and remained constant during data collection.

2.7. Statistical methods

Generated PLA_2 data consisted mostly of percentages, and for this reason an angular transformation (arcsin) was used to generate an approximated gaussian distribution of data. Detection of significant differences among groups was determined using ANOVA and the Tukey test as a post-hoc test. When angular transformation did not produce gaussian data, Kruskal Wallace ANOVA on Ranks was used. The IC_{50} values were calculated by using non-linear regression with sigmoidal modeling (SigmaPlot, V4). Data are presented as the means ± SEM from at least four different experiments conducted in triplicate. Two-tailed P values <0.05 were considered significant.

3. Results

3.1. Involvement of Ca^{2+}/calmodulin in PCB-induced activation of PLA_2

It has been reported previously that PCB-induced activation of PLA_2 is independent of extracellular calcium [22]. To investigate whether intracellular calcium is important for Aroclor 1242-induced PLA_2 activation, neutrophils were pretreated with the intracellular calcium release blocker TMB-8 [38]. TMB-8 (25–50 μM) produced a small but significant decrease in Aroclor 1242-elicited release of [^{3}H]AA (Table 1). This suggests that a small fraction of the total PLA_2 activity induced by Aroclor 1242 depends on the release of Ca^{2+} from intracellular stores.

Exposure of neutrophils to Aroclor 1242 caused a time-dependent increase in $[Ca^{2+}]_i$ (Fig. 1). This increase began after about 5 min of exposure to PCBs and continued through 18 min. This response was quite different from that observed for fMLP, a neutrophil stimulus for which the rise of $[Ca^{2+}]_i$ is known to be critical [39]. To determine if this increase in $[Ca^{2+}]_i$ was caused by the activation of PLA_2 by Aroclor 1242, calcium measurements were made in cells preincubated with the cell permeable PLA_2 inhibitor BEL (10 μM). Treatment with BEL did not abrogate the overall increase in $[Ca^{2+}]_i$ elicited by 10 μg/mL of Aroclor 1242 measured as area under the curve ($189 \pm 8 \times 10^3$ nM·sec and $164 \pm 6 \times 10^3$ nM·sec in the absence and presence of

Table 1
TMB-8-induced inhibition of Aroclor 1242-stimulated release of [^{3}H]AA from rat neutrophils

TMB-8 (μM)	Release of [^{3}H]AA from rat neutrophils (% of control)
0	100
1	98.0 ± 1.8
10	96.9 ± 3.4
25	87.1 ± 3.6*
50	83.3 ± 4.0*

Cells were preincubated with TMB-8 and then exposed to 10 μg/mL of Aroclor 1242. Release of [^{3}H]AA was determined as described in "Materials and methods." Release of [^{3}H]AA was about 5–10% in cells exposed to vehicle (DMF) alone and about 4–11% in cells treated with TMB-8 in the absence of Aroclor 1242. Exposure to Aroclor 1242 alone caused release of 25–35% (14,960 ± 4815 cpm) of the total incorporated radioactivity. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

* Significantly different ($P < 0.05$) from the control group (100%) when analyzed by ANOVA using the Tukey test as a post-hoc test.

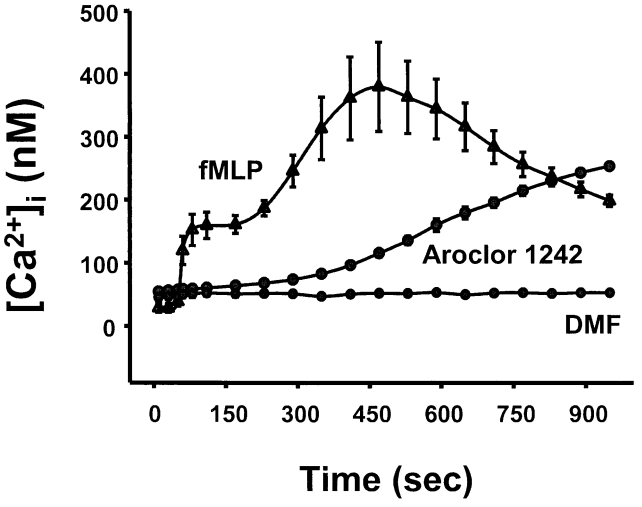

Fig. 1. Changes in intracellular free calcium ($[Ca^{2+}]_i$) in neutrophils exposed to Aroclor 1242. Cells were loaded with Fura-2/AM as described in "Materials and methods." Aroclor 1242 (10 μg/mL) was added to Fura-2-loaded neutrophils at 50 sec. Data for vehicle control (DMF) and the agonist fMLP are shown for comparison. Results are expressed as means ± SEM for four different experiments.

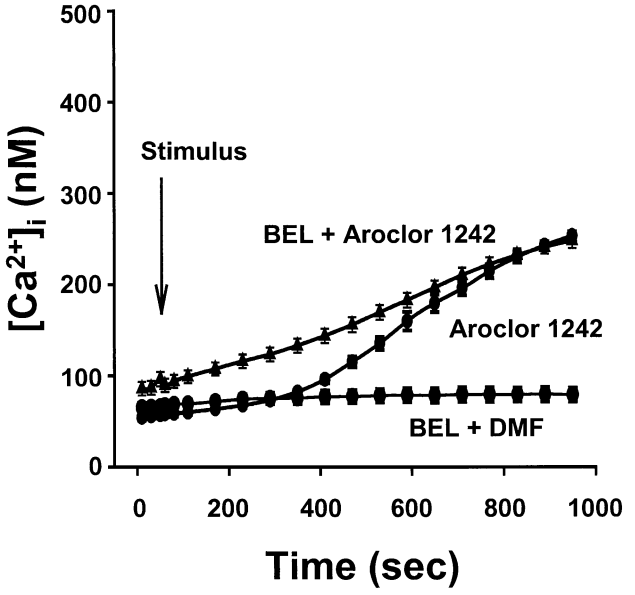

Fig. 2. Effects of BEL on the Aroclor 1242-induced increase of $[Ca^{2+}]_i$ in rat neutrophils. Cells were loaded with Fura-2/AM as described in "Materials and methods." Fura-2-loaded neutrophils were incubated with 10 μM BEL for 20 min, and Aroclor 1242 (10 μg/mL) was added at the time indicated by the arrow. Data for control (BEL + DMF) and Aroclor 1242 alone are shown for comparison. Results are expressed as means ± SEM for four different experiments.

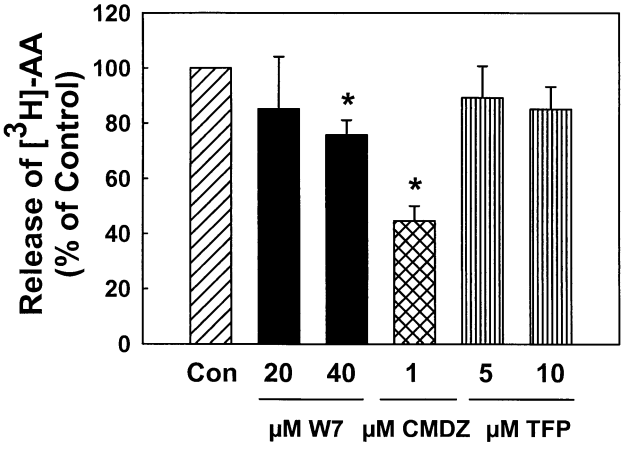

Fig. 3. Effects of calmodulin inhibitors on PLA_2 activity induced by Aroclor 1242. Neutrophils were labeled with [^{3}H]AA and incubated with different concentrations of the calmodulin inhibitors W7, CMDZ, and TFP for 20 min at 37°, followed by incubation with 10 μg/mL of Aroclor 1242 for 30 min at 37°. The release of [^{3}H]AA into the medium at each concentration of inhibitor was compared with the release by Aroclor 1242 alone (Con) and presented as a percentage of activity. Release of [^{3}H]AA in the presence of Aroclor 1242 alone was 25–35% (11,570 ± 1410 cpm) of the total radioactivity. Results are expressed as means ± SEM from four different experiments performed in triplicate. Key: (*) significantly different from control ($P < 0.05$).

BEL, respectively; no significant difference) but produced a variation in the kinetics of the release over a period of 18 min (Fig. 2).

Calmodulin is the main Ca^{2+} regulatory protein in eukaryotic cells. To examine its role in Aroclor 1242-induced PLA_2 activity, the effects of the calmodulin inhibitors TFP, W7, and CMDZ were examined. At noncytotoxic concentrations (lactate dehydrogenase release < 20%; not significantly different from vehicle control), both W7 and CMDZ induced a significant blockade of Aroclor 1242-induced PLA_2 activity (Fig. 3). On the other hand, noncytotoxic concentrations of TFP did not reduce Aroclor 1242-stimulated release of [^{3}H]-AA.

3.2. Similarity in action of TFP and Aroclor 1242 on neutrophil function

Aroclor 1242 induced a concentration-dependent inhibition of fMLP-induced neutrophil degranulation (Fig. 4A), consistent with a previous report [18]. The effect of TFP on neutrophil degranulation was also examined. Like Aroclor 1242, TFP almost completely blocked degranulation in response to fMLP (Fig. 4B).

4. Discussion

PCBs cause an increase in $[Ca^{2+}]_i$ in cerebellar granule cells [40], hepatocytes [41], endothelial cells [42], and human neutrophils [43]. The mechanisms associated with this increase in intracellular free calcium are unknown. In rat neutrophils, omission of extracellular Ca^{2+} or inhibition of intracellular Ca^{2+} reduced superoxide anion production and degranulation in response to PCBs, suggesting an important consequence to the increase in $[Ca^{2+}]_i$ [19]. We have demonstrated previously that Aroclor 1242 increased PLA_2 activity with release of AA and that this event, although necessary for superoxide anion production, did not require extracellular calcium [22]. Similarly, Aroclor 1242 induced an increase in $[Ca^{2+}]_i$ in rat neutrophils (Fig. 1). One question addressed in this study is whether there is a causal relationship between activation of PLA_2 and the increase in $[Ca^{2+}]_i$. For example, it is known that arachidonate [44] or its metabolites [45] can cause depletion of intracellular calcium stores, leading to a rise in $[Ca^{2+}]_i$. Following exposure of neutrophils to Aroclor 1242, AA was released within 2 min [22], whereas the increase in $[Ca^{2+}]_i$ began after 5 min (Fig. 1). Given this, it was hypothesized that inhibition of PLA_2 would block the Aroclor 1242-induced increase in $[Ca^{2+}]_i$. This was not the case (Fig. 2). We have demonstrated previously that BEL abrogated Aroclor 1242-stimulated release of AA [22]. The lack of effect of BEL on the overall increase in $[Ca^{2+}]_i$ elicited by Aroclor 1242 led us to conclude that PCBs act on $[Ca^{2+}]_i$ through a signal that is not downstream from activation of $iPLA_2$; however, $iPLA_2$ can modulate the kinetics of the process. This dissociation between $iPLA_2$ and Ca^{2+} has also been observed in aortic smooth muscle cells in which [Arg^8]vasopressin, an activator of $iPLA_2$, elicited a spike in $[Ca^{2+}]_i$ [30]. In these cells, BEL did not abolish this rise in $[Ca^{2+}]_i$ when

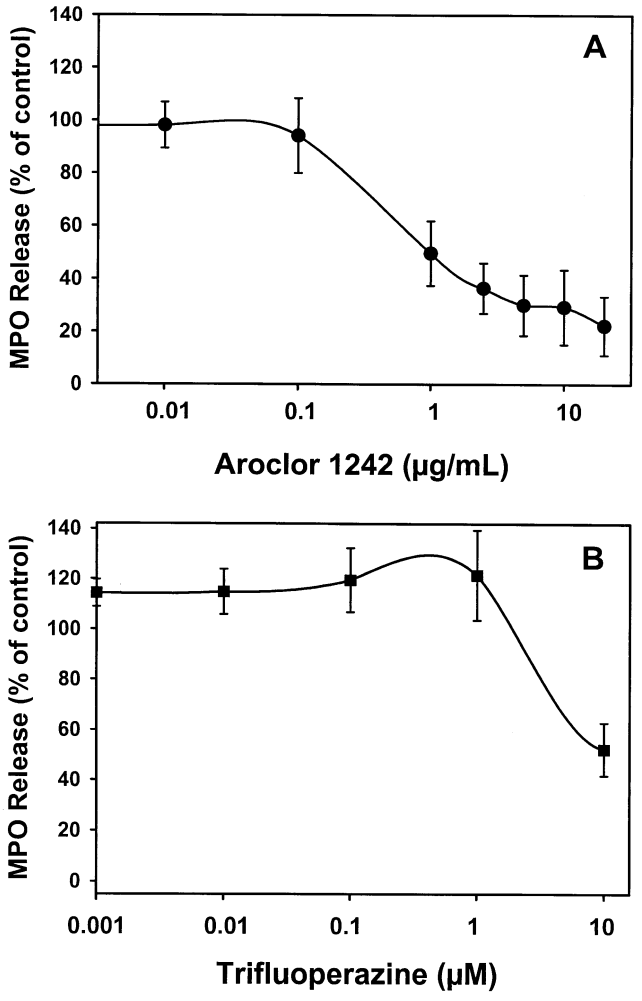

Fig. 4. Inhibitory effect of (A) Aroclor 1242 and (B) TFP on fMLP-induced neutrophil degranulation. Cytochalasin B-treated neutrophils were exposed to Aroclor 1242 or TFP at the indicated concentrations for 10 min and then were incubated for another 10 min with 100 nM fMLP. Degranulation was measured as described in "Materials and methods." Data are expressed as a percentage of the release of MPO in the presence of 100 nM fMLP alone (i.e. control) for each individual experiment. For all experiments, the average release of MPO in the presence of fMLP alone was 58 ± 12% of total cellular MPO activity. Data are presented as means ± SEM of four different experiments.

experiments were performed in the absence of extracellular Ca^{2+} but prolonged the duration of the response in the presence of extracellular Ca^{2+} [30].

It has been reported that the majority of AA released from neutrophils after PCB treatment comes from stimulation of $iPLA_2$; however, the partial reduction by TMB-8 of Aroclor 1242-induced release of AA suggests that release of Ca^{2+} from intracellular stores contributes to a small fraction of the PCB-stimulated PLA_2 activity. Previously, it was reported that PCB-induced activation of PLA_2 was insensitive to the intracellular calcium chelator BAPTA [1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) [22]. The TMB-8 data reported here are largely in agreement with this finding, although the small inhibition observed for this compound suggests differences in the mechanisms of intracellular calcium sequestration by TMB-8 and BAPTA. In comparison to BAPTA, the effects of TMB-8 on intracellular Ca^{2+} can be considered more local than global. It has been suggested that TMB-8 causes its effects on lysosomal or other intracellular Ca^{2+} pools, and on intracellular calcium pools that are associated with cytoskeletal elements and anchoring Ca^{2+} to its membranous storage sites [46, 47]. Neutrophils have at least two distinct Ca^{2+} storage and release sites, one located peripherally under the plasma membrane and the other deep and close to the juxtanuclear space. The latter pool is sensitive to fMLP [48,49], and TMB-8 decreases fMLP-induced responses [46], suggesting that this pool may be inhibited by TMB-8.

Despite the observations that the PCB-induced increase in $[Ca^{2+}]_i$ is independent of PLA_2 activity (Fig. 2) and that the stimulation of PLA_2 by PCBs is largely independent of calcium (Table 1), there appears to be some interaction between calcium signaling and release of [^{3}H]AA. This interpretation is based on the known relationship between calcium and calmodulin and the results of studies with the calmodulin inhibitors W7 and CMDZ in which these compounds decreased PCB-stimulated release of AA (Fig. 3). A third calmodulin inhibitor, TFP, did not affect PCB-induced activation of $iPLA_2$. This lack of effect could be related to the concentrations of TFP used: larger concentrations produced cytotoxicity and therefore were not used.

One explanation for the effects of W7 and CMDZ on the release of [^{3}H]AA is that Ca^{2+}/calmodulin regulates the $iPLA_2$ activated by PCBs in neutrophils. Activity of some enzymes is inhibited by calmodulin. For instance, in Limulus, Ca^{2+}/calmodulin-binding peptides and calmodulin itself block PLC activity, probably through a calmodulin-like structure present in the amino-terminal domain of PLC [50]. W7 binds tightly to calmodulin in solution [51,52]. W7 or CMDZ binding to calmodulin causes a change in tertiary structure from an elongated dumbbell with exposed hydrophobic surfaces to a compact globular form that can no longer interact with its target enzymes. Regulation of neutrophil $iPLA_2$ might be similar to that observed in myocytes, in which $iPLA_2$ forms a complex with calmodulin, and this binding inhibits activity [33]. If this model of enzyme regulation by calmodulin applies to neutrophil $iPLA_2$, then the interaction between PLA_2 and PCBs may require the presence of calmodulin. Prior treatment with W7 or CMDZ may alter the conformation of calmodulin bound to $iPLA_2$ such that the enzyme binding site for the PCBs is inaccessible but at the same time allowing calmodulin to remain bound to $iPLA_2$, thus maintaining inhibition of enzyme activity. Alternatively, PCBs may induce PLA_2 activity through a different mechanism, but inhibition of calmodulin by CMDZ or W7 interferes with this mechanism indirectly. For instance, a fraction of Aroclor 1242-induced activation of PLA_2 is dependent upon protein phosphorylation pathways

[53] that may include Ca^{2+}/calmodulin-dependent protein kinases as intracellular mediators [54].

Using a molecular modeling program, the three-dimensional structure of TFP can be superimposed on an *ortho*-substituted PCB, 2,2′,4,4′-tetrachlorobiphenyl, suggesting some structural similarity. Aroclor 1242 contains many lower chlorinated PCB congeners, like 2,2′,4,4′-tetrachlorobiphenyl, and inhibits neutrophil degranulation [20]. Accordingly, the effect of TFP on neutrophil degranulation was examined. It is interesting that both the calmodulin inhibitor TFP and Aroclor 1242 inhibited fMLP-induced neutrophil degranulation (Fig. 4). The concentration–response curve for TFP-elicited inhibition of fMLP-induced neutrophil degranulation measured as MPO release was similar to that reported for release of the enzyme β-glucuronidase [55], another marker of neutrophil degranulation. A noncytotoxic concentration of TFP (10 μM) inhibited approximately 50% of fMLP-induced neutrophil degranulation. This concentration of TFP is equivalent to that reported for TFP inhibition of calmodulin activity [56]. The role of calmodulin in neutrophil degranulation is extensive. It binds tightly to vacuoles during the post-docking phase of vacuole fusion, promoting bilayer mixing [57], and it mediates membrane fusion, an event essential for degranulation, by allowing the phosphorylation of trafficking proteins such as syntaxin 3 by protein kinase II [58]. Therefore, it is not unexpected that inhibition of calmodulin activity interferes with degranulation.

Although inhibition of neutrophil degranulation has been observed with other, chemically unrelated calmodulin and Ca^{2+}/calmodulin-coupling inhibitors [59,60], data presented here should be interpreted with care. The concentrations of TFP that inhibited neutrophil degranulation were not effective in reducing $iPLA_2$ activity, although the other calmodulin inhibitors were effective. Therefore, alternative mechanisms unrelated to calmodulin should be considered for inhibition of degranulation by TFP. For instance, TFP inhibits annexin I- and annexin II-mediated aggregation of liposomes by releasing them from the plasma membrane [61] and blocks annexin II tetramer-mediated membrane fusion [62]. Although calmodulin could participate in these processes, calmodulin inhibitors have other, non-calmodulin effects such as direct calcium channel blocking [63], alteration in microfilament function [64], and changes in membrane fluidity [65] that could contribute to inhibition of degranulation. The mechanisms by which PCBs inhibit degranulation are also unclear; however, some properties attributed to TFP, such as alterations in membrane fluidity [66] and inhibition of calcium influx induced by fMLP [67], have also been observed for PCBs. To date, no information is available regarding the interaction between PCBs and calmodulin. It has been proposed that one essential feature for optimal ligand binding to calmodulin is the presence of two hydrophobic, aromatic rings, such as are found in PCBs [56], which may suggest that calmodulin might be one relevant target that mediates cellular responses elicited by PCBs.

How is it that calmodulin inhibitors both mimic and inhibit the effects of Aroclor 1242 in neutrophils? Both effects may arise from an ability of Aroclor 1242 and the inhibitors to bind to calmodulin. For example, binding of Aroclor 1242 to calmodulin may alter the conformation of the protein, as described above for W7, leading to disinhibition of PLA_2 and release of AA. For calmodulin inhibitors, the change in conformation prevents Aroclor 1242 from binding to calmodulin and activating PLA_2. With respect to degranulation, binding of either TFP or Aroclor 1242 may cause a change in conformation that inhibits the degranulation response, perhaps through one of the mechanisms described in the previous paragraph.

In summary, the PCB mixture Aroclor 1242 activates neutrophils through different signal transduction pathways involving Ca^{2+}/calmodulin-dependent processes. The calmodulin inhibitor TFP mimics one of the effects of Aroclor 1242 on neutrophil function. Aroclor 1242-induced release of AA via $iPLA_2$ is not coupled to the increase in $[Ca^{2+}]_i$ elicited by Aroclor. The above findings add a new aspect toward the recognition of cellular targets for PCBs.

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References

[1] Fromberg A, Cleemann M, Carlsen L. Review on persistent organic pollutants in the environment of Greenland and Faroe Islands. Chemosphere 1999;38:3075–93.
[2] Guillette LJ Jr, Brock JW, Rooney AA, Woodward AR. Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch Environ Contam Toxicol 1999;36:447–55.
[3] Ramos L, Eljarrat E, Hernandez LM, Rivera J, Gonzalez MJ. Levels of PCBs, PCDDs and PCDFs in commercial butter samples in Spain. Chemosphere 1999;38:3141–53.
[4] Czaja K, Ludwicki JK, Goralczyk K, Strucinski P. Effect of changes in excretion of persistent organochlorine compounds with human breast milk on related exposure of breast-fed infants. Arch Environ Contam Toxicol 1999;36:498–503.
[5] Greizerstein HB, Stinson C, Mendola P, Buck GM, Kostyniak PJ, Vena JE. Comparison of PCB congeners and pesticide levels between serum and milk from lactating women. Environ Res 1999;80:280–6.
[6] Waid J. PCBs and the environment. Boca Raton: CRC Press 1987.
[7] Rice DC. Behavioral impairment produced by low-level postnatal PCB exposure in monkeys. Environ Res 1999;80:S113–21.
[8] Bae J, Peters-Golden M, Loch-Caruso R. Stimulation of pregnant rat uterine contraction by the polychlorinated biphenyl (PCB) mixture Aroclor 1242 may be mediated by arachidonic acid release through

activation of phospholipase A_2 enzymes. J Pharmacol Exp Ther 1999;289:1112–20.
[9] Arnold DL, Bryce F, Mes J, Tryphonas H, Hayward S, Malcolm S. Toxicological consequences of feeding PCB congeners to infant rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. Food Chem Toxicol 1999;37:153–67.
[10] Lagueux J, Pereg D, Ayotte P, Dewailly E, Poirier GG. Cytochrome P450 CYP1A1 enzyme activity, and DNA adducts in placenta of women environmentally exposed to organochlorines, Environ Res 1999;80:369–82.
[11] Schantz SL, Gardiner JC, Gasior DM, Sweeney AM, Humphrey HE, McCaffrey RJ. Motor function in aging Great Lakes fisheaters. Environ Res 1999;80:S46–56.
[12] Seegal RF. Are PCBs the major neurotoxicant in Great Lakes salmon? Environ Res 1999;80:S38–45.
[13] Dorgan JF, Brock JW, Rothman N, Needham LL, Miller R, Stephenson HE Jr, Schussler N, Taylor PR.Serum organochlorine pesticides and PCBs and breast cancer risk: results from a prospective analysis. Cancer Causes Control 1999;10:1–11.
[14] Kimbrough RD, Doemland ML, LeVois ME. Mortality in male and female capacitor workers exposed to polychlorinated biphenyls. J Occup Environ Med 1999;41:161–71.
[15] Kodavanti PR, Tilson HA. Comments on "Developmental neurotoxicity of PCBs in humans: what do we know and where do we go from here?" Neurotoxicol Teratol 1997;19:1–5.
[16] Wong PW, Joy RM, Alberston TE, Schantz SL, Pessah IN. Orthosubstituted 2,2′3,5′,6-pentachlorobiphenyl (PCB 95) alters rat hippocampal ryanodine receptors, and neuroplasticity in vitro: evidence for altered hippocampal function. Neurotoxicology 1997;18:443–56.
[17] Fischer LJ, Zhou HR, Wagner MA. Polychlorinated biphenyls release insulin from RINm5F cells. Life Sci 1996;59:2041–9.
[18] Olivero-Verbel J, Ganey PE. Effect of three polychlorinated biphenyls on f-met-leu-phe-induced degranulation in rat neutrophils. Toxicol Lett 1998;98:195–202.
[19] Brown AP, Ganey PE. Neutrophil degranulation and superoxide production induced by polychlorinated biphenyls are calcium dependent. Toxicol Appl Pharmacol 1995;131:198–205.
[20] Ganey PE, Sirois JE, Denison M, Robinson JP, Roth RA. Neutrophil function after exposure to polychlorinated biphenyls *in vitro*. Environ Health Perspect 1993;101:430–4.
[21] Tithof PK, Watts S, Ganey PE. Protein tyrosine kinase involvement in the production of superoxide anion by neutrophils exposed to Aroclor 1242, a mixture of polychlorinated biphenyls. Biochem Pharmacol 1997;53:1833–42.
[22] Tithof PK, Peters-Golden M, Ganey PE. Distinct phospholipases A_2 regulate the release of arachidonic acid for eicosanoid production and superoxide anion generation in neutrophils. J Immunol 1998;160: 953–60.
[23] Tithof PK, Contreras ML, Ganey PE. Aroclor 1242 stimulates the production of inositol phosphates in polymorphonuclear neutrophils. Toxicol Appl Pharmacol 1995;131:136–43.
[24] Dana R, Leto TL, Malech HL, Levy R. Essential requirement of cytosolic phospholipase A_2 for activation of the phagocyte NADPH oxidase. J Biol Chem 1998;273:441–5.
[25] Henderson LM, Moule SK, Chappell JB. The immediate activator of the NADPH oxidase is arachidonate not phosphorylation. Eur J Biochem 1993;211:157–62.
[26] Smolen JE, Weissmann G. Effects of indomethacin, 5,8,11,14-eicosatetraynoic acid, and *p*-bromophenacyl bromide on lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. Biochem Pharmacol 1980;29:533–8.
[27] Hazen SL, Gross RW. ATP-dependent regulation of rabbit myocardial cytosolic calcium-independent phospholipase A_2. J Biol Chem 1991;266:14526–34.
[28] Ma Z, Ramanadham S, Hu Z, Turk J. Cloning and expression of a group IV cytosolic Ca^{2+}-dependent phospholipase A_2 from rat pancreatic islets. Comparison of the expressed activity with that of an islet group VI cytosolic Ca^{2+}-independent phospholipase A_2. Biochim Biophys Acta 1998;1391:384–400.
[29] Gross RW, Rudolph AE, Wang J, Sommers CD, Wolf MJ. Nitric oxide activates the glucose-dependent mobilization of arachidonic acid in a macrophage-like cell line (RAW 264.7) that is largely mediated by calcium-independent phospholipase A_2. J Biol Chem 1995;270:14855–8.
[30] Lehman JJ, Brown KA, Ramanadham S, Turk J, Gross RW, Arachidonic acid release from aortic smooth muscle cells induced by [Arg^8]vasopressin is largely mediated by calcium-independent phospholipase A_2. J Biol Chem 1993;268:20713–6.
[31] Derrickson BH, Mandel LJ. Parathyroid hormone inhibits Na^+-K^+-ATPase through G_q/G_{11} and the calcium-independent phospholipase A_2. Am J Physiol 1997;272:F781–8.
[32] Hazen SL, Gross RW. The specific association of a phosphofructokinase isoform with myocardial calcium-independent phospholipase A_2. J Biol Chem 1993;268:9892–900.
[33] Wolf MJ, Gross RW. The calcium-dependent association and functional coupling of calmodulin with myocardial phospholipase A_2. J Biol Chem 1996;271:20989–92.
[34] Hewett JA, Roth RA. Dieldrin activates rat neutrophils *in vitro*. Toxicol Appl Pharmacol 1998;96:269–78.
[35] Cogliano VJ. Assessing the cancer risk from environmental PCBs. Environ Health Perspect 1998;106:317–23.
[36] Henson PM, Zanolari B. Schwartzman NA, Hong SR. C5a-induced stimulus-specific desensitization, and the effects of cytochalasin B J Immunol 1978;121:851–5.
[37] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[38] Kemmerich B, Pennington JE. Different calcium and oxidative metabolic responses in human blood monocytes during exposure to various agonists. J Leukoc Biol 1988;43:125–32.
[39] Lad PM, Olson CV, Grewal IS, Scott SJ. A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization, and lectin-induced capping. Proc Natl Acad Sci USA 1985;82:8643–7.
[40] Shafer TJ, Mundy WR, Tilson HA, Kodavanti PR. Disruption of inositol phosphate accumulation in cerebellar granule cells by polychlorinated biphenyls: a consequence of altered Ca^{2+} homeostasis. Toxicol Appl Pharmacol 1996;141:448–55.
[41] Atzori L, Flore C, Corriga AM, Cherchi P, Casula D, Congiu L. Mechanisms of PCBs mixture toxicity on isolated rat hepatocytes. Ind Health 1991;29:57–64.
[42] Toborek M, Barger SW, Mattson MP, Espandiari P, Robertson LW, Hennig B. Exposure to polychlorinated biphenyls causes endothelial cell dysfunction. J Biochem Toxicol 1995;10:219–26.
[43] Voie OA, Fonnum F. Ortho substituted polychlorinated biphenyls elevate intracellular [Ca^{2+}] in human granulocytes. Environ Toxicol Pharmacol 1998;5:105–12.
[44] Rzigalinski BA, Blackmore PF, Rosenthal MD. Arachidonate mobilization is coupled to depletion of intracellular calcium stores and influx of extracellular calcium in differentiated U937 cells. Biochim Biophys Acta 1996;1299:342–52.
[45] Reynaud D, Pace CR. 12-HETE, and 12-HPETE potently stimulate intracellular release of calcium in intact human neutrophils. Prostaglandins Leukot Essent Fatty Acids 1997;56:9–12.
[46] Weisdorf DF, Thayer MS. Occult intracellular calcium pools: relevance to neutrophil oxidant production. J Lab Clin Med 1989;114: 260–5.
[47] Rittenhouse-Simmons S, Deykin D. The activation by Ca^{2+} of platelet phospholipase A_2. Effects of dibutyryl cyclic adenosine monophosphate and 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate. Biochim Biophys Acta 1978;543:409–22.
[48] Davies EV, Hallett MB. Near membrane Ca^{2+} changes resulting from store release in neutrophils: detection by FFP-18. Cell Calcium 1996; 19:355–62.

[49] Pettit EJ, Hallett MB. Two distinct Ca^{2+} storage sites and release sites in human neutrophils. J Leukoc Biol 1998;63:225–32.
[50] Richard EA, Ghosh S, Lowenstein JM, Lisman JE. Ca^{2+}/calmodulin-binding peptides block phototransduction in Limulus ventral photoreceptors: evidence for direct inhibition of phospholipase C. Proc Natl Acad Sci USA 1997;94:14095–9.
[51] Osawa M, Kuwamoto S, Izumi Y, Yap KL, Ikura M, Shibanuma T, Yokokura H, Hidaka H, Matsushima N. Evidence for calmodulin inter-domain compaction in solution induced by W-7 binding. FEBS Lett 1999;442:173–7.
[52] Osawa M, Swindells MB, Tanikawa J, Tanaka T, Mase T, Furuya T, Ikura M. Solution structure of calmodulin-W-7 complex: the basis of diversity in molecular recognition. J Mol Biol 1998;276:165–7.
[53] Olivero J, Ganey PE. Role of protein phosphorylation in activation of phospholipase A_2 by the polychlorinated biphenyl mixture Aroclor 1242. Toxicol Appl Pharmacol 2000;163:9–16.
[54] Barrett PQ, Lu H-K, Colbran R, Czernik A, Pancrazio JJ. Stimulation of unitary T-type Ca^{2+} channel currents by calmodulin-dependent protein kinase II. Am J Physiol 2000;279:C1694–703.
[55] Smith RJ, Bowman BJ, Iden SS. Effects of trifluoperazine on human neutrophil function. Immunology 1981;44:677–84.
[56] Hait WN, Lazo JS. Calmodulin: a potential target for cancer chemotherapeutic agents. J Clin Oncol.;1986;4:994–1012.
[57] Peters C, Mayer A. Ca^{2+}/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature 1998;396:575–80.
[58] Risinger C, Bennett MK. Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. J Neurochem 1999;72:614–24.
[59] Alobaidi T, Naccache PH, Sha'afi RI. Calmodulin antagonists modulate neutrophil degranulation. aggregation and stimulated oxygen consumption. Biochim Biophys Acta 1981;675:316–21.
[60] Nagaji J. The role of protein kinase C and $[Ca^{2+}]_i$ in superoxide anion synthesis and myeloperoxidase degranulation of human neutrophils. Kurume Med J 1999;46:157–62.
[61] Blackwood RA, Hessler RJ. Effect of calcium on phenothiazine inhibition of neutrophil degranulation. J Leukoc Biol 1995;58:114–8.
[62] Liu L, Tao JQ, Li HL, Zimmerman UJ. Inhibition of lung surfactant secretion from alveolar type II cells and anexin II tetramer-mediated membrane fusion by phenothiazines. Arch Biochem Biophys 1997; 342:322–8.
[63] Sunagawa M, Yokoshiki H, Seki T, Nakamura M, Laber P, Sperelakis N. Direct block of Ca^{2+} channels by calmidazolium in cultured vascular smooth muscle cells. J Cardiovasc Pharmacol 1999;34:488–96.
[64] Connor CG, Brady RC, Brownstein BL. Trifluoperazine inhibits spreading and migration of cells in culture. J Cell Physiol 1981;108: 299–307.
[65] Franchi E, Camatini M. Evidence that a Ca^{2+} chelator and a calmodulin blocker interfere with the structure of inter-Sertoli junctions. Tissue Cell 1985;17:13–25.
[66] Xue JH, Wang JZ, Xin PZ. Effects of chloroform, chlorobenzene and polychlorinated biphenyls on erythrocyte lipid peroxidation and membrane fluidity in poisoned rabbits. Chung Hua Yu Fang I Hsueh Tsa Chih 1994;28:294–6.
[67] Ganey PE, Olivero J, Mahajerin A. Alterations in intracellular free calcium concentration induced by polychlorinated biphenyls in rat neutrophils. Toxicol Sci 1999;48:278.