This is to certify that the

dissertation entitled

Activation of Phospholipase A_2 By polychlorinated biphenyls (PCBs) and other chlorinated Compounds

presented by

Jesus Tadeo Olivero-Verbel

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology and Toxicology and Environmental Toxicology

Patricia E. Ganey, Ph.D
Major professor

Date 12/17/99

ACTIVATION OF PHOSPHOLIPASE A_2 BY POLYCHLORINATED BIPHENYLS (PCBs) AND OTHER CHLORINATED COMPOUNDS

By

JESUS TADEO OLIVERO VERBEL

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology
Institute for Environmental Toxicology

1999

ABSTRACT

ACTIVATION OF PHOSPHOLIPASE A_2 BY POLYCHLORINATED BIPHENYLS (PCBs) AND OTHER CHLORINATED COMPOUNDS

By

JESUS OLIVERO VERBEL

Neutrophils are immune cells that constitute the first line of defense against pathogens. Upon stimulation these cells undergo biochemical processes of importance in host defense and inflammation. One group of chemicals that stimulates neutrophils is the polychlorinated biphenyls (PCBs). PCBs are compounds of environmental concern because they are present in all the ecosystems and have a broad spectrum of biological effects. An important event during neutrophil stimulation by PCBs is the activation of phospholipase A_2 (PLA_2), an enzyme responsible for the release of arachidonic acid from cell membranes and required for degranulation and superoxide anion production, two of the major cellular responses associated with neutrophil activation. This study investigated the cellular and molecular mechanisms controlling the activation of PLA_2 by PCBs. *Ortho*-PCBs but not non-*ortho*-PCBs induced increases in intracellular Ca^{2+} concentration, phosphorylation of p44 MAPK and

activation of two isoforms of PLA_2, a Ca^{2+}-dependent ($cPLA_2$) and a Ca^{2+}-independent PLA_2 ($iPLA_2$). Changes in Ca^{2+} homeostasis and activation of $iPLA_2$ were independent events. PCBs targeted the same intracellular store as, and blocked Ca^{2+} influx induced by, the chemotactic agent formyl-methyonyl-leucyl-phenylalanine (fMLP). Pharmacological intervention of the PCB signalling suggested that activation of PLA_2 occurs through transduction pathways involving tyrosine kinases, protein kinase C, mitogen activated protein kinase and Ca^{2+}/calmodulin. Activation of PLA_2 was also triggered by a variety of other organochlorine compounds that share a particular substructure similar to that found in *ortho*-PCBs. This motif, called the OG motif, was present in *ortho*-PCBs, α-, δ-, and γ-hexachlorocyclohexane (HCCH), dieldrin and chlordane, and was absent in inactive organochlorines such as β-HCCH and non-*ortho*-PCBs. It consists of a planar, hydrophobic structure linked to a perpendicular, negatively charged atom through a rigid bridge.

To my parents, Rafael y Carmen
to my lovely wife Isabel
to my children, Maria and Catalina
to the honest people from my country

ACKNOWLEDGMENTS

I would love to give thanks to the following people and institutions:

Dr. Patricia Ganey, my professor and mentor

My thesis committee:

Dr. Robert Roth
Dr. Lawrence Fischer
Dr. Norbert Kaminski
Dr. B.V. Madhukar

Lab People: John Buchweitz, Bryan Copple, Charles Barton, Eva Barton, Steve Yee, Shawn Kinser, Manju Thabolingam, Ali Mahajerin , Jamie Morgan, Sara Jehl and Oliva Primera.

The Department of Pharmacology and Toxicology and the Institute of Environmental Toxicology of Michigan State University.

Colciencias, Fulbright and Laspau

Universidad de Cartagena

Republic of Colombia

My parents, my brother, my wife and my children

TABLE OF CONTENTS

Chapter V
BIOCHEMICAL SIGNALS INVOLVED IN THE ACTIVATION OF PLA_2 BY 2,2',4,4'-TETRACHLOROBIPHENYL 99

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$[^3H]$-AA	$[^3H]$-arachidonic acid
12-HETE	12-hydroxyeicosa (5Z, 8Z, 10E, 14Z) tetraenoic acid
12-HPETE	12-hydroperoxyeicosa (5Z, 8Z, 10E, 14Z) tetraenoic acid
AA	Arachidonic acid
AHH	Aryl hydrocarbon hydroxylase
ANOVA	Analysis of variance
BEL	Bromoenol lactone
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular free Ca^{2+} concentration
DAG	1,2-sn-diacylgycerol
DMF	N,N-Dimethylformamide
EROD	7-ethoxyresorufin-*o*-deethylase
fMLP	formyl-methionyl-leucyl-phenylalanine
HCCH	Hexachlorocyclohexane
HxA_8	Hepoxilin A_8
IP_3	Inositol-1,4,5-trisphosphate
$iPLA_2$	Calcium-independent PLA_2
LTB_4	Leukotriene B_4
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidase
OC	Organochlorine compound
PCB	Polychlorinated Biphenyl
PI	Phosphoinositide

PIP_2	Phophotidylinositol-4,5-bisphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
RyRs	Ryanodine receptors
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TFP	Trifluoroperazine
TK	Tyrosine Kinase

Chapter I

INTRODUCTION

I.1. Preface

Neutrophils are one of the most important cells in the innate immune system. They are critical in host defense and inflammation. They perform a complex variety of biochemical processes that involve different signaling cascades and lead to the production of oxidants and the release of enzymes which ultimately battle pathogens.

Polychlorinated biphenyls (PCBs), as well as other organochlorine compounds (OCs), are globally distributed in all of the ecosystems and have been shown to induce biochemical and functional changes in the neutrophil. The study of the mechanisms by which PCBs or OCs affect neutrophils is important to determine the risk of immune system impairment in people exposed to these chemicals. In previous studies it has been shown that in neutrophils PCBs interfere with signaling involving phospholipase A_2 (PLA_2) and calcium (Ca^{2+}), two components of the machinery required to fight pathogens. Accordingly, the overall goal of the work outlined in this dissertation was to test the hypothesis that PCBs cause changes in Ca^{2+} homeostasis and phospholipase A_2 by independent mechanisms and that these mechanisms can be triggered by any OC compound sharing a particular substructure similar to that found in *ortho*-chlorinated PCBs.

To provide the background and rationale for these studies, in the remainder of this Introduction what is known about the physiology and mechanisms of activation of neutrophils, with particular emphasis on PLA_2, will be discussed. In addition, a review of the literature relevant to PCBs, their chemistry and toxicity, and their effects on neutrophil function will be presented. Finally, the effects of other OCs on cell activation will be reviewed.

I.2. Neutrophils: Physiological function

Neutrophils constitute the first line of host defense against pathogens and are mediators of antiviral immunity (Rouse *et al.*, 1978). Neutrophils are one of the major cell types involved in inflammation. During tissue injury, locally released chemotactic factors recruit neutrophils from the bloodstream. Once at the site of tissue damage, neutrophils undergo a series of cellular changes leading to the killing and removal of microorganisms. These processes are the result of biochemical responses, and the most commonly observed during neutrophil activation are superoxide anion production and degranulation.

One of the most studied neutrophil functions is degranulation. It is characterized by the release of hydrolytic enzymes from membrane granules and intracellular vesicles and by the expression of receptors and proteins on the plasma membrane (Fletcher *et al.*, 1982; O'Shea *et al.*, 1985). The extracellular release of specific granular constituents appears to be crucial for the amplification of the initial and subsequent phases of the inflammatory response (Gallin *et al*, 1982). Degranulation can be triggered by a variety of stimuli, such as chemotactic factors including formyl-methionyl-leucyl-phenylalanine (fMLP) and C5a (Henson *et al.*, 1978).

Enzymes including lysozyme, β-glucuronidase and myeloperoxidase are released during degranulation. Neutrophils contain several types of granules with particular compositions and release kinetics. Azurophilic granules contain myeloperoxidase, lysozyme and the membrane protein CD63 (Kuijpers *et al.*, 1991). Myeloperoxidase generates hypochlorous acid and other chlorinated oxidants as both a host defense mechanisms and a means of invoking tissue injury (Hazen *et al.*, 1996; Weiss *et al.*, 1983). In comparison to the azurophilic granules, the secretory vesicles are readily mobilized, and they contain cytochrome b_{558} and the heterodimeric glycoprotein CD11b/CD18 (Calafat *et al.*, 1993). Tertiary granules store gelatinase, and their release upregulates CD11b/CD18 on the plasma membrane (Petrequin *et al.*, 1987; Lacal *et al.*, 1988).

During microbial phagocytosis and exposure to soluble stimuli, neutrophils undergo a rapid burst of oxygen consumption leading to the generation of oxidants. The main biochemical effector responsible for this respiratory burst is the superoxide-generating NADPH oxidase. This enzyme becomes activated when cytosolic and membrane bound proteins are assembled into a membrane complex that converts oxygen into superoxide anion. One of the second messengers responsible for the activation of NADPH oxidase is arachidonic acid (AA) (Henderson *et al*, 1993). The increase in NADPH oxidase activity elicited by AA is due to an increase in the number of assembled enzyme complexes and to the augmentation of its affinity for oxygen (Rubinek and Levy, 1993).

I. 3. Neutrophils: Mechanisms of activation

Neutrophil activation refers to the series of intracellular biochemical processes that follow exposure to stimuli that are responsible for the detectable changes in neutrophil function, such as degranulation and superoxide anion production. The elucidation of these processes is crucial to understand the mechanisms by which neutrophils participate in both host defense and inflammation.

Mechanisms of activation differ for different stimuli; however, similar biochemical pathways can be activated by chemically or physiologically unrelated stimuli (Lad *et al.*, 1996). One of the best documented compounds with respect to mechanism of activation of neutrophils is the chemotactic agent fMLP. FMLP activates neutrophils to undergo oxidative burst and degranulation. This process is mediated through a seven-transmembrane domain receptor coupled to a pertussis toxin-sensitive

GTP binding protein, which in turn regulates Ca^{2+} mobilization and neutrophil function (Lad *et al.*, 1985). The major components of activation of neutrophils by fMLP are Ca^{2+}, phospholipase C (PLC), tyrosine kinase (TK), protein kinase C (PKC), phospholipase D (PLD) and phospholipase A_2 (PLA_2). Each of these will be discussed below in the context of activation of neutrophils by fMLP. It should be noted however, that these events are not unique to fMLP. Thus, this discussion can also be considered a framework for understanding intracelullar events during neutrophil activation by a variety of stimuli, including some of those examined in subsequent chapters.

I. 3. A. Calcium

FMLP stimulation of human neutrophils leads to a rapid increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), an effect which is significantly reduced by removal of extracellular Ca^{2+}. These results indicate that the increase in $[Ca^{2+}]_i$ is in part mediated by an increase of the plasma membrane permeability to Ca^{2+}. The fMLP-dependent influx is not due to the activation of voltage-dependent Ca^{2+} channels since depolarization did not affect the resting $[Ca^{2+}]_i$ (Andersson *et al.*, 1986).

Influx of Ca^{2+} into fMLP-activated neutrophils is only detected on completion of efflux of the cation from intracellular stores (Anderson and Mahomed, 1997). The observed influx is initially slow and is detected at

30-60 s after addition of fMLP, accelerating around 2-3 min, and terminating at 5 min (Anderson and Mahomed, 1997). The long-sustained phase of intracellular Ca^{2+} increase elicited by fMLP is reduced markedly by flunarizine, a blocker of receptor-coupled Ca^{2+}-channels. ^{1}H-NMR studies have shown that flunarizine binds to cell membranes of neutrophils and prevents Ca^{2+} entry (Pasini, *et al.*, 1990). Similarly, adenosine causes a concentration-dependent inhibition of the reactive oxygen species generated by fMLP-activated neutrophils and reduces the influx of extracellular calcium induced by fMLP (Zhang *et al.*, 1996).

It is well established that human neutrophils have a least two distinct Ca^{2+} storage and release sites. One site is located peripherally next to the plasma membrane, and the other is in the juxtanuclear space. Confocal imaging has demonstrated that the non-peripheral Ca^{2+} storage site releases Ca^{2+} in response to fMLP (Pettit and Hallet, 1998). In addition, phagocytosis relies mainly on Ca^{2+} release from internal stores which are replenished from the extracellular Ca^{2+} pool. The neutrophil has some ionomycin-sensitive Ca^{2+} stores which are not solicited during phagocytic activity. A gradient of elevated $[Ca^{2+}]_i$ can be transiently observed around the early phagosome, and changes in $[Ca^{2+}]_i$ are not a prerequisite for phagocytic activity (Theler *et al.*, 1995).

In addition to fMLP, other agonists can trigger changes in $[Ca^{2+}]_{i.}$ Arachidonic acid derivatives such as hepoxilin A_3 (HxA_3), a 12-

lipoxygenase metabolite of arachidonic acid, also can induce release of Ca^{2+} in a concentration-dependent manner (Dho *et al.*, 1990). Furthermore, platelet-derived metabolites such as 12-HETE (12-hydroxyeicosa(5Z,8Z,10E,14Z)tetraenoic acid) and 12-HPETE (12-hydroperoxyeicosa (5Z,8Z,10E,14Z) tetraenoic acid) can stimulate intracellular Ca^{2+} release in neutrophils, with 12-HETE being more potent than 12-HPETE. This is an example of transcellular modulation of $[Ca^{2+}]_i$ in neutrophils (Reynaud and Pace-Asciak, 1997). Another metabolite of AA, leukotriene B_4 (LTB_4) induces a rise of $[Ca^{2+}]_i$ in human neutrophils without involving pertussis toxin-sensitive G proteins or neutrophil activation (Palmblad *et al.*, 1994). Finally, ryanodine, an agonist of the ryanodine receptor, produces an increase of $[Ca^{2+}]_i$ due to the liberation of Ca^{2+} from internal stores and to the influx of extracellular Ca^{2+}. Neutrophils contain ryanodine-sensitive Ca^{2+} stores that might be involved in receptor-mediated chemotaxis (Elferink and De Koster, 1995).

Interestingly, there is evidence suggesting that influenza A virus deactivates neutrophils via an increase in resting $[Ca^{2+}]_i$. It is suggested that high resting $[Ca^{2+}]_i$ has a negative effect on neutrophil function, specifically decreasing the ability to kill bacteria (Haag-Weber and Horl, 1992).

In short, after stimulation by agonist the biochemical behavior of diverse intracellular effector proteins requires changes in the $[Ca^{2+}]_i$ and compartmentalization within the neutrophil. The source, magnitude and duration of the Ca^{2+} oscillations ultimately determine the specificity in cellular function.

I. 3. B. Phospholipase C

Phospholipase C (PLC) activation results in hydrolysis of phosphotidylinositol-4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-trisphosphate (IP_3) and 1,2-sn-diacylglycerol (DAG). In neutrophils, fMLP-stimulated PLC activation is mediated by a pertussis toxin-sensitive GTP-binding protein (Smith *et al.*, 1985). IP_3 can directly stimulate the IP_3 receptor on the endoplasmic reticulum to release Ca^{2+}. Preincubation of neutrophils with an inhibitor of PLCγ, U73122, inhibits Ca^{2+} responses after neutrophil activation with fMLP, suggesting that PLC activation is necessary not only for Ca^{2+} release from intracellular stores but also for sustaining the extracellular influx of Ca^{2+} (Davies, *et al.*, 1994). PLC signalling may be coupled to both PKC and PLD. DAG produced by PLC can activate PKC, and in turn PKC can enhance the response elicited by PLD (Exton, 1990).

I. 3. C. Tyrosine kinases

Activation of tyrosine kinases in neutrophils mediates superoxide production (Tithof *et al.*, 1997), upregulation of CD11b/CD18, adherence and locomotion (Naccache *et al.*, 1994). It has been reported that stimulation of neutrophils with fMLP leads to a rapid and time-dependent increase in the tyrosine autophosphorylation activity of the *src*-related tyrosine kinases *lyn* (Gaudry *et al.*, 1995) and *syk* (Fernandez and Suchard, 1998), which may be involved in the activation and/or modulation of enzymes/receptors located in close proximity such as NADPH oxidase and PLD. FMLP induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein kinases (MAPKs) with apparent molecular weights of 40 kDa and 42 kDa, and treatment with genistein, a tyrosine kinase inhibitor, reduces phosphorylation of the 40 and 42 kDa proteins (Torres *et al*, 1993). FMLP also activates p38 MAPK, which is an upstream activator of NADPH oxidase (Lal *et al.*, 1999). Genistein suppresses the inositol phospholipid metabolism induced by the endoperoxide analog U-46619, suggesting that the turnover of inositol phospholipids is linked to tyrosine phosphorylation (Gaudette and Holub, 1990; Ozaki *et al.*, 1993). Accordingly, activation of tyrosine kinases may lead to release of IP_3 and subsequent rapid mobilization of intracellular Ca^{2+} in neutrophils (Siddiqui and English, 1996).

I. 3. D. Protein Kinase C

PKC accounts for the majority of the kinase activities of neutrophils, and its intracellular distribution is to some extent dependent on the state of the activation of the cell. Following stimulation, the cytosolic PKC and a Ca^{2+}-activated neutral proteinase (CANP) are translocated to the plasma membrane where active CANP promotes proteolytic conversion of PKC. Activated PKC is then released to the cytosol, and it is fully active in the absence of Ca^{2+} and phospholipids. PKC is involved in many steps of Ca^{2+} signaling. For instance, different PKC isoforms require Ca^{2+} for their activity, and in turn PKC can phosphorylate Ca^{2+}-ATPases (Becker, 1988).

I. 3. E. Phospholipase D

PLD catalyzes the hydrolysis of phosphatidylcholine containing either ester- or ether-linkage at the sn-1 position into phosphatidic acid and DAG. PLD is involved in chemotaxis and superoxide anion generation induced by fMLP (Wanikiat *et al.*, 1997). It has been shown that more than 90% of the diglyceride formed in neutrophils in response to fMLP is due to activation of PLD/phosphatidic acid phosphohydrolase (Billah *et al.*, 1989a) which is sensitive to a pertussis toxin-sensitive GTP-binding protein (Kanaho *et al.*, 1991). In HL-60 granulocytes, the activation of PLD requires both Ca^{2+} and GTP (Anthes *et al*, 1989) and can be both protein kinase-independent as well as protein kinase-dependent (Billah *et al.*, 1989b).

I. 3. F. Summary

Neutrophil activation by fMLP leads to a rapid tyrosine phosphorylation followed by an increase in $[Ca^{2+}]_i$, activation of phospholipases and protein phosphorylation, particularly stimulation of PKC and the MAPK pathway (Figure I.1). These events precipitate release of superoxide anion and degranulation. An additional pathway important in PMN stimulation is phospholipase A_2 (PLA_2) activation. Given the central role of this enzyme in the studies described in subsequent chapters, PLA_2 will be discussed in greater detail.

I. 4. Phospholipase A_2 and cellular function.

PLA_2 is the enzyme responsible for the release of arachidonic acid (AA) from the sn-2-position of phospholipids. Depending on the isoform, the substrate for PLA_2 can be phosphatidylcholine, phosphatidylethanolamine, or plasmalogen, among others. In neutrophils, most of the AA released during cellular stimulation is attributed to the stimulation of PLA_2 (Chilton and Murphy, 1986). The activation of PLA_2 is one of the primary steps in the production of a broad spectrum of inflammatory precursors in neutrophils. For instance, the release of AA by PLA_2 increases the activity of the assembled NADPH oxidase in cytoplasmic membranes of neutrophils to produce superoxide anion (Rubinek and Levy, 1993; Dana *et al.*, 1998), an oxidant required for killing

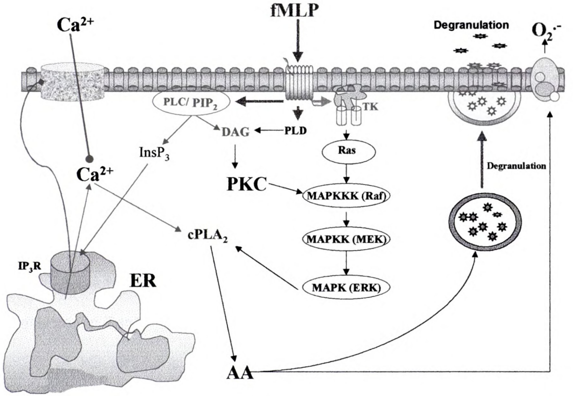


Figure I.1. ***Signal transduction during neutrophil activation by fMLP***

pathogens. PLA_2 activation has been linked to other cellular responses in stimulated neutrophils such as degranulation (Cockcroft, 1991) and adhesion and spreading (Chun and Jacobson, 1993). An excellent review about the regulatory functions of phospholipase A_2 was recently published (Murakami, *et al.*, 1997).

In general, PLA_2s can be divided into extracellular (secreted) and intracellular isoforms. Secreted PLA_2s ($sPLA_2$) are commonly called groups V and IIA secreted PLA_2s. Intracellular PLA_2s are present in the cytosol and include $cPLA_2$, referred to as group IV and the Ca^{2+}-independent PLA_2 ($iPLA_2$) named group VI PLA_2. The different types of phospholipases A_2 have been reviewed (Bauldry *et al.*, 1996; Bereziat, 1996; Murakami, *et al.*, 1997, 1998). A summary of their characteristics is presented in Table 1.

14 kD $sPLA_2$ is present in large quantities in human eosinophils (Blom *et al.*, 1998). $sPLA_2$ is secreted during ischemia-reperfusion injury (Sonnino et al., 1997). $sPLA_2$s can differ significantly in their pH-dependence (Murakami et al., 1997b). The microtubular system is necessary for the synthesis of $sPLA_2$ induced by TNF-α along with IL-1β (Pruzanski et al., 1997), and after removal of the cytokines the induced-$sPLA_2$ synthesis continues for over a period of 8 hours (Vervoordeldonk *et al.*, 1997).

Table I.1. Selected properties of Phospholipases A_2

PLA_2	MW (kDa)	Tissue	Ca^{2+} Requirement	References
$sPLA_2$	14 kDa	Neutrophils	Yes (mM)	Rosenthal *et al.*, 1995
	14 kDa	Pancreas		Chang *et al.*, 1999
	16 kDa	Colon		Lamura *et al.*, 1997
$cPLA_2$	85 kDa		Yes (nM)	De Carvalho *et al.*, 1995
$cPLA_2\alpha$	85 kDa		Yes	Pickard *et al.*, 1999
$cPLA_2\beta$	114 kDa		Yes	Pickard *et al.*, 1999
$cPLA_2\gamma$	61 kDa		No	Pickard *et al.*, 1999
$iPLA_2$	85 kDa	Pancreatic islets	No	Ma *et al.*, 1997, 1998
	80 kDa	Macrophage cell line P388D1		Ackermann *et al.*, 1994; 1995
	85 kDa	Myocardium		Hazen *et al.*, 1993
	88 kDa	Human B-lymphocytes		Ma *et al.*, 1999

Activity of $cPLA_2$ can be modulated by protein phosphorylation involving the MAPK pathway (Qiu and Leslie, 1994). The $cPLA_2$ sequence contains consensus phosphorylation sites for PKC, PKA, TK and MAPK (Sharp *et al.*, 1991). $cPLA_2$ can be phosphorylated *in vitro* by the serine-threonine-specific kinases p42 MAPK and PKC (Nemenoff *et al.*, 1993; Lin *et al.*, 1993). In mammalian cells the common phosphorylation sites on $cPLA_2$ are ser-505 and ser-727 (Borsch-Haubold, 1998). The activation of $cPLA_2$ is mediated by an N-terminal domain which is required for the Ca^{2+}-dependent translocation of the enzyme to the membranes. The $cPLA_2$

domain interacts primarily with the head group of the phospholipid and prefers Ca^{2+} over other group IIA cations (Nalefski *et al.*, 1998). This increase in $[Ca^{2+}]_i$ has to be prolonged in order to translocate to cell membranes (Ishimoto *et al.*, 1996). The binding motif of $cPLA_2$ surrounds two adjacent Ca^{2+}-binding sites together with an adjoining strip of basic residues, which suggests that electrostatic and hydrophobic forces are important for membrane binding (Perisic *et al.*, 1998). Interestingly, protein-protein interactions seem to play a major role in regulation of PLA_2. For instance, in neutrophils, $cPLA_2$ is directly inhibited by interaction with annexin V (Mira *et al.*, 1997) and lipocortin I (Haigler *et al.*, 1987). $cPLA_2$ can also be regulated both transcriptionally and translationally, as seen for IL-1 β in rat mesanglial cells (Schalkwijk et al., 1993).

$cPLA_2$ can be phosphorylated by a diverse number of agonists. $sPLA_2$ can trigger the phosphorylation of $cPLA_2$ through a PKC/MAPK pathway (Huwiler *et* al., 1997). Macrophage $cPLA_2$ can be phosphorylated through a MAPK pathway by stimulation with phorbol 12-myristate 13-acetate (PMA), zymosan and LPS but not with the Ca^{2+}-ionophore A23187 (Ambs *et al.*, 1995), although there are MAPK-dependent and independent mechanisms of activation of $cPLA_2$ by LPS (Fouda *et al.*, 1995). In platelets, collagen is able to stimulate the phosphorylation of $cPLA_2$ through both p38 and p42/p44 MAPK (Borsch *et al.*, 1997).

In myocytes $iPLA_2$ exists as a 400-kDa complex made by a 40-kDa PLA_2 catalytic subunit polypeptide and a tetrameric phosphofructokinase-related regulatory subunit (Hazen and Gross, 1993). In this system, $iPLA_2$ activity is strongly inhibited by calmodulin (Wolf and Gross, 1996). Despite the lack of understanding of the regulation of $iPLA_2$, a relatively selective pharmacological inhibitor exists. Hazen *et al.* (1991b) have found that BEL is >1000-fold more specific for inhibition of $iPLA_2$ than for the Ca^{2+}-dependent PLA_2s. This inhibition is both irreversible and time-dependent. Although BEL is selective for $iPLA_2$ among PLA_2s, BEL also inhibits cellular phosphatidic acid phosphohydrolase (PAP) activity in $P388D_1$ macrophages. This inhibition results in the blockage of triacylglycerol biosynthesis (Balsinde and Dennis, 1996).

Annexins are proteins that participate in the modulation of PLA_2s. Annexin VI inhibits $sPLA_2$ by sequestering the phospholipid substrate (Koumanov *et al.*, 1997). Epidermal and dermal PLA_2 can be inhibited by annexin V, annexin II and annexin I by a mechanism which implies that there is no interaction between PLA_2 and annexins (Bastian *et al.*, 1993). At least for annexin I, the inhibition of PLA_2 is dependent on the concentration of Ca^{2+} in the media (Buckland and Wilton, 1998). Interestingly, annexin V uses the same mechanism to inhibit PKC (Dubois *et al.*, 1998).

The differences in Ca^{2+} requirements, the biochemical specificity in terms of regulation and substrates and the stability to certain agents such as disulfide-reducing chemicals has allowed the analytical differentiation of all the intracellular and extracellular PLA_2s (Yang *et al.*, 1999).

The work described in the following chapters tests the hypothesis that polychlorinated biphenyls alter cell signaling pathways dependent on Ca^{2+} and phospholipase A_2 in rat neutrophils. Accordingly, background information on PCBs will be reviewed before presentation of those studies.

I. 5. Polychlorinated biphenyls

I. 5. A. Structure

Polychlorinated biphenyls (PCBs) are halogenated aromatic hydrocarbons with a biphenyl moiety in which hydrogens are substituted by chlorines (Figure I.2). Chlorine substitution in the biphenyl leads to the formation of 209 compounds named as congeners. Only about 130 individual congeners have been identified in commercial PCBs mixtures at concentrations $\geq 0.05\%$ (Giesy and Kannan, 1998).

I. 5. B. Sources, distribution and occurrence

PCBs were commercially produced beginning in 1929 and have been widely used industrially as dielectric fluids for transformers, plasticizers, cutting oils, pesticide extenders, flame retardants, etc.

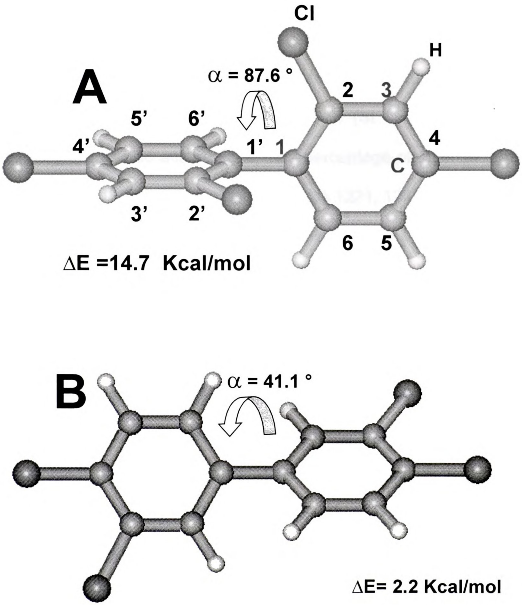


Figure I.1. ***Molecular structure of polychlorinated biphenyls***. Computer modelling using the semiempirical method AM1 was used to fully optimize the structure of PCB congeners. A. Ortho-chlorinated 2,2',4,4'-tetrachlorobiphenyl. B. Non-ortho-chlorinated 3,3',4,4,-tetrachlorobiphenyl. Figure A shows the numbering position for the different atoms in the biphenyl, the torsional angle (α) generated between the two phenyl groups and the rotational energy barrier (ΔE) calculated as the difference between the energy of the conformer in the planar versus the non-planar state.

(Hutzinger *et al.*, 1974). In the environment PCBs are found as mixtures whose source is mainly commercial preparations (Tanabe *et al.*, 1987). Commercial PCB mixtures known as Aroclors were formerly produced by the Monsanto Chemical Company in the U.S. (St Louis, MO), and in general they are named according to their percentage of chlorine content by weight (Waid, 1987). For example, Aroclors 1221, 1242 and 1248 have 21, 42 and 48% chorine content by weight, respectively.

PCBs exhibit a broad range of physicochemical properties that account for their diverse biological and environmental distribution profile. PCBs have low water solubility, relatively low vapor pressure and extreme resistance to chemical reactions (Shiu and Mackay, 1986). These three properties are inversely related to chlorine content (Waid, 1987). Due to their lipophilicity and different rates of metabolism, PCBs can be bioaccumulated and biomagnified in the food chain (Kannan *et at.*, 1998; Matthews and Anderson, 1975), and this accounts for their persistance, widespread distribution, and mobilization to remote areas (Subramanian *et al.*, 1983) such as the Himalayan lakes (Galassi *et al.*, 1997) and Antarctica (Kallenborn *et al.*, 1998).

Humans can accumulate PCBs from direct exposure and by bioaccumulation processes through the diet (Patandin et *al.*, 1999). PCBs have been found in breast milk (Ramos *et al.*, 1997; Schecter *et al.*, 1998), blood, adipose tisssue, and placenta (Laden *et al.*, 1999), among other tissues.

1. 5. C. Toxicokinetics of PCBs

In mammalian systems, PCBs can be metabolized via cytochrome P450s 1A1, 1A2, 2B1 and 2B2. These enzymes catalyze the formation of intermediate arene oxides that are converted to hydroxyl or methyl sulphone metabolites and subsequently into sulfate or glucuronide conjugates (Bergman *et al.*, 1994).

1. 5. D. Toxicology of PCBs

The toxic effects of PCBs have been discussed in different reviews (Safe, 1984; Silberhorn *et al.*, 1990) and are widely diverse. PCBs can cause immunosuppression (Vos and Loveren, 1998), neurodevelopmental deficiencies (Jacobson and Jacobson, 1997), changes in sex hormone levels and sweet preference behavior (Hany *et al.*, 1999a), hearing deficits (Goldey *et al.*, 1995), periodontal diseases (Hashiguchi *et al.*, 1999), dermatological problems (Nakayama *et al.*, 1999) and neutrophilia (Hansen *et al.*, 1995) among other effects. Although extensive epidemiological studies have revealed an association between PCBs and cancer (Cogliano, 1998), there is still an ongoing controversy about this effect (Moysich *et al.*, 1999; Dorgan *et al.*, 1999).

It has been shown that the presence of congener mixtures in biological samples is similar to that present in different commercial preparations (Dewailly *et al.*, 1996). Different Aroclors have been reported to exert toxicological effects. Aroclor 1242 alters the levels of thyroid hormones in rats (Morse *et al*, 1996a). Aroclor 1254 induces long-term changes in neuronal and glial cell proteins such as synaptophysin, glial fibrillary protein and calcineurin (Morse *et al.*, 1996b) and alters serotonin metabolism (Morse *et al.*, 1996c). Aroclors 1254, 1260, 1242 and 1016 induced hepatocellular neoplasms in animals (Mayes *et al.*, 1998).

Metabolism, toxic potential and mechanism of action vary with physicochemical properties of the congeners (Seegal, 1996; McFarland and Clarke, 1989). It has been proposed that the presence of substitutions at particular sites on the phenyl ring determines the mechanism of action for each congener. PCB congeners that are not *ortho*-substituted, such as those substituted in both para and two or more meta positions, appear to have greater affinity for binding to the Ah-receptor (AhR) and elicit effects similar to those observed for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the compound with the highest affinity for the AhR (Kafafi *et al.*, 1993a). These compounds are commonly referred to as dioxin-like PCBs. On the other hand, congeners with *ortho*-substitutions have low affinity for the AhR and consequently are called non-dioxin like PCBs. The differences in activity observed with *ortho* and non-*ortho*- PCBs are in part due to structural and electronic properties derived from the three-dimensional conformations generated by the presence of chlorines in *ortho* positions. A chlorine in the *ortho* position of a ring in the biphenyl skeleton interacts sterically with an hydrogen or another chlorine in the other ring of the biphenyl creating a steric force that shifts one of the rings out of the plane increasing the torsional angle between the two phenyl groups (Figure I.1). This is the reason why *ortho-* and non-*ortho*-PCBs are called coplanar and non-coplanar PCBs, respectively. Although this shifting occurs both in *ortho-* and non-*ortho*-PCBs, it is greater in *ortho*-PCBs because the force of steric interaction between chlorines and chlorine/hydrogens is greater than for hydrogens alone. In fact, using computer modelling, it has been demonstrated that the majority of PCBs are likely to interact with the AhR in their non-planar conformation (Kafafi *et al.*, 1993a) and that their interaction with the AhR depends on their lipophilicities, electron affinities, entropies and differences in energy of frontier orbitals (Kafafi *et al.*, 1993b). The structure of representative *ortho-* and non-*ortho* PCBs is shown in Figure 1.

I.5.D.i. Toxicology of dioxin-like PCBs

Non-*ortho* chlorinated PCBs bind directly to the AhR and elicit a variety of responses (Bandiera *et al.*, 1982). These include induction of hepatic drug metabolizing enzymes such as aryl hydrocarbon hydroxylase (AHH), 7-ethoxyresorufin O-deethylase (EROD) (Kafafi *et al.*, 1993b; Sanderson *et al.*, 1996), and arylamine oxidase (Marczylo and Ioannides, 1997), and alterations of hepatic glucose 6-phosphate dehydrogenase (Hori *et al.*, 1997). In environmental and human samples, several dioxin-like congeners are present, including 3,3',4,4'- and 3,4,4',5-tetrachlorobiphenyl, and 2,3,3',4,4'- and 3,3',4,4',5-, pentachlorobiphenyl (Hong *et al.*, 1993; Safe *et al.*, 1985). 3,3',4,4'-tetrachlorobiphenyl produces both an induction and inhibition of cytochrome P4501A in a dose-dependent manner *in vitro* (Hahn *et al.*, 1993). This congener causes a decrease in the anti-sheep red blood cells antibody forming cell response and also produces thymic atrophy, hepatomegaly, hepatic centrilobular hypertrophy and extensive hepatic fatty infiltration (Silkworth and Grabstein, 1982).

I. 5. D.ii. Toxicology of non-dioxin like PCBs

Although most *ortho*-substituted PCBs have low affinity for the AhR, they do have biological activity. Cell stimulation by *ortho*-chlorinated PCBs involves the activation of a series of subcellular systems. Although a receptor-mediated mechanism has not been discovered, some studies have suggested that in fact *ortho*-PCBs can interact directly with cellular receptors (Angus and Contreras, 1995). A recent symposium dealing with the mechanisms of toxicity induced by of *ortho*-substituted PCBs has been published (Fischer *et al.*, 1998).

Effects of *ortho*-substituted PCBs have been documented in cells from the central nervous system (Kodavanti *et al.*, 1997). Structure-activity relationships have shown that congeners with one, two or three chlorine substitutions and having a log K_{ow} between 5.2 and 6.6 are more active than other congeners (Svendsgaard *et al.*, 1997). Aroclor 1016 and *ortho*-substituted PCB congeners decrease dopamine concentration in the caudate, putamen, substantia nigra, and hypothalamus (Seegal *et al.*, 1990). Aroclor 1254 has the same effect in PC12 cells (Angus and Contreras, 1994).

PCB mixtures Aroclors 1016, 1254 and 1260 and individual congeners with *ortho/meta-* or *ortho/para-* chlorine substitutions inhibit cerebellum microsomal and mitochondrial Ca^{2+}-sequestration (Kodavanti *et al.*, 1996). In cerebellar granule cells, 2,2'-dichlorobiphenyl causes a concentration-dependent increase in $[Ca^{2+}]_i$, an inhibition of microsomal and mitochondrial Ca^{2+}-sequestration (Kodavanti *et al.*, 1993; Shafer *et al.*, 1996), a biphasic effect on receptor-mediated phosphatidylinositol hydrolysis and translocation of PKC (Kodavanti *et al.*, 1994).

PCBs containing two or more *ortho*-chlorine substituents activate ryanodine receptors (RyRs) in mammalian brain, mobilizing Ca^{2+} in a dose-dependent manner, whereas the non-*ortho*-substituted PCBs are completely inactive (Wong *et al.*, 1997a). The mechanism responsible for this effect has been proposed to involve the disruption of the modulating effect of immunophilin FKBP12 on the distinct conformations of the Ry_1R complexes (Wong and Pessah, 1997).

At the transcriptional level, the *ortho*chlorinated 2,3,4,2',4',5' hexachlorobiphenyl (PCB-138) induced overexpression of ras, jun, and myc in the 3T3-L1 cell line (Gribaldo *et al.*, 1998). 2,3',4,4',5-pentachlorobiphenyl induced methylcholanthrene- and phenobarbital-inducible isoenzymes of cytochrome P450 monooxygenases and enhanced foci growth in rat liver (Haag-Gronlund *et al.*, 1997).

Ortho-chlorinated PCBs can abrogate some of the toxic responses observed with non-*ortho*-chlorinated PCBs. For instance, 2,2',4,4',5,5'-hexachlorobiphenyl protects from 3,3',4,4',5-pentachlorobiphenyl-induced embryo malformations (Zhao *et al.*, 1997).

Other non-dioxin-like responses of PCBs include estrogenic activity both *in vivo* and *in vitro* (Arcaro *et al.*, 1999). Interestingly, hydroxylated, non-*ortho* and *ortho*-chlorinated PCBs have been reported to have antiestrogenic activity *in vitro* (Kramer *et al.*, 1997) and *in vivo* (Connor *et al.*, 1997). In the immune system, the effects of PCB have been extensively studied in neutrophils. The next section will present a review of what is known about the effects of *ortho*-chlorinated PCBs in neutrophils.

1.5.E. *Ortho*-chlorinated PCBs and neutrophil function

Ortho-chlorinated PCBs cause superoxide anion production, degranulation and inhibition of fMLP-induced degranulation in rat neutrophils (Ganey *et al.*, 1993). These processes are mediated through Ca^{2+}-dependent mechanisms (Brown and Ganey, 1995) and are elicited only by *ortho*-chlorinated biphenyls (Olivero and Ganey, 1998; Brown *et al.*, 1998). The PCB mixture Aroclor 1242 induces an increase in intracellular calcium in rat neutrophils (See Chapter II). The time course of the increase in $[Ca^{2+}]_i$ shows a delay and starts only after 5 minutes incubation with Aroclor 1242, reaching a maximum after approximately 15 minutes. Furthermore, Aroclor 1242 or 2,2',4,4'-tetrachlorobiphenyl but not 3,3',4,4'-tetrachlorobiphenyl induces the production of inositol phosphates in neutrophils, suggesting activation of PLC (Tithof *et al.,* 1995).

PCBs induce the activation of Ca^{2+}-independent PLA_2 in neutrophils. This effect has been observed for *ortho*-substituted PCBs but has not been detected for non-*ortho*-substituted PCBs (Tithof *et al.*, 1998). This Aroclor 1242-induced PLA_2 activation is linked to generation of superoxide anion from rat neutrophils, and inhibition of PLA_2 activity with BEL also inhibits superoxide anion production (Tithof *et al*, 1996). Conversely, TCDD, which binds specifically to neutrophils from Ah-responsive mice, does not impair the production of superoxide anion and hydrogen peroxide or degranulation, measured as the release of β-glucoronidase (Ackerman *et al.*, 1989). The production of superoxide anion in neutrophils exposed to *ortho*-PCBs has been linked to activation of PLC (Tithof *et al.*, 1995), TK (Tithof *et al.*, 1997; Voie *et al.,* 1998) and PLD (Voie *et al.,* 1998).

1. 6. Cell activation by other chlorinated chemicals

Other chlorinated chemicals affect signal transduction pathways similar to those activated in neutrophils by PCBs. Hexachlorocyclohexanes (HCCHs) are a group of chemicals widely used as pesticides. Theoretically there are eight possible conformational isomers for these compounds, the most common being α, β, δ and γ. The γ-isomer is commonly known as lindane and has been shown to induce hepatocellular carcinoma and lung cancer (Wolff et al., 1993). In humans, symptoms of intoxication with lindane include seizures of the mixed type, intention tremors, memory impairment, and irritability.

The α-, γ-, and δ-, but not β-HCCH, induce superoxide anion production and release of intracellular Ca^{2+} in human neutrophils (Kuhns *et al.*, 1986). γ-HCCH-induced superoxide production is sensitive to the intracellular antagonist of Ca^{2+} release, TMB-8, suggesting a role for intracellular Ca^{2+} in superoxide production by γ-HCCH. Lindane induces superoxide anion release by a mechanism that does not involve a physical interaction of the agonist with the NADPH-dependent superoxide-generating system (English *et al.*, 1986). It has been reported that γ-, and δ-HCCH isomers, which stimulate superoxide formation, inhibit chemotaxis to other mediators as a result of widespread and dysfunctional changes in intracellular calcium that can no longer effect the coordinated cytoskeletal actions required for cell movement (Kaplan *et al.* 1988).

Structurally, HCCHs resemble analogs of inositol. For instance, muco- and myo-inositol have the stereochemical configuration of γ and δ isomers of HCCH, respectively. Mohr *et al.* (1995) demonstrated that the δ-isomer of HCCH releases Ca^{2+} from IP_3-sensitive Ca^{2+} stores, and that this is not due to its similarity to myo-inositol-1,4,5-trisphosphate. δ-HCCH did not compete with IP_3 for the IP_3 receptor, suggesting the presence of a binding site for δ-HCCH in IP_3 sensitive Ca^{2+} channels.

Lindane induces profound effects on the phosphatidylinositol cycle through the generation of phosphatidic acid and consumption of PIP_2 (English *et al.*, 1986) and decreases the incorporation of myo-[2-^{3}H]inositol into phosphoinositides (PI) with some degree of specificity depending on the different PI classes (Carrero *et al.*, 1996). In myocytes, the disruption of PI-generated second messengers by lindane can lead to inhibition of gap junctional communication (Criswell *et al.*, 1995), as observed in other cell types (Guan and Ruch, 1996). Because EDTA prevented the effects of lindane on PI synthesis, it was hypothesized that lindane inhibits phosphatidylinositol synthesis by increasing intracellular calcium. This increase in intracellular calcium may be due to two factors. First, in macrophages, lindane can directly trigger a Ca^{2+} influx (Pinelli *et al.*, 1994). Second, this Ca^{2+} can in turn activate PLC to generate a sustained increase in cytoplasmic free Ca^{2+} after IP_3 generation. Furthermore, EGTA blocks the lindane-induced $[Ca^{2+}]_i$ increase, suggesting a role for extracellular Ca^{2+} during neutrophil activation by this compound (Grigorian *et al.*, 1988). The increase in $[Ca^{2+}]_i$ together with the inhibition of Ca^{2+},K^+-ATPases may account for the toxic effects of lindane in the cardiovascular system of rats (Anand, *et al.*, 1995). At concentrations greater than 50 μM, lindane activates a phospholipase A_2 (PLA_2) which prefers phosphatidyl inositol rather than phosphatidyl choline as substrate (Lopez-Aparicio *et al.*, 1995). PLA_2 activity in both soluble and membrane fractions was not modified by lindane (30-300 μM) over a 120 min period, suggesting a role for particular intracellular messengers in PLA_2 activation. On the other hand, lindane can directly stimulate PKC activity (Bagchi, *et al.*, 1997). Interestingly, Enan and Matsumura (1998) have shown that c-neu tyrosine kinase is activated by β-HCCH in cell-free and intact cell preparations from MCF-7 human breast cancer cells. The effects of dieldrin on neutrophil function are not as well documented as those for lindane. Dieldrin causes a concentration-dependent increase in superoxide anion production by neutrophils, which is regulated by extracellular calcium (Hewett and Roth, 1988). It has been suggested that dieldrin activates PLC (De Schroeder and De D'angelo, 1995) and inhibits forskolin-induced stimulation of adenyl cyclase (Carrero *et al.*, 1993).

I. 7. Summary

In summary, PCBs stimulate neutrophils and in doing so invoke a number of signal transduction pathways involved in activation of the cells by other agents. These include PLC, TKs, PLA_2 and increased intracellular calcium. Similar pathways may be important in HCCH- and dieldrin-induced stimulation of neutrophils. Activation of PLA_2 is critical and appears to be an early event after exposure to PCBs. The PLA_2 activated is Ca^{2+}-independent, and regulation of $iPLA_2$ is not understood. Accordingly, studies have been undertaken to elucidate the mechanisms of activation of $iPLA_2$ during neutrophil activation by PCBs and other organochlorine compounds. These studies are described in detail in the following chapters. The overall hypothesis tested by these studies is that PCBs cause changes in Ca^{2+} homeostasis and phospholipase A_2 by independent mechanisms and that these mechanisms can be triggered by any OC compound sharing a particular substructure similar to that found in *ortho*-chlorinated PCBs.

Chapter II

REGULATION OF CALCIUM HOMEOSTASIS BY POLYCHLORINATED BIPHENYLS IN RAT NEUTROPHILS: EFFECTS ON REPONSES TO THE CHEMOTACTIC AGENT FMLP

II.1 Summary

Polychlorinated biphenyls (PCBs) activate polymorphonuclear neutrophils (PMNs) acting on different subcellular targets. The objective of this study was to characterize the effects of PCBs on Ca^{2+} homeostasis in rat PMNs. Aroclor 1242, a technical PCB mixture, induced a dose- and time-dependent increase in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in Fura 2/AM-labeled PMNs. A significant increase in $[Ca^{2+}]_i$ from basal levels was observed only after 5 min and reached a plateau after 10-15 min. To examine whether the effects of PCBs on Ca^{2+} homeostasis are structure-dependent, studies were performed with individual congeners. 2,4'-dichorobiphenyl, an *ortho*-PCB, produced the same effect on Ca^{2+} as Aroclor 1242, whereas 3,3',4,4'-tetrachlorobiphenyl, a non-*ortho* PCB congener, was inactive. N-formyl-methionyl-leucyl-phenylalanine (fMLP) induced a biphasic increase in intracellular Ca^{2+}. The first phase is due to release of Ca^{2+} from intracellular stores, while the second one is due to extracellular Ca^{2+} influx. The rapid increase in intracellular Ca^{2+} elicited by fMLP during the first phase was abrogated in a time-dependent manner by Aroclor 1242, suggesting that the intracellular calcium stores activated by both Aroclor 1242 and fMLP are the same. Furthermore, Aroclor 1242 blocked Ca^{2+} influx induced by fMLP. In short, the PCB-induced increase in $[Ca^{2+}]_i$ in PMNs was dose-, time- and structure-dependent, and the Ca^{2+} pool released was the same store as that released by fMLP.

II.2. Introduction

Among environmental pollutants, PCBs are of great concern due to their global distribution, high persistence, and the toxicological diversity in terms of congener specificity. PCBs can be categorized in two different groups depending on the presence or absence of a chlorine atom in the 2-position of either of the phenyl groups composing the biphenyl. In general, it is accepted that PCBs lacking the chlorine atom in the 2-position can obtain a quasi-planar conformation allowing for their interaction with the Ah receptor (Ah-R) (McKinney and Waller, 1994). On the other hand, those PCBs with a chlorine group in the 2-position (*ortho*-chlorinated PCBs) are commonly referred to as non-coplanar and do not exhibit high affinity for the AhR. Although the ortho-chlorinated PCBs have low potency in inducing AhR-mediated effects, their toxicological profile is diverse and includes neurotoxicity (Kodavanti *et al.*, 1994; Choksi, *et al.*, 1997; Tilson and Kodavanti, 1997), cancer promotion (Wolfle, 1997-98), inhibition of cell-cell communication (Kato *et al.*, 1998) and neutrophil dysfunction (Ganey *et al*, 1993).

One of the Ah-R-independent biochemical effects of PCBs is the alteration of calcium (Ca^{2+}) homeostasis in various cell types. PCBs cause changes in microsomal Ca^{2+} transport by direct interaction with ryanodine receptors in mammalian brain (Wong *et al.*, 1997b), inhibition of Ca^{2+} sequestration in rat cerebellum (Kodavanti, *et al.*, 1996) and Ca^{2+}-

dependent induction of insulin release from RINm5F cells (Fischer *et al*, 1996). In neutrophils, activation by PCBs is a Ca^{2+}-dependent process that also involves other subcellular systems such as tyrosine kinases (Tithof *et al.*, 1997), phospholipase A_2 (Tithof *et al.*, 1996) and phospholipase C (PLC) (Tithof, *et al.*, 1995).

This recognized role of Ca^{2+} in cell homeostasis can be attributed to its interaction with many proteins to guarantee optimal cell function. Proteins that directly interact with Ca^{2+} include calmodulin, annexins, the EF-hand calcium-binding protein S-100, the Ca^{2+} binding C-2 domains and other extracellular proteins such as blood clotting and immune system proteins (Willians *et al.*, 1998). The objectives of this work were to examine the effects of the commercial PCB mixture, Aroclor 1242, on Ca^{2+} homeostasis in neutrophils and to determine the effects of these changes on the response elicited by the bacterial chemotactic compound fMLP. Results of these studies will offer clues about the role of alterations in Ca^{2+} homeostasis in neutrophil dysfunction induced by PCBs.

II. 3. Materials and Methods

II.3.A. Chemicals

PCB congeners 8 (2,4'-dichlorobiphenyl) and 77 (3,3',4,4'-tetrachlorobiphenyl), (>99% pure) and the PCB mixture Aroclor 1242 were purchased from Chemservice (West Chester, PA). Glycogen (Type

II from oysters) and fMLP were purchased from Sigma Chemical Co. (St. Louis, MO).

II.3.B. Isolation of rat, peritoneal neutrophils

After glycogen elicitation into the peritoneum of male, Sprague-Dawley, retired breeder rats, neutrophil isolation was conducted as described (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt solution (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. Concentration of cells in all of the assays was 2×10^6 cells/mL. The isolation procedure was performed at room temperature.

II.3.C. Measurement of $[Ca^{2+}]_i$ in rat neutrophils

Neutrophils (2.5×10^6 cells/mL) were labeled by incubation for 25 min at 37 °C with 5 μM Fura-2/AM in HBSS. After loading cells were washed with HBSS, and the cell count was readjusted to 2×10^6 cells/mL. Fluorescence emission at 505 nm was monitored at room temperature with constant stirring, using a dual wavelength spectrofluorometer system with excitation at 340 and 380 nm. The $[Ca^{2+}]_i$ was calculated from fluorescence intensity readings using the following equation: $[Ca^{2+}]=K_d*Q(R-R_{min})/(R_{max}-R)$. R is the ratio of emission intensities at 340 and 380 nm excitation

(340/380), R_{min} is the 340/380 ratio under Ca^{2+}-free conditions, R_{max} is the ratio under saturating Ca^{2+} concentrations; Kd is the dissociation constant of the Ca^{2+}: Fura-2 complex; and Q is the ratio of the 380 nm fluorescence under conditions of minimum and maximum $[Ca^{2+}]_i$ conditions (Shao *et al.*, 1998). The equilibrium dissociation constant, Kd, was taken from literature, 224 nM (Kankaanranta *et al.*, 1995a). R_{max} and R_{min} values for each assay were determined from the fluorescence intensities in the presence of 0.01% Triton X-100 or 10 mM EGTA, respectively. These two parameters did not change significantly in the presence of Aroclor 1242. Fluorescence emission after excitation of 360 nm was monitored and in all studies conducted remained constant during data collection. Increases in resting $[Ca^{2+}]_i$ evoked by agonists were detected by measuring the change in fluorescence ratio after exposure with the vehicle N,N-dimethyllformamide (DMF). Typical values for quiescent neutrophils ranged from 50 to 100 nM.

PCBs were added to labeled cells 50 sec after the start of data collection. PCB stock solutions were prepared by dissolution of the neat chemical in DMF, and 1 µL/mL of the respective stock solutions was added to the cells to achieve the desired concentration in a final cell volume of 3 mL. When inhibitors were used, labeled cells were pre-incubated with the inhibitor for 20 minutes at 37 °C before PCBs were added.

II.3.D. Statistical analysis

Data are presented as the means ± standard error of the mean (SEM) for at least four different replicates. Comparisons between the calcium concentrations for different agonists at a particular time were done using the Student's t-test. The criterion for statistical significance was $p<0.05$.

II.4. Results

II.4.A. Aroclor 1242-stimulated increases in $[Ca^{2+}]_i$ in rat neutrophils are both time- and dose-dependent

Aroclor 1242 and the PCB congeners 2,4'-dichlorobiphenyl (PCB 8) and 3,3',4,4'-tetrachlorobiphenyl (PCB 77) were investigated to determine their capacity to induce changes in $[Ca^{2+}]_i$ in rat neutrophils. Aroclor 1242 induced a time-dependent increase in $[Ca^{2+}]_i$ in rat neutrophils. Significant changes from basal levels were observed after 5-6 minutes incubation, and $[Ca^{2+}]_i$ continued to increase through 15 minutes (Figure II.1). This increase in $[Ca^{2+}]_i$ was also dose-dependent (Table II.1). Addition of EGTA 50 seconds before treatment with agonists abolished the second phase of fMLP-induced increase in $[Ca^{2+}]_i$ but did not eliminate the Aroclor 1242-induced response (Figure II.2), although the net increase was reduced (Figure II.2, compare to Figure II.1). These results

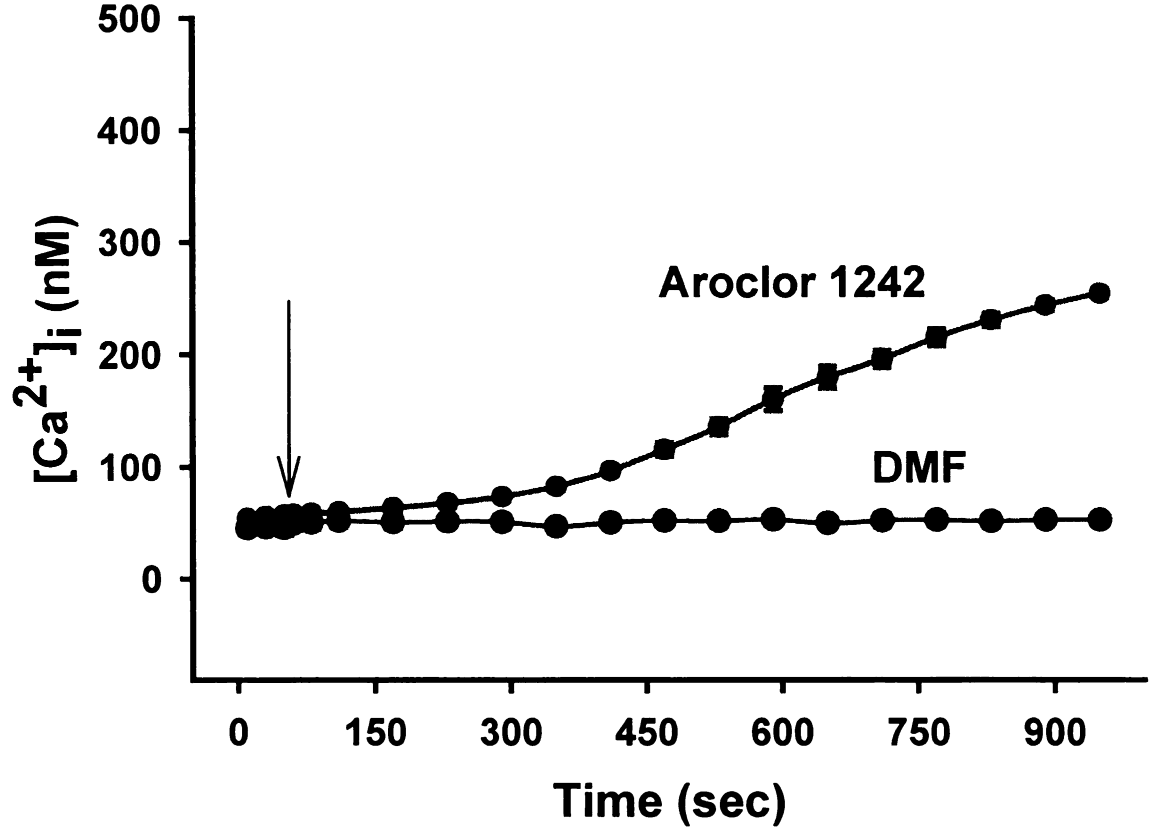


Figure II.1. ***Effect of 10 µg/mL Aroclor 1242 on $[Ca^{2+}]_i$ in rat neutrophils.*** Cells were loaded with fura-2/AM as described in Materials and Methods. Fura-2-loaded neutrophils were stimulated with 10 µg/mL Aroclor 1242 at the time indicated by the arrow. Data for control (DMF) are shown for comparison. Results are expressed as means ± SEM for four different experiments.

Agonist Concentration	Incubation time (minutes) 5	 10	 15
Aroclor 1242 1 μg/mL	4.4±8.4	29.0±10.2	59.1±13.7
5 μg/mL	20.6±5.4	60.6±7.1	83.0±12.7
10 μg/mL	28.3±1.6	125.5±8.5	199.4±4.4
FMLP 2 μM	284.9±49.5	287.4±38.8	170.1±12.6

Table II.1. Net changes in basal $[Ca^{2+}]_i$ (nM) in rat neutrophils exposed to different concentrations of Aroclor 1242. Data are presented as the mean ± standard error for at least three different experiments conducted as presented in Materials and methods. FMLP data are presented for comparison. The maximal change in increase in $[Ca^{2+}]_i$ induced by fMLP was observed at 7 min treatment (351.2±71.8 nM).

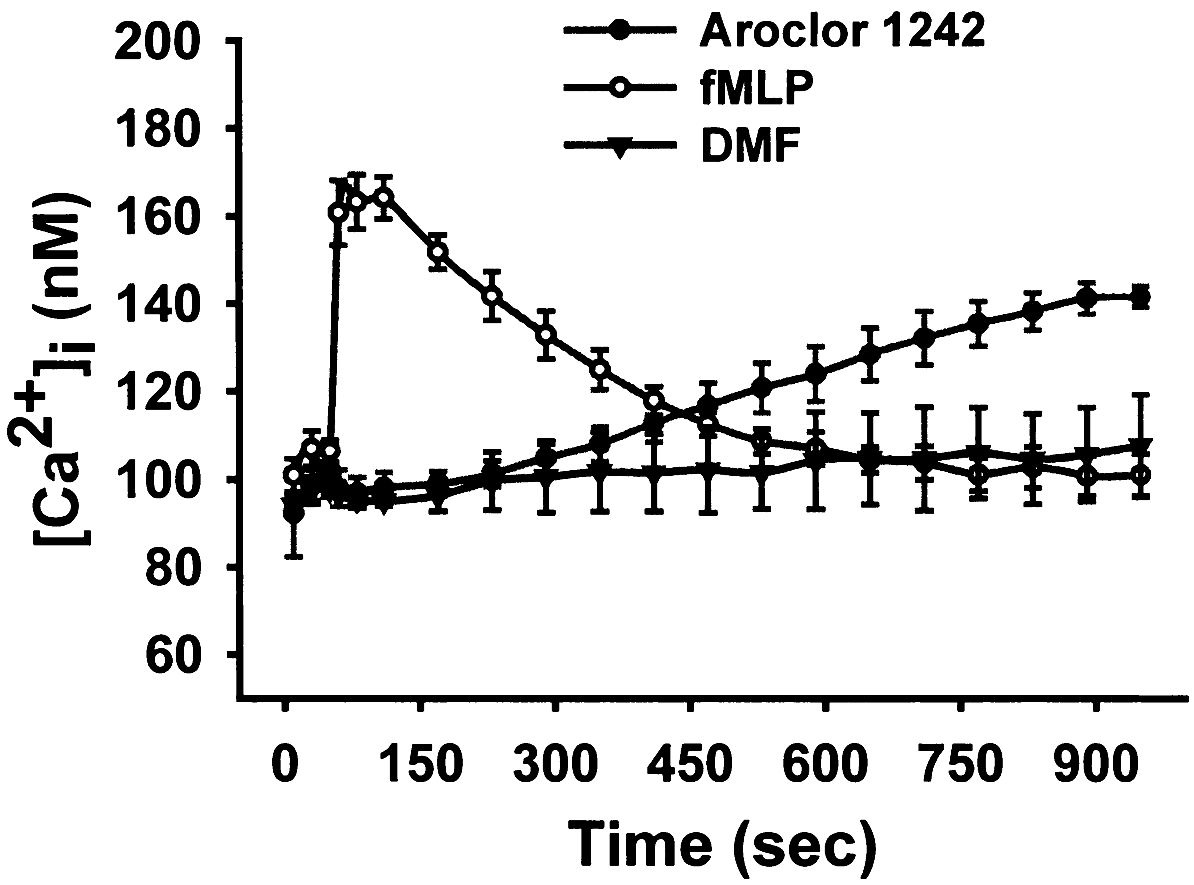


Figure II.2. ***Effect of EGTA on Aroclor 1242- and fMLP-induced increase on $[Ca^{2+}]_i$ in rat neutrophils.*** Cells were labeled with fura-2/AM as described in Materials and methods. Fura-2-loaded neutrophils were treated with 5 mM EGTA and 50 seconds later with either 2 µM fMLP, 10 µg/mL Aroclor 1242 or 1 µL/mL DMF (vehicle control). Results are expressed as mean ± SEM for four different experiments.

show that Aroclor 1242-induced increase in $[Ca^{2+}]_i$ is both dose- and time-dependent and is decreased in magnitude, but not abolished, in the absence of extracellular Ca^{2+}.

II.4.B. *Ortho*-chlorinated but not non-*ortho*-chlorinated PCBs reproduce the effects of Aroclor 1242

The *ortho*-chlorinated PCB 2,4'-dichlorobiphenyl caused an increase in $[Ca^{2+}]_i$ similar to that observed with Aroclor 1242; however, the non-*ortho*--chlorinated PCB 3,3',4,4'-TCB, failed to induce a change in $[Ca^{2+}]_i$ (Figure II.3). These results are in agreement with previously reported data showing that in human neutrophils *ortho*-substituted PCBs but not non-*ortho*-PCBs increase $[Ca^{2+}]_i$ (Voie *et al.*, 1998).

II.4.C. Aroclor 1242 interferes with fMLP-induced increases in $[Ca^{2+}]_i$

Treatment of neutrophils with the chemotactic agent fMLP causes a characteristic, time-dependent, biphasic increase in $[Ca^{2+}]_i$. Data from our lab represent this well (Figure II.4). The first phase, which results from a rapid release of intracellular Ca^{2+}, lasts for approximately 50-80 seconds. The second phase, which starts at about 2-3 min (Figure II.4), is characterized by a bell-shaped increase in $[Ca^{2+}]_i$ which lasts for about 13 minutes, reaching a maximum at 7. It is well recognized that the first phase

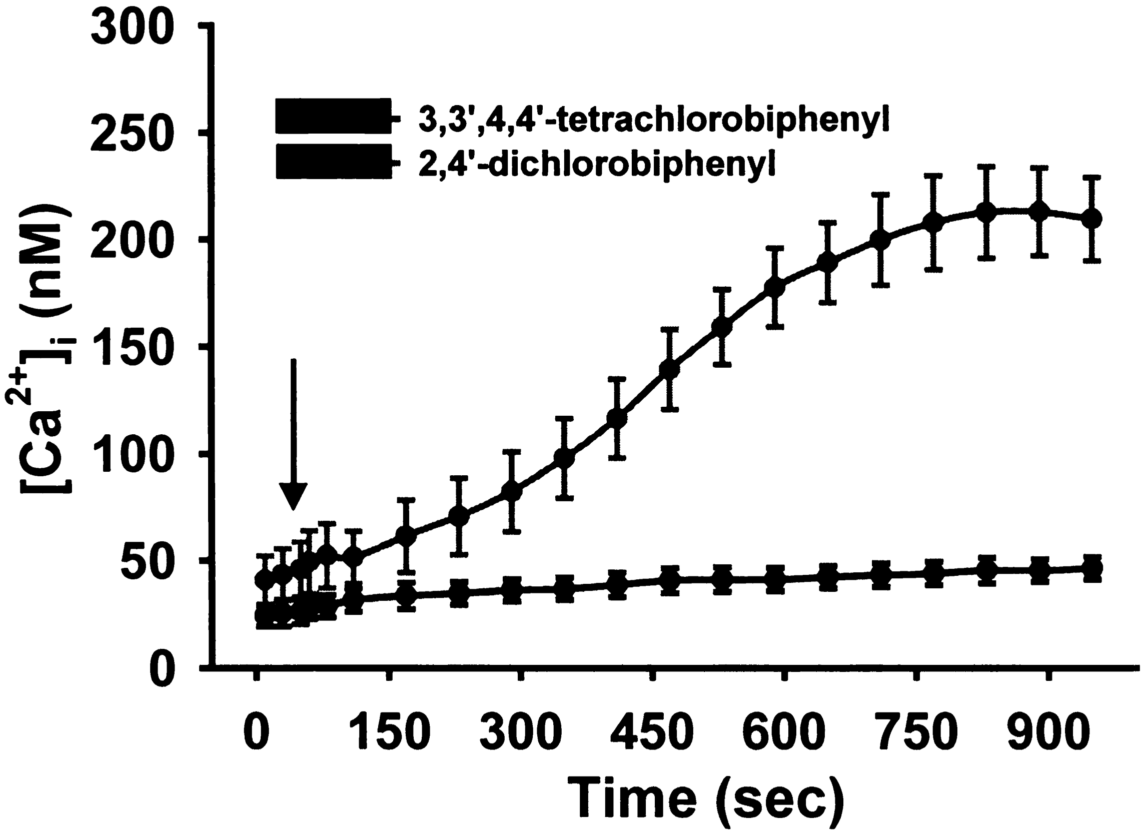


Figure II.3. ***Effects of 2,4'-dichlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl on [Ca^{2+}]$_i$ in rat neutrophils.*** Cells were labeled with fura-2/AM as described in Materials and Methods. Fura-2-loaded neutrophils were stimulated with 25 µM of the indicated congener at the time indicated by the arrow. Results are expressed as means ± SEM for four different experiments.

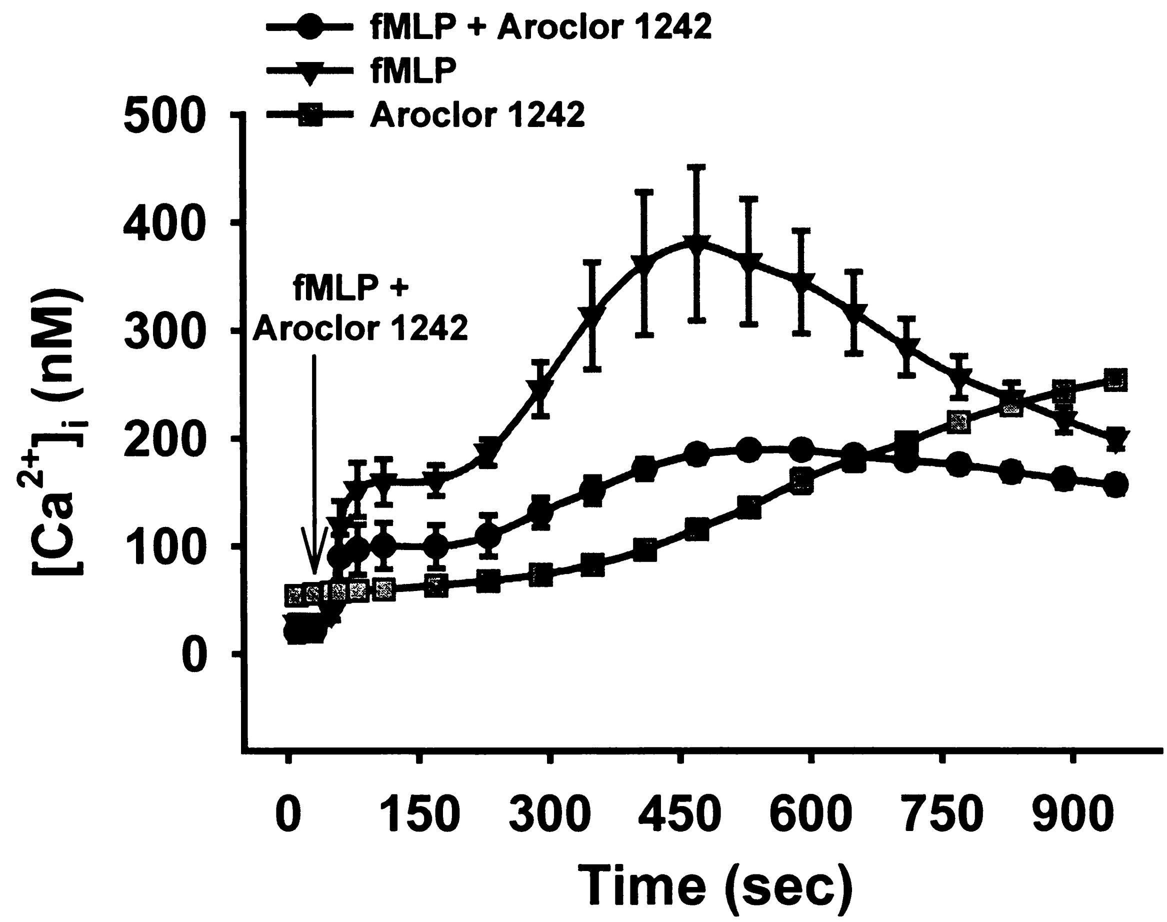


Figure II.4. ***Effects of simultaneous addition of fMLP and Aroclor 1242 on [Ca^{2+}]$_i$ in rat neutrophils.*** Cells were loaded with fura-2/AM as described in Materials and Methods. Fura-2-loaded cells were stimulated simultaneously with 2 µM fMLP and 10 µg/mL Aroclor 1242. Data for 2 µM fMLP and 10 µg/mL Aroclor 1242 are shown for comparison. Results are expressed as means ± SEM for four different experiments.

is due to the release of Ca^{2+} from an inositol trisphosphate (IP_3)-sensitive Ca^{2+} pool and the second phase is the result of Ca^{2+} influx from extracellular Ca^{2+} upon discharge of the intracellular stores (Anderson and Mahomed, 1997; Montero *et al.*, 1991; Krause *et al.*, 1993). These reports are supported by our observation that EGTA abolished the second phase of the fMLP-stimulated increase in $[Ca^{2+}]_i$ but did not affect the first phase (Figure II.2).

To determine whether changes in $[Ca^{2+}]_i$ induced by Aroclor 1242 would affect the increase in $[Ca^{2+}]_i$ stimulated by fMLP, experiments were performed in which cells were initially exposed to Aroclor 1242 and then treated with fMLP or vice-versa. In the absence of Aroclor 1242, $[Ca^{2+}]_i$ increased in a biphasic manner after addition of fMLP as described (Figure II.4). Simultaneous addition of Aroclor 1242 and fMLP induced an increase in $[Ca^{2+}]_i$ during the first phase that was similar to, but less pronounced than, that observed with fMLP alone and a significantly decreased rise in the second phase (Figure II.4). Addition of fMLP two minutes after exposure to Aroclor 1242 induced an immediate increase in $[Ca^{2+}]_i$ which emulated the first phase seen with fMLP alone. This was followed by a very small, second increase (Figure II.5.A). A similar but less pronounced effect was observed when fMLP was added five minutes after Aroclor 1242 (Figure II.5.B). However, when fMLP was added after

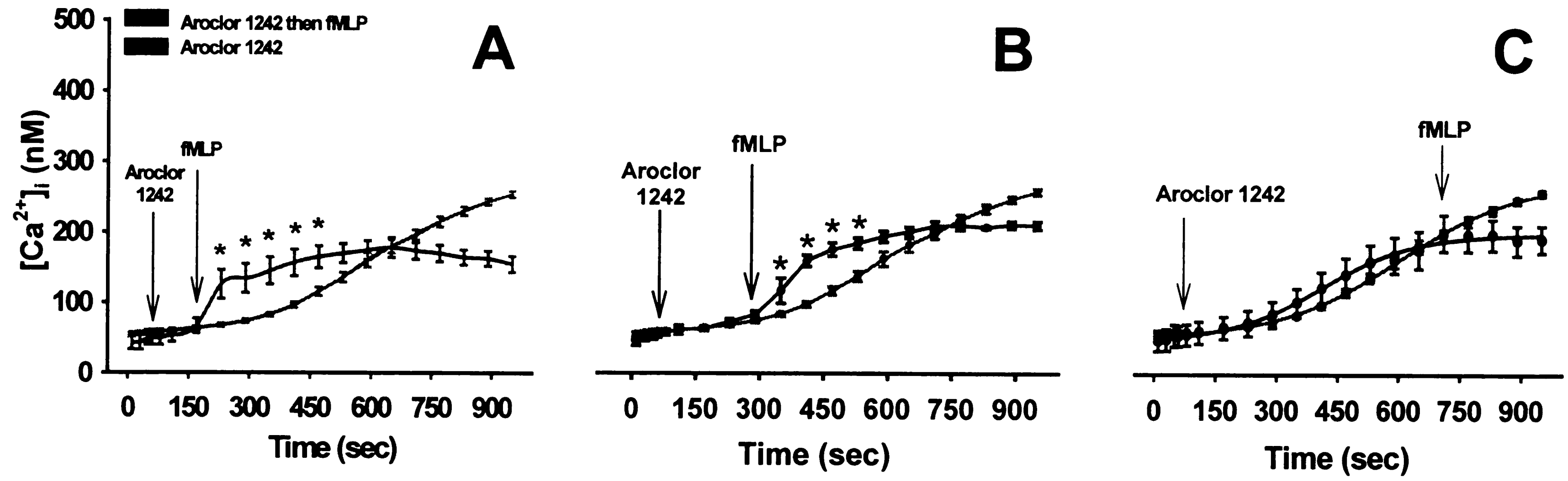


Figure II.5. ***Effect of fMLP applied at different times after exposure to Aroclor 1242 on [Ca^{2+}]$_i$ in rat neutrophils.*** Cells were loaded with fura-2/AM as described in Materials and Methods. Fura-2-loaded cells were stimulated with 10 µg/mL Aroclor 1242 and two (A), five (B) or ten (C) minutes later with 2 µM fMLP as indicated by the arrows. Results are expressed as means ± SEM for four-five different experiments. Results with Aroclor 1242 alone are repeated on each graph for comparison.

*. Significantly different from respective Aroclor 1242 time ($p<0.05$).

ten minutes of exposure to Aroclor 1242 (Figure II.5.C) no significant early increase in $[Ca^{2+}]_i$ was detected.

Reversing the order of agonist incubation showed that when Aroclor 1242 was applied exactly at the end of the first phase elicited by fMLP, most of the second phase of the response (Ca^{2+} influx) was blocked (Figure II.6). This observation suggests that Aroclor 1242 is interfering with the calcium channel functioning on the cell membrane.

II.5. Discussion

In neutrophils, increases in $[Ca^{2+}]_i$ have been observed not only with the bacterial chemotactic agent fMLP but also with other pathogen-related agonists including influenza virus (Hartshorn *et al.*, 1995), influenza A nucleoprotein (Cooper *et al.*, 1996), virus T21/DP107, and a synthetic leucine zipper-like domain of the HIV-1 envelope gp41 (Su *et al.*, 1999).

Results presented here demonstrate that PCBs increase $[Ca^{2+}]_i$ in rat neutrophils in a time-, concentration-, and structure-dependent way. Because Aroclor 1242-induced increases in $[Ca^{2+}]_i$ were significantly different from basal levels only after approximately 4-5 minutes and increased progressively up to 15 minutes, it is reasonable to speculate that this event may be associated with the generation of superoxide anion, which is significant after 10 min of cell treatment with Aroclor 1242 (Ganey *et al.*, 1993). This interpretation is consistent with the observation that

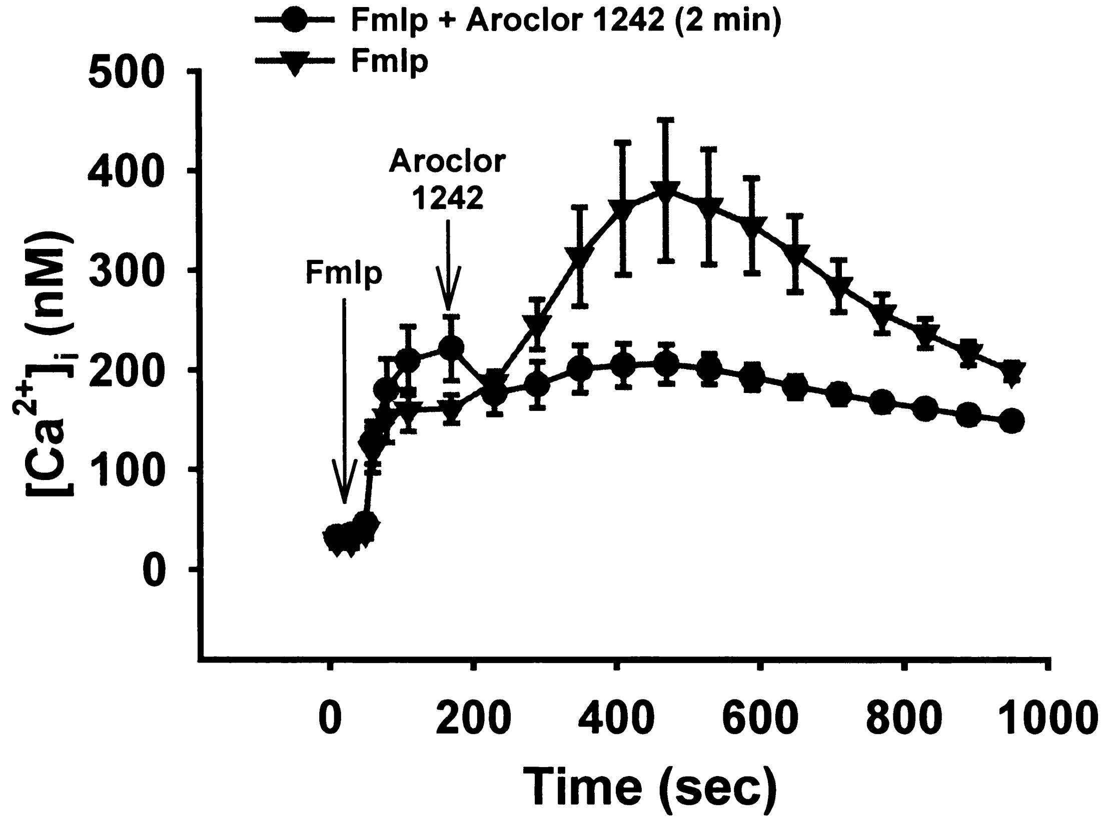


Figure II.6. ***Effect of Aroclor 1242 applied two minutes after exposure to fMLP on $[Ca^{2+}]_i$ in rat neutrophils.*** Cells were loaded with fura-2/AM as described in Materials and Methods. Fura-2-loaded cells were stimulated with 2 μM fMLP and two minutes later with 10 μg/mL Aroclor 1242 as indicated by the arrows. Results are expressed as means ± SEM for five different experiments.

PCB-stimulated superoxide anion generation is Ca^{2+}-dependent (Brown and Ganey, 1995).

One of the best characterized mechanisms of increasing $[Ca^{2+}]_i$ by an agonist is that elicited by fMLP, which releases Ca^{2+} from intracellular stores. It has been well documented that neutrophils have at least two distinct Ca^{2+} storage and release sites. One site is located peripherally under the plasma membrane, and the other is in the juxtanuclear space. Confocal imaging has demonstrated that the central Ca^{2+} storage site releases Ca^{2+} in response to fMLP (Pettit and Hallet, 1998) after PLCγ activation and release of IP_3 (Davies *et al.*, 1994). The Ca^{2+} store released by fMLP is deep within the neutrophil, and the cloud of elevated Ca^{2+} released from the store does not significantly affect the concentration of Ca^{2+} near the membrane (Davies and Hallett, 1996).

During the first 5 min of exposure to Aroclor 1242, co-treatment with fMLP triggers an increase in intracellular calcium that is significantly different from that observed with Aroclor 1242 alone. However, when the release of $[Ca^{2+}]_i$ by Aroclor 1242 has reached a plateau (10-15 minutes), addition of fMLP does not produce a further increase in $[Ca^{2+}]_i$. One interpretation for this observation is that the Aroclor 1242-sensitive Ca^{2+} store is the same that is targeted by fMLP. As a result of a progressive depletion of these stores by Aroclor 1242, the effect of fMLP in producing a direct release of intracellular Ca^{2+} from the same store is abrogated in a time-dependent manner. That the source of Ca^{2+} released upon Aroclor 1242 stimulation is intracellular is also suggested by the observation that $[Ca^{2+}]_i$ does not increase when Aroclor 1242 is added to fMLP-treated cells at a time when fMLP has caused intracellular stores to empty and before refilling of these from extracellular sources of Ca^{2+} has begun (Figure II.6). Further support arises from the lack of effectiveness of EGTA to block completely the Aroclor 1242-induced response. It is important, however, to point out that the use of EGTA data to rule out the intra- or extracellular-dependent source of Ca^{2+} in cell signaling should be interpreted with care. Neutrophils transferred to Ca^{2+}-free medium progressively lose their responsiveness to fMLP (Gabler *et al.*, 1986), suggesting that there exists a rapid, passive mobilization from intracellular stores to the extracellular space when a concentration gradient is present.

An alternative explanation for the lack of effect of fMLP after 10 mins incubation with Aroclor 1242 is that increases in $[Ca^{2+}]_i$ coming from a different store or extracellularly might regulate the IP_3 receptor to inhibit further stimulation by IP_3. The presence of Ca^{2+} binding sites in the IP_3 receptor (Patel *et al.*, 1999) permit this possibility. However, this is unlikely because Aroclor 1242 failed to increase $[Ca^{2+}]_i$ further after depletion of intracellular Ca^{2+} stores by fMLP (Figure II.6).

As mentioned before, fMLP releases Ca^{2+} from an IP_3-sensitive intracellular Ca^{2+} store. Although the results discussed above seem in agreement with the interpretation that Aroclor 1242 shares the same, central Ca^{2+} pool as fMLP, the time course associated to each chemical suggests different mechanisms are involved in release from the pools by these two stimuli. Despite this, both fMLP and Aroclor 1242 induce a very early (15-20 sec) and similar release of IP_3 (Tithof, *et al.*, 1995). It can be speculated that the absence of an early rise in $[Ca^{2+}]_i$ in Aroclor 1242-treated neutrophils in the face of early release of IP_3 is due to a change in the IP_3 receptor (IP_3R) functioning mediated by Aroclor 1242. However, the increases of $[Ca^{2+}]_i$ induced by fMLP during the first 5 minutes of treatment with Aroclor 1242 suggests that the IP_3R is functional, and consequently Aroclor 1242 may not be interfering with the interaction of IP_3 and its receptor. An alternative hypothesis could be compartmentalization for different PLCs stimulated by Aroclor 1242 and by fMLP. Accordingly, although an early release of IP_3 will be observed with both agonists, the IP_3 released by Aroclor 1242 will not diffuse rapidly into the cytosol and will require accumulation over time to reach the IP_3R. On the other hand, the IP_3 released by fMLP could be localized in the same compartment as the IP_3R and elicit a rapid release of Ca^{2+}. Another possibility is that Aroclor 1242 acts directly on ryanodine receptors (RyRs), which have been identified in the neutrophil (Rardon and Krause, 1992; Elferink and De Koster, 1995), to release Ca^{2+}. This has been observed in other tissues such as the hippocampus and skeletal and cardiac muscles (Wong and Pessah, 1996; Wong *et al.*, 1997a; Wong *et al.*, 1997b).

Aroclor 1242 blocked most of the Ca^{2+} influx characteristic of the second phase of the fMLP response. Because this phase arises from influx of extracellular Ca^{2+}, this observation suggests that Aroclor 1242 is interfering with the Ca^{2+} channel functioning on the cell membrane. This observation may shed light on the mechanisms by which Aroclor 1242 and *ortho*-chlorinated PCBs block fMLP-induced degranulation as reported previously (Ganey *et al.*, 1993; Olivero and Ganey, 1997). Although fMLP-induced degranulation is not totally abrogated by chelators of extracellular Ca^{2+}, the granule release process is optimal when extracellular Ca^{2+} is present. The inhibition of the fMLP-induced Ca^{2+} influx and the consequent decrease in neutrophil degranulation has been observed for other compounds as well, such as adenosine (Pasini *et al.*, 1990; Thiel and Bardenheuer, 1992), fenamates (Kankaanranta, *et al.*, 1995a-b), trifluoperazine and W-7 (Capuozzo *et al.*, 1997; Smith *et al.*, 1981; Blackwood *et al.*, 1995).

Increases in $[Ca^{2+}]_i$ are considered to be essential for activation (Korchak *et al.*, 1984) and subsequent bacteria killing by neutrophils (Wilsson *et al.*, 1996); however, these events by themselves are not sufficient for the optimal neutrophil capacity for degranulation, superoxide anion generation and aggregation (Korchak *et al.*, 1984). In this context, the physiological relevance of PCB-induced increases in $[Ca^{2+}]_i$ is not only associated with neutrophil activation but can be important in terms of impairment of neutrophil function. Depletion of intracellular Ca^{2+} stores by PCBs may lead to a decrease in neutrophil response when a subsequent stimulus is applied. For instance, it is known that reduction of $[Ca^{2+}]_i$ resulted in abrogation of neutrophil activation by macrophage-derived neutrophil chemotactic factor produced after Japanese encephalitis virus infection in mice (Srivastava *et al.*, 1994). Experimental evidence suggests that Influenza A virus deactivates neutrophils via an increased resting $[Ca^{2+}]_i$ (Hartshorn *et al.*, 1988). Furthermore, the requirement for extracellular Ca^{2+} during neutrophil phagocytosis (Sawyer *et al.*, 1985) suggests that PCBs could impair bacterial clearing given the blockage by Aroclor 1242 of Ca^{2+} influx in fMLP-treated neutrophils. Thus, taken together the results presented here suggest that Aroclor 1242 mobilizes Ca^{2+} from IP_3-sensitive intracellular stores and also blocks Ca^{2+} influx following IP_3R-mediated depletion of intracellular stores. These events can have implications in the activity of the immune system to battle pathogens.

Chapter III

ROLE OF PROTEIN PHOSPHORYLATION IN ACTIVATION OF PHOSPHOLIPASE A_2 BY THE POLYCHLORINATED BIPHENYL MIXTURE AROCLOR 1242

III.1. Summary

Polychlorinated biphenyls (PCBs) activate neutrophils to induce degranulation and undergo superoxide anion production through a mechanism that involves stimulation of phospholipase A_2 (PLA_2). Since the biochemical processes leading to the PCB-induced activation of PLA_2 are unknown, the objective of this study was to determine whether or not protein phosphorylation, a common intracellular pathway, has a role in this mechanism. Isolated, rat neutrophils were labeled with $[^3H]$-arachidonic acid ($[^3H]$-AA), and activation of PLA_2 was determined from release of radioactivity into the medium. Exposure to the PCB mixture Aroclor 1242 induced release of $[^3H]$-AA, and pretreatment with bromoenol lactone (BEL), an inhibitor of Ca^{2+}-independent PLA_2, diminished release by 80%. Genistein, an inhibitor of tyrosine kinases, caused a small but significant decrease in Aroclor 1242-stimulated release of $[^3H]$-AA. Daidzein, a genistein analog with no activity to inhibit tyrosine kinases, had no effect on $[^3H]$-AA release. An inhibitor of p38 mitogen-activated protein kinase (MAPK), SB203580, did not affect Aroclor 1242-induced PLA_2 activity at concentrations selective for p38 MAPK; however, PD 98059, which inhibits MAPK kinase (MEK), decreased $[^3H]$-AA release to about the same extent as genistein. Treatment of neutrophils with Aroclor 1242 induced phosphorylation of p44 MAPK, and this phosphorylation was unaffected by

BEL but was inhibited by PD 98059. Staurosporine, a non-selective inhibitor of protein kinase C (PKC), inhibited PCB-induced release of [^{3}H]-AA. Ro 32-0432, a selective inhibitor of PKC_{α} and $PKC_{\beta1}$, produced the greatest degree of inhibition (40%) among the tested protein kinase inhibitors. These results suggest that tyrosine kinases, PKC, and the MEK/MAPK pathway are involved in a fraction of Aroclor 1242-induced activation of PLA_2.

III. 2. Introduction

Polychlorinated biphenyls are widespread, persistent chemicals present in almost all the ecosystems and trophic levels. Theoretically, chlorine substitutions in the aromatic ring lead to the existence of 209 congeners. Congeners with chlorine substitution in the *ortho* position are termed non-coplanar, whereas congeners without *ortho* chlorines are considered coplanar PCBs. This chemical characteristic defines many of the toxicological profiles of PCBs as non-dioxin-like or dioxin-like for *ortho*- and non-*ortho*-substituted congeners, respectively. In humans, about 130 individual PCB congeners have been detected in blood, milk and other tissue samples (Geisy and Kannan, 1998). Of interest is the fact that *ortho*-chlorinated PCBs are the most commonly found in human tissue after environmental or occupational exposure (Korrick and Altshul, 1998).

The toxicology of PCBs has been extensively studied worldwide in diverse species. Some of the effects that have been attributed to PCBs include neurotoxicity (Choksi *et al.*, 1997; Seegal, 1999; Rice *et al.*, 1999; Hany *et al.*, 1999b), endocrine disruption (Desaulniers *et al.*, 1999; Kato *et al.*, 1999; Gould *et al.*, 1999), impairment of reproduction (Hugla and Thome, 1999), immunosuppression (Chang *et al.*, 1982; Arnold *et al.*, 1999; Wu *et al.*, 1999), and cancer-related pathologies (Moysich *et al.*, 1999; Nakanishi *et al.*, 1999), although there is controversy around this latter effect (Dorgan *et al.*, 1999).

Immunosuppression has been one of the most studied and polemical toxic effects of PCBs (Lahvis *et al.*, 1995; Harper *et al.*, 1995; Chang *et al.*, 1982). The presence of these contaminants in different species has been associated with impairment of non-specific immunity, including changes in natural killer function (De Swart *et al.*, 1996) and neutrophil counts (Wu *et al.*, 1999), and detriment of macrophage phagocytosis (Ville *et al.*, 1995). A well known effect of PCBs in the immune system is the activation of neutrophils (Ganey *et al*, 1993). Exposure *in vitro* to the PCB mixture Aroclor 1242, which contains mostly *ortho*-chlorinated biphenyls (Geisy and Kannan, 1998), activates several intracellular signals in neutrophils including phospholipase A_2 (PLA_2), tyrosine kinases (TKs) and phospholipase C (PLC) (Tithof *et al.*, 1997; Tithof *et al.*, 1998, Tithof *et al.*, 1995). The interdependence of these pathways in PCB-induced neutrophil stimulation is not known. One of the early events during exposure of neutrophils to PCBs is the activation of a Ca^{2+}-independent PLA_2 ($iPLA_2$), and inhibition of this enzyme abrogates PCB-induced production of superoxide anion by the cells (Tithof *et al.*, 1998).

In general, PLA_2 releases arachidonic acid (AA) from membrane phospholipids to function as a second messenger and a substrate for cyclooxygenases and lipoxygenases. Several isoforms of PLA_2 have been identified (See Table I.1), raising questions about the specific functions of each of them. Among its functions, $iPLA_2$ participates in phospholipid remodeling by regulating the incorporation of AA into membrane phospholipids by providing the lysophospholipid acceptor employed in the acylation reaction (Balsinde *et al.*, 1995). $iPLA_2$ can exist both in the cytosol (Hazen *et al*, 1990) and associated with membranes (Hazen *et al.*, 1991). The regulation of this isoform of PLA_2 is incompletely understood; however, ATP has been identified as one of the regulators of its enzymatic activity (Hazen and Gross, 1991; Ma *et al.*, 1998). Furthermore, $iPLA_2$ can be found forming complexes with other proteins such as phosphofructokinase (Hazen and Gross, 1993) and calmodulin (Wolf and Gross, 1996), which may represent other levels of regulation.

Given that $iPLA_2$ plays an important role in neutrophil stimulation by PCBs, it is of interest to understand its regulation during exposure to

PCBs. Protein kinases also play a critical role in PCB-induced activation of neutrophils (Tithof *et al.*, 1997), therefore we began exploring whether activation of $iPLA_2$ depends on phosphorylation pathways. Accordingly, the purpose of the present study was to examine the effects of pharmacological intervention of phosphorylation cascades on activation of $iPLA_2$ by PCBs.

III. 3. Materials and methods

III. 3. A. Chemicals

PCBs were purchased from ChemService (West Chester, PA). BEL was purchased from Biomol (Plymouth Meeting, PA). [^{3}H][5,6,8,9,11,12,14,15]-AA ([^{3}H]-AA; 180-240 Ci/mmol) was purchased from DuPont NEN (Boston, MA). Genistein and daidzein were obtained from Sigma Chemical Company (St. Louis, MO). PD 98059, SB 203580, staurosporine, and Ro 32-0432 were purchased from Calbiochem (San Diego, CA). Concentrations of inhibitors were selected based on literature reports of effective concentrations and low cytotoxicity.

III. 3. B. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats by glycogen elicitation (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt

solution (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.

III. 3. C. Exposure to PCBs

PCB stock solutions were prepared by dissolution in N,N-dimethylformamide (DMF). Neutrophils (2×10^6) were suspended in HBSS (1 mL) in borosilicate glass test tubes, 12 x 75 mm (VWR, Chicago, IL), and 1 µL of the PCB stock solution was added to the tubes to achieve the desired concentration. Control neutrophils received 1 µL of DMF.

III. 3. D. 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 [^{3}H]-AA for two hours, gently shaking at 37°C. Neutrophils were then washed twice with Mg^{2+}- and Ca^{2+}-free HBSS. The cell count was adjusted so that the final concentration of neutrophils was 2×10^6/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 routinely between 80-88%. PLA_2 activity was measured as the release of [^{3}H]-AA from labeled neutrophils (Tithof *et al.*, 1998) incubated with inhibitors for 20 minutes (37°C) and then

exposed to Aroclor 1242 at 37°C for 30 min, unless otherwise stated. At the end of each incubation, neutrophils were placed on ice and spun in a centrifuge 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.

III. 3. E. Detection of phosphorylated p42/p44 MAPKs

Neutrophils (3×10^6 /mL) were suspended in HBSS and incubated with inhibitors for 20 min at 37°C before stimulation with 10 µg/mL Aroclor 1242 or vehicle and incubated for an additional 30 min at 37°C. After incubation, samples were spun in a centrifuge at 4°C for 10 min. The pellets were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in 300 µL lysing buffer (20% sodium dodecyl sulfate, SDS) for one hour and then sonicated for 30 sec. Samples were denatured by boiling for 5 min at 100°C and separated on a denaturing, 10% SDS polyacrylamide gel. Approximately 50 µg of protein were added to each lane. Proteins were transferred electrophoretically to nitrocellulose membranes. After transfer, membranes were blocked for 3-4 hours in Tris-buffered saline (TBS) + Tween 20 (1%) (TBS-T) containing 4% chicken ovalbumin and 0.25% sodium azide. Membranes were incubated with mouse anti-phosphorylated MAPK antibody (New England BioLabs) (1:2500) in blocker for two hours with constant rocking. Blots were washed three times with TBS-T (30, 5 and

5 min) and once with TBS (5 min). Goat anti-mouse IgG linked to horseradish peroxidase (1:7500) in TBS was added for 1 hour. Blots were washed using the same protocol as described above. Enhanced chemiluminiscence using Amersham reagents was performed to visualize labeled, phosphorylated proteins.

III. 3. F. Cytotoxicity assay

Release by neutrophils of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as an indicator of cytotoxicity. The method for sample preparation was the same as that described above. LDH activity present in the supernatant fluid was measured according to the method of Bergmeyer and Bernt (1974).

III. 3. G. Statistical analysis

Data are presented as the mean ± SEM from at least 4 different experiments conducted in triplicate. To calculate percent of control for release of [^{3}H]-AA in studies using inhibitors, first the values for release of [^{3}H]-AA in the absence of Aroclor 1242 at each concentration of inhibitor (including vehicle control) were subtracted from the corresponding values in the presence of Aroclor 1242 to calculate the specific Aroclor 1242-induced release. Second, the value for Aroclor 1242-induced release in the absence of inhibitor (vehicle control) was taken to be 100%. Angular transformation

(arcsin) was used on percentage data to generate an approximated gaussian distribution. Detection of significant differences among treatments was determined using analysis of variance (ANOVA) and Tukey test as a post hoc test. When angular transformation did not produce gaussian data, Kruskal Wallace ANOVA on Ranks was used. Two-tailed p-values <0.05 were considered significant.

III. 4. RESULTS

III.4.A. Aroclor 1242 induced release of [^{3}H]-AA from neutrophils

The time course of cumulative release of [^{3}H]-AA (ie, PLA_2 activity) induced in rat neutrophils by 10 μg/mL Aroclor 1242 is presented in Figure III.1A. [^{3}H]-AA release increased exponentially for the first 30 min, after which little additional [^{3}H]-AA was observed. Based on this graph, all the experiments measuring PLA_2 activity were conducted using a 30-min incubation period.

An important feature of $iPLA_2$ is that it can be selectively, irreversibly inhibited by BEL (Hazen *et al.*, 1991) with 1000-fold greater specificity than for Ca^{2+}-dependent PLA_2. BEL inhibited Aroclor 1242-induced

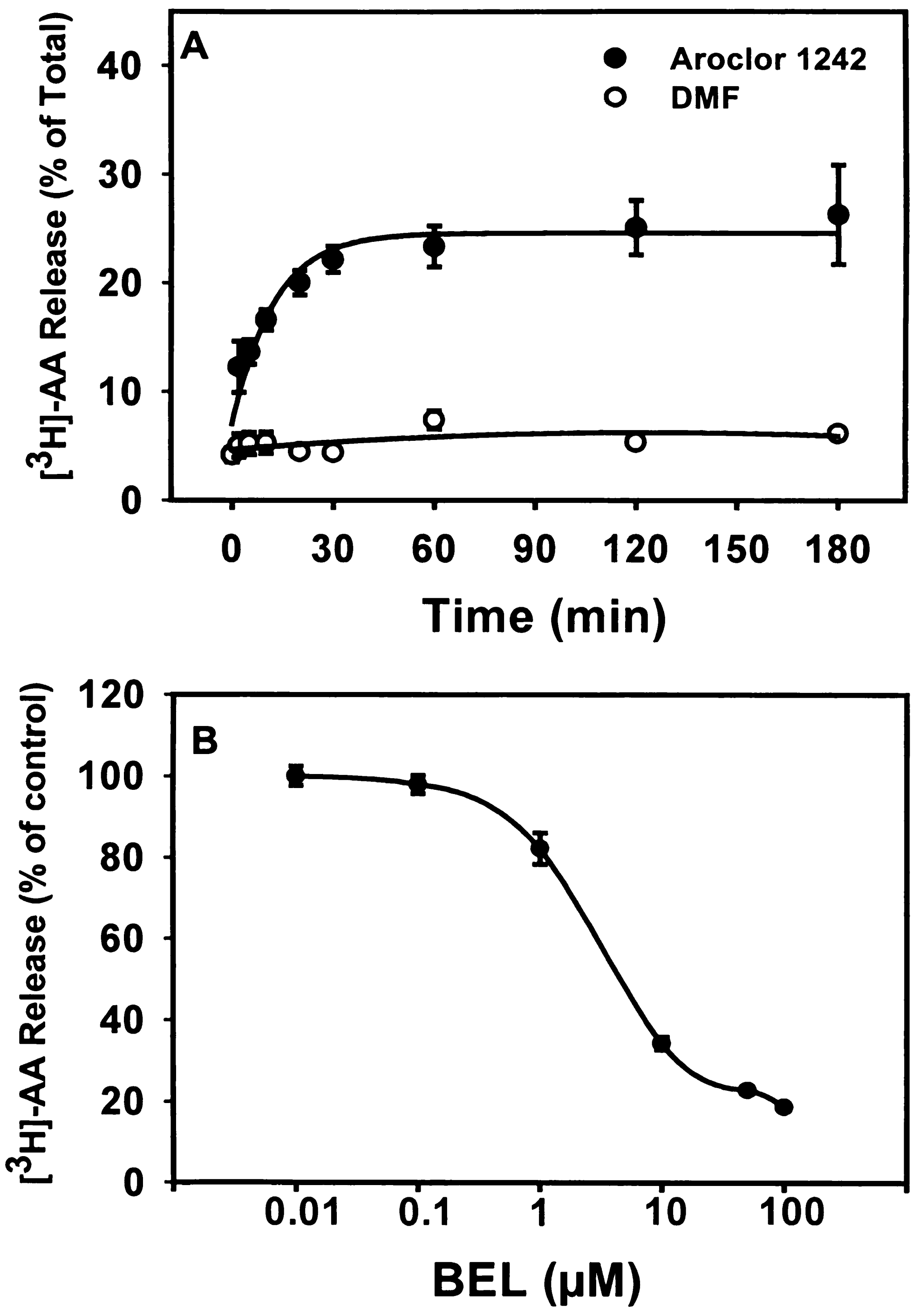


Figure III.1. ***A. Time-course for Aroclor 1242-induced release of [^{3}H]-AA from labeled neutrophils. B. Dose-response curve for BEL-induced inhibition of Aroclor 1242- elicited release of [^{3}H]-AA.*** Cells were exposed to 10 µg/mL Aroclor 1242, and release of [^{3}H]-AA was determined as described in Materials and Methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

release of [^{3}H]-AA (Figure III.1.B), consistent with previous results (Tithof *et al.*, 1998). At 100 μM BEL, total inhibition was not achieved.

III.4.B. Involvement of protein kinase pathways in Aroclor 1242-induced activation of PLA$_2$

Aroclor 1242 induces a rapid protein tyrosine phosphorylation in neutrophils (Tithof *et al.*, 1997). Furthermore, either inhibition of tyrosine kinase with genistein or inhibition of iPLA$_2$ with BEL diminished production of superoxide anion by Aroclor 1242-treated neutrophils (Tithof *et al.*, 1997, 1998). It was of interest to examine whether these pathways are interrelated; ie, is iPLA$_2$ activation dependent on tyrosine kinase activity? Genistein significantly inhibited Aroclor 1242-induced PLA$_2$ activity in a dose-dependent manner (Figure III.2) with a maximal inhibition of approximately 20%. After 5 min exposure to Aroclor 1242, inhibition was observed with concentrations of genistein ≥ 1 μM. After 30 min of Aroclor 1242 exposure larger concentrations of genistein (≥ 50 μM) were required to inhibit a fraction of the release of [^{3}H]-AA. The concentrations of genistein which produced significant inhibition also blocked respiratory burst in fMLP-stimulated neutrophils (Combadiere *et al.*, 1993) and are consistent with those reported to inhibit phosphorylation of cPLA$_2$ (Ambs *et al.*, 1995). Daidzein, a genistein analog without effect in tyrosine phosphorylation, had no significant effect on release of [^{3}H]-AA in

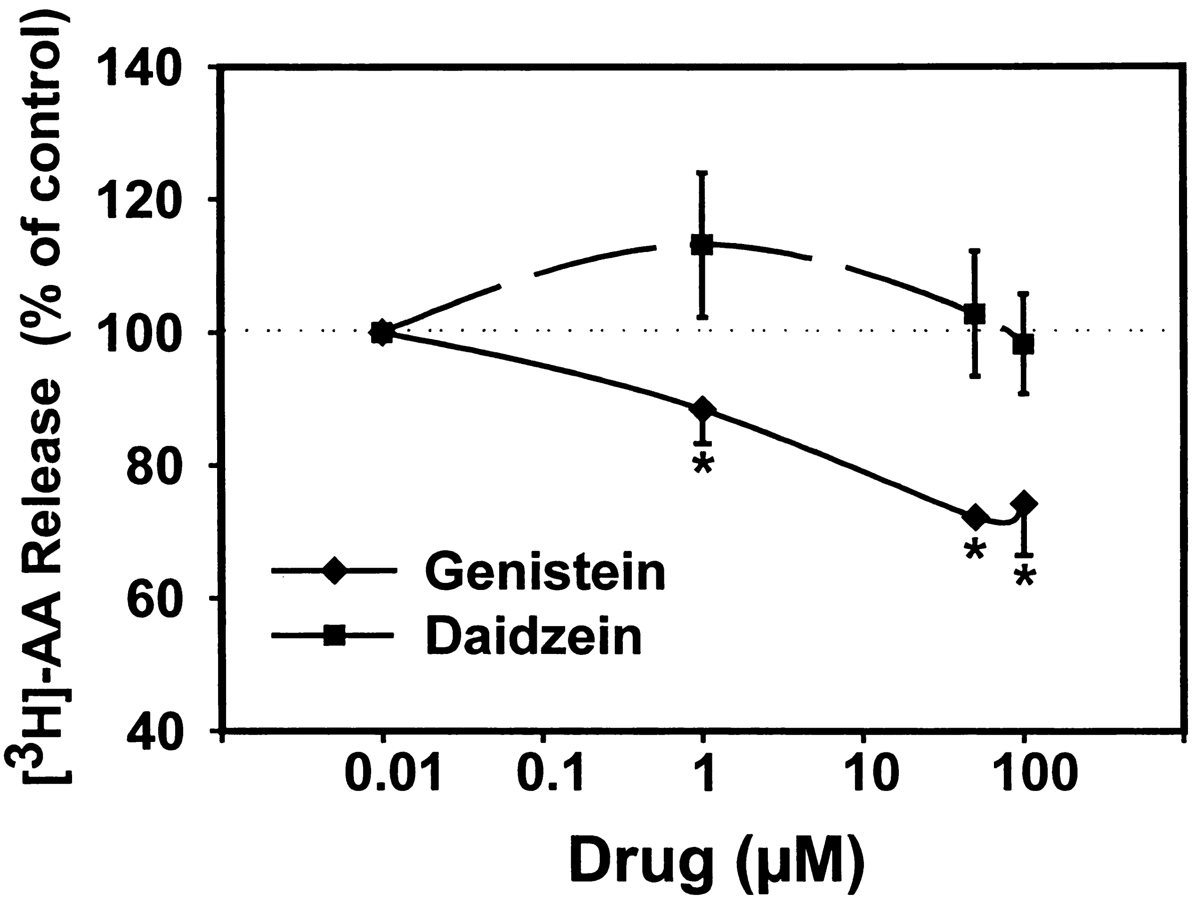


Figure III.2. ***Dose-response curve for inhibition of Aroclor 1242 (10 µg/mL)-induced release of [^{3}H]-AA by Genistein or Daidzein.*** Cells were incubated with the indicated concentrations of Genistein or Daidzein as described in Materials and Methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 10 µg/mL Aroclor 1242 alone.

Aroclor 1242-treated neutrophils. These results suggest that tyrosine phosphorylation is responsible for a fraction of Aroclor 1242-induced PLA_2 activation.

To investigate the possible involvement of a phosphorylation cascade leading to PLA_2 activation induced by Aroclor 1242, the effects of SB 203580, an inhibitor of p38 mitogen activated protein kinase (MAPK), and PD 98059, a MAPK kinase (MEK) inhibitor, were examined. At concentrations below 50 μM, SB 203580 did not affect Aroclor 1242-induced [^{3}H]-AA release (Figure III.3.A). At 50 and 100 μM, SB 203580 caused about a 20% decrease in release of [^{3}H]-AA. PD 98059 caused a small but significant decrease in [^{3}H]-AA release at 1 μM (Figure III.3.B), but the extent of inhibition at larger concentrations (50 and 100 μM) was about 20% (Figure III.3.B). These results suggest that a MAPK pathway involving MEK and p42/p44 MAPK is important for a fraction of the AA release observed upon stimulation of neutrophils with PCBs.

To examine the interdependence of PCB-induced activation of PLA_2 and MAPK, Western immunoblotting assays were conducted. Aroclor 1242 induced the phosphorylation of p44 MAPK, commonly referred to as extracellular signal-regulated kinase (ERK-1), and increased the phosphorylation of the constitutively expressed, phosphorylated p42 MAPK (ERK-2) (Figure III.4). This event was insensitive to BEL but was blocked by PD 98059.

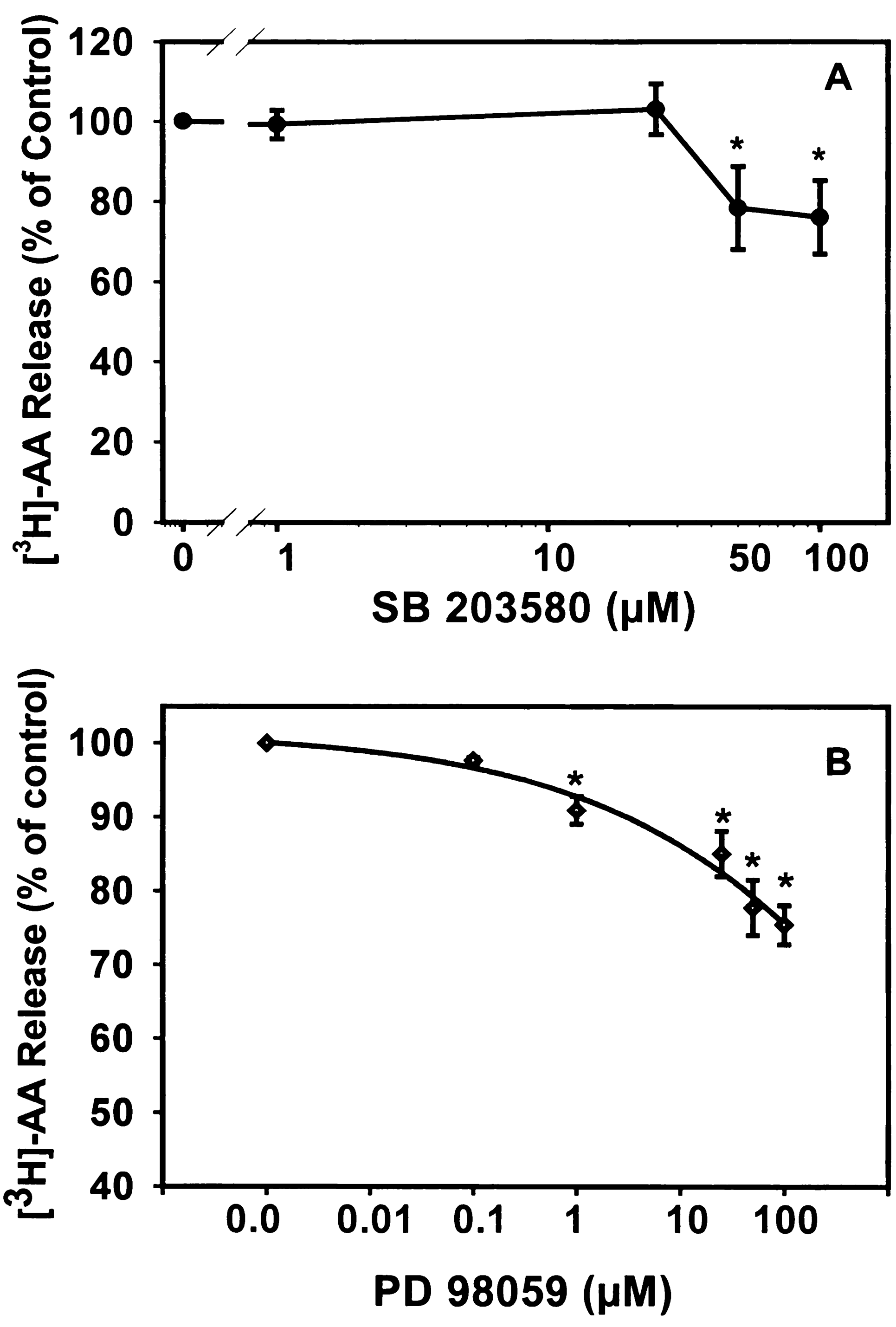


Figure III.3. ***A. Dose-response curves for (A) SB 203580- and (B) PD 98059-induced inhibition of Aroclor 1242 (10 µg/mL)-stimulated release of [^{3}H]-AA.*** Cells were incubated with the indicated concentrations of SB 203580 or PD 98059 as described in Materials and Methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 10 µg/mL Aroclor 1242 alone.

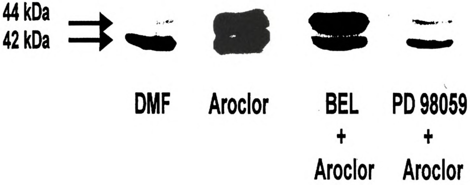


Figure III.4. ***Aroclor 1242-stimulated MAPK phosphorylation in rat neutrophils and effects of BEL and PD 98059.*** Cells were treated with DMF or with Aroclor 1242 (10 µg/mL) in the absence or presence of BEL (10 µM) or PD 98059 (50 µM). Phosphorylated MAPKs were detected as described in Materials and Methods. Results are representative of two separate experiments.

In BEL-treated neutrophils, treatment with either genistein or PD 98059 increased slightly the inhibition of [^{3}H]-AA release (63% for either genistein plus BEL or PD 98059 plus BEL vs 56% with 10 μM BEL alone). This minor increase in inhibition suggests that genistein and PD 98059 inhibit the same pathway as BEL, providing further support for the hypothesis that tyrosine kinases contribute in part to the activation of iPLA$_2$. Although Western analysis does not measure activity, increased phosphorylation is in general accompanied by increased activity (Hashimoto *et al.*, 1999; Qiu and Leslie, 1994) so that these results imply that Aroclor 1242 increased activity of p44 MAPK.

Staurosporine, a non-specific PKC inhibitor, slightly increased [^{3}H]-AA at 20 nM and had no effect at concentrations between 20 and 500 nM; however, at 1 μM it decreased Aroclor 1242-induced release of [^{3}H]-AA by 35% (Figure III.5.A). Ro 32-0432, a highly specific inhibitor of PKC$_\alpha$ and PKC$_{\beta 1}$ (Wilkinson *et al.*, 1993), decreased release of [^{3}H]-AA in a dose-related manner with significant inhibition ($\approx$ 40%) at 5-10 μM (Figure III.5.B). Ro 32-0432 also increased inhibition of [^{3}H]-AA release in BEL-treated neutrophils (80% vs. 56% BEL alone). These data suggest that PKC may be involved not only in iPLA$_2$ activation but also in the mechanism which accounts for the residual [^{3}H]-AA release not sensitive to BEL.

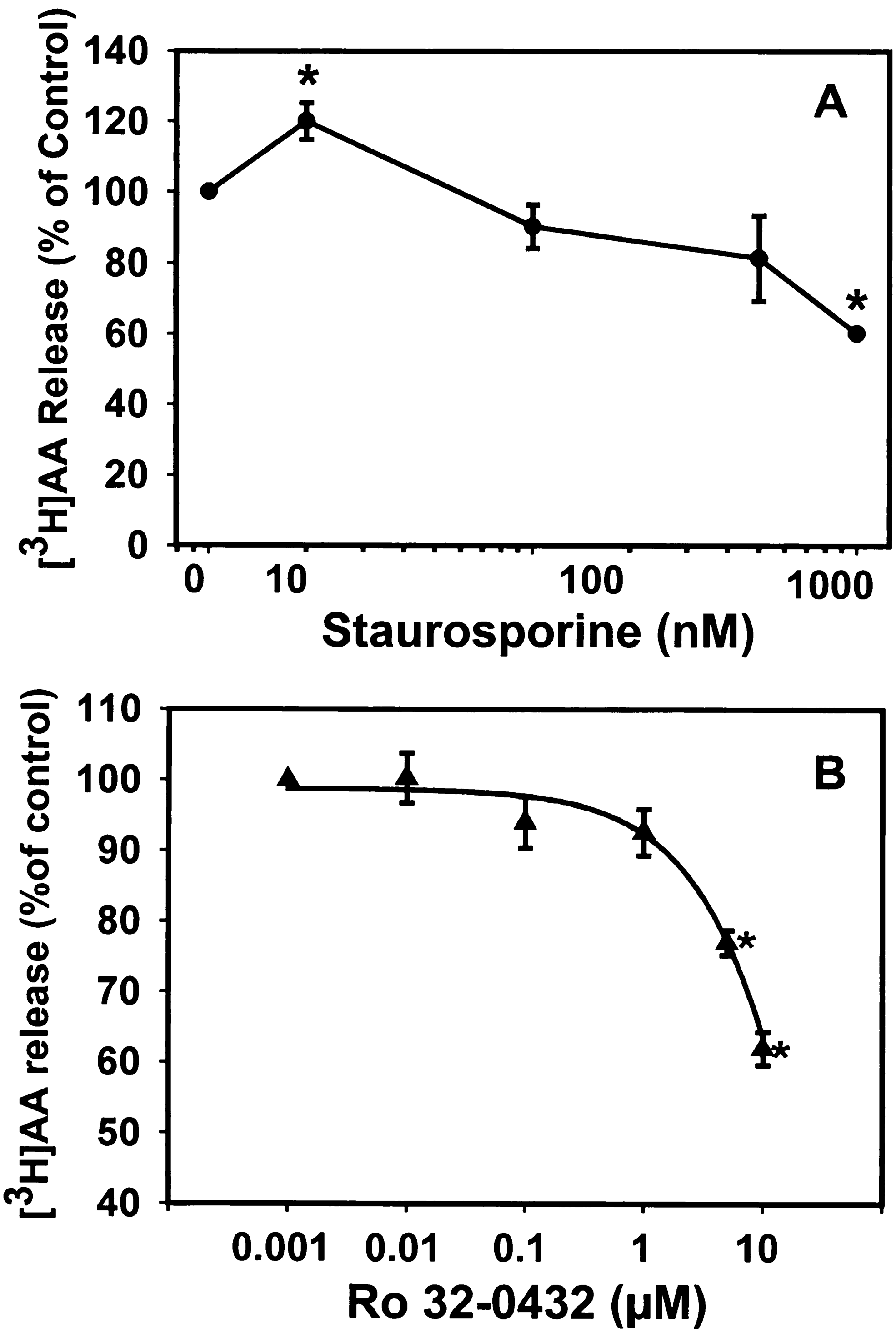


Figure III.5. ***Dose-response curves for (A) Staurosporine- and (B) Ro 32-0432-induced inhibition of Aroclor 1242 (10 μg/mL)-stimulated release of [^{3}H]-AA.*** Cells were incubated with the indicated concentrations of Staurosporine or Ro 32-0432 as described in Materials and Methods. Data are expressed as mean ± S.E.M of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 10 μg/mL Aroclor 1242 alone.

III. 5. Discussion

Previous studies have shown that Aroclor 1242-mediated neutrophil activation involves the participation of PLA_2 and tyrosine kinases (Tithof *et al.*, 1998; Tithof *et al.*, 1997). New findings reported here suggest that part of the Aroclor 1242-induced PLA_2 activity is dependent on a phosphorylation cascade involving MEK, and that Aroclor 1242-induced phosphorylation of MAPK is independent from the activation of $iPLA_2$.

Inhibition of $iPLA_2$ activity by BEL was maximal at 50 µM (Fig III.1B), which is consistent with saturation of inhibition observed by Lehman *et al.*, (1993). The maximal inhibition of $[^3H]$-AA release was 80-90%; the remaining 10-20% of $[^3H]$-AA release might be associated with the activation of other phospholipases including Ca^{2+}-dependent isoforms of PLA_2 which are not sensitive to BEL at these concentrations.

It has been demonstrated previously that inhibition of tyrosine kinase with genistein diminished production of superoxide anion by Aroclor 1242-treated neutrophils (Tithof *et al.*, 1997). Similarly, BEL inhibited Aroclor 1242-stimulated superoxide anion generation (Tithof *et al*, 1998). It was of interest to examine whether these pathways are interrelated; ie, is $iPLA_2$ activation dependent on tyrosine kinase activity? Treatment with genistein reduced $[^3H]$-AA release induced by Aroclor 1242 by about 20% (Fig. 2), suggesting that tyrosine kinases may play a role in activation of PLA_2.

MAPKs, particularly p38 MAPK, are activated by tyrosine phosphorylation (Krump *et al.*, 1997), and a MAPK activity dependent on tyrosine phosphorylation has been observed in fmlp-activated neutrophils (Torres *et al.*, 1993). Both p38 MAPK (Borsch *et al.*, 1998) and ERK1/ERK2 (Nemenoff *et al.*, 1993, Milella *et al.*, 1997) can directly phosphorylate and activate the cytosolic PLA_2 ($cPLA_2$). The use of SB 203580 and PD 98059 has been considered a powerful tool to differentiate between different MAPK activities (Gould *et al.*, 1995). SB 203580 is a potent and selective inhibitor of p38 MAPK, whereas PD 98059 inhibits phosphorylation of ERK1/ERK2 (Zu *et al.*, 1998). SB 203580 specifically abolishes the enzymatic activity of p38 MAPK (Ridley *et al.*, 1997) at concentrations as low as 1-10 µM (Foltz *et al.*, 1997; Cuenda *et al.*, 1995). The SB 203580 concentrations that significantly decreased Aroclor 1242-induced PLA_2 activity (50-100 µM) (Fig. 3A) presume a minor role for p38 MAPK in this response; however, a total lack of participation of p38 MAPK cannot be ruled out from these data.

In contrast to SB 203580, inhibition of $[^3H]$-AA release was observed with concentrations of PD 98059 selective for MEK (Alessi *et al.*, 1995). The dose-response curve observed for PD 98059 suggests that MAPK is involved in about 20-25% of $[^3H]$-AA released during Aroclor 1242-induced activation of PLA_2 (Fig. 3B). The lack of inhibition of Aroclor 1242-induced phosphorylation of p44 MAPK by BEL (Fig. 4) also indicates that

activation of this MAPK occurs upstream of activation of PLA_2. Tyrosine kinase activation probably occurs prior to stimulation of PLA_2 as well because genistein inhibited [^{3}H]-AA release. Activation of tyrosine kinases may contribute to activation of ERK1/ERK2 as has been described (Peavy and Conn, 1998; Luconi *et al.*, 1998). Interestingly, the MEK pathway can be activated through ras proteins including H-ras, K-ras, N-ras, and R-ras (Waddick and Uckun, 1998), and the function of these proteins has been implicated in the etiology of large variety of human tumors (Beaupre and Kurzrock, 1999; Kato *et al.*, 1999). If these same pathways activated by Aroclor 1242 in neutrophils are also involved in other cell types and if a ras-dependent pathway is involved in Aroclor 1242-induced cell activation, it may explain some of the reported associations between *ortho*-chlorinated biphenyls and cancer (Gribaldo *et al.*, 1998; Haag *et al.*, 1997) and promotion activity observed for some PCB mixtures (Nakanishi *et al.*, 1999). For instance, it has been suggested that PLA_2 activity is increased in colorectal tumors (Hendrickse *et al.*, 1995). Moreover, it is known that inhibition of the MEK pathway can suppress as much as 80% of tumor growth in mice (Sebolt *et al.*, 1999; Seufferlein *et al.*, 1999). Thus, it may be that activation of both PLA_2 and the MEK pathway induced by Aroclor 1242 play an important role in some of the effects observed in other tissues following exposure to PCB mixtures.

Among the protein kinase inhibitors, the greatest degree of inhibition of Aroclor 1242-induced PLA_2 activity was produced by the PKC inhibitors staurosporine and Ro 32-0432, which suggests a key role for PKC (Figs. III.5A and III.5B). Ro 32-0432 is highly selective for PKC_{α} and $PKC_{\beta1}$ (Wilkinson *et al.*, 1993), two Ca^{2+}-dependent isoforms of PKC (Lin and Chen, 1998). The effectiveness of Ro 32-0432 at blocking Aroclor 1242-induced activation of PLA_2 suggests that specific isoforms of PKC may be critical to cell activation. These data are consistent with reports that Aroclor 1242 stimulates PKC in cerebellar granule cells (Kodavanti *et al.*, 1995).

PLA_2 activation can be triggered following activation of the MEK/MAPK pathway (Milella *et al.*, 1997; Wheeler *et al.*, 1997) and in turn, the MEK pathway can be stimulated in a PKC-dependent manner (Zhang *et al.*, 1998; Kumar *et al.*, 1997; Slevin *et al.*, 1998), and PKC activation can be initiated by tyrosine phosphorylation (Bradford and Soltoff, 1998). These observations can be synthesized to a model involving the phosphorylation cascade: tyrosine kinase→PKC→MEK→ERK (Aharonovitz *et al.*, 1998; Muthusamy and Leiden, 1998) that might lead to subsequent phosphorylation and activation of PLA_2 (Huwiler *et al.*, 1997). However, activation of PLA_2 by PKC can be independent from p42/p44 MAPK (Husain and Abdel, 1998).

The data presented here suggest that tyrosine kinase, PKC, MEK and ERK contribute to a fraction of total PLA_2 activity induced by Aroclor 1242. An interesting possibility is that the phosphorylation cascade does not target PLA_2 directly but indirectly through other proteins. For instance, MAPK (Mizutani *et al.*, 1993) and PKC (Regnouf *et al.*, 1995) can each phosphorylate annexins, proteins with known inhibitory activity on PLA_2 (Mira *el al.*, 1997; Haigler *et al.*, 1987). Phosphorylation may initiate conformational changes or subcellular translocations leading to disinhibition of PLA_2.

An interesting question deals with the mechanism of the initial activation of the phosphorylation-dependent pathway. At least three possibilities can be considered. First, Aroclor 1242 may activate tyrosine kinases or PLA_2 directly. PCBs have been shown to interact directly with PKC, inducing its activation and membrane translocation (Kodavanti *et al.*, 1995; Kodavanti and Ward, 1998). In addition, Aroclor 1242 elicited activation of PLA_2 in cell lysates (Tithof *et al.*, 1998), although it is unknown whether this was a direct activation of the enzyme or occurred through other cytosolic factors. A second possibility is the direct activation of a receptor by PCBs, leading to enzyme phosphorylation. Receptor-mediated modulation of $iPLA_2$ has been reported to occur through a G_q/G_{11}-coupled mechanism (Derrickson and Mandel, 1997). Although G_q can be coupled to activation of PKC, tyrosine kinase and MAPK (Naor *et al.*, 1998), it has

been reported that in neutrophils production of superoxide activation is unlikely to proceed through a G protein-dependent mechanism (Tithof *et al.*, 1997). Structure-activity relationships with PCBs suggest that such a receptor is not the Ah receptor, but its identity is unknown (Olivero and Ganey, 1998). There is some evidence, however, that PCBs interact with nerve growth factor receptors (Angus and Contreras, 1995), so the possibility for receptor interaction beyond the AhR exists.

A third possibility may be related to PCB-dependent autocrine activation of neutrophils. Recently it has been reported that secretory PLA_2 ($sPLA_2$) can activate neutrophils (Zallen *et al.*, 1998) and other cell types (Hernandez *et al.*, 1998) as a ligand which interacts with specific receptors on the membrane surface and independently of its enzymatic activity. Neutrophils store $sPLA_2$ in granules and secrete it in response to soluble stimuli (Rosenthal *et al.*, 1995). Neutrophil degranulation after exposure to PCBs occurs in a structure-dependent manner (Olivero and Ganey, 1998). Thus, PCBs may initially induce degranulation, releasing $sPLA_2$ which activates the phosphorylation cascade as described above.

In summary, the PCB mixture Aroclor 1242 activates neutrophils through multiple mechanisms. PLA_2 activation by Aroclor 1242 occurs through different signal transduction pathways involving tyrosine kinase, PKC, MEK and ERK1/ERK2. The above findings add a new aspect toward the recognition of cellular targets for PCBs.

Chapter IV

CALCIUM/CALMODULIN-DEPENDENT REGULATION OF PHOSPHOLIPASE A_2 DURING NEUTROPHIL ACTIVATION BY POLYCHLORINATED BIPHENYLS

IV.1. Summary

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 was examined. The commercial PCB mixture Aroclor 1242 induced activation of PLA_2 and promoted an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). Bromoenol lactone (BEL), an inhibitor of the Ca^{2+}-independent PLA_2 ($iPLA_2$) isoform activated by PCBs, did not abrogate the increase in $[Ca^{2+}]_i$, suggesting that this change in $[Ca^{2+}]_i$ is not downstream from the activation of $iPLA_2$. TMB-8, a blocker of the release of intracellular Ca^{2+}, induced a small but significant decrease in Aroclor 1242-induced stimulation of PLA_2 in a dose-dependent manner 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 $[Ca^{2+}]_i$. Trifluoperazine (TFP), W7 and calmidazolium, three chemically distinct calmodulin inhibitors, inhibited significantly Aroclor 1242-induced PLA_2 activity in a dose-dependent manner. Moreover, both Aroclor 1242 and TFP blocked the degranulation induced by the chemotactic agent fMLP. These results suggest the possibility that Aroclor 1242 alters PMN function through interaction with calmodulin.

IV.2. Introduction

The chemical stability of polychlorinated biphenyls (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 (Fromberg *et al.,* 1999), wildlife refuges (Guillette *et al.*, 1999), dairy products (Ramos *et al.,* 1999) and human breast milk (Czaja *et al.*, 1999, Greizerstein *et al.*, 1999). 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 (Rice, 1999), stimulation of oscillatory uterine contraction (Bae *et al.*, 1999), changes in immunoglobulins M and G (Arnold *et al.*, 1999), and enzyme induction (Lagueux *et al.*, 1999). Some evidence has suggested a relationship between PCB exposure and effects on motor functioning (Schantz *et al.*, 1999), neurotoxicity (Seegal *et al.*, 1999), cancer (Dorgan *et al.*, 1999) and cancer-derived mortality (Kimbrough *et al.*, 1999).

The mechanisms of action of PCBs are multiple and structure-dependent. Many of the PCBs can bind with relatively high affinity to the aryl hydrocarbon (Ah) receptor and elicit toxic responses similar to those

observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Another group of PCBs has low affinity for the Ah receptor and exhibits different biological activities such as neurotoxicity (Kodavanti *et al.*, 1997, Wong *et al.*, 1997), stimulation of insulin release (Fischer *et al.*, 1996), and effects on neutrophil function (Olivero *et al.*, 1998, Brown *et al.*, 1995). With respect to neutrophils, PCBs both activate these cells and alter activation in response to other stimuli (Olivero *et al.*, 1998).

In addition to alterations in cellular function, several intracellular signals are activated by PCBs in neutrophils including phospholipase A_2 (PLA_2), tyrosine kinases (TKs) and phospholipase C (PLC) (Tithof *et al.*, 1997,Tithof *et al.*, 1998, Tithof *et al.*, 1995). PLA_2 hydrolyzes phospholipids at the *sn*-2 position to release the second messenger arachidonic acid. This enzyme regulates neutrophil function by modulating superoxide anion production (Dana *et al.*, 1998, Henderson *et al.*, 1993) and degranulation (Smolen *et al.*, 1980). Indeed, inhibition of PLA_2 prevents PCB-induced stimulation of these cells (Tithof *et al.*, 1998). In rat neutrophils, both Ca^{2+}-dependent and Ca^{2+}-independent isoforms of PLA_2 have been identified, and most of the PCB-induced activity is attributed to activation of Ca^{2+}-independent PLA_2 ($iPLA_2$) (Tithof *et al.*, 1998).

The biochemical mechanisms underlying the regulation of $iPLA_2$ are not known. It has been shown that $iPLA_2$ can be regulated by ATP (Hazen *et al.*, 1991, Ma *et al.*, 1998), nitric oxide (Gross *et al.*, 1995) and peptides

such [Arg^8] vasopressin (Lehman *et al.*, 1997) and parathyroid hormone (Derrickson *et al.*, 1997). An intriguing observation is that iPLA$_2$ can be modulated by direct protein-protein interactions with biomolecules such as phosphofructokinase (Hazen *et al.*, 1993) and calmodulin (Wolf and Gross, 1996).

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 signalling pathways involved in activation of neutrophils by PCBs. In particular, the roles of intracellular Ca^{2+} and calmodulin in activation of PLA$_2$ were explored.

IV.3. Materials and methods

IV.3.A. Chemicals

Aroclor 1242 was purchased from ChemService (West Chester, PA). BEL was purchased from Biomol (Plymouth Meeting, PA). [^{3}H][5,6,8,9,11,12,14,15]-arachidonic acid ([^{3}H]-AA; 180-240 Ci/mmol) was acquired from DuPont NEN (Boston, MA). Formyl-methionyl-leucyl-phenylalanine (fMLP), cytochalasin B and trifluoperazine were obtained from Sigma Chemical Company (St. Louis, MO). TMB-8, W-7, and calmidazolium were bought from Calbiochem (San Diego, CA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR).

IV.3.B. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats by glycogen elicitation as described (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt solution (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.

IV.3.C. Exposure to PCBs

PCB stock solutions were prepared by dissolution in N,N-dimethylformamide (DMF). Neutrophils ($2x10^6$) were suspended in HBSS (1 mL) in borosilicate glass test tubes, 12 x 75 mm (VWR, Chicago, IL), and 1 µL of the PCB stock solution was added to the tubes to achieve the desired concentration. Control neutrophils received 1 µL of DMF.

IV.3.D. 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 [^{3}H]-AA for two hours, gently shaking at 37°C. Neutrophils were then washed twice with Mg^{2+}- and Ca^{2+}-free HBSS. The cell count was adjusted so that the final concentration of neutrophils was 2x10^6/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 routinely between 80-88%. Release of [^{3}H]-AA from labeled neutrophils was measured in cells incubated with inhibitors for 20 minutes (37°C) and then exposed to Aroclor 1242 at 37°C for 30 min, unless otherwise stated. At the end of each incubation, neutrophils were placed on ice and spun in a centrifuge 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.

IV.3.E. Neutrophil degranulation

Degranulation was measured by the release from neutrophils of the enzyme myeloperoxidase (MPO). Neutrophils (2x10^6 cells/mL) were suspended in HBSS and pretreated with 5 μg/mL cytochalasin B for 5 minutes at room temperature. The quiescent neutrophils were then exposed to PCBs or vehicle for 10 min at 37°C followed by incubation for an additional 10 min at 37°C with 100 nM fMLP. Cells were centrifuged at 4°C, and the cell-free supernatant fluids were collected. Activity of MPO in the medium is determined using the method of Henson *et al.* (1978).

IV.3.F. Intracellular Free Ca^{2+} Measurements

Neutrophils (2.5 x 10^6 cells/mL) were labeled by incubation for 25 min at 37 °C with 5 μM Fura-2/AM in HBSS. After loading, cells were washed with HBSS, and the cell count was readjusted to 2 x 10^6 cells/mL. Fluorescence emission at 505 nm was monitored at room temperature with constant stirring, using a dual wavelength spectrofluorometer system with excitation at 340 and 380 nm. The $[Ca^{2+}]_i$ was calculated from fluorescence intensity readings using the following equation: $[Ca^{2+}]=K_d*Q(R-R_{min})/(R_{max}-R)$. R is the ratio of emission intensities at 340 and 380 nm excitation (340/380), R_{min} is the 340/380 ratio under Ca^{2+}-free conditions, R_{max} is the ratio under saturating Ca^{2+} concentrations; Kd is the dissociation constant of the Ca^{2+}: Fura-2 complex; and Q is the ratio of the 380 nm fluorescence under conditions of minimum and maximum $[Ca^{2+}]$ conditions (Shao *et al.*, 1998). The equilibrium dissociation constant, Kd, was taken from literature, 224 nM (Kankaanranta *et al*, 1995a). R_{max} and R_{min} values for each assay were determined from the fluorescence intensities in the presence of 0.01% Triton X-100 or 10 mM EGTA, respectively. These two parameters did not change significantly in the presence of Aroclor 1242. Fluorescence emission after excitation at 360 nm was monitored and in all studies conducted remained constant during data collection. Increases in resting $[Ca^{2+}]_i$ evoked by agonists were detected by measuring the change in fluorescence ratio and comparing this to fluorescence ratio after exposure

with the vehicle N,N-dimethyllformamide (DMF). Typical values for quiescent neutrophils ranged from 50 to 100 nM.

PCBs were added to labeled cells 50 sec after the start of data collection. PCB stock solutions were prepared by dissolution of the neat chemical in DMF, and 1 µL/mL of the respective stock solutions was added to the cells to achieve the desired concentration in a final cell volume of 3 mL. When BEL was used, labeled cells were pre-incubated with the inhibitor for 20 minutes at 37 °C before PCBs were added.

IV.3.G. Cytotoxicity assay

Release by neutrophils of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as an indicator of cytotoxicity. The method for sample preparation was the same as that described above. LDH activity present in the supernatant fluid was measured according to the method of Bergmeyer and Bernt (1974).

IV.3.H. Statistical methods

Data are presented as the means ± SEM from at least 4 different experiments conducted in triplicate. To calculate percent of control for release of [^{3}H]-AA in studies using BEL, first the values for release of [^{3}H]-AA in the absence of Aroclor 1242 at each concentration of inhibitor (including vehicle control) were subtracted from the corresponding values in

the presence of Aroclor 1242 to calculate the specific Aroclor 1242-induced release. Second, the value for Aroclor-induced release in the absence of inhibitor (vehicle control) was taken to be 100%. Angular transformation (arcsin) was used on percentage data to generate an approximated gaussian distribution. Detection of significant differences among treatments was determined using analysis of variance (ANOVA) and Tukey test as a post hoc test. When angular transformation did not produce gaussian data, Kruskal Wallace ANOVA on Ranks was used (SigmaPlot, V4. Jandel Scientific. San Rafael CA. 1996). Two-tailed p-values <0.05 were considered significant.

IV.4. Results

IV.4.A. 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 Ca^{2+} (Tithof *et al.*, 1998). To investigate whether intracellular Ca^{2+} is important for Aroclor 1242-induced PLA_2 activation, neutrophils were pretreated with the intracellular Ca^{2+} release blocker, TMB-8 (Kemmerich and Pennington, 1988). TMB-8 (25-50 µM) produced a small but significant decrease in Aroclor 1242-elicited release of $[^3H]$-AA (Table IV.1). This suggests that a fraction of the total

PLA_2 activity induced by Aroclor 1242 depends on the release of Ca^{2+} from intracellular stores.

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

Table IV.1. TMB-8-induced inhibition of Aroclor 1242-stimulated release of [^{3}H]-AA from rat neutrophils. Cells were preincubated with TMB-8 and then exposed to 10 μg/mL Aroclor 1242. Release of [^{3}H]-AA was determined as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay. a, Significantly different ($p<0.05$) from the control group (100%) when analyzed by ANOVA using Tukey test as a post hoc test.

Exposure of neutrophils to Aroclor 1242 caused a time-dependent increase in $[Ca^{2+}]_i$ (See Figure II.1). This increase began after about 5 minutes of exposure to PCBs and continued through 18 minutes. In order to determine if this increase in $[Ca^{2+}]_i$ was caused by the activation of PLA_2 by Aroclor 1242, $[Ca^{2+}]_i$ measurements were made in cells preincubated with the cell permeable PLA_2 inhibitor BEL (10 μM). Treatment with BEL

did not abrogate the overall release of $[Ca^{2+}]_i$ elicited by 10 μg/mL Aroclor 1242 measured as area under the curve but produced a change in the kinetics of the release over a period of 18 minutes (Figure IV.1).

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, trifluoperazine (TFP), W7 and calmidazolium were determined. W7 induced a dose-dependent decrease in the Aroclor 1242-stimulated PLA_2 activity with minimal cytotoxicity (Figure IV.2). TFP also caused a decrease in AA release, but only at significant cytotoxic concentrations (data not shown). About 50% inhibition was obtained with calmidazolium, a very potent inhibitor of calmodulin ($IC_{50} \approx 0.05$ μM) (Hait and Lazo, 1986).

IV.4.B. TFP and Aroclor 1242-induced changes in neutrophil function

Aroclor 1242 caused a dose-dependent inhibition of fMLP-induced neutrophil degranulation (Figure IV.3.A) consistent with a previous report with ortho-chlorinated PCBs (Olivero *et al.*, 1998). A similar effect of TFP on neutrophil degranulation was observed. Like Aroclor 1242, TFP blocked most of the degranulation in response to fMLP (Figure IV.3.B).

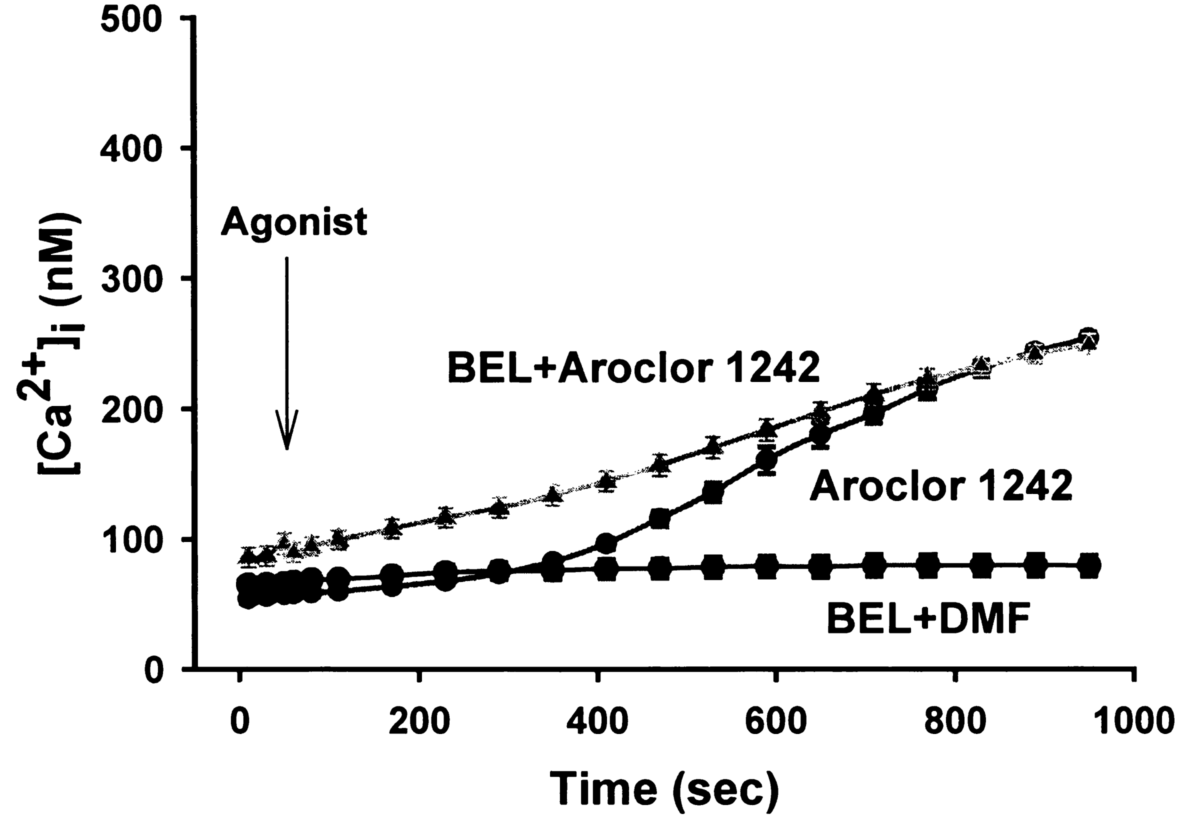


Figure IV.1. ***Effects of BEL on 10 µg/mL Aroclor 1242-induced increase in [Ca^{2+}]$_i$ in rat neutrophils.*** Cells were loaded with fura-2/AM as described in Materials and Methods. Fura-2-loaded neutrophils were incubated with 10 µM BEL for 20 min and then stimulated with 10 µg/mL Aroclor 1242 at the time indicated by the arrow. Data for control (DMF) are shown for comparison. Results are expressed as means ± SEM for four different experiments.

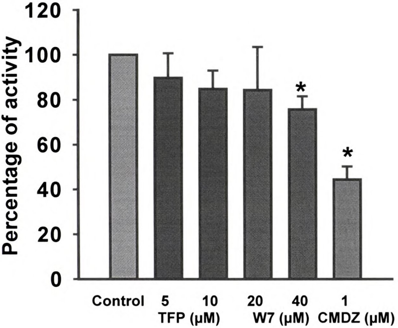


Figure IV.2. ***Effects of calmodulin inhibitors on phospholipase A_2 activity induced by 10 µg/mL Aroclor 1242***. Neutrophils were labeled with $[^3H]$-AA and incubated with different concentrations of the calmodulin inhibitors TFP, W7 and calmidazolium (CMDZ) for 20 min at 37 °C followed by incubation with 10 µg/mL Aroclor 1242 for 30 min at 37 °C. The release of $[^3H]$-AA into the medium at each concentration was compared with the release by the same compound in absence of the calmodulin inhibitor and presented as percentage of activity. Results are expressed as means ± SEM from four different experiments performed in triplicates.

*, $p<0.05$ vs $[^3H]$-AA released by neutrophils exposed to 10 µg/mL Aroclor 1242 alone.

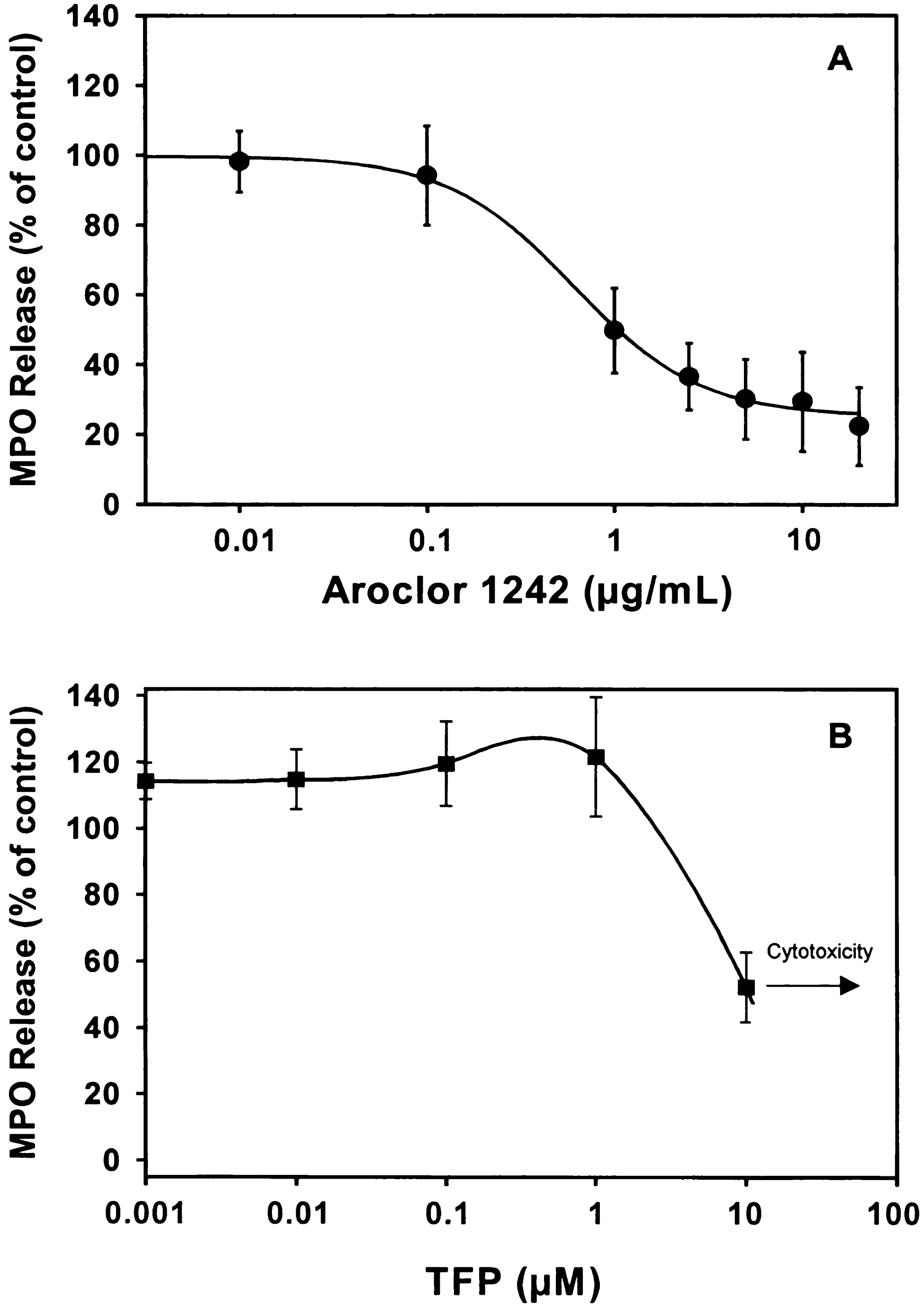


Figure IV.3. ***Inhibitory effect of (A) Aroclor 1242 and (B) Trifluoperazine on fMLP-induced neutrophil degranulation.*** Cytochalasin-treated neutrophils were exposed to Aroclor 1242 or TFP for 10 min then incubated for another 10 min with 100 nM fmlp, and degranulation was measured as described in Materials and methods. Data are expressed as means ± SEM of four different experiments.

control

IV.5. Discussion

PCBs cause an increase in $[Ca^{2+}]_i$ in cerebellar granule cells (Shafer *et al.*, 1996), hepatocytes (Atzori *et al* ., 1991), endothelial cells (Toborek *et al.*, 1995) and human neutrophils (Voie *et al.,*1998). The mechanisms associated with this increase in intracellular Ca^{2+} are structure-dependent and, at least in neutrophils, have been associated with activation of PLC (Voie and Fonnum, 1998). We have demonstrated that Aroclor 1242 increases PLA_2 activity with release of arachidonic acid (Tithof *et al.*, 1998). Similarly, Aroclor 1242 induces an increase in $[Ca^{2+}]_i$ in rat neutrophils (Figure IV.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 (Rzigalinski et al., 1996) or its metabolites (Reynaud and Pace, 1997) can cause depletion of intracellular calcium stores leading to a rise in $[Ca^{2+}]_i$.

Following exposure of neutrophils to Aroclor 1242, arachidonic acid is released within 2 minutes (Tithof *et al.*, 1998), whereas the increase in $[Ca^{2+}]_i$ begins after 5 minutes (Figure IV. 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 (Figure IV.2). We have demonstrated previously that BEL abrogates Aroclor 1242-stimulated release of arachidonate acid (Tithof *et al.*, 1998), and 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 in the absence of extracellular Ca^{2+} the spike in $[Ca^{2+}]_i$ elicited by vasopressin was not abolished by treatment with BEL, however, in presence of extracellular Ca^{2+}, BEL prolonged the duration of the response (Lehman *et al.*, 1993).

In previous studies it has been demonstrated that the majority of arachidonic acid released from neutrophils after PCB treatment comes from stimulation of a Ca^{2+}-independent PLA$_2$ and that this activation is not abrogated by the intracellular Ca^{2+} chelator BAPTA-AM (Tithof *et al.*, 1998). Studies presented here demonstrated that TMB-8 caused a small reduction of Aroclor 1242-induced release of arachidonic acid. This suggests that release of Ca^{2+} from intracellular stores contributes to a fraction of the PCB-stimulated PLA$_2$ activity. Moreover, these results presume that BAPTA/AM and TMB-8 target different intracellular Ca^{2+} pools. It has been reported that TMB-8 inhibits fMLP- and PMA-stimulated superoxide anion production in neutrophils, and that these responses can be restored upon Ca^{2+} loading with A23187 (Weisdorf and Thayer, 1989), supporting that effects of TMB-8 are due to interference with normal Ca^{2+} responses. It has been suggested that TMB-8 causes its effects on

intracellular Ca^{2+} pools that may be associated with cytoskeletal elements that sequester intracellular Ca^{2+}, or on lysosomal or other intracellular pools (Weisdorf and Thayer, 1989; Khan *et al.*, 1985).

Despite the observation that the PCB-induced increase in $[Ca^{2+}]_i$ is independent of PLA_2 activity (Figure IV.2), and that the stimulation of PLA_2 by PCBs is largely independent of Ca^{2+}, there appears to be an interaction between Ca^{2+} signaling and release of [^{3}H]-AA. This interpretation is based on the known relationship between Ca^{2+} and calmodulin and the results of studies with TFP, W7 and calmidazolium in which these calmodulin inhibitors diminished PCB-stimulated release of AA (Figure IV. 3). One explanation for these results is that calmodulin may regulate 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 (Richard *et al.*, 1997). The fact that calmidazolium strongly blocks the activation of $iPLA_2$ by Aroclor 1242 suggests the participation of calmodulin as regulator of $iPLA_2$ activity. TFP, which also inhibited PCB-increased PLA_2 activity by ~15%, binds tightly to calmodulin in solution as determined by nuclear magnetic resonance studies (Craven *et al.*, 1996; Vandonselaar *et al.*, 1994). TFP and W7 binding to calmodulin causes a change in tertiary structure from an elongated dumb bell with exposed hydrophobic surfaces

to a compact globular form which can no longer interact with its target enzymes (Vandonselaar *et al.*, 1994; Ozawa *et al.*, 1999)

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 (Wolf and Gross, 1996). If this model of enzyme regulation by calmodulin applies to neutrophil $iPLA_2$, then PCBs may activate neutrophil $iPLA_2$ through interaction with calmodulin. The rank order of potency for the inhibition of calmodulin appeared to be calmidazolium > TFP > W7 (Ambudkar *et al.*, 1989), which is consistent with the results presented here. TFP, W7 or calmidazolium may alter the conformation of calmodulin such that the binding site for Aroclor 1242 is inaccessible. Alternatively, PCBs may activate $iPLA_2$ through a different mechanism, but inhibition of calmodulin by TFP, W7 or calmidazolium interferes with this mechanism indirectly. For instance, it has been shown that W7 inhibits fMLP stimulation of PLD in neutrophils with an IC_{50} value of $\approx$ 50 μM (Takahashi *et al.*, 1996). Similarly, it was recently reported that the PCB-stimulated release of insulin from RINm5F cells was blocked by an inhibitor of Ca^{2+}/calmodulin-dependent kinase (Fischer *et al.*, 1999) suggesting that calmodulin is required for PCB-mediated signaling.

Both TFP and Aroclor 1242 inhibited fMLP-induced neutrophil degranulation (Figure IV.4). The dose-response curve obtained for TFP-elicited inhibition of fMLP-induced neutrophil degranulation measured as

MPO release is very similar to that previously reported for the enzyme ß-glucuronidase (Smith *et al.*, 1981), another marker of neutrophil degranulation. Remarkably, 10 µM TFP elicited approximately 50% inhibition of fMLP-induced neutrophil degranulation; this same concentration has been reported to inhibit calmodulin activity (Hait *et al.*, 1986). Although, TFP is cytotoxic at concentrations greater than 10 µM, the lack of massive release of MPO by this compound suggests that it targets the extracellular membrane and not the granule membrane. TFP interacts with annexin proteins and is capable of inhibiting annexin I- and annexin II-mediated aggregation of liposomes by releasing them from the plasma membrane (Blackwood *et al.*, 1995). In addition, TFP blocks annexin II tetramer-mediated membrane fusion (Liu *et al.*, 1997). Annexins link secretory vesicles to the plasma membrane (Klee *et al.*, 1998) and are linked to calmodulin. Remarkably, calmodulin binds tightly to vacuoles during the post-docking phase of vacuole fusion promoting bilayer mixing (Peters *et al.*, 1998). Thus, this activity provides a potential mechanism by which TFP may inhibit degranulation of neutrophils through calmodulin-dependent processes.

Another possibility that may link the inhibition of fMLP-induced degranulation to an inhibitory effect of TFP or PCBs on calmodulin is that membrane fusion requires the phosphorylation of trafficking proteins such as syntaxin 3, a process proposed to be mediated by Ca^{2+}- and

calmodulin-dependent protein kinase II (Risinger *et al.*, 1999). In consequence, the binding of PCB to calmodulin would reduce the activity of the calmodulin-dependent kinase and abrogate the degranulation process.

These effects of TFP and PCBs on fMLP-induced degranulation are not the only analogy between these two compounds. In neutrophils, Aroclor 1242 has been found to block Ca^{2+} after depletion of intracellular stores (See Chapter I). Similarly, calmodulin inhibitors block the same effect in several cell types (Mene *et al.*, 1996; Haverstick *et al.*, 1998).

To date, no information is available regarding the interaction between PCBs and calmodulin. However, it has been proposed that one essential feature for optimal ligand binding to calmodulin is the presence of two hydrophobic, aromatic rings, such as is present in PCBs (Hait *et al.*, 1986). From these results, it is evident that calmodulin might be one relevant target that mediates cellular responses elicited by PCBs.

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

Chapter V

BIOCHEMICAL SIGNALS INVOLVED IN THE ACTIVATION OF PLA_2 BY 2,2',4,4'-TETRACHLOROBIPHENYL

V.1. Summary

The PCB mixture Aroclor 1242 stimulates neutrophils to produce superoxide anion and release different enzymes. The activation of neutrophils by PCBs require the cooperation of diverse enzymes, particularly phospholipase A_2 (PLA_2). The objectives of this study were to determine whether or not the stimulation of PLA_2 by Aroclor 1242 was shared by 2,2',4,4'-tetrachlorobiphenyl, an *ortho*-chlorinated PCB, and investigate the mechanisms leading to its activation. Rat neutrophils were labeled with $[^3H]$-arachidonic acid ($[^3H]$-AA), and stimulation of PLA_2 was measured from release of radioactivity into the medium. Exposure to the *ortho*-PCB congener 2,2',4,4'-tetrachlorobiphenyl, but not to the non-*ortho* congener 3,3',4,4'-tetrachlorobiphenyl induced a dose-dependent activation of PLA_2 and phosphorylation of $cPLA_2$ and p44 MAPK. About 50% of the PLA_2 activity was sensitive to BEL, an inhibitor of Ca^{2+}-independent PLA_2. Pharmacological intervention using inhibitors showed that tyrosine kinases, ras, protein kinase C (PKC) and p42/p44 MAPK contributed to a fraction (20%) of the total activity induced by 2,2',4,4'-tetrachlorobiphenyl. In addition, the calmodulin inhibitors W7 and calmidazolium blocked up to 40% of the total activity. These results suggest that the *ortho*-chlorinated PCBs activate PLA_2 through mechanisms involving tyrosine kinases, PKC, ras, the MAPK pathway and calmodulin.

V.2. Introduction

Polychlorinated biphenyls are man-made chemicals that disrupt function of a variety of cells by interfering with multiple biochemical mechanisms. In the last two decades the importance of these compounds has grown considerably due to their detection in human tissues (Angulo *et al.*, 1999; Stewart *et al*). These contaminants are normally found in complex mixtures having both *ortho*- and non-*ortho* chlorinated congeners. As a consequence, toxicological assessment is complicated by the differences in mechanisms of toxicity, potency and other factors among congeners. The study of individual congeners is important not only for risk assessment but also for determination of the mechanisms of action. To simplify the toxicological profile of PCBs they have been divided in two groups: the *ortho*-PCBs and the non-*ortho* PCBs. The *ortho*-PCBs have dioxin-like properties and are active at sub-micromolar concentrations, whereas the non-*ortho*-PCBs have no dioxin-like activity and require micromolar concentrations to interfere with biochemical pathways. The aim of the present study was to determine the molecular signaling involved in the activation of PLA_2 by the *ortho*-chlorinated PCB congener 2,2',4,4'-tetrachlorobiphenyl.

V.3. Materials and methods

V.3.A. Chemicals

PCBs were purchased from ChemService (West Chester, PA). [^{3}H][5,6,8,9,11,12,14,15]-arachidonic acid ([^{3}H]-AA; 180-240 Ci/mmol) was purchased from DuPont NEN (Boston, MA). W7, calmidazolium and genistein were obtained from Sigma Chemical Company (St. Louis, MO). PD 98059, Ro-32-0432, manumycin, and UO126 were purchased from Calbiochem (San Diego, CA).

V.3.B. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats by glycogen elicitation as described (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt solution (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.

V.3.C. Exposure to PCBs

PCB stock solutions were prepared by dissolution in N,N-dimethylformamide (DMF). Neutrophils ($2x10^6$) were suspended in HBSS (1 mL) in borosilicate glass test tubes, 12 x 75 mm (VWR, Chicago, IL), and 1

V.3. Materials and methods

V.3.A. Chemicals

PCBs were purchased from ChemService (West Chester, PA). [^{3}H][5,6,8,9,11,12,14,15]-arachidonic acid ([^{3}H]-AA; 180-240 Ci/mmol) was purchased from DuPont NEN (Boston, MA). W7, calmidazolium and genistein were obtained from Sigma Chemical Company (St. Louis, MO). PD 98059, Ro-32-0432, manumycin, and UO126 were purchased from Calbiochem (San Diego, CA).

V.3.B. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats by glycogen elicitation as described (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt solution (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.

V.3.C. Exposure to PCBs

PCB stock solutions were prepared by dissolution in N,N-dimethylformamide (DMF). Neutrophils ($2x10^6$) were suspended in HBSS (1 mL) in borosilicate glass test tubes, 12 x 75 mm (VWR, Chicago, IL), and 1 μL of the PCB stock solution was added to the tubes to achieve the desired concentration. Control neutrophils received 1 μL of DMF.

V.3.D. 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 [^{3}H]-AA for two hours, gently shaking at 37°C. Neutrophils were then washed twice with Mg^{2+}- and Ca^{2+}-free HBSS. The cell count was adjusted so that the final concentration of neutrophils was $2x10^6$/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 routinely between 80-88%. Release of [^{3}H]-AA from labeled neutrophils was measured in cells incubated with inhibitors for 20 minutes (37°C) and then exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl at 37°C for 30 min, unless otherwise stated. At the end of each incubation, neutrophils were placed on ice and spun in a centrifuge 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.

V. 3. E. Detection of phosphorylated $cPLA_2$ and p42/p44 MAPKs

Neutrophils (3×10^6 /mL) were suspended in HBSS and incubated with inhibitors for 20 min at 37°C before stimulation with 25 µM 2,2',4,4'-tetrachlorobiphenyl or vehicle and incubated for an additional 30 min at 37°C. After incubation, samples were spun in a centrifuge at 4°C for 10 min. The pellets were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in 300 µL lysing buffer (20% sodium dodecyl sulfate, SDS) for one hour and then sonicated for 30 sec. Samples were denatured by boiling for 5 min at 100°C and separated on a denaturing, 10% SDS polyacrylamide gel. Approximately 50 µg of protein were added to each lane. Proteins were transferred electrophoretically to nitrocellulose membranes. After transfer, membranes were blocked for 3-4 hours in Tris-buffered saline (TBS) + Tween 20 (1%) (TBS-T) containing 4% chicken ovalbumin and 0.25% sodium azide. Membranes were incubated with mouse anti-phosphorylated MAPK antibody (New England BioLabs) (1:2500) or the anti-$cPLA_2$ antibody (Santa Cruz Biotechnology) (1:500) in blocker for two hours with constant rocking. Blots were washed three times with TBS-T (30, 5 and 5 min) and once with TBS (5 min). Goat anti-mouse IgG linked to horseradish peroxidase (1:7500) in TBS was added for 1 hour. Blots were washed using the same protocol as described above. Enhanced chemiluminiscence using Amersham reagents was performed to visualize labeled, phosphorylated proteins.

V.3.F. Cytotoxicity assay

Release by neutrophils of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as an indicator of cytotoxicity. The method for sample preparation was the same as that described above. LDH activity present in the supernatant fluid was measured according to the method of Bergmeyer and Bernt (1974).

V.3.G. Statistical Methods

Data are presented as the means ± SEM from at least 4 different experiments conducted in triplicate. To calculate percent of control for release of [^{3}H]-AA in studies using inhibitors, first the values for release of [^{3}H]-AA in the absence of 2,2',4,4'-tetrachlorobiphenyl at each concentration of inhibitor (including vehicle control) were subtracted from the corresponding values in the presence of 2,2',4,4'-tetrachlorobiphenyl to calculate the specific 2,2',4,4'-tetrachlorobiphenyl-induced release. Second, the value for 2,2',4,4'-tetrachlorobiphenyl-induced release in the absence of inhibitor (vehicle control) was taken to be 100%. Angular transformation (arcsin) was used on percentage data to generate an approximated gaussian distribution. Detection of significant differences among treatments was determined using analysis of variance (ANOVA) and Tukey test as a post hoc test. When angular transformation did not produce gaussian data, Kruskal Wallace ANOVA on Ranks was used. Two-tailed p-values <0.05 were considered significant.

V.4. Results

V.4.A. Activation of PLA_2 by 2,2',4,4'-tetrachlorbiphenyl and 3,3',4,4'-tetrachlorobiphenyl.

Dose-response curves for the activation of neutrophil PLA_2 by 2,2',4,4'-tetrachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl are presented in Figures V.1 and V.2, respectively. Only the *ortho*-chlorinated congener activated PLA_2 and did so in a dose-dependent manner. At concentrations greater than 50 µM, 2,2',4,4'-tetrachlorobiphenyl induced a statistically significant release of LDH from neutrophils that was less than 20% of total activity. BEL (25 µM) decreased $\approx$ 50% of the total PLA_2 activity induced by 2,2',4,4'-tetrachlorobiphenyl, suggesting that this congener targets the Ca^{2+}-independent PLA_2 isoform ($iPLA_2$).

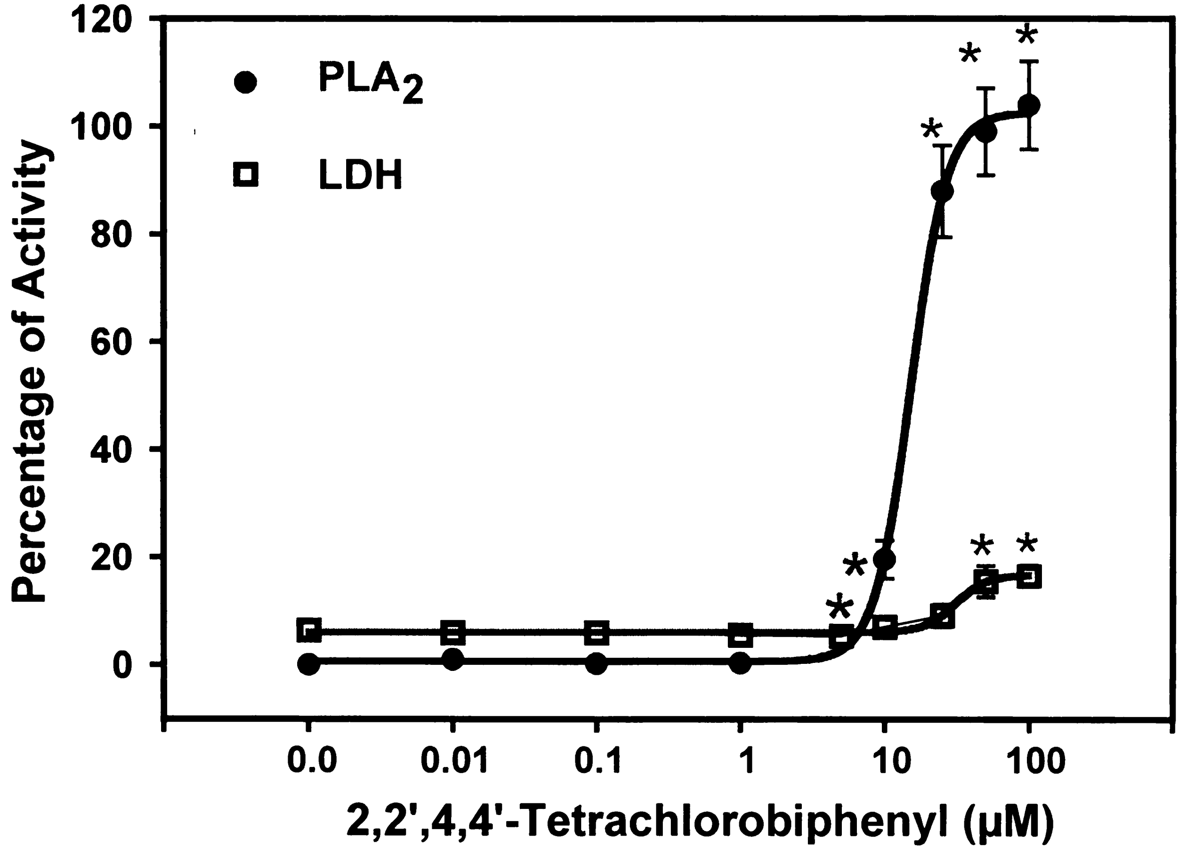


Figure V.1. ***Effects of 2,2',4,4'-tetrachlorobiphenyl on neutrophil PLA$_2$ activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. For this and all subsequent graphs, the activity is plotted against the log of the concentration. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 μg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. Aroclor 1242 under these conditions caused release of 25-35% of total incorporated [^{3}H]-AA. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

*. Significantly different from vehicle control (DMF).

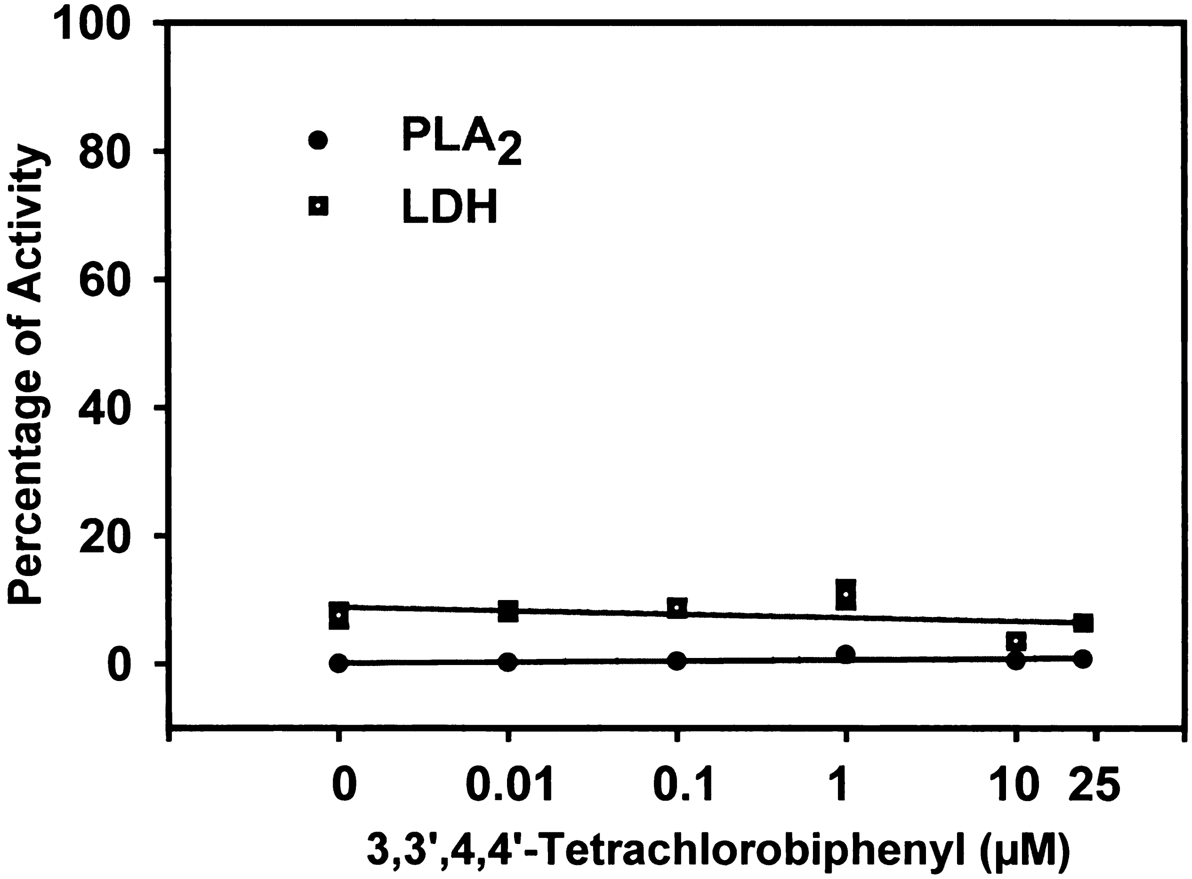


Figure V.2. ***Effects of 3,3',4,4'-tetrachlorobiphenyl on neutrophil PLA$_2$ activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 µg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

V.4.B. Role of protein kinase pathways and calmodulin in 2,2',4,4'-tetrachlorobiphenyl-induced activation of PLA_2

To study different signaling cascades that could be involved in the activation of PLA_2 induced by 2,2',4,4'-tetrachlorobiphenyl, various protein inhibitors and antagonists were tested.

The tyrosine kinase (TK) inhibitor, genistein, inhibited a small fraction of the 2,2',4,4'-tetrachlorobiphenyl-induced PLA_2 activity in a dose-dependent manner (Figure V.3). Maximal inhibition (≈20%) was reached at 50-100 μM. PD 98059 and UO126, two inhibitors of the mitogen activated protein kinase (MAPK) kinase (MEK) produced a decrease in 2,2',4,4'-tetrachlorobiphenyl-stimulated PLA_2 activity which was only significant for UO173 and reached approximately 20% inhibition (Figure V.4). Ro-32-0432 (10 μM), an inhibitor of protein kinase C (PKC), produced an inhibition of stimulated PLA_2 activity that was similar in magnitude to that observed for genistein and UO173 (Figure V.5). Finally, manumycin, an inhibitor of ras farnesylation, produced a greater degree of inhibition (approximately 35%) at 10 μM (Figure V.6). These inhibitors were not cytotoxic (< 25% LDH release) at the concentrations tested.

The effects of two chemically distinct calmodulin inhibitors were tested. At noncytotoxic concentrations, W7 and calmidazolium, each

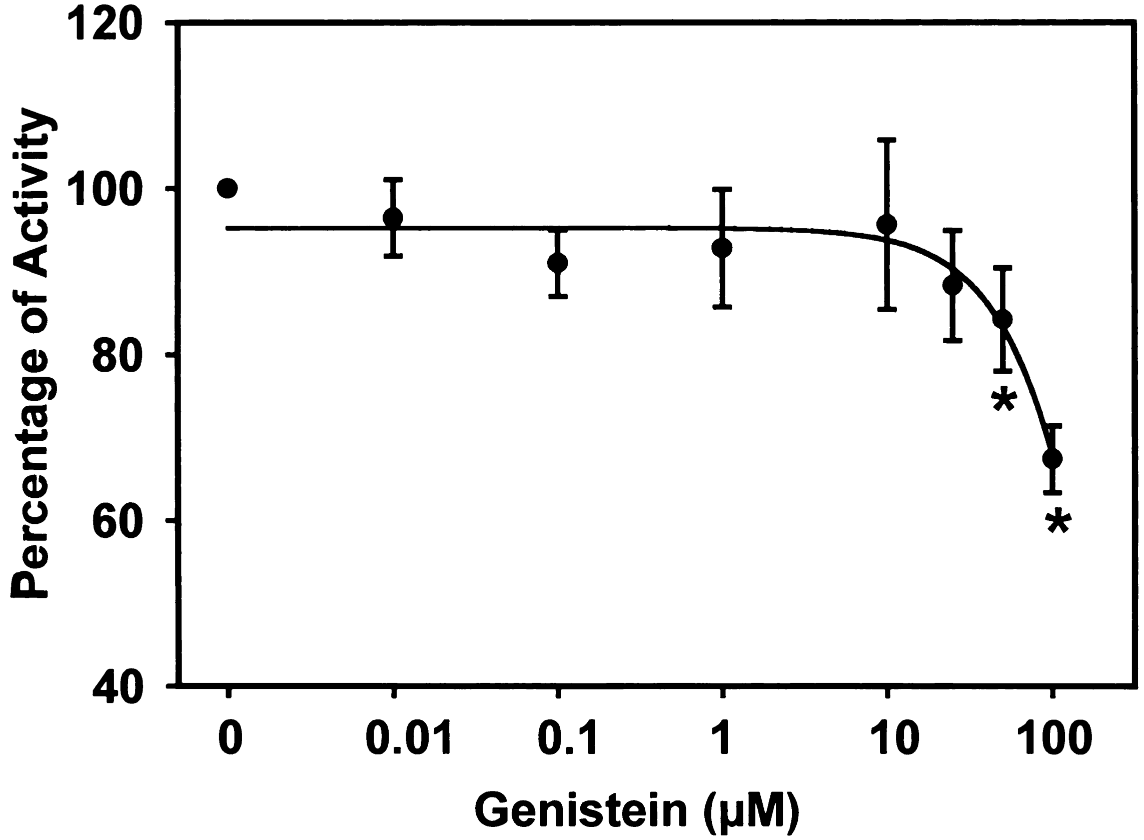


Figure V.3. ***Dose-response curve for inhibition of 2,2',4,4'-tetrachlorobiphenyl (25 μM)-induced release of [^{3}H]-AA by genistein.*** Cells were incubated with the indicated concentrations of genistein as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl alone.

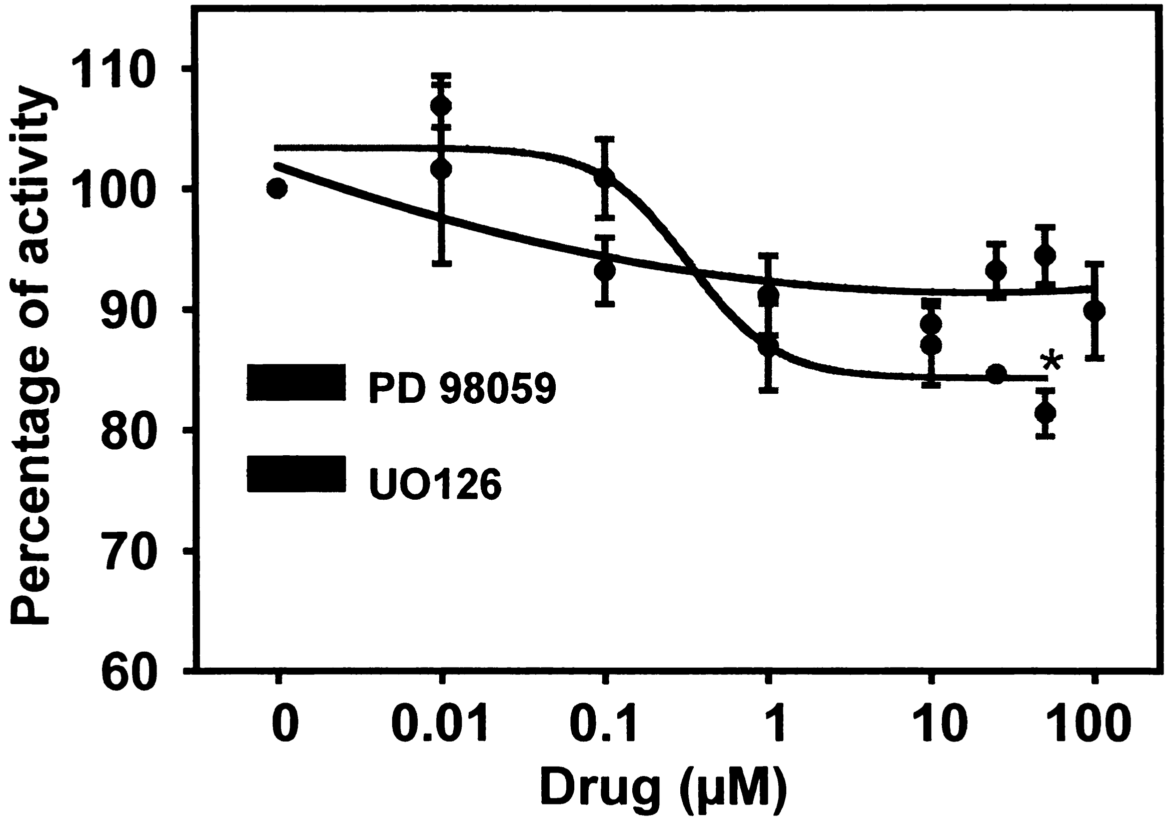


Figure V.4. ***Dose-response curves for inhibition of 2,2',4,4'-tetrachlorobiphenyl (25 μM)-induced release of [^{3}H]-AA by PD 98059 and UO126.*** Cells were incubated with the indicated concentrations of PD 98059 and UO126 as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl alone.

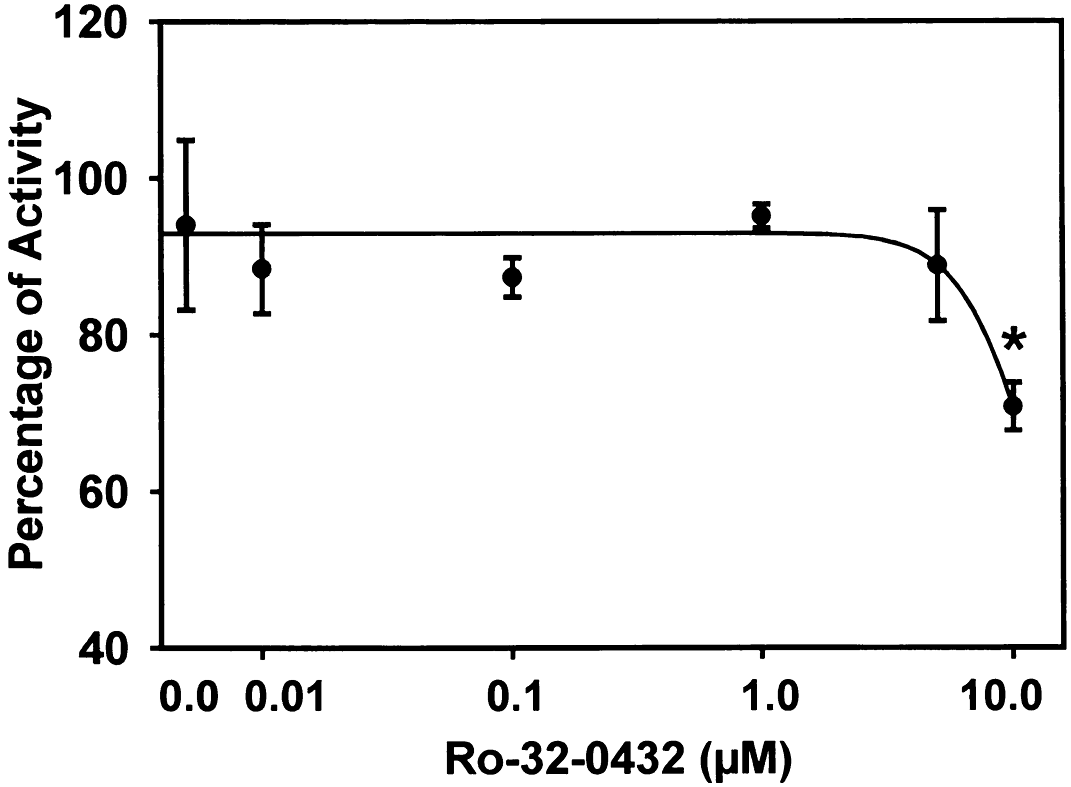


Figure V.5. ***Dose-response curve for inhibition of 2,2',4,4'-tetrachlorobiphenyl (25 μM)-induced release of [^{3}H]-AA by Ro-32-0432.*** Cells were incubated with the indicated concentrations of Ro-32-0432 as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl alone.

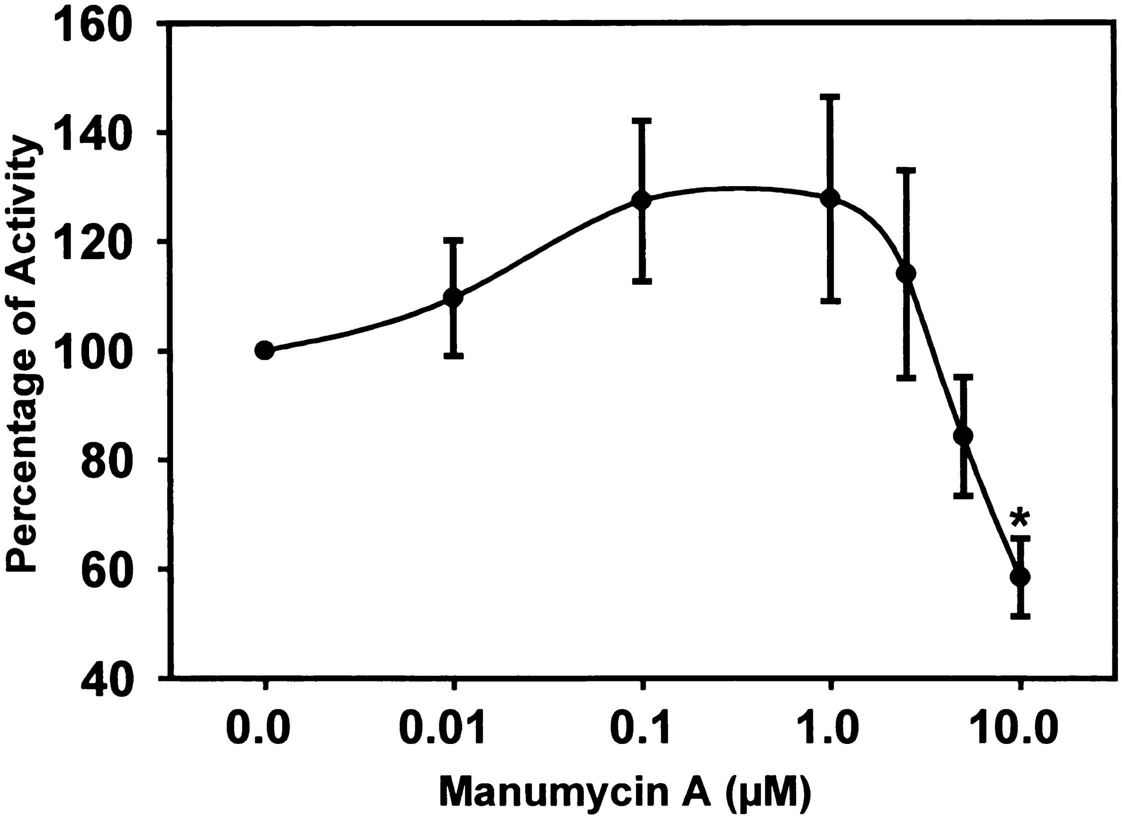


Figure V.6. ***Dose-response curve for inhibition of 2,2',4,4'-tetrachlorobiphenyl (25 μM)-induced release of [^{3}H]-AA by manumycin A.*** Cells were incubated with the indicated concentrations of manumycin A as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl alone.

induced a significant decrease (≈40%) in the 2,2',4,4'-tetrachlorobiphenyl-induced PLA_2 activity (Figure V.7).

V.4.C. Effects of 2,2',4,4'-tetrachlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl on $cPLA_2$ and p42/p44 MAPK phosphorylation.

2,2',4,4'-tetrachlorobiphenyl induced the phosphorylation of $cPLA_2$ in rat neutrophils. This was not observed with the non-*ortho*-congener 3,3',4,4'-tetrachlorobiphenyl (Figure V.8). Similarly, 2,2',4,4'-tetrachlorobiphenyl induced the phosphorylation of p44 MAPK, commonly referred to as extracellular signal-regulated kinase (ERK-1), and increased the phosphorylation of the constitutively expressed, phosphorylated p42 MAPK (ERK-2) (Figure V.9). Induction of phosphorylation of p44 MAPK was blocked by PD 98059 and attenuated by genistein.

Although western analysis does not measure activity, increased phosphorylation of p42/p44 MAPK is in general accompanied by increased activity (Hashimoto *et al.*, 1999; Qiu and Leslie, 1994) so that these results imply that 2,2',4,4'-tetrachlorobiphenyl increased activity of $cPLA_2$ and p44 MAPK.

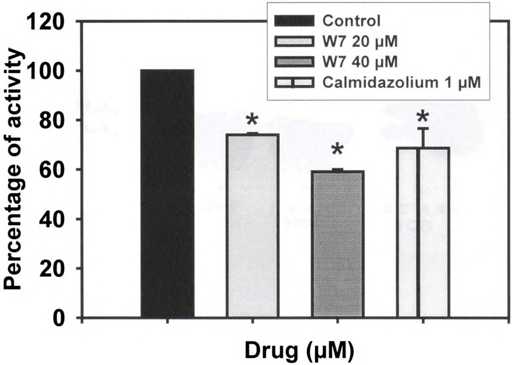


Figure V.7. ***Effects of calmodulin inhibitors on 2,2',4,4'-tetrachlorobiphenyl-induced PLA_2 activity.*** Cells were incubated with the indicated concentrations of calmodulin inhibitors W7 or calmidazolium as described in Materials and methods. Data are expressed as means ± SEM of four different experiments with triplicates per assay.

*, $p<0.05$ vs. [^{3}H]-AA released by neutrophils exposed to 25 μM 2,2',4,4'-tetrachlorobiphenyl alone.

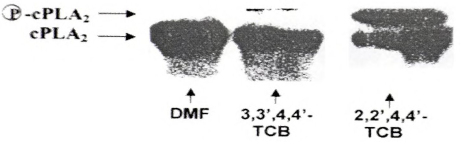


Figure V.8. 2,2',4,4'-tetrachlorobiphenyl but not 3,3',4,4'-tetrachlorobiphenyl phosphorylates cPLA$_2$. 3x10^6 cells/mL were incubated with 1 µL vehicle control (DMF) or with 25 µM of each PCB congener for 30 min at 37 °C. cPLA$_2$ phosphorylation was detected by Western blotting as described in Materials and methods. Western blot is representative of two independent experiments.

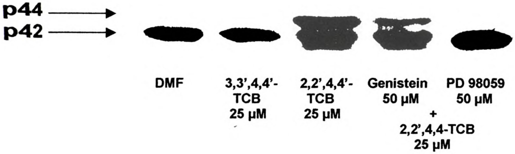


Figure V.6. ***p42/p44 MAPK phosphorylation response by PCBs: Effects of genistein and PD 98059 in 2,2'4,4'-tetrachlorobiphenyl-induced phosphorylation of p42/p44 MAPK.*** 3×10^6 cells/mL were incubated with 1 µL vehicle control (DMF) or with 25 µM of each PCB congener for 30 min at 37 °C. Experiments with inhibitors were conducted by incubating the cells with the shown inhibitor concentration for 20 min (37 °C) and then with 2,2',4,4-tetrachlorobiphenyl for additional 30 min. p42/p44 MAPK phosphorylation was detected by Western blotting as described in Materials and methods. Western blot is representative for two independent experiments.

V.5. Discussion

It has been demonstrated that the PCB mixture Aroclor 1242 activates PLA_2. Results reported here show that 2,2',4,4'-tetrachlorobiphenyl but not 3,3',4,4'-tetrachlorobiphenyl activates PLA_2, suggesting that it is the *ortho*-chlorinated congeners in Aroclor 1242 which are responsible for the activity. Furthermore, results suggest that 2,2',4,4'-tetrachlorobiphenyl activates two isoforms of PLA_2: a BEL-sensitive PLA_2 known to be Ca^{2+}-independent ($iPLA_2$) and $cPLA_2$.

In this study, the effects of different signaling pathways leading to activation of PLA_2 by 2,2',4,4'-tetrachlorobiphenyl were studied using pharmacological inhibitors. It was shown that TKs, PKC, ras and MEK, as well as calmodulin are important for a fraction of the observed PLA_2 activity induced by this PCB congener. Among PLA_2s, only $cPLA_2$ has been shown to be regulated by phosphorylation (Lin *et al.*, 1998; Qiu and Leslie, 1994; Borsch-Haubold *et al.*, 1998). Due to the fact that activation of $cPLA_2$ may occur following a signal transduction pathway involving TK, PKC, ras and MEK (Lin *et al.*, 1998; Miura *et al.*, 1999; Sa and Das, 1999; Li *et al.*, 1997), it is likely that inhibition of these pathways prevents activation of this isoform of PLA_2. Consequently, it is possible that the fraction of the observed PLA_2 activity inhibited by interference with these pathways represents the contribution of $cPLA_2$.

Previous studies have shown that PCBs can induce tyrosine phosphorylation in rat neutrophils (Tithof *et al.*, 1997). In this study, genistein (50-100 µM), a nonspecific tyrosine kinase inhibitor, abrogated the PLA_2 response elicited by 2,2',4,4'-tetrachlorobiphenyl. Different genistein concentrations have been used to block some effects induced by tyrosine kinase activation, such as prostaglandin production (5-10 µM) (Kniss *et al.*, 1996), adherence (30-200 µM) (Gozin *et al.*, 1998; Ozaki *et al.*, 1993), superoxide anion release (50-300 µM) (Hazan *et al.*, 1997), and degranulation (5-200 µM) (Foster *et al.*, 1999).

Inhibition of ras attenuated activation of PLA_2 by 2,2',4,4'-tetrachlorobiphenyl. Ras is a GTP-binding protein that regulates a variety of cellular processes such as cell growth and differentiation (Satoh *et al.*, 1992). Ras protein acts as a signal transducer in which signaling from tyrosine kinases, intrinsic to or associated with receptors, activates ras in response to an extracellular stimulus. Ras activates several downstream effectors, including the Raf-1/mitogen-activated protein kinase pathway and the Rac/Rho pathway. Ras activity requires its attachment to the inner surface of the plasma membrane, which is obtained by the addition of a farnesyl isoprenoid moiety in a reaction catalyzed by the enzyme protein farnesyltransferase (Rowinsky *et al.*, 1999). Manumycin is an antibiotic with recognized activity to inhibit protein farnesyltransferase (Hara *et al.*, 1993). It has been used to block p21(ras)-dependent pathways (Lee-Kwon *et al.*, 1998; Flamigni *et al.*, 1999; Smith *et al.*, 1999) such as phosphorylation of p42/p44 MAPK (Kouchi *et al.*, 1999). *In vitro* studies have shown that manumycin can block human protein farnesyltransferase activity with an IC_{50} of 1.8 μM (Del Villar *et al.*, 1999). Inhibitors of farnesyltransferases have emerged as potential anti-cancer drugs (Sattler and Tamonoi, 1996; Waddick and Uckun, 1998), based on the involvement of ras in cell growth and morphogenesis (Gibbs and Oliff, 1997). The activation of a ras-dependent pathway can be a link between PCBs and tumor formation, particularly because the PCB mixture Aroclor 1254 (Borlak *et al.*, 1996) and the *ortho*-chlorinated PCB 2,3,4,2',4',5'-hexachlorobiphenyl (Gribaldo *et al.*, 1998) have been shown to induce overexpression of ras.

The PKC inhibitor Ro-32-0432 partially inhibited PLA_2 activity induced by 2,2',4,4'-tetrachlorobiphenyl. Ro 32-0432 is a bisindolylmaleimide molecule that selectively inhibits PKC_α or PKC_β with low selectivity for PKC_ε (Wilkinson *et al.*, 1993). The involvement of PKC in PLA_2 activation by PCBs is not surprising because it is known that PCBs can interact directly with PKC and promote its translocation (Kodavanti *et al.*, 1998).

2,2',4,4'-Tetrachlorobiphenyl phosphorylated p44 MAPK and increased the phosphorylation of p42 MAPK. This effect on p44 MAPK was partially blocked by genistein and abrogated by PD 98059. In rat neutrophils, activation of MAPK induced by fMLP is sensitive in a dose-dependent manner to inhibitors of TK, PKC, PLC and chelators of intracellular Ca^{2+} (Chang *et al.*, 1999), suggesting that MAPKs are a converged downstream target for extracellular signaling by fMLP. p42/p44 MAPKs can be phosphorylated by MAPK kinase (MEK), an enzyme inhibited by PD 98059 (Dudley et al., 1995). This compound prevents the activation of MAPK kinase 1 by Raf or MEK kinase *in vitro* with an IC_{50} of 2-7 μM. However, PD 98059 inhibited the activation of MAPK kinase 2 by Raf at a larger IC_{50} value (50 μM) (Alessi *et al.*, 1995). Another inhibitor of the MEK pathway used in this study was UO126. This compound selectively inhibits MEK-1 and MEK-2 (Favata *et al.*, 1998) and had similar effects on PLA_2 activity as PD 98059.

Taken together, the observed phosphorylation of $cPLA_2$ and the effects of the protein inhibitors in the 2,2',4,4'-tetrachlorobiphenyl-induced PLA_2 activity suggest that phosphorylation/activation of this enzyme occurs following the signal transduction cascade: PKC or TK⇒ras (Raf)⇒MEK⇒MAPK⇒$cPLA_2$, as a typical response to cellular stress or mitogenic signals (Norris and Baldwin., 1999; Takahashi *et al.*, 1999; Qiu and Leslie, 1994).

In contrast to $cPLA_2$, the Ca^{2+}-independent PLA_2 ($iPLA_2$) is not regulated by phosphorylation but can be modulated by protein-protein interaction with calmodulin (Wolf and Gross, 1996). As observed with

Aroclor 1242 (Chapter IV), non cytotoxic concentrations of the calmodulin inhibitors calmidazolium and W7 were slightly more effective than the kinase inhibitors to inhibit 2,2',4,4'-tetrachlorobiphenyl-induced activation of PLA_2. In bovine tracheal smooth muscle strips W7 and calmidazolium inhibit the Ca^{2+}-calmodulin-induced activation of myosin light chain kinase with IC_{50} values of 25 μM and 1 μM, respectively (Asano, 1989), which closely match the effective inhibitory concentrations used in this study. Molecular structure of the calmodulin complexed with W7 has been reported (Osawa *et al.*, 1998). W7 binds to hydrophobic amino acid residues which occur in the vicinity of Ca^{2+} binding sites and elicits structural changes similar to trifluoperazine and oxmetidine (Akiyama and Sutoo, 1988). Upon binding, W7 tends to form a globular structure in solution (Osawa *et al.*, 1999). Studies of calmodulin with W7 have suggested that this protein can accommodate a variety of bulky, aromatic rings into the two hydrophobic pockets (Osawa *et al.*, 1998), supporting a hypothesis that PCBs may be ligands for this Ca^{2+}-regulatory protein.

Many molecules can bind calmodulin, for instance tamoxifen (McCague *et al.*, 1994), the antitumor drugs KAR-2 and vinblastine (Vertessy *et al.*, 1997; Orosz *et al.*, 1997), and cyclosporin-A (Knott *et al.*, 1994) among others. It has been recently demonstrated that calmodulin can bind simultaneously different drugs such as trifluoperazine and KAR-2 or vinblastine and KAR-2 (Vertessy *et al.*, 1998). An interesting possibility

is that calmodulin can be a carrier protein for PCBs, allowing PCBs to interact directly with the $iPLA_2$ while the calmodulin-$iPLA_2$ complex is formed through a different binding site. Experimental evidence showing the binding of PCBs to calmodulin will be necessary to prove some of the effects observed with calmodulin inhibitors. However, it is likely that PCBs are binding to calmodulin because of their hydrophobic nature and presence of aromatic rings, two structural requirements for drug binding to this protein (Hait *et al.*, 1986).

In short, the *ortho*-PCB 2,2',4,4'-tetrachlorobiphenyl, but not the non-*ortho*-PCB 3,3',4,4'-tetrachlorobiphenyl, activated neutrophil PLA_2. This PLA_2 activity was sensitive to BEL, suggesting the stimulation of $iPLA_2$. Experiments with calmodulin inhibitors indicate that calmodulin can be an important regulator of the $iPLA_2$ activity induced by *ortho*-PCBs. Phosphorylation of $cPLA_2$ and p42/p44 MAPK and inhibition of a fraction of the observed PLA_2 activity by different kinase inhibitors presumes that in addition to $iPLA_2$, $cPLA_2$ is also activated by 2,2',4,4'-tetrachlorobiphenyl through a mechanism involving TK, PKC, ras, and the MEK pathway.

Chapter VI

STRUCTURE-ACTIVITY RELATIONSHIPS FOR THE ACTIVATION OF RAT NEUTROPHIL PHOSPHOLIPASE A_2 BY ORGANOCHLORINE COMPOUNDS

VI.1. Summary

Organochlorine compounds (OCs) are some of the main toxicants present in the food web and target different cellular systems including the non-specific immune system. The objective of this study was to test the hypothesis that OCs that activate neutrophils share common structural features. Using activation of phospholipase A_2 (PLA_2) as a marker of neutrophil activation, isolated rat neutrophils were exposed to a variety of OCs. The *ortho*-substituted polychlorinated biphenyl 2,2',4,4'-tetrachlorobiphenyl, the α-, δ- and γ-hexachlorocyclohexanes (HCCHs), *p,p'*-diorthodichlorotrichloroethane (DDT), dieldrin and chlordane, but not the non-*ortho* substituted 3,3',4,4'-tetrachlorobiphenyl or β-HCCH induced activation of PLA_2 in neutrophils. This activation of PLA_2 was sensitive to bromoenolactone (BEL), suggesting that a Ca^{2+}-independent isoform of PLA_2 is activated by these compounds. Molecular modeling techniques were used to develop structure-activity relationships for the activation of PLA_2 by OC compounds. Superimposing three-dimensional structures we have identified an electrotopological motif shared by all the active compounds, including the $iPLA_2$ inhibitor BEL. This motif is absent in the inactive β-HCCH and non-*ortho* chlorinated PCBs. These results suggest that the bioactivity of organochlorine compounds in neutrophils may be due to the presence of a specific substructure that fits into a receptor-like structure, allowing the activation of the neutrophil PLA_2. This motif, which

we have called the OG motif, consists of a planar hydrophobic domain connected rigidly at a perpendicular angle to a negatively charged atom.

VI.2. Introduction

One of the forces that moved the industrial age was the extensive use of new chemicals which allowed the production of large quantities of food supplies and the decrease in child mortality. Among these chemicals, organochlorine (OC) compounds were of particular importance due to their versatility and low cost. These same chlorinated chemicals such as polychlorinated biphenyls and chlorinated pesticides such as DDT, dieldrin, hexachlorocyclohexanes and chlordane are now recognized as food contaminants due to their accumulation in the food chain. Adding to concern about OCs is their diverse toxicity. The immune system, and particularly the neutrophil, has been shown to be a target for OC compounds such as PCBs (Ganey *et al*, 1993), hexachlorocyclohexanes (HCCHs) (Dunstan *et al.*, 1996), DDT (Sitarska *et al.*, 1990), dieldrin (Hewett and Roth, 1988) and chlordane (Miyagi *et al.*, 1998), among others. For example, PCBs activate neutrophils through complex biochemical mechanisms to induce superoxide anion release and degranulation. These processes are mediated through the activation of PLA_2.

Toxicological testing of all the known OC food contaminants for their activity on neutrophil function is not practical because it is expensive and time consuming. A knowledge of the molecular structure and the biological activity of groups of related compounds will allow the development of structure-activity relationships (SARs) which can be critical not only to predict the activity of unknown, related compounds but also for risk assessment. Different SAR studies have been performed for PCBs (Van der Burght *et al.*, 1999; Brown *et al.*, 1998; Kodavanti *et al.*, 1997; Mekenyan *et al.*, 1996), cyclodienes (Rohr *et al.*, 1985), DDT (Coats, 1990), and other halogenated pesticides (Moser *et al.*, 1993). In neutrophils, SAR studies have been conducted for the activity of PCBs to induce superoxide anion production (Brown *et al.*, 1998) and degranulation (Olivero and Ganey, 1998). This work presents the results of an SAR study conducted to find molecular features responsible for the activation of PLA_2 by OC compounds.

VI. 3. Materials and methods

VI. 3. A. Chemicals

$[^3H]$[5,6,8,9,11,12,14,15]-AA ($[^3H]$-AA; 180-240 Ci/mmol) was purchased from DuPont NEN (Boston, MA), BEL was purchased from Biomol (Plymouth Meeting, PA). Organochlorine compounds 2,2',4,4'-tetrachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl, α-, β-, δ-, and γ-hexachlorocyclohexane, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene (dieldrin), p,p'-dichlorodiphenyltrichloroethane (DDT) and 1,2,4,5,6,7,8,8-octachlor-2,3,3A,4,7,7A-hexahydro-4,7-methanoindane (chlordane) were purchased from ChemService (West Chester, PA).

VI. 3. B. Isolation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneum of male, Sprague-Dawley, retired breeder rats by glycogen elicitation (Hewett and Roth, 1988). Isolated neutrophils were resuspended in Hanks' balanced salt solution (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.

VI. 3. C. Exposure to organochlorine compounds

Organochlorine (OC) stock solutions were prepared by dissolution in N,N-dimethylformamide (DMF). Neutrophils ($2x10^6$) were suspended in HBSS (1 mL) in borosilicate glass test tubes, 12 x 75 mm (VWR, Chicago, IL), and 1 μL of the OC stock solution was added to the tubes to achieve the desired concentration. Control neutrophils received 1 μL of DMF.

VI. 3. D. 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 [^{3}H]-AA for two hours, gently shaking at 37°C. Neutrophils were then washed twice with Mg^{2+}- and Ca^{2+}-free HBSS. The cell count was adjusted so that the final concentration of neutrophils was $2x10^6$/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 routinely between 80-88%. Release of [^{3}H]-AA from labeled neutrophils was measured in cells treated with OCs for 30 minutes (37 °C). Studies with the $iPLA_2$ inhibitor BEL were conducted by incubating the cells with BEL for 20 mins (37 °C) and then with OC compounds for an additional 30 minutes at the same temperature. At the end of each incubation, neutrophils were placed on ice and spun in a centrifuge 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.

IV. 3. E. Cytotoxicity assay

Release by neutrophils of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium was used as an indicator of cytotoxicity. LDH activity present in 10 µL of the supernatant fluid from the

PLA_2 assay was measured according to the method of Bergmeyer and Bernt (1974).

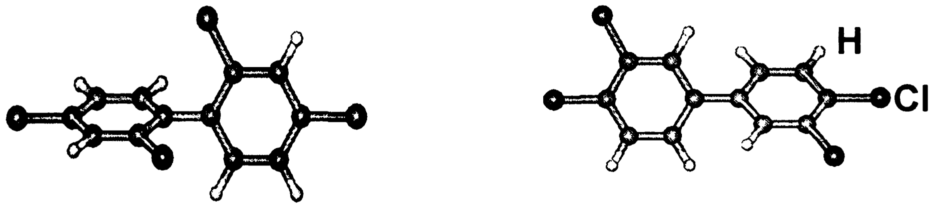
VI. 3. F. Structure-activity relationships

VI.3.F.i Data set

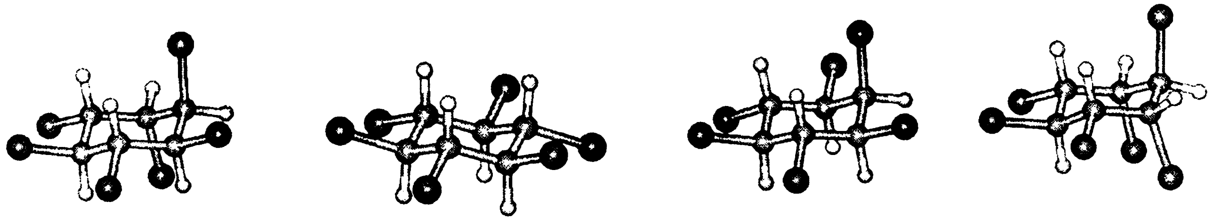
Organochlorine compounds tested for their ability to activate PLA_2 were 2,2',4,4'-tetrachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl, α-, β-, δ-, and γ-hexachlorocychlohexane, DDT, dieldrin and chlordane. Compounds that induce a statistically significant release of arachidonic acid at 25 μM when compared to that elicited by the vehicle control (DMF) were considered active. Molecular structures for the compounds in the data set appear in Figure VI.1.

VI.3.F.ii. Computational details.

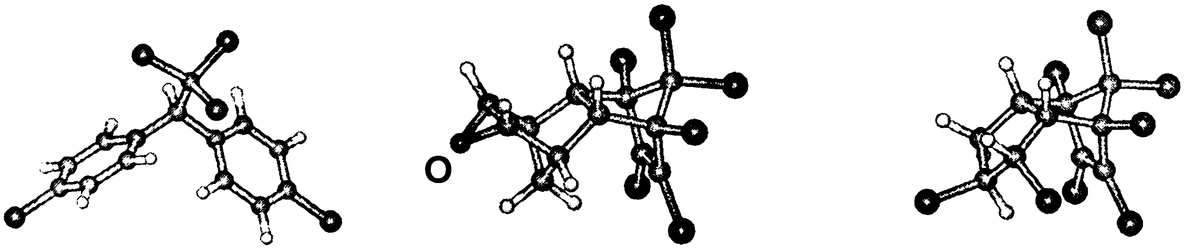
Molecular modelling procedures used in this study were performed using Hyperchem 5.1 (Hypercube Inc, 1996). The compounds in the data set were entered as two-dimensional sketches into Hyperchem and stored as atomic coordinates. The presence of a torsional angle in the PCB structures and DDT generates different conformers, among which some are less energetically favorable. To search for the most stable structure, molecule geometries for these compounds were submitted to a conformational search to obtain a conformer with the lowest energy (i.e.,



2,2',4,4'-tetrachlorobiphenyl **3,3',4'4-tetrachlorobiphenyl**



α-**HCCH** β-**HCCH** δ-**HCCH** γ-**HCCH**



DDT **Dieldrin** **Chlordane**

Figure VI.I. ***Molecular structure of organochlorine compounds used in this study.***

the most stable conformer). Full optimization geometry for the best conformer for PCBs, DDT, and the other OC compounds was performed using the semiempirical method AM1 (Dewar, 1985) running on Hyperchem. Electronic properties were calculated from single point calculations at the ab initio level STO-3G (Hehre *et al.*, 1969). Other properties from the energetically minimized structure, such as the molecular weight and the total solvent-accessible surface area were calculated using the subroutine QSAR properties implemented in ChemPlus/ Hyperchem 5.1.

VI.3.F.iii. Model construction

Establishment of relationships between the molecular structure and the PLA_2 activity for the OC compounds in the data set was done following a structure-based design approach. The intrinsic biological/toxicological activity or potency can be a function of the three-dimensional shape of the molecule and of the electric charge distribution and lipophilicity. These are also determinant factors in the molecular affinity for a receptor. Accordingly, comparison of conformational profiles of various molecules to a template may reveal those overlapping or similar conformational spaces and electronic features which are common to the molecules that elicit a particular effect. General similarities between the OC were searched by superposition of selected atoms of an OC compound on their chemically equivalent atoms on the 2,2',4,4'-

tetrachlorobiphenyl used as a template. The Hyperchem subroutine ChemPlus was used to perform the calculations. Initially, the file for the optimized 2,2,'4,4'-tetrachlorobiphenyl molecule was open and merged with the targeted optimized geometry. For each molecule six atoms were selected, and the function "RMS overlay" was used to perform the superpositions. The program translates the centers of the atoms to be fitted to the centroid of the corresponding atom in the reference molecule. The best fit was obtained when the structure superposition gave the lowest root-mean-square deviation (RMSD) value between selected pairs of equivalent atoms using the 2,2',4,4'-tetrachlorobiphenyl as a reference. After visual comparison of the superpositions of 2,2',4,4'-tetrachlorobiphenyl with the active vs. the inactive OCs, the molecular features, both topological and electronical, present or absent in both groups were determined (SAR model). In order to validate the model, non-cytotoxic doses of different organochlorine and organobromine compounds were tested to see if they activated PLA_2 following the structural rules presented by the SAR model. The general procedure followed to obtain the SAR is presented in Figure VI.2.

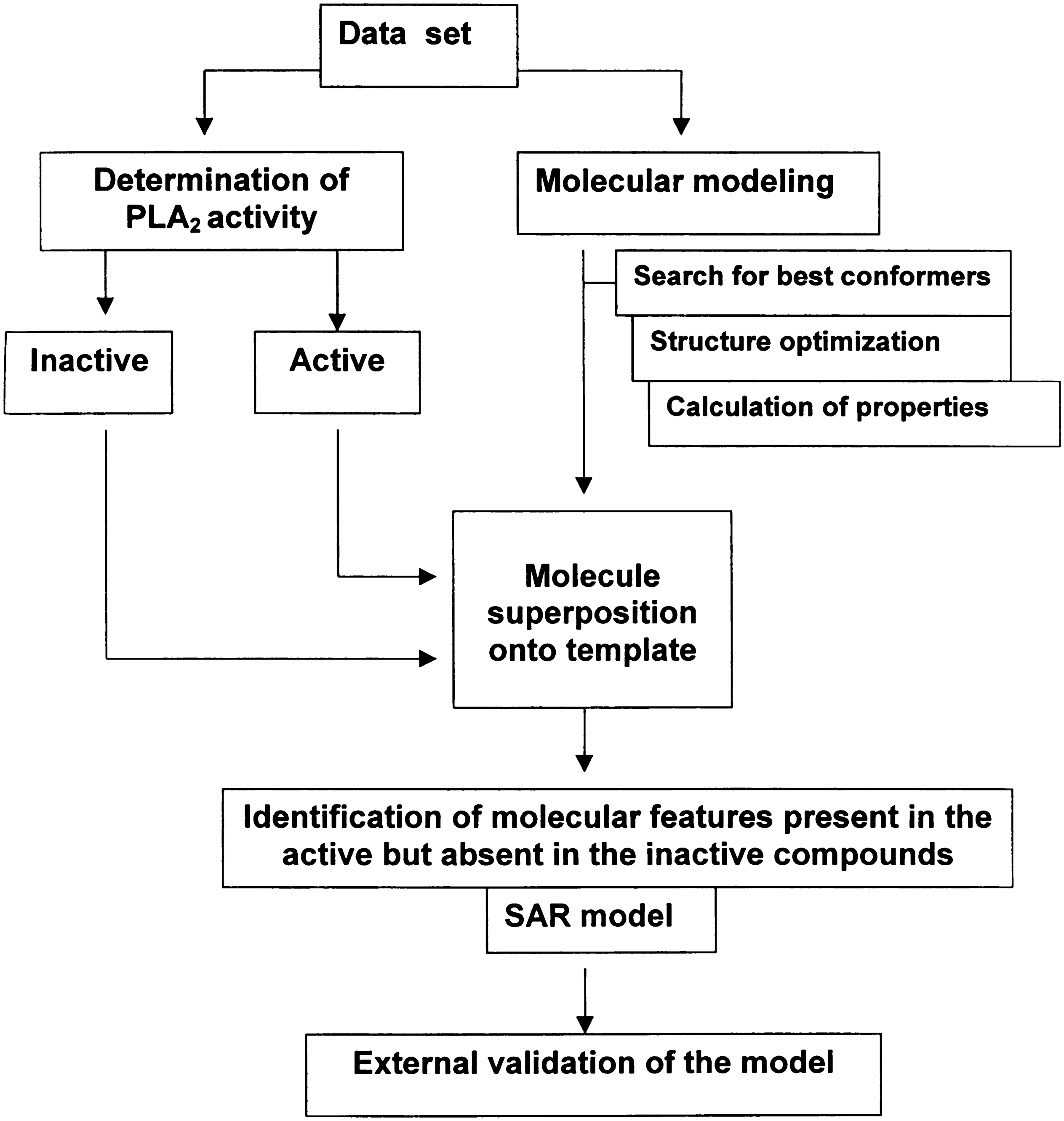


Figure VI.2. *General scheme for the development of structure-activity relationships for organochlorine compounds and activation of rat PLA$_2$.*

VI. 4. Results

VI.4.1. PLA_2 activity induced by organochlorine compounds.

The dose-response curve for the activation of rat neutrophil PLA_2 by different OC compounds showed clear differences in activity. 2,2',4,4'-tetrachlorobiphenyl activated PLA_2 at concentrations ≥ 5 µM. Its activity at concentrations ≥ 10 µM was similar to that elicited by 10 µg/mL Aroclor 1242 (See Chapter V, Figure V.1). Concentrations greater than 50 µM produced some cytotoxicity (≈20% LDH release) (Figure V.1). 3,3',4,4'-tetrachlorobiphenyl failed to activate PLA_2 at concentrations up to 25 µM (Figure V.2). Greater concentrations were not compatible with the buffer and precipitated. α-, δ-, and γ-HCCH activated PLA_2 at concentrations equal to or greater than 10 µM (Figures VI.3, VI.4, and VI.5). Of these hexachlorocyclohexanes, α-HCCH was the least efficacious. No cytotoxicity was observed for any of these OCs at the maximal concentrations tested (100 µM). Conversely, β-HCCH (Figure VI.6) was unable to activate PLA_2 over the concentration range tested (0-100 µM).

DDT, dieldrin and chlordane each activated PLA_2 at concentrations equal to or greater than 10 µM (Figures VI.7, VI.8 and VI.9). Only dieldrin

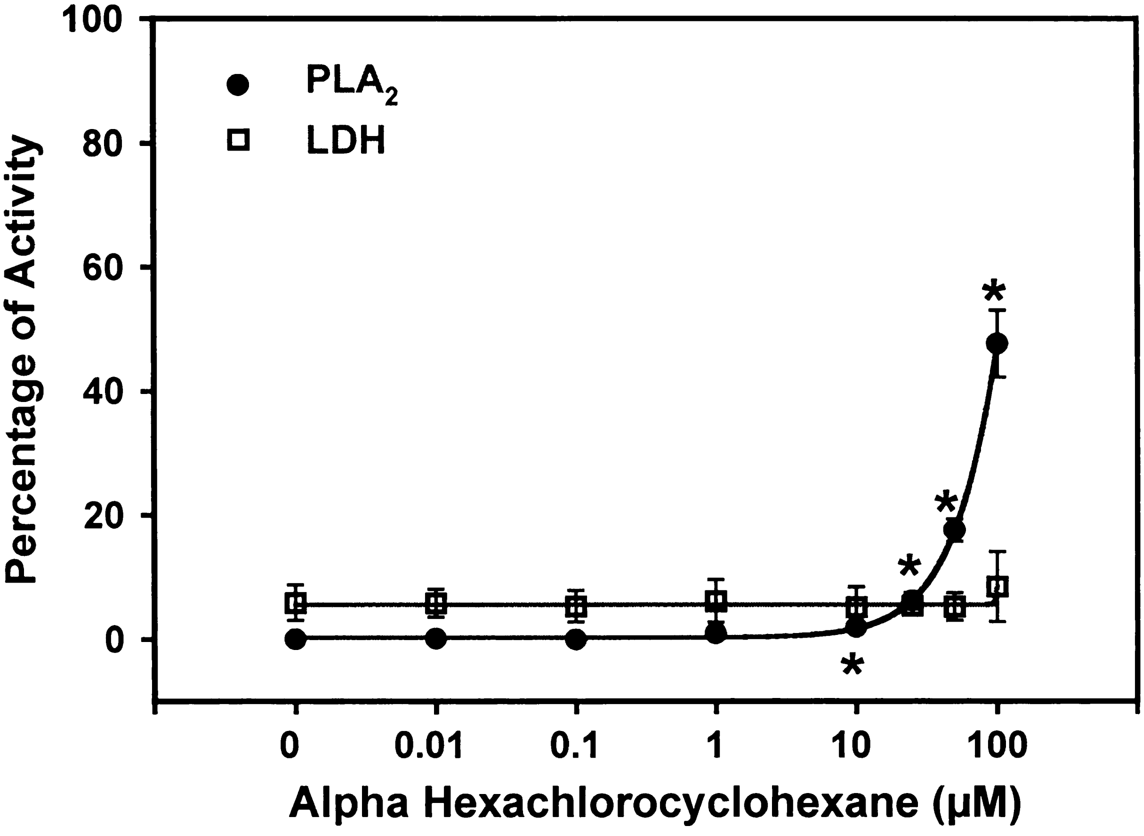


Figure VI.3. ***Effects of α-HCCH on neutrophil PLA$_2$ activity and cytotoxicity***. Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 μg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. Under these conditions, Aroclor 1242 released 25-35% of total [^{3}H]-AA. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.
*. Significantly different from vehicle control (DMF).

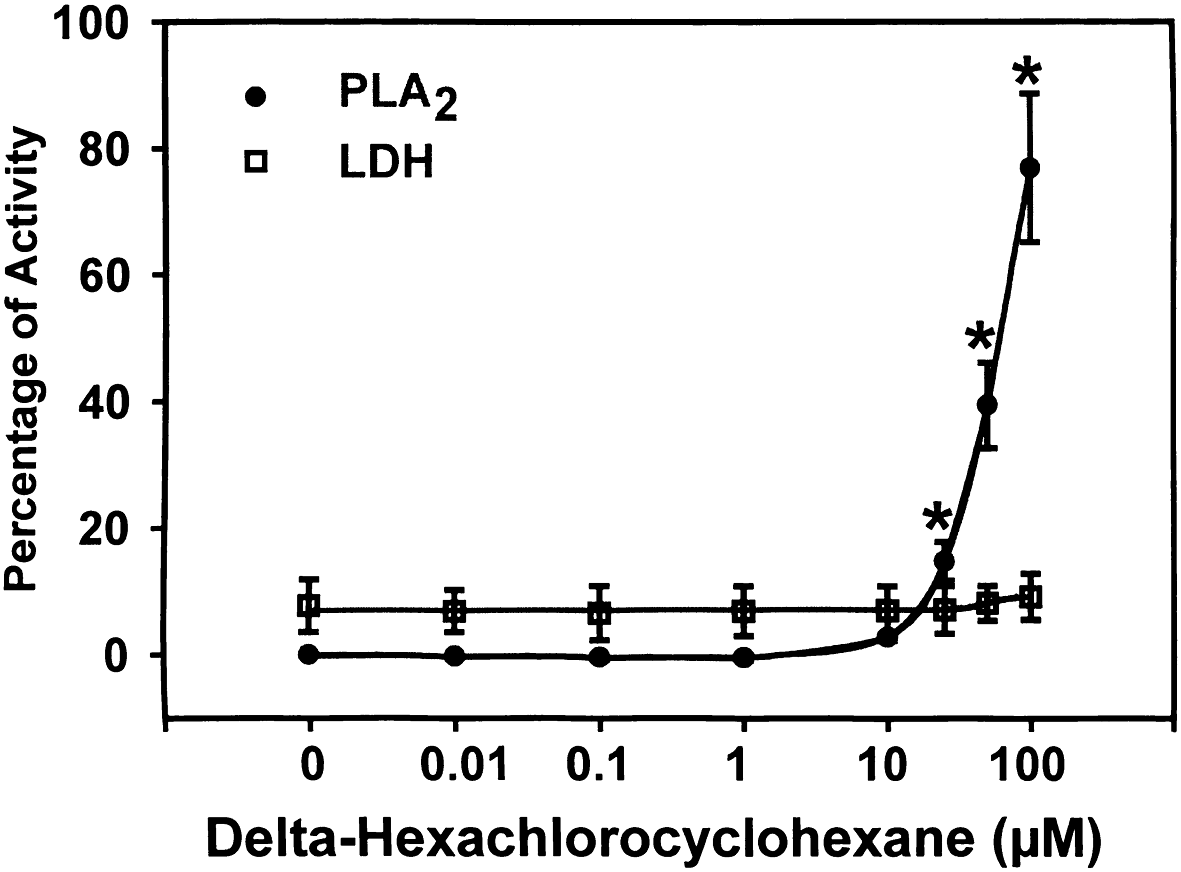


Figure V ***and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 µg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

*. Significantly different from vehicle control (DMF).

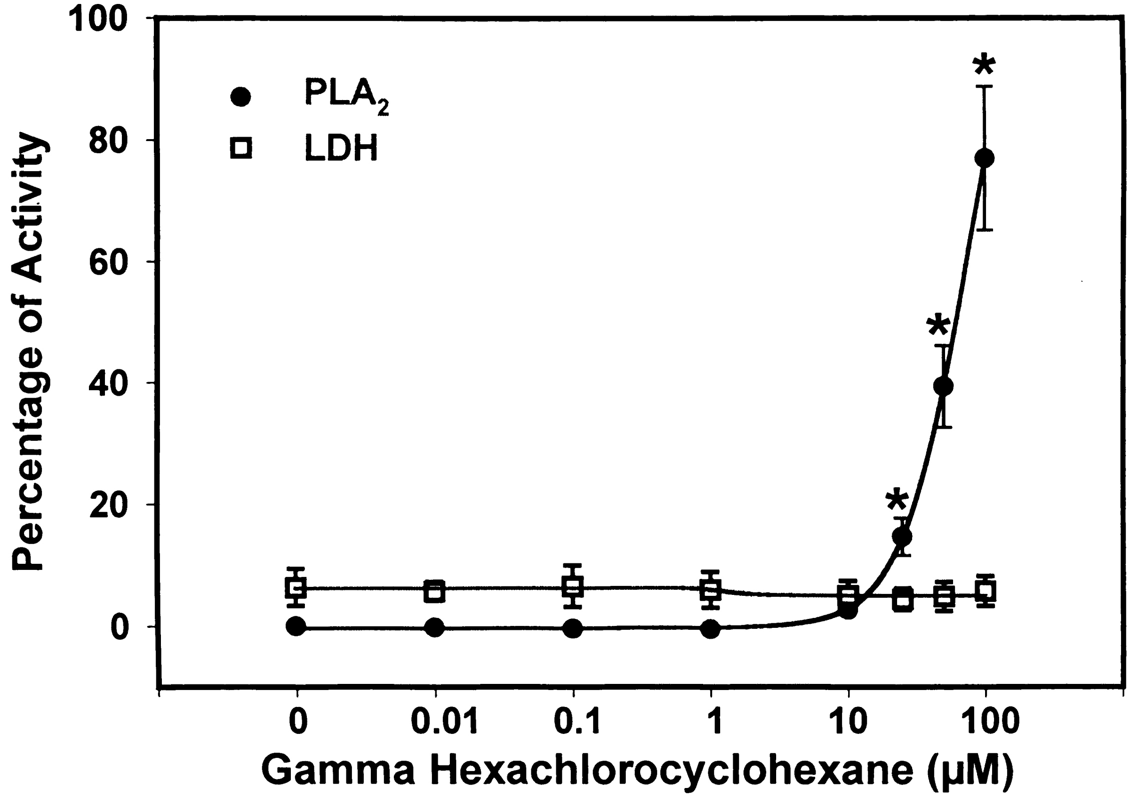


Figure VI.5. ***Effects of γ-HCCH on neutrophil PLA$_2$ activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 µg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

*. Significantly different from vehicle control (DMF).

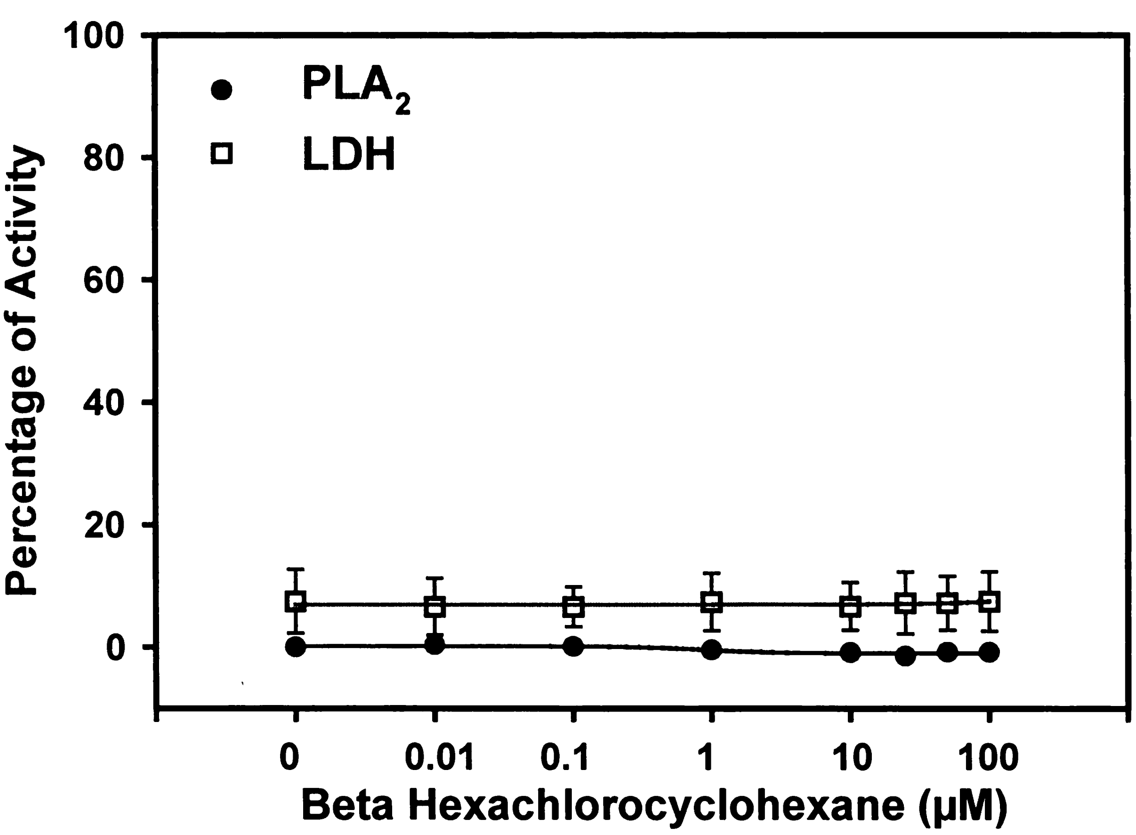


Figure VI.6. ***Effects of β-HCCH on neutrophil PLA$_2$ activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 μg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.
*. Significantly different from vehicle control (DMF).

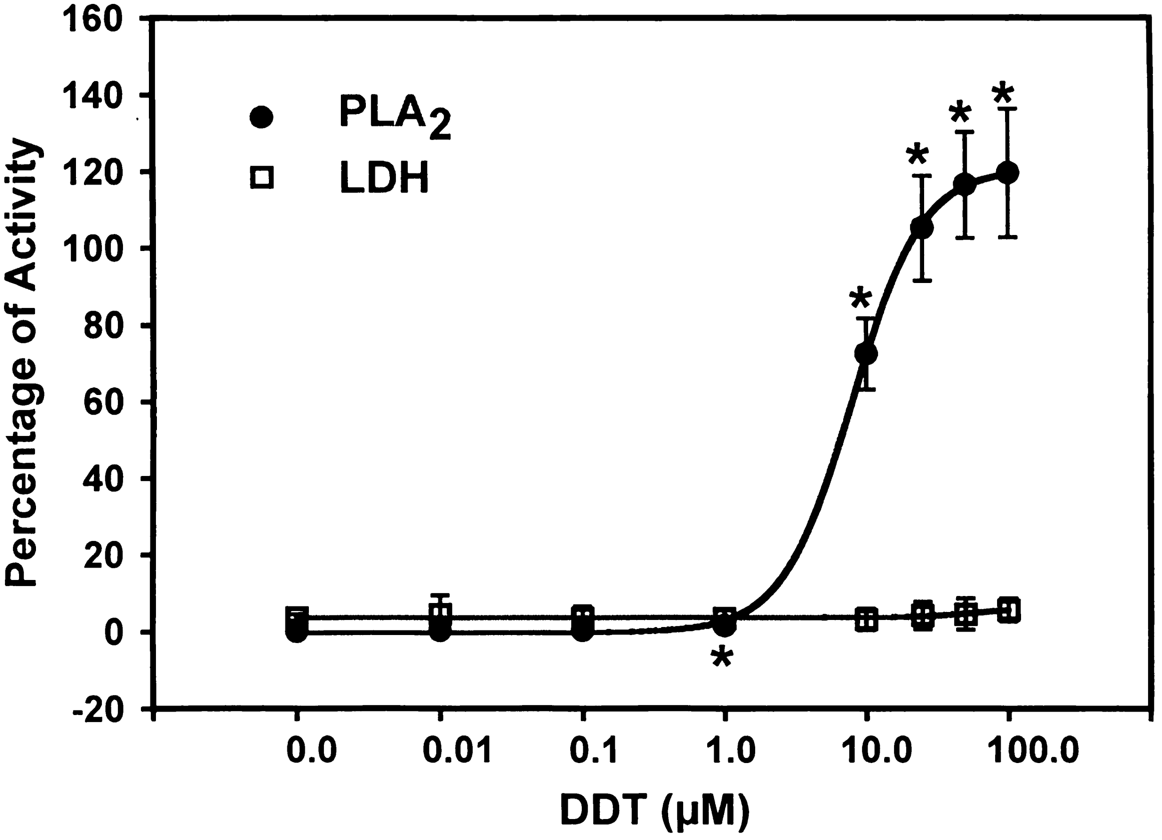


Figure VI.7. ***Effects of DDT on neutrophil phospholipase A_2 activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 μg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

*. Significantly different from vehicle control (DMF).

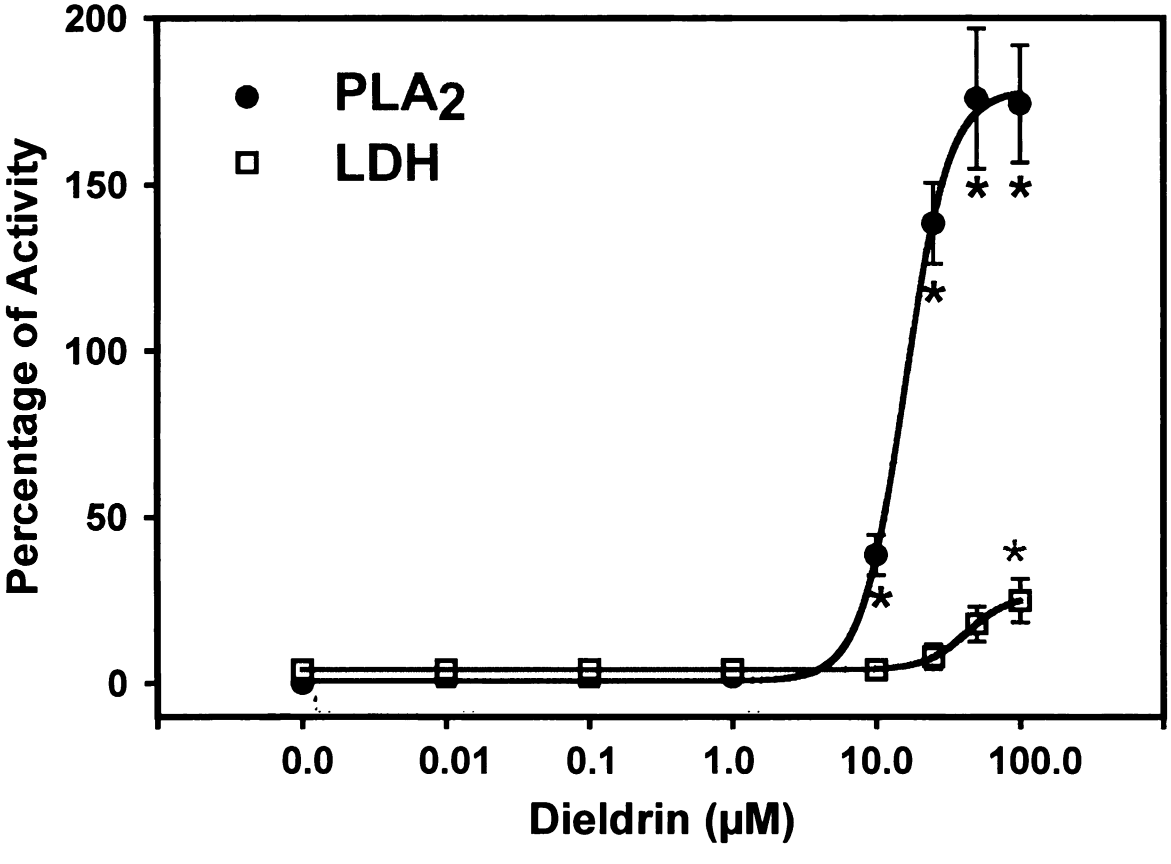


Figure VI.8. ***Effect of Dieldrin on neutrophil PLA$_2$ activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 µg/mL Aroclor 1242-treated neutrophils under the same experimental conditions and are presented as percentage of activity of Aroclor 1242. LDH released at each concentration was compared with the LDH released from neutrophils lysed with 0.01% Triton X-100 and are presented as percentage of total LDH activity. Results are expressed as means ± SEM for four different experiments performed in triplicate.

*. Significantly different from vehicle control (DMF).

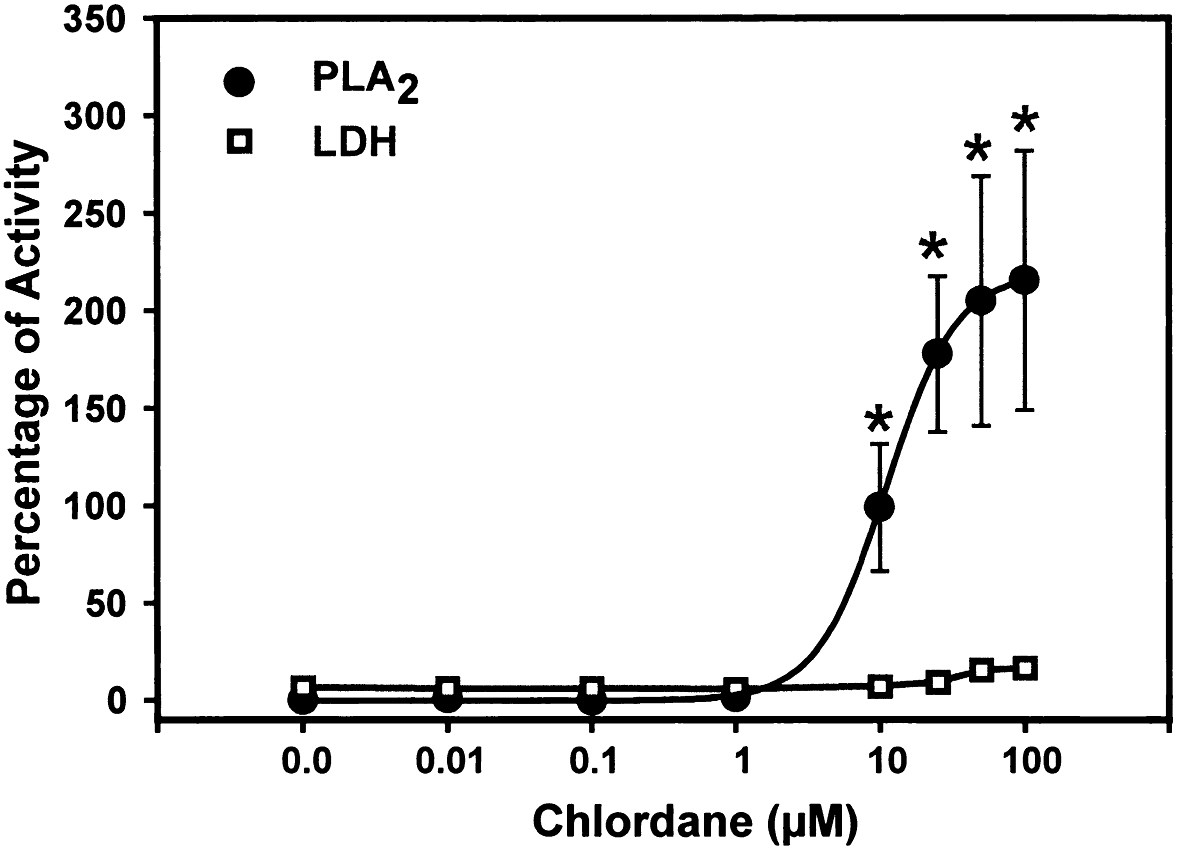


Figure VI.9. ***Effects of Chlordane on neutrophil phospholipase A_2 activity and cytotoxicity.*** Neutrophils were labeled with [^{3}H]-AA and incubated with the concentrations shown, as stated in Materials and methods. The release of [^{3}H]-AA into the medium at each concentration was compared with the release from 10 μg/mL Aroclor 1242- treated neutrophils under the same experimental conditions, and are presented as percentage of activity of Aroclor 1242. LDH release at each concentration was compared with the LDH release from 0.01% Triton X-100-lysed neutrophils and are presented as percentage of activity of Triton X-100. Results are expressed as means ± SEM from four different experiments performed in triplicates.

*. Significantly different from vehicle control (DMF).

presented a significant cytotoxicity (>20%). This occurred at concentrations greater than 50 μM.

PLA_2 activity elicited by the active compounds was abrogated more than 50% by BEL (Figure VI.10), suggesting that a Ca^{2+}-independent PLA_2 is targeted by these OCs. BEL treatment did not induce significant toxicity for any of the tested compounds. These results are consistent with previous reports for PCBs.

VI.4.2. Structure-activity relationships

Molecular structures for the OCs used in this study are diverse, and apparently they have few particular similarities. Calculation of selected electronic and topological properties of these compounds is shown in Table VI.1. It is evident that global properties such as the energy of the frontier orbitals highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) and dipolar moment vary considerably among compounds. Interestingly, the inactive compounds 3,3',4,4'-tetrachlorobiphenyl and β-HCCH have the lowest dipolar moment values.

In order to look for common features responsible for the activity of OC compounds on PLA_2, the structure of the *ortho*-chlorinated PCB

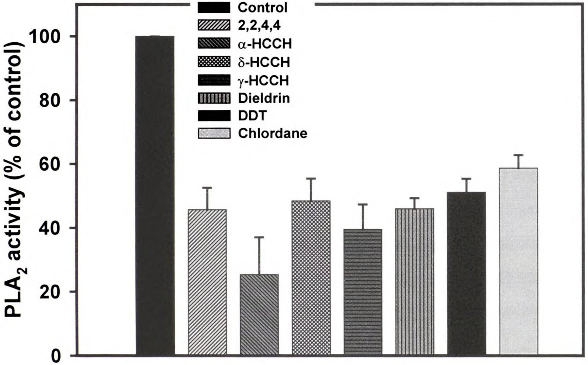


Figure VI.10. ***Effects of BEL on PLA_2 activity induced by OC compounds.*** Neutrophils were labeled with $[^3H]$-AA and incubated with 25 µM BEL for 20 min at 37 °C followed by incubation with the OC for 30 min at 37 °C. A. The release of $[^3H]$-AA into the medium at each concentration was compared with the release by the same compound in absence of BEL. Results are expressed as means ± SEM from four different experiments performed in triplicate.

Table VI.1. *Molecular descriptors for OCs in the data set.*

Molecules	Molecular descriptors*						
	MW (g/mol)	SA (Å^2)	DM (Debye)	MPC	MNC	HOMO (eV)	LUMO (eV)
2,2’,4,’4-TCB**	291.99	395.6	2.573	0.098	-0.108	-8.053	5.645
3,3’,4,’4-TCB	291.99	418.9	1.273	0.088	-0.095	-7.430	4.608
α-HCCH	290.83	396.4	2.707	0.124	-0.134	-10.943	7.103
β-HCCH	290.83	411.4	0.000	0.114	-0.116	-10.990	7.012
δ-HCCH	290.83	402.2	2.788	0.125	-0.126	-10.966	7.150
γ-HCCH	290.83	388.3	3.782	0.127	-0.146	-10.656	6.781
DDT	354.49	460.4	1.490	0.118	-0.117	-7.757	5.578
Dieldrin	380.91	370.5	2.953	0.095	-0.215	-8.733	5.302
Chlordane	409.78	445.20	2.934	0.112	-0.145	-9.088	5.048

*. **MW**: Molecular weight; **SA**: solvent accessible surface area; **DM**: dipolar moment; **MPC** and **MNC**: Most positive and most negative charge in the molecule, respectively; **HOMO**: energy of the highest occupied molecular orbital. **LUMO**: Energy of the lowest unoccupied molecular orbital.

**. TCB, tetrachlorobiphenyl

2,2',4,4'-tetrachlorobiphenyl, an activator of PLA_2 (See chapter V), was used as a template to superimpose the structures of the other OCs. Superposition of α-HCCH and DDT on 2,2',4,4'-tetrachlorobiphenyl is shown in Figure VI.11. Clearly, α-HCCH can fit on one phenyl group of the PCB and position a chlorine in close proximity to the *ortho* chlorine on the other phenyl ring. Interestingly, DDT and 2,2',4,4'-tetrachlorobiphenyl are superimposed almost perfectly. Both superpositions showed a planar structure connected to a chlorine perpendicular to the plane. Superposition of dieldrin and chlordane on 2,2',4,4'-tetrachlorobiphenyl is presented in Figure VI.12. Although dieldrin and chlordane lack phenyl rings, they each have a planar-like domain which fits onto one phenyl group of the PCB, and they also can position a chlorine atom in the vicinity of the *ortho*-chlorine of the other phenyl group on the PCB.

Superposition of the inactive compounds 3,3',4,4'-tetrachlorobiphenyl and β-HCCH on 2,2',4,4'-tetrachlorobiphenyl is shown in Figure VI.13. Although both compounds can be superimposed to 2,2',4,4'-tetrachlorobiphenyl through their planar motifs, they failed to match a chlorine with the chlorine in the *ortho-* position of the template PCB. Finally, BEL, a known inhibitor of the Ca^{2+}-independent PLA_2, could also be almost perfectly superimposed onto 2,2',4,4'-tetrachlorobiphenyl (Figure VI.14). In summary, despite differences in structure, all the active

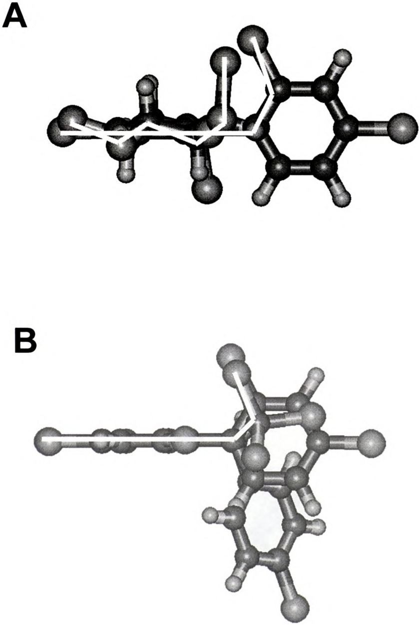


Figure VI.11. ***Molecular structures of superimposed organochlorine compounds***. A. 2,2',4,4'-Tetrachlorobiphenyl is superimposed on α-HCCH. B. 2,2',4,4'-Tetrachlorobiphenyl is superimposed on DDT. White lines indicate the substructure shared by both compounds.

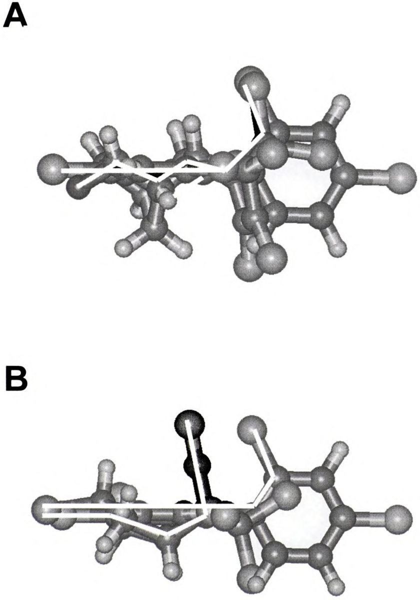


Figure VI.12. ***Molecular structures of superimposed organochlorine compounds.*** A. 2,2',4,4'-Tetrachlorobiphenyl is superimposed on dieldrin B. 2,2',4,4'-Tetrachlorobiphenyl is superimposed on chlordane. White lines indicate the substructure shared by both compounds.

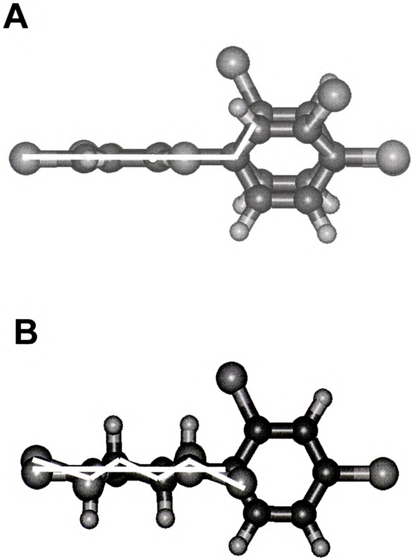


Figure VI.13. ***Molecular structures of superimposed organochlorine compounds.*** A. 2,2',4,4'-Tetrachlorobiphenyl is superimposed to 3,3',4,4'-tetrachlorobiphenyl. B. 2,2',4,4'-Tetrachlorobiphenyl is superimposed to β-HCCH. White lines indicate the substructure shares by both compounds.

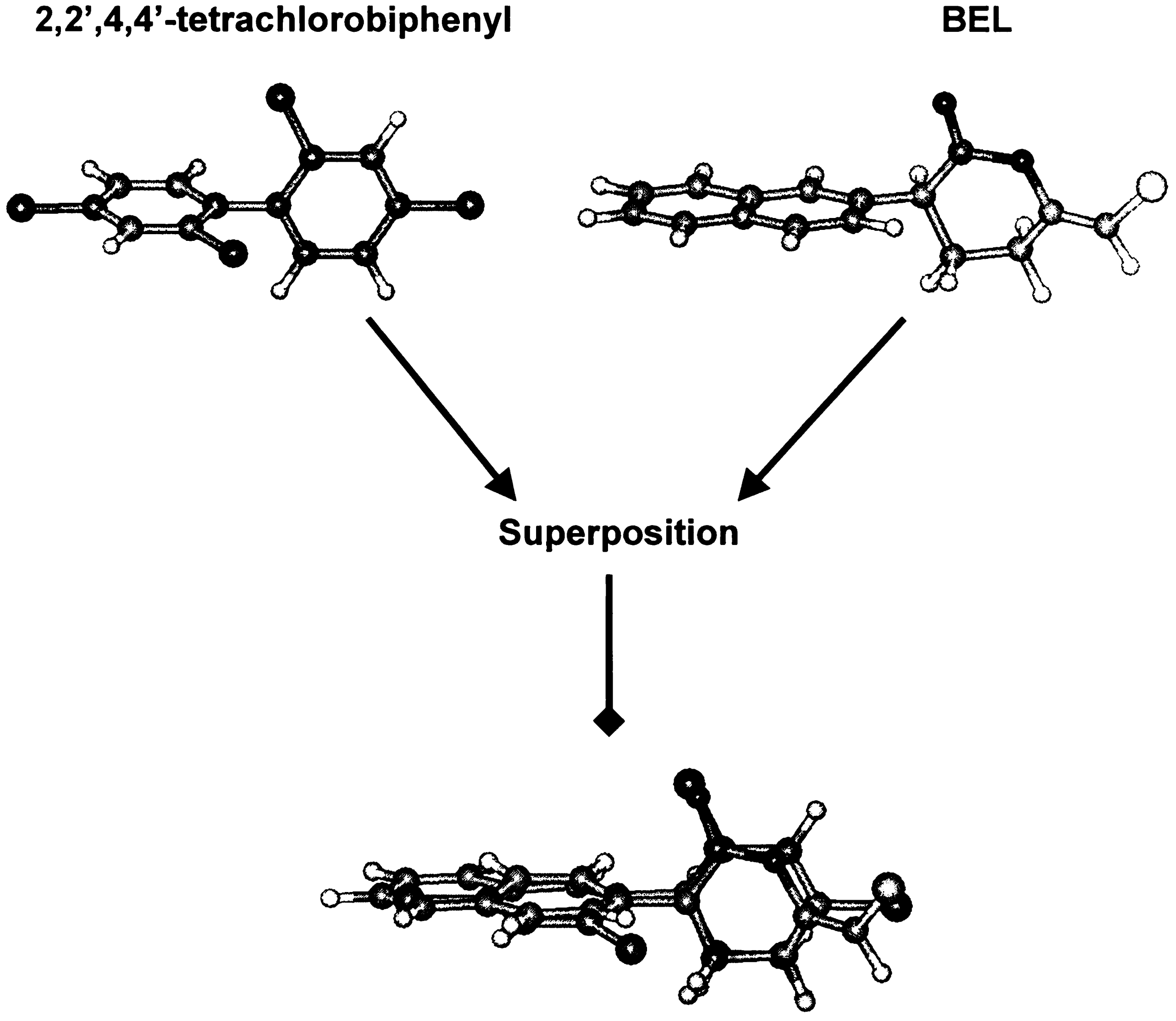


Figure VI.14. ***Superposition of 2,2',4,4'-tetrachlorobiphenyl and BEL, an inhibitor of Ca^{2+}-independent PLA$_2$.***

compounds could be superimposed with 2,2',4,4'-tetrachlorobiphenyl revealing the presence of a motif characteristic to all of them. We have called this substructure the OG motif. The OG motif was absent in the inactive OCs.

External validation of the model with some halogenated compounds revealed that the OG motif is present in the active compounds 2,4'-diclorobiphenyl, 2,2'-dibromobiphenyl and aldrin. However, it is absent in the inactive compounds 4,4'-dichlorobiphenyl and 4,4'-dibromobiphenyl.

VI. 5. Discussion

In this chapter the use of an empirical model to classify chlorinated compounds as activators of the neutrophil, BEL-sensitive PLA_2 has been described. The results of this SAR study suggest that OC molecules use a single, molecular substructure, the OG motif, to activate the Ca^{2+}-independent isoform of PLA_2. It is tempting to summarize the SAR study in terms of structural requirements that determine the activity of OCs toward PLA_2. Three factors are necessary to define the OG motif present in OC compounds. First is the presence of a planar like-structure that is mainly hydrophobic (PH) and measures between 4.6 Å (α-HCCH) and 7.6 Å (BEL). The second is the presence of an electronegative (ENR) region located perpendicular to one corner of the planar structure within 1.8 Å (α-HCCH) to 2.7 Å (2,2',4,4'-tetrachlorobiphenyl). The third part is a rigid connector or bridge between PH and ENR. This bridge is important to generate some rigidity to position the ENR in the binding site. This steric requirement may suggest the presence of a protein receptor. A diagram of the OG motif is shown in Figure VI.15. It is important to mention that one advantage of this approach is that it considers simultaneously both topological and electronic features of the molecule and does not require additional interpretation as from molecular descriptors obtained from classical QSARs. SARs can provide good activity prediction for compounds in homologous series. Although the compounds presented here are of diverse molecular structure, they may be considered as an homologous series, and that emphasizes the usefulness of the model.

This approach of looking at sub-structures associated with toxicity of OCs has been applied successfully to study the effects of dieldrin in GABA channel functioning (Matsumura, 1985) and the assessment of carcinogenicity by OC compounds (King and Srinivasan, 1996).

The activation of PLA_2 by the compounds having the OG motif would occur by assuming a model in which each compound interacts with a hydrophobic pocket within PLA_2 and with an electropositive charge nearby. Given that *ortho*-chlorinated and not non-*ortho*-chlorinated PCBs activate PLA_2, the steric rotational impediment of *ortho*-PCBs may be necessary for their activity. It is consistent with the model that activation may depend on the possibility of the 2-chlorine of PCBs to interact with a positive site in the

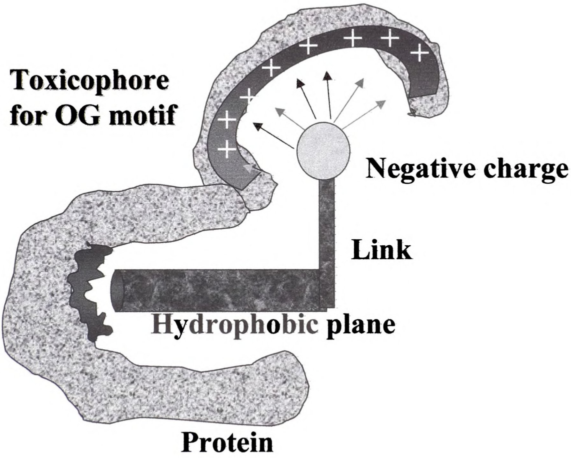


Figure VI.15. ***Representation of the OG motif of OC compounds interacting with the toxicophore.***

toxicophore. This toxicophore, presumably present in PLA_2, is the critical local molecular fragment that is responsible for the activity of OCs in neutrophils.

OC compounds which are able to penetrate cell membranes and interact with PLA_2 are good probes to study the biochemical properties of this enzyme. Direct binding studies are necessary to understand fully the mechanism of activation of PLA_2 by OCs. It is likely that OCs interact directly with the active site of the PLA_2 due to their molecular similarity to BEL. It is known that BEL binds covalently to the enzyme in the active site inhibiting its activity (Hazen *et al.*, 1991c).

One shortcoming of this SAR model is that is cannot provide information on the different potencies of the compounds. Consequently, secondary parameters in addition to the OG motif, such as energy of orbitals, charge density and number of chlorines, are necessary for potency prediction.

One interesting and important observation from these studies is the possibility of similar biological activity among OCs for which structural similarity is not obvious from 2-D rendering. The molecular modelling data showed that DDT, dieldrin, chlordane, α-HCCH, δ-HCCH and γ-HCCH, are likely to behave as PCB like-compounds in some biochemical systems and vice-versa.

It should be stated that the activity predicted from SAR methods is an estimate and that the geometry from quantum calculations may not be entirely correct. However, the concept that quantum chemical approaches can help to clarify mechanistic questions involving drug-receptor interactions has been reinforced (Randic, 1991). In addition, it has been suggested that SARs could and should be used in the hazard assessment process (Fiedler *et al.*, 1990). Accordingly, the biochemical activity of OC compounds having the OG motif should be considered when assessing potential toxicological effects.

Based on this model we predict that other chemicals having the OG motif substructure such as toxaphene and in general all the *ortho*-PCBs and *ortho*-brominated biphenyls are likely to activate neutrophil PLA_2. On the other hand, compounds such as polychlorinated naphthalenes, dioxins or other non-ortho-PCBs are not likely to activate PLA_2. It may be concluded that organochlorine and organobromine compounds having the OG motif have a good likelihood to activate neutrophil PLA_2 and consequently induce neutrophil activation to produce superoxide anion and release enzymes.

CHAPTER VII

Summary

One of the legacies of the twentieth century is the creation of new compounds probably unknown to nature. Although we have managed to produce and use millions of tons of the new substances, we know little about their relationship with our living biochemistry. There is no doubt that the world as we see it today would have not been possible without the widespread use of pesticides and related chemicals. Among the arsenal of compounds developed this century, organochlorine (OC) compounds have proven important for the food industry, polymerization processes, military activities and human health.

Compounds such as polychlorinated biphenyls, DDT, hexachlorocyclohexanes and dieldrin, among other organochlorines, were extensively used without the knowledge of their potential toxicity and effects on the ecosystems. Many years later, their legal production has been halted or banned in many countries. However, these compounds remain accumulated in all the ecosystems and probably are permanently flowing in our own blood. Although during the last twenty years a great amount of information on the toxicity of these compounds has been compiled, there are still many effects which remain to be understood.

PCBs have been extensively studied and some of their physiological targets identified. Alterations in the chemistry of the central nervous

system, impairment of neutrophil function, and cancer are some of the effects attributed to these compounds.

Polychlorinated biphenyls are defined as biphenyls having their hydrogens substituted by chlorines, theoretically producing 209 different congeners. It has been proposed that the presence or absence of chlorines in the 2-position of one of the phenyls is sufficient to toxicologically classify these compounds. Accordingly, PCBs substituted on the 2-position (*ortho*-PCBs) have low affinity for the Ah receptor and are considered to have non-dioxin-like toxicity. On the other hand, the absence of the chlorine in this position allows the compound to have dioxin-like toxicity with higher affinity for the Ah receptor.

In the immune system, neutrophils have been the focus of diverse studies including this one. PCBs cause activation of NADPH oxidase and degranulation. The biochemical mechanisms leading to these changes in neutrophil function include interference with signaling involving Ca^{2+}, tyrosine kinases, phospholipase C and phospholipase A_2 (PLA_2). The goal of this thesis was to test the hypothesis that in neutrophils PCBs cause changes in Ca^{2+} homeostasis and PLA_2 by independent mechanisms and that PLA_2 activation mechanisms can be triggered by any OC compound sharing a particular substructure similar to that found in *ortho*-chlorinated PCBs.

The PCB mixture Aroclor 1242 and 2,2',4,4'-tetrachlorobiphenyl induced increases in $[Ca^{2+}]_i$. These effects were independent of the activity of PLA_2 based on the lack of effect of BEL to inhibit the Aroclor 1242-induced increase in $[Ca^{2+}]_i$. In addition, Aroclor 1242 blocked Ca^{2+} influx elicited by the bacterial product fMLP and failed to increase $[Ca^{2+}]_i$ after intracellular store depletion by this chemotactic agent. Given that the changes in $[Ca^{2+}]_i$ caused by fMLP may be important in the ability of the neutrophil to defend against bacteria, these results suggest that the PCB mixture Aroclor 1242 may lead to neutrophil dysfunction in presence of pathogens.

Changes in the $[Ca^{2+}]_i$ are generally associated with enzyme activation, particularly for those enzymes having Ca^{2+}-binding domains. One of these enzymes is $cPLA_2$. PCBs, particularly *ortho*-PCBs, are known to target the neutrophil $iPLA_2$; however, the observation that PCBs also increase $[Ca^{2+}]_i$ was the first suggestion that more than one isoform of PLA_2 may be activated by PCBs.

Inhibition of TK, p42/p44 MAPK, or PKC decreased around 20-30% the PLA_2 activity induced by Aroclor 1242. Similar results were observed for the *ortho*-PCB 2,2',4,4'-tetrachlorobiphenyl, which also showed sensitivity to inhibition of ras farnesylation. These results suggest that a phosphorylation cascade involving TK, PKC, ras and MAPK regulate a fraction of PCB-stimulated PLA_2 activity. Currently, the only PLA_2 known to be activated by phosphorylation is $cPLA_2$. Thus, these results suggest that in addition to $iPLA_2$, Aroclor 1242 may activate $cPLA_2$. This suggestion was confirmed with the observation that 2,2',4,4'-tetrachlorobiphenyl induced phosphorylation of $cPLA_2$. In addition, this PCB also phosphorylates p44 MAPK, an upstream event in $cPLA_2$ activation.

Little is known about the regulation of $iPLA_2$. In some systems, this enzyme is present in a complex with calmodulin. Calmodulin inhibitors decreased Aroclor 1242-induced PLA_2 activity. Interestingly, calmodulin inhibitors also blocked fMLP-induced neutrophil degranulation, as did Aroclor 1242. These results suggest that calmodulin-dependent processes are also involved in the activity of PCBs in this system. The conceptual framework regarding the different signalling pathways involved in the activation of neutrophils by PCB is summarized in Figure VII.1.

In the environment, PCBs are not found alone. In general, they are present with other organochlorine compounds such as DDT, dieldrin and chlordane among others. Some of these compounds have been shown to activate neutrophils (Hewett and Roth, 1988). Despite their diversity in structure, we wondered if those compounds would not only activate PLA_2 but also present some common molecular characteristics responsible for that activity. Molecular modeling techniques uncovered a common motif present in *ortho*-chlorinated PCBs, dieldrin, and α-, *ortho*-

chlorinated PCBs and β-hexachlorocyclohexane. This motif has been called the OG motif. It has three parts, a planar hydrophobic structure, a negatively charged atom perpendicular to the plane and a rigid bridge connecting these two. The OG motif might be responsible for all the biological activities shared by *ortho*-PCBs and pesticides such as DDT, dieldrin and chlordane. Accordingly, this molecular characteristic can be the link between the effects of PCBs in neutrophils and other cells, such as neurons where *ortho*-PCBs have shown similar biological properties. These results demonstrate the utility of molecular modeling for identifying substructures with potential toxicity, and open a door to better understand the relationships between molecular recognition and biological activity for organochlorine compounds. This will allow the prediction of the biological activity of these compounds in different cell systems using data from a particular cell type, which shares the biochemical machinery influenced by these compounds.

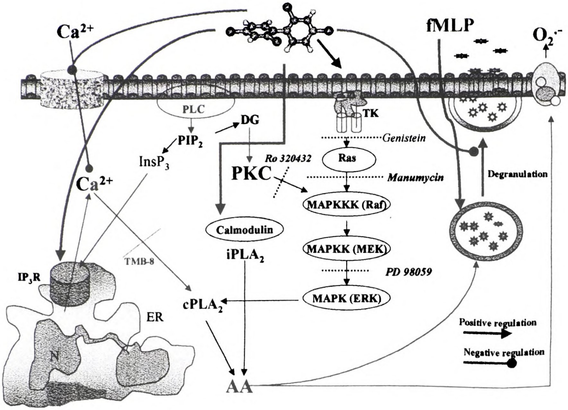


Figure VII.1. *Signal transduction pathways altered by ortho-chlorinated biphenyls.*

BIBLIOGRAPHY

BIBLIOGRAPHY

Ackermann, E., Kempner, E., and Dennis, A. (1994). Ca^{2+}-independent cytosolic phospholipase A_2 from macrophage-like P388D1 cells. Isolation and characterization. *J. Biol. Chem.* **269,** 9227-9233.

Ackermann, E., Conde-Frieboes, K., and Dennis, E. (1995) Inhibition of macrophage Ca^{2+}-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.* **270,** 445-450.

Ackerman, M., Gasiewicz, T., Lamm, K., Germolec, D., and Luster, M. (1989). Selective inhibition of polymorphonuclear neutrophil activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Appl. Pharm.* **101,** 470-480.

Aharonovitz, O., Aboulafia, S., Leor, J., Battler, A., and Granot, Y. (1998). Stimulation of 42/44 kDa mitogen-activated protein kinases by arginine vasopressin in rat cardiomyocytes. *Biochim. Biophys. Acta.* **1401,** 105-111.

Akiyama, K., and Sutoo, D. (1988). ^{1}H-NMR studies of calmodulin: the modifying effect of W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) on the calcium-induced conformational changes of calmodulin. *Jpn. J. Pharmacol.* **48**, 157-164.

Alessi, D., Cuenda, A., Cohen, P., Dudley, D., and Salt, A. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and in vivo. *J. Biol. Chem.* **270(46),** 27489-27494.

Ambs, P., Baccarini, M., Fitzke, E., and Dieter, P. (1995). Role of cytosolic phospholipase A_2 in arachidonic acid release of rat-liver macrophages: regulation by Ca^{2+} and phosphorylation. *Biochem. J.* **311,** 189-195.

Ambudkar, I., Horn, V., and Baum, B. (1989). ATP-dependent Ca^{2+} transport in the rat parotid basolateral plasma membrane is regulated by calmodulin. *Arch. Biochem. Biophys.* **268**, 576-584.

Anand, M., Meera, P., Kumar, R., Gupta, G., Tripathi, O., and Srimal, R. (1995). Possible role of calcium in the cardiovascular effect of prolonged administration of gamma-HCH (lindane) in rats. *J. Appl. Toxicol.* **15,** 245-248.

Anderson, R., and Mahomed, G. (1997). Calcium efflux and influx in f-met-leu-phe (fMLP)-activated human neutrophils are chronologically distinct events. Clinical and Experimental Immunology. *Clin. Exp. Immunol.* **110,** 132-138.

Andersson, T., Dahlgren, C., Pozzan, T., Stendahl, O., and Lew, P. (1986). Characterization of fMet-Leu-Phe receptor-mediated Ca^{2+} influx across the plasma membrane of human neutrophils. *Mol. Pharmacol.* **30,** 437-443.

Angulo, R., Martinez, P., and Jodral, M. (1999). PCB congeners transferred by human milk, with an estimate of their daily intake. *Food Chem. Toxicol.* **37**, 1081-1088.

Angus, W., and Contreras, M. (1994). The effects of Aroclor 1254 on undifferentiated and NGF-stimulated differentiating PC12 cells. *Neurotoxicology.* **15,** 809-818.

Angus, W., and Contreras, M. (1995). Aroclor 1254 alters the binding of ^{125}I-labeled nerve growth factor in PC12 cells. *Neurosci. Lett.* **191,** 23-26.

Anthes, J., Eckel, S., Siegel, M., Egan, R., and Billah, M. (1989). Phospholipase D in homogenates from HL-60 granulocytes: implications of calcium and G protein control. *Biochem. Biophys. Res. Commun.* **163,** 657-664.

Arcaro, K., Yi, L., Seegal, R., Vakharia, D., Yang, Y., Spink, D., Brosch, K., and Gierthy, J. (1999). 2,2',6,6'-tetrachlorobiphenyl is estrogenic *in vitro* and in vivo. *J. Cell. Biochem.* **72,** 94-102.

Arnold, D., Bryce, F., Mes, J., Tryphonas, H., Hayward, S., and Malcolm, S. (1999). Toxicological consequences of feeding PCB congeners to infant rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. *Food Chem. Toxicol.* **37,** 153-167.

Asano, M. (1989). Divergent pharmacological effects of three calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine and calmidazolium, on isometric tension development and myosin light chain phosphorylation in intact bovine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.* **251,** 764-773.

Atzori, L., Flore, C., Corriga, A., Cherchi, P., Casula, D., and Congiu, L. (1991). Mechanisms of PCBs mixture toxicity on isolated rat hepatocytes. *Ind. Health.* **29,** 57-64.

Bae, J., Peters-Golden, M., and Loch-Caruso, R. (1999). 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.* **289,** 1112-1120.

Bagchi, D., Bagchi, M., Tang, L., and Stohs, S. (1997). Comparative *in vitro* and in vivo protein kinase C activation by selected pesticides and transition metals salts. *Toxicol. Lett.* **91,** 31-37.

Balsinde, J., Bianco, I., Ackermann, I., Conde-Frieboes, K., and Dennis, E. (1995). Inhibition of calcium-independent phospholipase A_2 prevents arachidonic acid incorporation and phospholipid remodeling in $P388D_1$ macrophages. *Proc. Natl. Acad. Sci. USA.* **92,** 8527-8531.

Balsinde, J., and Dennis, E. (1996). Bromoenol lactone inhibits magnesium-dependent phosphatidate phosphohydrolase and blocks triacylglycerol biosynthesis in mouse $P388D_1$ macrophages. *J. Biol. Chem.* **271,** 31937-31941.

Bandiera, S., Safe, S., and Okey, A. (1982). Binding of polychlorinated biphenyls classified as either phenobarbitone-, 3-methylcholanthrene- or mixed-type inducers to cytosolic Ah receptor. *Chem. Biol. Inter.* **39,** 259-277.

Bastian, B., Sellert, C., Seekamp, A., Romisch, J., Paques, E., and Brocker, E. (1993). Inhibition of human skin phospholipase A_2 by "lipocortins" is an indirect effect of substrate/lipocortin interaction. *J. Inves. Derm.* **3,** 359-363.

Bauldry, S., Wooten, R., and Bass, D. (1996). Activation of cytosolic phospholipase A_2 in permeabilized human neutrophils. *Biochim. Biophys. Acta.* **1299,** 223-234.

Beaupre, D., Kurzrock, R. (1999). RAS and leukemia: from basic mechanisms to gene-directed therapy. *J. Clin. Oncol.* **17-3,** 1071-9.

Becker, E. (1988). Membrane activation in immunological relevant cells. *Prog. Allergy.* **42.** Karger (Eds). 1-64.

Bereziat, G. (1996). Diversity of phospholipases A_2 and their functions. *C. R. Seances. Soc. Biol. Fil.* **190**, 409-416.

Bergman, A., Klasson, E., and Kuroki, H. (1994). Selective retention of hydroxylated PCB metabolites in blood. *Environ. Health Perspect.* **102,** 464-469.

Bergmeyer, H., and Bernt, E. (1974). Lactate dehydrogenase UV assay with pyruvate and NADH. In: Bergmeyer, H.V., (Ed), Methods of Enzymatic Analysis. Academic Press, New York, pp. 524-579.

Bernard, A., Hermans, C., Broeckaert, F., De Poorter, G., De Cock, A., and Houins, G. (1999). Food contamination by PCBs and dioxins. *Nature.* **401,** 231-232.

Billah, M., Eckel, S., Mullmann, T., Egan, R., and Siegel, M. (1989a). Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction. *J. Biol. Chem.* **264,** 17069-17077.

Billah, M., Pai, J., Mullmann, T., Egan, R., and Siegel, M. (1989b). Regulation of phospholipase D in HL-60 granulocytes. Activation by phorbol esters, diglyceride, and calcium ionophore via protein kinase-independent mechanisms. *J. Biol. Chem.* **264,** 9069-9076.

Blackwood, R., and Hessler, J. (1995). Effect of calcium on phenothiazine inhibition of neutrophil degranulation. *J. Leukoc. Biol.* **58,** 114-118.

Blom, M., Tool, A., Wever, P., Wolbink, G., Brouwer, M., Calafat, J., Egesten, A., Knol, E., Hack, C., Roos, D., and Veroeven, A. (1998). Human eosinophils express, relative to other circulating leukocytes, large amounts of secretory 14-kD phospholipase A_2. *Blood.* **91,** 3037-3043.

Borlak, J., Scott, A., Henderson, C., Jenke, H., and Wolf, C. (1996). Transfer of PCBs via lactation simultaneously induces the expression of P450 isoenzymes and the protooncogenes c-Ha-ras and c-raf in neonates. *Biochem. Pharmacol.* **23,** 517-529.

Borsch, A., Kramer, R., and Watson, S. (1997). Phosphorylation and activation of cytosolic phospholipase A_2 by 38-kDa mitogen –activated protein kinase in collagen-stimulated human platelets. *Eur.J.Biochem.* **245,** 751-759.

Borsch-Haubold, A., Bartoli, F., Asselin, J., Dudler, T., Kramer, R., Apitz-Castro, R., Watson, S., and Gelb, M. (1998). Identification of the phosphorylation sites of cytosolic phospholipase A2 in agonist-stimulated human platelets and HeLa cells. *J. Biol. Chem.* **273,** 4449-4458.

Borsch-Haubold, A., Kramer, R., and Watson, S. (1997). Phosphorylation and activation of cytosolic phospholipase A_2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. *Eur. J. Biochem.* **245,** 751-759.

Bradford, M., and Soltoff, S. (1998). Involvement of protein kinases and phosphatases in tyrosine phosphorylation of PKCdelta in rat parotid acinar cells exposed to secretory stimuli. *Eur. J. Morphol.* **36,** Suppl. 176-80.

Brown, A., and Ganey, P. (1995). Neutrophil degranulation and superoxide production induced by polychlorinated biphenyls are calcium dependent. *Toxicol. Appl. Pharmacol.* **131,** 198-205.

Brown, A., Olivero-Verbel, J., Holdan, W., and Ganey, P. (1998). Neutrophil activation by polychlorinated biphenyls: structure-activity relationship. *Toxicol. Sci.* **46,** 308-316.

Buckland, A., and Wilton, D. (1998). Inhibition of human cytosolic phospholipase A_2 by human annexin V. *Biochem. J.* **329,** 369-372.

Calafat, J., Kuijpers, T., Janssen, H., Borregaard, N., Verhoeven, A., and Roos, D. (1993). Evidence for small intracellular vesicles in human blood phagocytes containing cytochrome b558 and the adhesion molecule CD11b/CD18. *Blood.* **81,** 3122-3129.

Capuozzo, E., Verginelli, D., Crifo, C., and Salerno, C. (1997). Effects of calmodulin antagonists on calcium pump and cytosolic calcium level in human neutrophils. *Biochim. Biophys. Acta.* **1357,** 123-127.

Carrero, I., Recio, M., Prieto, J., and Perez, M. (1996). Effect of γ-hexachlorohexane in phosphoinositide synthesis in rat enterocytes is calcium-mediated. *Pesticide Biochemistry and Physiology.* **56,** 79-87.

Carrero, I., Rodriguez, N., Guijarro, L., Recio, M., Perez, M., and Prieto, J. (1993). Lindane decreases forskolin-stimulated cyclic AMP accumulation but does not modify Gs in rat enterocytes. *Cell Signal.* **5,** 453-462.

Chang, L., and Wang, J. (1999). Examination of the signal transduction pathways leading to activation of extracellular signal-regulated kinase by formyl-methyonyl-leucyl-phenylalanine in rat neutrophils. *FEBS Lett.* **454**, 165-168.

Chang, T., Chang C., Wagner, D., and Chey, W. (1999). Porcine pancreatic phospholipase A_2 stimulates secretin release from secretin-producing cells. *J. Biol. Chem.* **274**, 10758-10764.

Chang, K., Hsieh, K., Tang, S., Tung, T., and Lee, T. (1982). Immunologic evaluation of patients with polychlorinated biphenyl poisoning: evaluation of delayed-type skin hypersensitive response and its relation to clinical studies. *J. Toxicol. Environ. Health.* **9,** 217-223.

Chilton, F., and Murphy, R. (1986). Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. *J. Biol. Chem.* **261,** 7771-7777.

Choksi, N., Kodavanti, P., Tilson, H., and Booth, R. (1997). Effects of polychlorinated biphenyls (PCBs) on brain tyrosine hydroxylase activity and dopamine synthesis in rats. *Fundam. Appl. Toxicol.* **39(1),** 76-80.

Chun, J., and Jacobson, B. (1993). Requirement for diacylglycerol and protein kinase C in HeLa cell-substratum adhesion and their feedback amplification of arachidonic acid production for optimum cell spreading. *Mol. Biol. Cell.* **4,** 271-281.

Coats, J. (1990). Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environ. Health Perspect.* **87,** 255-262.

Cockcroft, S. (1991). Relationship between arachidonate release and exocytosis in permeabilized human neutrophils stimulated with formylmethionyl-leucyl-phenylalanine (fMetleuPhe), guanosine 5'[gamma-thio] triphosphate GTP[S] and Ca^{2+}. *Biochem. J.* **275,** 127-131.

Cogliano, V. (1998). Assessing the cancer risk from environmental PCBs. *Environ. Health Perspect.* **106,** 317-323.

Combadiere, C., el Benna, J., Pedruzzi, E., Hakim, J., and Perianin, A. (1993). Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine. *Blood.* **82,** 2890-2898.

Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balaguer, P. (1997). Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: Structure-activity relationships. *Tox. Appl. Pharmacol.* **145,** 111-123.

Cooper, G., Germolec, D., Heindel, J., and Selgrade, M. (1999). Linking environmental agents and autoimmune diseases. *Environ. Health Perspect* **107 (Suppl 5)**, 695-660.

Cooper, J., Carcelen, R., and Culbreth, R. (1996). Effects of influenza A nucleoprotein on polymorphonuclear neutrophil function. *J. Infect. Dis.* **173,** 279-284.

Craven, J., Whitehead, B., Jones, S., Thulin, E., Blackburn, M., and Waltho, J. (1996). Complexes formed between calmodulin and the antagonists J-8 and TFP in solution. *Biochemistry.* **35,** 10287-10299.

Criswell, K., Loch-Caruso, R., and Stuenkel, E. (1995). Lindane inhibition of gap junctional communication in myometrial myocytes is partially dependent on phosphoinositide-generated second messengers. *Toxicol. Appl. Pharmacol.* **130,** 280-293.

Cuenda, A., Rouse, J., Doza, Y., Meier, R., Cohen, P., Gallagher, T., Young P., and Lee, J. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364,** 229-233.

Czaja, K., Ludwicki, J., Goralczyk, K., and Strucinski, P. (1999). Effect of changes in excretion of persistent organochlorine compounds with human breast milk on related exposure of breast-fed infants. *Arch. Environ. Contam. Toxicol.* **36,** 498-503.

Dana, R., Leto, T., Malech, H., and Levy, R. (1998). Essential requirement of cytosolic phospholipase A_2 for activation of the phagocyte NADPH oxidase. *J. Biol. Chem.* **273,** 441-445.

Dana, R., Malech, H., and Levy, R. (1994). The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils. *Biochem. J.* **297,** 217-223.

Davies, E., Campbell, A., and Hallett, M. (1994). Ca^{2+} oscillations in neutrophils triggered by immune complexes result from Ca^{2+} influx. *Immunology.* **82,** 57-62.

Davies, E., and Hallett, M. (1996). Near membrane Ca^{2+} changes resulting from store release in neutrophils: detection by FFP-18. *Cell Calcium.* **19,** 355-362.

De Carvalho, M., Garritano, J., and Leslie, C. (1995). Regulation of lysophospholipase activity of the 85-kDa phospholipase A2 and activation in mouse peritoneal macrophages. *J. Biol. Chem.* **270,** 20439-20446.

De Schroeder, F., and De D'angelo, P. (1995). Dieldrin modifies the hydrolysis of PIP_2 and decreases the fertilization rate in bufo arenarum oocytes. *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol.* **112,** 61-67.

De Swart, R., Ross, P., Vos, J., and Osterhaus, A. (1996). Impaired immunity in harbour seals (Phoca vitulina) fed enviornmentally contaminated hearring. *Vet. Q.* **18 Suppl 3,** S127- S128.

Del Villar, K., Urano, J., Guo, L., and Tamanoi, F. (1999). A mutant form of human protein farnesyltransferase exhibits increased resistance to farnesyltransferase inhibitors. *J. Biol. Chem.* **274**, 27010-27017.

Derrickson, B., and Mandel, L. (1997). Parathyroid hormone inhibits Na^+-K^+-ATPase through G_q/G_{11} and the calcium-independent phospholipase A_2. *Am. J. Physiol.* **272,** F781-F788.

Desaulniers, D., Leingartner, K., Wade, M., Fintelman, E., Yagminas, A., and Foster, W. (1999). Effects of acute exposure to PCBs 126 and 153 on anterior pituitary and thyroid hormones and FSH isoforms in adult Sprague Dawley male rats. *Toxicol. Sci.* **47,** 158-169.

Dewailly, E., Ayotte, P., Laliberte, C., Weber, J., Gingras, S., and Nantel, A. (1996). Polychlorinated biphenyl (PCB) and dichlorodiphenyl dichloroethylene (DDE) concentrations in the breast milk of women in Quebec. *Am. J. Public Health.* **86,** 1241-1246.

Dewar, M., Zoebisch, E., Healy, E., and Stewart, J. (1985). AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **107**, 3902-3909.

Dho, S., Grinstein, S., Corey, E., Su, W., Pace-Asciak, C. (1990). Hepoxilin A_3 induces changes in cytosolic calcium, intracellular pH and membrane potential in human neutrophils. *Biochem. J.* **266,** 63-68.

Dorgan, J., Brock, J., Rothman, N., Needham, L., Miller, R., Stephenson, H., Schussler, N., and Taylor, P. (1999). Serum organochlorine pesticides and PCBs and breast cancer risk: results from a prospective analysis (USA). *Cancer Causes Control.* **10,** 1-11.

Dubois, T., Mira, J., Feliers, D., Solito, E., Russo-Marie, F., and Oudinet, J. (1998). Annexin V inhibits protein kinase C activity via a mechanism of phospholipid sequestration. *Biochem. J.* **330,** 1277-1282.

Dudley, D., Pang, L., Decker, S., Bridges, A., and Saltiel, A. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci.* **92,** 7686-7689.

Dustan, R., Roberts, t., Donohoe, M., McGregor, N., Hope, D., Taylor, W., Watkins, J., Murdoch, R., and Butt, H. (1996). Bioaccumulated chlorinated hydrocarbons and red/white blood cell parameter. *Biochem. Mol. Med.* **58,** 77-84.

Elferink, J., and De Koster, B. (1995). Ryanodine as inhibitor of chemotactic peptide-induced chemotaxis in human neutrophils. *Biochem. Pharmacol.* **50,** 975-979.

Enan, E., and Matsumura, F. (1998). Activation of c-neu tyrosine kinase by o,p'-DDT and β-HCH in cell-free and intact cell preparations from MCF-7 human breast cancer cells. *J. Biochem. Molecular Toxicol.* **12,** 83-92.

English, D., Schell, M., Siakotos, A., and Gabig, T. (1986). Reversible activation of the neutrophil superoxide generating system by hexachlorocyclohexane: correlation with effects on a subcellular superoxide-generating fraction. *J. Immunol.* **137,** 283-290.

Exton, J., (1990). Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265,** 1-4.

Favata, M., Horiuchi, K., Manos, E., Daulerio, A., Stradley, D., Feeser, W., Van Dyk, D., Pitts, W., Earl, R., Hobbs, F., Copeland, R., Magolda, R., Scherle, P., and Trzaskos, J. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273,** 18623-18632.

Fernandez, R., and Suchard, S. (1998). Syk activation is required for spreading and H_2O_2 release in adherent human neutrophils. *J. Immunol.* **160,** 5154-5162.

Fischer, L., Wagner, M., and Madhukar, BV. (1999). Potential involvement of calcium, CaM Kinase II, and MAP kinases in PCB-stimulated insulin release from RINm5F cells. *Toxicol. Appl. Pharmacol.* **159**, 194-203.

Fischer, L., Seegal, R., Ganey, P., Pessah, I., and Kodavanti, P. (1998). Symposium overview: toxicity of non-coplanar PCBs. *Toxicol. Sci.* **41**, 49-61.

Fischer, L., Zhou, H., and Wagner, M. (1996). Polychlorinated biphenyls release insulin from RINm5F cells. *Life Sci.* **59,** 2041-2049.

Flamigni, F., Facchini, A., Capanni, C., Stefanelli, C., Tantini, B., and Caldarera, C. (1999). P44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells. *Biochem. J.* **341,** 363-369.

Fletcher, M., Seligmann, B., Gallin, J. (1982). Correlation of human neutrophil secretion, chemoattractant receptor mobilization, and enhanced functional capacity. *J. Immunol.* **128,** 941-948.

Foltz, I., Lee, J., Young, P., Schrader, J. (1997). Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J. Biol. Chem.* **272,** 3296-3301.

Foster, F., and Conigrave, A. (1999). Genistein inhibits lysosomal enzyme release by suppressing Ca^{2+} influx I HL-60 granulocytes. *Cell Calcium.* **25,** 69-76.

Fouda, S., Thaddeus, F., Molski, T., Ashour, M., and Sha'afl, R. (1995). Effect of lipopolysaccharide on mitogen-activated protein kinases and cytosolic phospholipase A_2. *Biochem. J.* **308,** 815-822.

Fromberg, A., Cleemann, M., and Carlsen, L. (1999). Review on persistent organic pollutants in the environment of Greenland and Faroe Islands. *Chemosphere.* **38,** 3075-3093.

Gabler, W., Creamer, H., and Bullock, W. (1986). Role of extracellular calcium in neutrophil responsiveness to chemotactic tripeptides. *Inflammation.* **10**, 281-292.

Galassi, S., Valsecchi, S., and Tartari, G. (1997). The distribution of PCB's and chlorinated pesticides in two connected Himalayan lakes. *Water, Air, Soil Poll.* **99,** 717-725.

Gallin, J., Fletcher, M., Seligmann, B., Hoffstein, S., Cehrs, K., and Mounessa, N. (1982). Human neutrophil-specific granule deficiency: a model to assess the role of neutrophil-specific granules in the evolution of the inflammatory response. *Blood.* **59,** 1317-1329.

Ganey, P., Sirois, J., Denison, M., Robinson, J., and Roth, R. (1993). Neutrophil function after exposure to polychlorinated biphenyls *in vitro. Environ. Health Perspect.* **101,** 430-434.

Gaudette, D., and Holub, B. (1990). Effect of genistein, a tyrosine kinase inhibitor, on U46619-induced phosphoinositide phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.* **170**, 238-242.

Gaudry, M., Gilbert, F., Barabe, P., Poubelle, P., and Naccache, P. (1995). Activation of Lyn is a common element of the stimulation of human neutrophils by soluble and particulate agonists. *Blood.* **86,** 3567-3574.

Geisy, J., and Kannan, K. (1998). Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. *Crit. Rev. Toxicol.* **28,** 511-569.

Gibbs, J., and Oliff, A. (1997). The potential of pharnesyltransferase inhibitors as cancer chemotherapeutics. *Annu. Rev. Pharmacol. Toxicol.* **37**, 143-166.

Goldey, E., Kehn, L., Lau, C., Rehnberg, G., and Crofton, K. (1995). Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. *Toxicol. Appl. Pharmacol.* **135,** 77-88.

Gould, G., Cuenda, A., Thomson, F., and Cohen P. (1995). The activation of distinct mitogen-activated protein kinase cascades is required for the stimulation of 2-deoxyglucose uptake by interleukin-1 and insulin-like growth factor-1 in KB cells. *Biochem. J.* **311,** 735-738.

Gould, J., Cooper, K., and Scanes, C. (1999). Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeiodinase in the chick embryo. *Ecotoxicol. Environ. Saf.* **43,** 195-203.

Gozin, A., Franzini, E., Andrieu, V., Da Costa, L., Rollet-Labelle, E., and Pasquier, C. (1998). Reactive oxygen species activate focal adhesion kinase, paxillin and p130cas tyrosine phosphorylation in endothelial cells. *Free Radic. Biol. Med.* **25,** 1021-1032.

Greizerstein, H., Stinson, C., Mendola, P., Buck, G., Kostyniak, P., and Vena, J. (1999). Comparison of PCB congeners and pesticide levels between serum and milk from lactating women. *Environ. Res.* **80,** 280-286.

Gribaldo, L., Sacco, M., Casati, S., Zucchi, I., Dosanjh, M., Catalani, P., Marafante, E. (1998). Modulation of proto-oncogene expression by polychlorinated biphenyls in 3T3-L1 cell line. *J. Toxicol. Environ. Health.* **55-2,** 121-31.

Grigorian, M., Akopov, S., and Gabrielian, E. (1988). Regulation by the protein kinase C activator phorbol ester of calcium channels in polymorphonuclear leukocytes. *Biokhimiia.* **53,** 1462-1466.

Gross, R., Rudolph, A., Wang, J., Sommers, C., and Wolf, M. (1995). 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.* **270,** 14855-14858.

Guan, X, and Ruch, R. (1996). Gap junction endocytosis and lysosomal degradation of connexin43-P2 in WB-F344 rat liver epithelial cells treated with DDT and Lindane. *Carcinogenesis.* **17,** 1791-1798.

Guillette, L., Brock, J., Rooney, A. and Woodward, A. (1999). Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile american alligators. *Arch. Environ. Contam. Toxicol.* **36,** 447-455.

Haag-Gronlund, M., Warngard, L., Flodstrom, S., Scheu, G., Kronevi, T., Ahlborg, U., and Fransson-Steen, R. (1997). Promotion of altered hepatic foci by 2,3',4,4',5-pentachlorobiphenyl in Sprague-Dawley female rats. *Fundam. Appl. Toxicol.* **35,**120-30.

Haag-Weber M., and Horl, W. (1992). Calcium-dependent neutrophil activation. The Kidney Today. Selected Topics in Renal Science. Berlyne GM (ed). **100**, 269-285.

Hahn, M., Lamb, T., Schultz, M., Smolowitz, R., and Stegeman. (1993). Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquat. Toxicol.* **26,** 185-208.

Haigler, H., Schlaepfer, D., Burgess, W. (1987). Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A_2 inhibitors. *J. Biol. Chem.* **262,** 6921-6930.

Hait, W., and Lazo, J. (1986). Calmodulin: A potential target for cancer chemotherapeutic agents. *J. Clin. Oncol.* **4,** 994-1012.

Hansen, L., Li, M., Saeed, A., and Bush, B. (1995). Environmental polychlorinated biphenyls: Acute toxicity of landfill soil extract to female prepubertal rats. *Arch. Environ. Contam.Toxicol.* **29,** 334-343.

Hany, J., Lilienthal, H., Sarasin, A., Roth, A., Fastabend, A., Dunemann, L., Lichtensteiger, W., and Winneke, G. (1999a). Developmental exposure of rats to a reconstituted PCB mixture or aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. *Toxicol. Appl. Pharmacol.* **158,** 231-243.

Hany, J., Lilienthal, H., Roth, A., Ostendorp, G., Heinzow, B., Winneke, G. (1999b). Behavioral effects following single and combined maternal exposure to PCB 77 (3,4,3',4'-tetrachlorobiphenyl) and PCB 47 (2,4,2',4'-tetrachlorobiphenyl) in rats. *Neurotoxicol. Teratol.* **21,** 147-156.

Hara, M., Akasaka, K., Akinaga, S., Okabe, M., Nakano, H., Gomez, R., Wood, D., Uh, M., and Tamanoi, F. (1993). Identification of Ras farnesyltransferase inhibitors by microbial screening. *Proc. Natl. Acad. Sci.* **90,** 2281-2285.

Harper, N., Connor, K., Steinberg, M., and Safe, S. (1995). Immunosuppressive activity of polychlorinated biphenyl mixtures and congeners; non-additive (antagonistic) interactions. *Fundam. Appl. Toxicol.* **27,** 131-139.

Hartshorn, K., Collamer, M., Auerbach, M., Myers, J., Pavlotsky, N., and Tauber, A. (1988). Effects of influenza A virus on human neutrophil calcium metabolism. *J. Immunol.* **141**. 1295-1301.

Hartshorn, K., Liou, L., White, M., Kazhdan, M., Tauber, J., and Tauber, A. (1995). Neutrophil deactivation by influenza A virus. Role of hemagglutinin binding to specific sialic acid-bearing cellular proteins. *J. Immunol.* **154,** 3952-3960.

Hashiguchi, I., Furukawa, K., Akamine, a., Fukuyama, H., and Okumura, H. (1999). An epidemiologic examination on the prevalence of the periodontal diseases and oral pigmentation in Yusho patients in 1998. *Fukuoka Igaku Zasshi.* **90,** 150-153.

Hashimoto, S., Maruoka, S., Gon, Y., Matsumoto, K., Takeshita, I., and Horie, T. (1999). Mitogen activated protein kinase involves neutrophil elastase-induced morphological changes in human bronchial epithelial cells. *Life Sciences.* **64,** 1465-1471.

Haverstick, D., Densmore, J., and Gray, L. (1998). Calmodulin regulation of Ca^{2+} entry in Jurkat T cells. *Cell Calcium.* **23,** 361-367.

Hazan, I., Dana, R., Granot, Y., and Levy R. (1997). Cytosolic phospholipase A_2 and its mode of activation in human neutrophils by opsonized zymosan. *Biochem. J.* **326,** 867-876.

Hazen, S., and Gross, R. (1991). ATP-dependent regulation of rabbit myocardial cytosolic calcium-independent phospholipase A_2. *J. Biol. Chem.* **266,** 14526-14534.

Hazen, S., and Gross, R. (1993). The specific association of a phosphofructokinase isoform with myocardial calcium-independent phospholipase A_2. *J. Biol. Chem.* **268,** 9892-9900.

Hazen, S., Ford, D., and Gross, R (1991a). Activation of a membrane-associated phospholipase A_2 during rabbit myocardial ischemia which is highly selective for plasmalogen substrate. *J. Biol. Chem.* **266,** 5629-5633.

Hazen, S., Zupan, L., Weiss, R., Getman, D., and Gross, R. (1991b). Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A_2. *J. Biol. Chem.* **266,** 7227-7232.

Hazen, S., Hsu, F., Mueller, D., Crowley, J., Heinecke, J. (1996). Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J. Clin. Invest.* **98,** 1283-1289.

Hazen, S., Stuppy, R., and Gross, R. (1990). Purification and characterization of canine myocardial cytosolic phospholipase A_2. A calcium-independent phospholipase with absolute f1-2 regiospecificity for diradyl glycerophospholipids. *J. Biol. Chem.* **265,** 10622-10630.

Hehre, W., Stewart, R., and Pople, J. (1969). *J. Chem. Phys.* **51,** 2657.

Henderson, L., Moule, S., and Chappell, J. (1993). The immediate activator of the NADPH oxidase is arachidonate not phosphorylation. *Eur. J. Biochem.* **211,** 157-162.

Hendrickse, C., Radley, S., Donovan, I., Keigley, M., and Neoptolemos, J. (1995). Activities of phospholipase A_2 and diacylglycerol lipase are increased in human colorectal cancel. *Br. J. Surg.* **82,** 475-478.

Henson, P., Zanolari, B., Schwartzman, N., and Hong, S. (1978). C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J. Immunol.* **121,** 851-855.

Hernandez, M., Burillo, S., Crespo, M., and Nieto, M. (1998). Secretory phospholipase A_2 activates the cascade of mitogen-activated protein kinases and cytosolic phospholipase A_2 in the human astrocytoma cell line 1321N1. *J. Biol. Chem.* **273,** 606-612.

Hewett, J., and Roth, R. (1988). Dieldrin activates rat neutrophils *in vitro*. *Toxicol. Appl. Pharmacol.* **96,** 269-278.

Hong, C., Bush, B., Xiao, J., and Qiao, H. (1993). Toxic potential of non-*ortho* and mono-*ortho* coplanar polychlorinated biphenyls in Aroclors, seals and humans. *Arch. Environ. Contam. Toxicol.* **25,** 118-123.

Hori, M., Kondo, H., Ariyoshi, N., Yamada, H., Oguri, K. (1997). Species-specific alteration of hepatic glucose 6-phosphate dehydrogenase activity with coplanar polychlorinated biphenyl: evidence for an Ah-receptor-linked mechanism. *Chemosphere.* **36,** 951-958.

Hugla, J., and Thome, J. (1999). Effects of polychlorinated biphenyls on liver structure, hepatic monooxygenases, and reproductive success in the barbel. *Ecotoxicol. Environ. Saf.* **42,** 265-273.

Husain, S., and Abdel-Latif, A. (1998). Role of protein kinase C α in endothelin-1 stimulation of cytosolic phospholipase A2 and arachidonic acid release in cultured cat iris sphincter smooth muscle cells. *Biochim. Biophys. Acta.* **1392**, 127-144.

Hutzinger, O., Jamieson, W., Safe, S., Paulmann, L., and Ammon, R. (1974). Identification of metabolic dechlorination of highly chlorinated biphenyl in rabbit. *Nature.* **252,** 698-699.

Huwiler, A., Staudt, G., Kramer, R., and Pfeilschifter, J. (1997). Cross-talk between secretory phospholipase A_2 and cytosolic phospholipase A_2 in rat renal mesenglial cells. *Biochim. Biophys. Acta.* **1348,** 257-272.

Ishimoto, T., Arisato, K., Akiba, S., Sato, T. (1996). Requirement of calcium influx for hydrolytic action of membrane phospholipids by cytosolic phospholipase A_2 rather then mitogen-activated protein kinase activation in Fc epsilon RI-stimulated rat peritoneal mast cells. *J. Biochem. (Tokyo).* **120,** 1247-1252.

Jacobson, J., and Jacobson, S. (1997). Evidence for PCBs as neurodevelopmental toxicants in humans. *Neurotoxicology.* **18** ,415-424.

Kafafi, S., Afeefy, H., Ali, A., Said, H., and Kafafi, A. (1993a). Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. *Environ. Health. Perspect.* **101,** 422-428.

Kafafi, S., Afeefy, H., Ali, A., Said, H., Abd-Elazem, I., and Kafafi, A. (1993b). Affinities for the aryl hydrocarbon receptor, potencies as aryl hydrocarbon hydroxylase inducers and relative toxicities of polychlorinated biphenyls. A congener specific approach. *Carcinogenesis.* **14,** 2063-2071.

Kallenborn, R., Oehme, M., Williams, D., Schlabach, M., and Harris, J. (1998). Ambient air levels and atmospheric long-range transport of

persistent organochlorines to Signy Island, Antarctica. *Sci. Tot. Environ.* **220,** 167-180.

Kanaho, Y., Kanoh, H., Nozawa, Y. (1991). Activation of phospholipase D in rabbit neutrophils by fMet-Leu-Phe is mediated by a pertussis toxin-sensitive GTP-binding protein that may be distinct from a phospholipase C-regulating protein. *FEBS Lett.* **279,** 249-252.

Kankaanranta, H., Moilanen, E., Lindberg, K., and Vapaatalo, H. (1995a). Pharmacological control of human polymorphonuclear leukocyte degranulation by fenamates and inhibitors of receptor-mediated calcium entry and protein kinase C. *Biochem. Pharm.* **50,** 197-203.

Kankaanranta, H., Wuorela, H., Siltaloppi, E., Vuorinen, P., Vapaatalo, H., and Moilanen, E. (1995b). Inhibition of human neutrophil function by tolfenamic acid involves inhibition of Ca^{2+} influx. *Euro.J.Pharm.* **291,** 17-25.

Kannan, K., Nakata, H., Stafford, R., Masson, G., Tanabe, S., and Giesy, J. (1998). Bioaccumulation and toxic potential of extremely hydrophobic polychlorinated biphenyl congeners in biota collected at a superfund site contaminated with Aroclor 1268. *Environ. Sci. Technol.* **32,** 1214-1221.

Kaplan, S., Zdziarski, U., Kuhns, D., and Basford, R. (1988). Inhibition of cell movement and associated changes by hexachlorocyclohexanes due to unregulated intracellular calcium increases. *Blood.* **71,** 677-683.

Kato, M., Shimada, Y., Tanaka, H., Hosotani, R., Ohshio, G., Ishizaki, K., and Imamura, M. (1999). Characterization of six cell lines established from human pancreatic adenocarcinomas. *Cancer.* **85,** 832-840.

Kato, Y., Haraguchi, K., Shibahara, T., Yumoto, S., Masuda, Y., and Kimura, R. (1999). Reduction of thyroid hormone levels by methylsulfonyl metabolites of tetra- and pentachlorinated biphenyls in male Sprague-Dawley rats. *Toxicol. Sci.* **48,** 51-54.

Kato, Y., Kenne, K., Haraguchi, K., Masuda, Y., Kimura, R., and Warngard, L. (1998). Inhibition of cell-cell communication by methylsulfonyl metabolites of polychlorinated biphenyl congeners in rat livwe epithelial IAR 20 cells. *Arch. Toxicol.* **72,** 178-82.

Kemmerich, B., and Pennington, J. (1988). Different calcium and oxidative metabolic responses in human blood monocytes during exposure to various agonists. *J. Leukoc. Biol.* **43,** 125-132.

Khan, S., Lane, P., and Smith, A. (1985). Desaggregation of PAF-acether-aggregated platelets by verapamil and TMB-8 with reversal of phosphorylation of 40K and 20K proteins. *Eur. J. Pharmacol.* **107,** 189-198.

Kimbrough, R., Doemland, M., and LeVois, M. (1999). Mortality in male and female capacitor workers exposed to polychlorinated biphenyls. *J. Occup. Environ. Med.* **41,** 161-171.

King, R., and Srinivasan, A. (1996). Prediction of rodent carcinogenicity bioassays from molecular structure using inductive logic programming. *Environ. Health Prospect.* **104,** 1031-1040.

Klee, C., (1998). Annexins link secretory vesicles to the plasma membrane, *Biochemistry.* **27,** 6645-6653.

Kniss, D., Zimmerman, P., Su, H., and Fertel, R. (1996). Genistein supresses EGF-induced prostaglandin biosynthesis by a mechanism independent of EGF receptor tyrosine kinase inhibition. *Prostaglandins.* **51**, 87-105.

Knott, R., Hansen, S., and Henderson, S. (1994). A small angle X-ray scattering study of the binding of cyclosporin-A to calmodulin. *J. Struct. Biol.* **112,** 192-198.

Kodavanti, P., and Tilson, H. (1997a). Structure-activity relationships of potentially neurotoxic PCB congeners in the rat. *Neurotoxicology.* **18,** 425-442.

Kodavanti, P., and Tilson, H. (1997b). Comments on "Development neurotoxicity of PCBs in humans: what do we know and where do we go from here?". *Neurotoxicol. Teratol.* **19,** 1-5.

Kodavanti, P., and Ward, T. (1998). Interactive effects of environmentally relevant polychlorinated biphenyls and dioxins on [^{3}H]phorbol ester binding in rat cerebellar granule cells. *Environ. Health. Perspect.* **106,** 479-486.

Kodavanti, P., Shafer, T., Ward, T., Mundy, W., Freudenrich, T., Harry, J., and Tilson, H. (1994). Differential effects of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. *Brain Research.* **662,** 75-82.

Kodavanti, P., Shin, D., Tilson, H., and Harry, J. (1993). Comparative effects of two polychlorinated biphenyl congeners on calcium homeostasis in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol.* **123,** 97-106.

Kodavanti, P., Ward, T., McKinney, J., and Tilson, H. (1996a). Inhibition of microsomal and mitochondrial Ca^{2+}-sequestration in rat cerebellum by polychlorinated biphenyl mixtures and congeners. Structure-activity relationships. *Arch. Toxicol.* **70,** 150-157.

Kodavanti, P., Ward, T., McKinney, J., Waller, C., and Tilson, H. (1996b). Increased [^{3}H] phorbol ester binding in rat cerebellar granule cells and inhibition of $^{45}Ca^{2+}$ sequestration in rat cerebellum by polychlorinated diphenyl ether congeners and analogs: structure-activity relationships. *Toxicol. Appl. Pharmacol.* **138,** 251-261.

Kodavanti, P., Ward, T., McKinney, J., and Tilson, H. (1995). Increased [^{3}H] phorbol ester binding in rat cerebellar granule cells by polychlorinated biphenyls mixtures and congeners: structure-activity relationships. *Toxicol. Appl. Pharmacol.* **130,** 140-148.

Korchak, H., Vienne, K., Rutherford, L., Wilkenfield, C., Finkelstein, M., and Weissmann, G. (1984). Stimulus response coupling in the human neutrophil. *J. Biol. Chem.* **259,** 4076-7082.

Korrick, S., and Altshul, L. (1998). High breast milk levels of polychlorinated biphenyls (PCBs) among four women living adjacent to a PCB-contaminated waste site. *Environ. Health Perspect.* **106,** 513-518.

Kouchi, H., Nakamura, K., Fushimi, K., Sakaguchi, M., Miyazaki, M., Ohe, T,. and Namba, M. (1999). Manumycin A, inhibitor of ras farnesyltransferase, inhibits proliferation and migration of rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **264,** 915-920.

Koumanov, K., Wolf, C., and Bereziat, G. (1997). Modulation of human type II secretory phospholipase A_2 by sphingomyelin and annexin VI. *Biochem. J.* **326,** 227-233.

Kramer, V., Gelferich, W., Bergman, A., Wehler, E., and Giesy, J. (1997). Hydroxylated polychlorinated biphenyl metabolites are anti-estrogenic in a stably transfected human breast adenocarcinoma (MCF7) cell line. *Toxicol. Appl. Pharmacol.* **144,** 363-376.

Krause, K., Demaurex N., Jaconi, M., and Lew, D. (1993). Ion channels and receptor-mediated Ca^{2+} influx in neutrophil granulocytes. *Blood Cells.* **19,** 165-175.

Krump, E., Sanghera, J., Pelech, S., Furuya, W., and Grinstein, S. (1997). Chemotactic peptide N-formyl-met-leu-phe activation of p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase-2 in human neutrophils. *J. Biol. Chem.* **272,** 937-944.

Kuhns, D., Kaplan, S., and Basford, R. (1986). Hexachlorocyclohexanes, potent stimuli of O_2^-production and calcium release in human polymorphonuclear leukocytes. *Blood.* **68,** 535-540.

Kuijpers, T., Tool, A., van der Schoot, C., Ginsel, L., Onderwater, J., Roos, D., Verhoeven, A. (1991). Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood.* **78,** 1105-1111.

Kumar, A., Chambers, T., Cloud, B., and Mehta, K. (1997). Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation. *J. Lipid Res.* **38,** 2240-2248.

Lacal, P., Pulido, R., Sanchez, F., and Mollinedo, F. (1988). Intracellular location of T200 and Mo1 glycoproteins in human neutrophils. *J. Biol. Chem.* **263,** 9946-9951.

Lad, P., Kaptein, J., and Lin, C. (1996). Signal Transduction in Leukocytes. G-protein related and other pathways. CRC Press. Boca Raton.

Lad, P., Olson, C., Grewal, I., and Scott, S. (1985). A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization, and lectin-induced capping. *Proc. Natl. Acad. Sci.* **82,** 8643-8647.

Laden, F., Neas, L., Spiegelman, D., Hankinson, S., Willett, W., Ireland, K., Wolff, M., Hunter, D. (1999). Predictors of plasma concentration of DDE and PCBs in a group of U.S. Women. *Environ. Health. Perspect.* **107,** 75-81.

Lagueux, J., Pereg, D., Ayotte, P., Dewailly, E., and Poirier, G. (1999). Cytochrome P450 CYP1A1 enzyme activity and DNA adducts in placenta

of women environmentally exposed to organochlorines. *Environ. Res.* **80,** 369-382.

Lahvis, G., Wells, R., Kuehl, D., Stewart, J., Rhinehart, H., and Via, C. (1995). Decreased lymphocyte responses in free-ranging bottlenose dolphins (Tursiops truncatus) are associated with increased concentrations of PCBs and DDT in peripheral blood. *Environ. Health Perspect.* **103 Suppl 4**, 67-72.

Lal, A., Clifton, A., Rouse, J., Segal, A., and Cohen, P. (1999). Activation of the neutrophil NADPH oxidase is inhibited by SB 203580, a specific inhibitor of SAPK2/p38. *Biochem. Biophys. Res. Commun.* **259**, 465-470.

Lamura, E., Hillier, K., Kinkaid, A., and Wilton, D. (1997). Compartmentalisation and characteristics of a Ca^{2+}-dependent phospholipase A_2 in human colon mucosa. *Biochem. Pharmacol.* **53**, 1323-1332.

Lee-Kwon, W., Park, D., and Bernier, M. (1998). Involvement of the Ras/extracellular signal-regulated kinase signalling pathway in the regulation of ERCC-1 mRNA levels by insulin. *Biochem. J.* **331,** 591-597.

Lehman, J., Brown, K., Ramanadham, S., Turk, J., and Gross, R. (1993). 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.* **268,** 20713-20716.

Li, J., Li, M., and Tsang, B. (1997). Regulation of cytosolic phospholipase A_2 in hen granulosa cells by transforming growth factors at different stages of follicular development. *Biol. Reprod.* **57,** 92-935.

Lin, L., Wartmann, M., Lin, A., Knopf, J., Seth, A., and Davis, R. (1993). $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell.* **72,** 269-278.

Lin, W., and Chen, B. (1998). Distinct PKC isoforms mediate the activation of $cPLA_2$ and adenylyl cyclase by phorbol ester in RAW264.7 macrophages. *Br. J. Pharmacol.* **125,** 1601-1609.

Liu, L., Tao, J., Li, H., and Zimmerman, U. (1997). Inhibition of lung surfactant secretion from alveolar type II cells and anexin II tetramer-mediated membrane fusion by phenothiazines. *Arch. Biochem. Biophys.* **342,** 322-328.

Lopez-Aparicio, P., Merino, M., Recio, M., and Perez, M. (1995). Lindane enhances a specific phospholipase A_2 activity in rat renal tubular cell culture. *Pest. Biochem. Physiol.* **52,** 90-97.

Luconi, M., Barni, T., Vannelli, G., Krausz, C., Marra, F., Benedetti, P., Evangelista, V., Francavilla, S., Properzi, G., Forti, G., and Baldi, E. (1998). Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biol. Reprod.* **58,** 1476-1489.

Ma, Z., Wang, X., Nowatzke, W., Ramanadham, S., and Turk, J. (1999). Human pancreatic islets express mRNA species encoding two distinct catalytically active isoforms of group VI phospholipase A_2 ($iPLA_2$) that arise from an exon-skipping mechanism of alternative splicing of the transcript from the $iPLA_2$ gene on chromosome 22q13.1. *J. Biol. Chem.* **274**, 9607-9616.

Ma, Z., Ramanadham, S., Hu, Z., and Turk, J. (1998). 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.* **1391,** 384-400.

Ma, Z., Ramanadham, S., Kempe, K., Chi, X., Ladenson, J., and Turk, J. (1997). Pancreatic islets express a Ca^{2+}-independent phospholipase A_2 enzyme that contains a repeated structural motif homologous to the integral membrane protein-binding domain of ankyrin. *J. Biol. Chem.* **272,** 11118-11127.

Matsumura, F. (1985). Involvement of picrotoxin receptor in the action of cyclodiene insecticides. *Neurotoxicology.* **6**, 139-163.

Matthews, H., and Anderson, M. (1975). Effect of chlorination on the distribution and excretion of polychlorinated biphenyls. *Drug. Metab. Dispos.* **3,** 371-380.

Mayes, B., McConnell, E., Neal, B., Brunner, M., Hamilton, S., Sullivan, T., Peters, A., Ryan, M., Toft, J., Singer, A., Brown, J., Menton, R., and Moore, J. (1998). Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254 and 1260. *Toxicol. Sci.* **41,** 62-76.

McCague, R., Rowlands, M., Grimshaw, R., and Jarman, M. (1994). Evidence that tamoxifen binds to calmodulin in a conformation different to that when binding to estrogen receptors, through structure-activity study on ring-fused analogues. *Biochem. Pharmacol.* **48,**1355-1361.

McFarland, V., and Clarke, J. (1989). Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. *Environ. Health. Perspect.* **81,** 225-239.

McKinney, J., and Waller, C. (1994). Polychlorinated Biphenyls as hormonally active structural analogues. *Environ. Health Perspect.* **102,** 290-297.

Mekenyan, O., Veith, G., Call, D., and Ankley, G. (1996). A QSAR evaluation of Ah receptor binding of halogenated aromatic xenobiotics. *Environ. Health Perspect.* **104,** 1302-1310.

Mene, P., Pugliese, F., and Cinotti, G. (1996). Regulation of capacitative calcium influx in cultured human mesangial cells: roles of protein kinase C and calmodulin. *J. Am. Soc. Nephrol.* **7,** 983-990.

Milella, M., Gismondi, A., Roncaioli, P., Bisogno, L., Palmieri, G., Frati, L., Cifone, M., and Santoni, A. (1997). CD16 cross-linking induces both secretory and extracellular signal-regulated kinase (ERK)-dependent cytosolic phospholipase A_2 (PLA_2) activity in human natural killer cells: involvement of ERK, but not PLA_2, in CD16-triggered granule exocytosis. *J. Immunol.* **158,** 3148-3154.

Mira, J., Dubois, T., Oudinet, J., Lukowski, S., Russo, F., and Geny, B. (1997). Inhibition of cytosolic phospholipase A_2 by annexin V in differentiated permeabilized HL-60 cells. *J. Biol. Chem.* **272,** 10474-10482.

Miura, K., Schroeder, J., Hubbard, W, and MacGlashan, D.(1999). Extracellular signal-regulated kinases regulate leukotriene C4 generation, but not histamine release or IL-4 production from human basophils. *J. Immmunol.* **162,** 4198-4206.

Miyagi, T., Lam, K., Chuang, L., and Chuang, R. (1998). Suppression of chemokine-induced chemotaxis of monkey neutrophils and monocytes by chlorinated hydrocarbon insecticides. *In Vivo.* **12,** 441-446.

Mizutani, A., Tokumitsu, H., Kobayashi, R., Hidaka, H. (1993). Phosphorylation of annexin XI (CAP-50) in SR-3Y1 cells. *J. Biol. Chem.* **268,** 15517-15522.

Mohr, C., Alojipan, S., Dunston, S., and Pessah, I. (1995). The δ isomer of hexachlorocyclohexane induces rapid release of the myo-inositol-1,4,5-triphosphate-sensitive Ca^{2+} store and blocks capacitative Ca^{2+}entry in rat basophilic leukemis cells. *Mol. Pharmacol.* **48,** 512-522.

Montero, M., Alvarez, J., and Garcia-Sancho, J. (1991). Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores. *Biochem. J.* **277,** 73-79.

Morse, D., Seegal, R., Borsch, K., and Brouwer, A. (1996a). Long-term alterations in regional brain serotonin metabolism following maternal polychlorinated biphenyl exposure in the rat. *Neurotoxicology.* **17,** 631-638.

Morse, D., Plug, A., Wesseling, W., van den Berg, K., and Brouwer, A. (1996b). Persistent alterations in regional brain glial fibrillary acidic protein and synaptophysin levels following pre- and postnatal polychlorinated biphenyl exposure. *Toxicol. Appl. Pharmacol.* **139,** 252-261.

Moser, G., Robinette, C., and Smart, R. (1993). Characterization of skin tumor promotion by mirex: structure-activity relationships, sexual dimorphism and presence of Ha-ras mutation. *Carcinogenesis.* **14,** 1155-1160.

Moysich, K., Shields, P., Freudenheim, J., Schisterman, E., Vena, J., Kostyniak, P., Greizerstein, H., Marshall, J., Graham, S., and Ambrosone, C. (1999). Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **8,** 41-44.

Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997a). Regulatory functions of phospholipase A_2. *Crit. Rev. Immunol.* **17,** 225-283.

Murakami, M., Tada, K., Shimbara, S., Kambe, T., Sawada, H., and Kudo, I. (1997b). Detection of secretory phospholipase A_2s related but not identical to type IIA isozyme in cultured mast cells. *FEBS Lett.* **413,** 249-254.

Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M., Tischfield, J., and Kudo, I. (1998). The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release- type IIA and type V secretory phospholipase A_2s are functionally redundant and act in concert with cytosolic phospholipase A_2. *J. Biol. Chem.* **273-23,** 14411-14423

Muthusamy, N., and Leiden, J. (1998). A protein kinase C-, Ras-, and RSK2-dependent signal transduction pathway activates the cAMP-responsive element-binding protein transcription factor following T cell receptor engagement. *J. Biol. Chem.* **273,** 22841-22847.

Naccache, P., Jean, N., Liao, N., Bator, J., McColl, S., and Kubes, P. (1994). Regulation of stimulated integrin surface expression in human neutrophils by tyrosine phosphorylation. *Blood.* **84,** 616-624.

Nakanishi, Y., Bai, F., Takayama, K., Pei, X., Inoue, K., Osaki, S., Izumi, M., Takaki, Y., Hara, N., Tokiwa, H., and Masuda, Y. (1999). Effect of PCBs on mouse lung tumorigenesis induced by 1-nitropyrene: a preliminary report. *Fukuoka Igaka Zasshi.* **90,** 231-237.

Nakayama, J., Toshitani, S., Asahi, M., and Furue, M. (1999). Evaluation of dermatological symptoms of Yusho patients in the annual examination in 1997-1998. *Fukuoka Igaku Zasshi.* **90,**143-146.

Nalefski, E., McDonagh, T., Somers, W., Seehra, **J., Falke, J., and Clark, J.** (1998). Independent folding and ligand specificity of the C2 calcium-dependent lipid binding domain of cytosolic phosphlipase A_2. *J. Biol. Chem.* **273-3,** 1365-1372.

Naor, Z., Harris, D., and Shacham, S. (1998). Mechanisn of GnRH receptor signaling: combinatorial cross-talk of Ca^{2+} and protein kinase C. *Front Neuroendocrinol.* **19,** 1-19.

Nemenoff, R., Winitz, S., Qian, N., Putten, V., Johnson, G., and Heasley, L. (1993). Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.* **268-3,** 1960-1964.

Norris, J., and Baldwin, A. (1999). Oncogenic ras enhances NF-κB transcriptional activity through raf-dependent and raf-independent mitogen-activated protein kinase signaling pathways. *J. Biol. Chem.* **274,** 13841-13846.

O'Shea, J., Brown, E., Seligmann, B., Metcalf, J., Frank, M., and Gallin, J. (1985). Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J. Immunol.* **134,** 2580-2587.

Olivero, J., and Ganey, P. (1998). Effects of three polychlorinated biphenyls on f-met-leu-phe-induced degranulation in rat neutrophils. *Toxicol. Letters.* **98,** 195-202.

Olivero-Verbel, J., and Ganey, P. (1998). Effect of three polychlorinated biphenyls on f-met-leu-phe-induced degranulation in rat neutrophils. *Toxicol. Lett.* **98,** 195-202.

Orosz, F., Vertessy, B., Salerno, C., Crifo, C., Capuozzo, E., and Ovadi, J. (1997). The interaction of a new anti-tumour drug, KAR-2 with calmodulin. *Br. J. Pharmacol.* **121,** 955-962.

Ozaki, Y., Yatomi, Y., Jinnai, Y., and Kume, S. (1993). Effects of genistein, a tyrosine kinase inhibitor, on platelet functions. Genistein attenuates thrombin-induced Ca^{2+} mobilization in human platelets by affecting polyphosphoinositide turnover. *Biochem. Pharmacol.* **46,** 395-403.

Osawa, M., Kuwamoto, S., Izumi, Y., Yap, K., Ikura, M., Shibanuma, T., Yokokura, H., Hidaka, H., and Matsushima, N. (1999). Evidence for calmodulin inter-domain compaction in solution induced by W-7 binding. *FEBS Letters.* **442**. 173-177.

Osawa, M., Swindells, M., Tanikawa, J., Tanaka, T., Mase, T., Furuya, T., and Ikura, M. (1998). Solution structure of calmodulin-W-7 complex: the basis of diversity in molecular recognition. *J. Mol. Bio*l. **276**, 165-176.

Palmblad, J., Lerner, R., and Larsson, S. (1994). Signal transduction mechanisms for leukotriene B_4 induced hyperadhesiveness of endothelial cells for neutrophils. *J. Immunol.* **152,** 262-269.

Pasini, F., Capecchi, P., Pasqui, A., Ceccatelli, L., Perri, T., Valensin, G., and Gaggelli, E. (1990). Adenosine blocks calcium entry in activated neutrophils and binds to flunarizine-sensitive calcium channels. *Immunopharmacol. Immunotoxicol.* **12,** 77-91.

Patandin, S., Dagnelie, P., Mulder, P., de Coul, E., van der Veen, J., Weisglas, N., Sauer, P. (1999). Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: a comparison between breast-feeding, toddler, and long-term exposure. *Environ. Health Perspect.* **107,** 45-51.

Patel, S., Joseph, S., and Thomas, A. (1999). Molecular properties of inositol 1,4,5-triphosphate receptors. *Cell Calcium.* **25,** 247-264.

Peavy, R., and Conn, P. (1998). Phosphorylation of mitogen-activated protein kinase in cultured rat cortical glia by stimulation of metabotropic glutamate receptors. *J. Neurochem.* **71**, 603-612.

Perisic, O., Fong, S., Lynch, D., Bycroft, M., Williams, R. (1998). Crystal structure of a calcium-phospholipid binding domain from cytosolic phospholipase A_2. (1998) *J. Biol. Chem.* **273-3,** 1596-1604.

Peters, C., and Mayer, A. (1998). Ca^{2+}/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature.* **396,** 575-580.

Petrequin P., Todd, R., Devall, L., Boxer, L., and Curnutte, J. (1987). Association between gelatinase release and increased plasma membrane expression of the Mo1 glycoprotein. *Blood.* **69,** 605-610.

Pettit, E., and Hallett, M. (1998). Two distinct Ca^{2+} storage and release sites in human neutrophils. *J. Leukoc. Biol.* **63,** 225-232.

Pickard, R., Strifler, B., Kramer, R., Sharp, J. (1999). Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A_2. *J. Biol. Chem.* **274-13**, 8823-8831

Pinelli, E., Cambon, C., Tronchere, H., Chap, H., Teissie, J., and Pipy, B. (1994). Ca^{2+}-dependent activation of phospholipase C and D from mouse peritoneal macrophages by a selective trigger of Ca^{2+} influx, γ-hexachlorocyclohexane. *Biochem. Biophys. Res. Commun.* **199,** 699-705.

Pruzanski, W., Kennedy, B., van den Bosch, H., Stefanski, E., and Vadas, P. (1997). Microtubule depolymerization selectively down-regulates the synthesis of proinflammatory secretory non-pancreatic phospholipase A_2. *Lab. Invest.* **76-2**, 171.

Qiu, Z., and Leslie, C. (1994). Protein kinase C-dependent and -independent pathways of mitogen-activated protein kinase activation in

macrophages by stimuli that activate phospholipase A_2. *J. Biol. Chem.* **269,** 19480-19487.

Ramos, L., Eljarrat, E., Hernandez, L., Rivera, J., and Gonzalez, M. (1999). Levels of PCBs, PCDDs and PCDFs in commercial butter samples in Spain. *Chemosphere* **38,** 3141-3153.

Ramos, L., Hernandez, L., and Gonzalez, M. (1997). Variation of PCB congener levels during lactation period and relationship to their molecular structure. *Arch. Environ. Contam. Toxicol.* **33,** 97-103.

Rardon, D., and Krause, P. (1992). Purification of a ryanodine-sensitive channel protein from human neutrophils. *Adv. Exp.Med. Biol.* **311,** 405-406.

Regnouf, F., Sagot, I., Delouche, B., Devilliers, G., Cartaud, J., Henry, J., Pradel, L. (1995). *In vitro* phosphorylation of annexin 2 heterotetramer by protein kinase C. *J. Biol. Chem.* **270(45),** 27143-27150.

Reynaud, D., and Pace, C. (1997). 12-HETE and 12-HPETE potently stimulate intracellular release of calcium in intact human neutrophils. *Prostaglandins Leukot. Essent. Fatty Acids.* **56,** 9-12.

Rice, D. (1999). Behavioral impairment produced by low-level postnatal PCB exposure in monkeys. *Environ. Res.* **80,** 2 pt 2. S113-S121.

Richard, E., Ghosh, S., Lowenstein, J., and Lisman, J., (1997). Ca^{2+}/calmodulin-binding peptides block phototransduction in *Limulus* ventral photoreceptors: Evidence for direct inhibition of phospholipase C. *Proc. Natl. Acad. Sci. USA* **94,** 14095-14099.

Ridley S., Sarsfield, S., Lee, J., Bigg, H., Cawston, T., Taylor, D., DeWitt, D., and Saklatvala, J. (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J. Immunol.* **158,** 3165-3173.

Risinger, C., and Bennett, M. (1999). Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. *J. Neurochem.* **72,** 614-624.

Rohr, U., Konig, W., and Selenka, F. (1985). Effect of pesticides on the release of histamine, chemotactic factors and leukotrienes from rat mast cells and human basophils. *Zentralbl Bakteriol Mikrobiol. Hyg.* **181**, 469-486.

Rosenthal, M., Gordon, M., Buescher, E., Slusser, J., Harris, L., and Franson, R. (1995). Human neutrophils store type II 14-kDa phospholipase A_2 in granules and secrete active enzyme in response to soluble stimuli. *Biochem. Biophys. Res. Commun.* **208,** 650-656.

Rouse, B., Babiuk, L., and Henson, P. (1978). Neutrophils are mediators of antiviral immunity. *Experientia.* **15,** 346-348.

Rowinsky, E., Windle, J., and Von Hoff, D. (1999). Ras protein farnesyltransferase: A strategic target for anticancer therapeutic development. *J. Clin.Oncol.* **17,** 3631-3652.

Rubinek, T., and Levy, R. (1993). Arachidonic acid increases the activity of the assembled NADPH oxidase in cytoplasmic membranes and endosomes. *Biochim. Biophys. Acta.* **1176,** 51-58.

Rzigalinski, B., Blackmore, P., and Rosenthal, M. (1996). Arachidonate mobilization is coupled to depletion of intracellular calcium stores and influx of extracellular calcium in differentiated U937 cells. *Biochim. Biophys. Acta.* **1299,** 342-352.

Sa, D., and Das, T. (1999). Basic fibroblast growth factor stimulates cytosolic phospholipase A2, phospholipase C-gamma1 and phospholipase D through distinguishable signaling mechanisms. *Mol. Cell Biochem.* **198**, 19-30.

Safe, S. (1984). Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology, and mechanism of action. *CRC. Crit. Rev. Tox.* **13,** 319-395.

Safe, S., Bandiera, S., Sawyer, T., Zmudzka, B, Mason, G., Romkes, M., Denomme, M., Sparling, J., Okey, A., and Fujita, T. (1985). Effects of structure on binding to the 2,3,7,8-TCDD receptor protein and AHH induction-halogenated biphenyls. *Environ. Health Perspect.* **61,** 21-33.

Sanderson, J., Aarts, J., Brouwer, A., Froese, K., Denison, M., and Giesy, J. (1996). Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. *Toxicol. Appl. Pharm.* **137,** 316-325.

Satoh, T., and Kaziro, Y. (1992). Ras in signal transduction. *Semin. Cancer. Biol.* **3,** 169-177.

Sattler, I., and Tamanoi, F. (1996). In: Regulation of the ras signaling network (Maruta, H., and Burgess, A. W., eds), pp. 95-137, R.G. Landes, Austin, TX.

Sawyer, D., Sullivan, J., and Mandell, G. (1985). Intracellular free calcium localization in neutrophils during phagocytosis. *Science.* **230,** 663-666.

Schalkwijk, C., Vervoordeldonk, M., Pfeilschifter, J., and van der Bosch, H. (1993). Interleukin-1 Beta-induced cytosolic phospholipase A_2 activity and protein synthesis is blocked by dexamethasone in rat mesangial cells. *FEBS Lett.* **333-3,** 339-343.

Schantz, S., Gardiner, J., Gasior, D., Sweeney, A., Humphrey, H., and McCaffrey, R. (1999). Motor function in aging Great Lakes fisheaters. *Environ. Res.* **80,** 2 Pt 2. S46-S56.

Schecter, A., Kassis, I., and Papke, O. (1998). Partitioning of dioxins, dibenzofurans, and coplanar PCBs in blood, milk, adipose tissue, placenta and cord blood from five american women. *Chemosphere.* **37,** 1817-1823.

Sebolt, J., Dudley, D., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R., Tecle, H., Barret, S., Bridges, A., Przybranowski, S., Leopold, W., Saltiel, A. (1999). Blockage of the MAP kinase pathway supresses growth of colon tumors in vivo. *Nat. Med.* **5,** 810-816.

Seegal, R. (1996). Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Crit. Rev. Toxicol.* **26,** 709-737.

Seegal, R. (1999). Are PCBs the major neurotoxicant in Great Lakes salmon? *Environ. Res.* **80 (2 Pt 2),** S38-S45.

Seegal, R., Bush, B and Shain, W. (1990). Lightly chlorinated *ortho*-substituted PCB congeners decrease dopamine in non-human primate brain and in tissue culture. *Toxicol. Appl. Pharmacol.* **106,** 136-144.

Seufferlein, T., Van, J., Liptay, S., Adler, G., and Schmid, R. (1999). Transforming growth factor alpha activates Ha-Ras in human pancreatic cancer cells with Ki-ras mutations. *Gastroenterology.* **116,** 1441-1452.

Shafer, T., Mundy, W., Tilson, H., and Kodavanti, P. (1996). Disruption of inositol phosphate accumulation in cerebellar granule cells by polychlorinated biphenyls: a consequence of altered Ca^{2+} homeostasis. *Toxicol. Appl. Pharmacol.* **141,** 448-455.

Shao, Q., Saward, L., Zahradka, P., and Dhalla, N. (1998). Ca^{2+} mobilization in adult rat cardiomyocytes by angiotensin type 1 and 2 receptors. *Biochem. Pharmacol.* **55,** 1413-1418.

Sharp, J., White, D., Chiou, X., Goodson, T., Gamboa, G., McClure, D., Burgett, S., Hoskins, J., Skatrud, P., Sportsman, J., et al. (1991). Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A_2. *J. Biol. Chem.* **266,** 14850-14853.

Shiu, W., and Mackay, D. (1986). A critical review of aqueous solubilities, vapor pressure, Henry's law constants, and octanol-water partition coefficients of the polychlorinated biphenyls. *J. Phys. Chem. Ref. Data.* **15,** 911-929.

Siddiqui, R., and English, D. (1996). Phosphatidic acid binding to human neutrophils: effects on tyrosine kinase-regulated intracellular Ca^{2+} mobilization. *Cell Signal.* **8,** 349-354.

Silberhorn, E., Glauert, H., and Robertson, L. (1990). Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *CRC Crit. Rev. Tox.* **20**, 440-496.

Silkworth, J., and Grabstein, E. (1982). Polychlorinated biphenyl immunotoxicity: Dependence on isomer planarity and the Ah gene complex. *Toxicol. Appl. Pharm.* **65**, 109-115.

Sitarska, E., Winnicka, A., Klucinski, W. (1990). Effect of organochlorine pesticides on the phagocytic activity of bovine milk cells. *Zentralbl Veterninarmed.* **37,** 471-476.

Slevin, M., Krupinski, J., Kumar, S., and Gaffney, J. (1998). Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation. *Lab. Invest.* **78,** 987-1003.

Smith, C., Lane, B., Kusaka, I., Verghese, M., Snyderman, R. (1985). Chemoattractant receptor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in human polymorphonuclear leukocyte membranes.

Requirement for a guanine nucleotide regulatory protein. *J. Biol. Chem.* **260,** 5875-5878.

Smith, J., Bowman, B., and Iden, S. (1981). Effects of trifluoperazine on human neutrophil function. *Immunology.* **44,** 677-684.

Smith, R., Baukal, A., Dent, P., and Catt, K. (1999). Raf-1 kinase activation by angiotensin II in adrenal glomerulosa cells: roles of Gi, phosphatidylinositol 3-kinase, and Ca2+ influx. *Endocrinology.* **140,** 1385-1391.

Smolen, J., and Weissmann, G. (1980). 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.* **29,** 533-538.

Sonnino, R., Pigatt, L., Schrama, A., Burchett, S., and Franson, R. (1997). Phospholipase A_2 secretion during intestinal graft ischemia. *Dig. Dis. Sci.* **42-5,** 972-981.

Srivastava, S., Khanna, N, Mathur, A., and Chaturvedi, U. (1994). Role of calcium in neutrophil activation by Japanese encephalitis virus-induced macrophage derived factor. *Acta Virol.* **38,** 111-116.

Stewart, P., Darvill, T., Lonky, E., Reihman, J., Pagano, J., and Bush, B. (1999). Assesement of prenatal exposure to PCBs from maternal consumption of Great lakes fish: an analysis of PCB pattern and concentration. *Environ. Res.* **80**(2 Pt 2): S87-S96.

Su, SB., Gao, J., Gong, W., Dunlop, N., Murphy P., Oppenheim, J., and Wang, J. (1999). T21/DP107, a synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. *J. Immunol.* **162,** 5924-5930.

Subramanian B., Tanade, S., Hidaka, H., and Tatsukawa, R. (1983). DDT and PCB isomers and congeners in antarctic fish. *Arch. Environ. Contam. Toxicol.* **12,** 621-626.

Svendsgaard, D., Ward, T., Tilson, H., and Kodavanti, P. (1997). Empirical modeling of an *in vitro* activity of polychlorinated biphenyl congeners and mixtures. *Environ. Health Perspect.* **105,** 1106-1115.

Takahashi, K., Tago, K., Okano, H., Ohya, Y., Katada, T., and Kanaho, Y. (1996). Augmentation by calmodulin of ADP-ribosylation factor-stimulated phospholipase D activity in permeabilized rabbit peritoneal neutrophils. *J. Immun.* **156,** 1229-1234.

Takahashi, T., Ueno, H., and Shibuya, M. (1999). VEGF activates protein kinase C-dependent, but ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene.* **18**, 2221-2230.

Tanabe, S., Kannan, N., Subramanian, A., Watanabe, S., and Tatsukawa, R. (1987). Highly toxic coplanar PCBs: occurrence, source, persistency and toxic implications to wildlife and humans. *Environ. Pollut.* **47,** 147-163.

Theler, J., Lew, D., Jaconi, M., Krause, K., Wollheim, C., and Schlegel, W. (1995). Intracellular pattern of cytosolic Ca^{2+} changes during adhesion and multiple phagocytosis in human neutrophils. Dynamics of intracellular Ca^{2+} stores. *Blood.* **85,** 2194-2201.

Thiel, M., and Bardenheuer, H. (1992). Regulation of oxygen radical production of human polymorphonuclear leukocytes by adenosine: the role of calcium. *Pflugers Arch.* **420,** 522-528.

Tilson, H., and Kodavanti, P. (1997). Neurochemical effects of polychlorinated biphenyls: an overview and identification of research needs. *Neurotoxicology.* **18,** 727-744.

Tithof, P., Contreras, M., and Ganey, P. (1995). Aroclor 1242 stimulates the production of inositol phosphates in polymorphonuclear neutrophils. *Toxicol. Appl. Pharmacol.* **131,** 136-143.

Tithof, P., Peters-Golden, M., and Ganey, P. (1998). Distinct phospholipases A_2 regulate the release of arachidonic acid for eicosanoid production and superoxide anion generation in neutrophils. *J. Immunol.* **160,** 953-960.

Tithof, P., Schiamberg, E., Peters, M., and Ganey, P. (1996). Phospholipase A_2 is involved in the mechanism of activation of neutrophils by polychlorinated biphenyls. *Environ. Health. Perspect.* **104,** 52-58.

Tithof, P., Watts, S., and Ganey, P. (1997). Protein tyrosine kinase involvement in the production of superoxide anion by neutrophils exposed to

Aroclor 1242, a mixture of polychlorinated biphenyls. *Biochem. Pharmacol.* **53,** 1833-1842.

Toborek, M., Barger, S., Mattson, M., Espandiari, P., Robertson, L., and Hennig, B. (1995). Exposure to polychlorinated biphenyls causes endothelial cell dysfunction. *J. Biochem. Toxicol.* **10,** 219-226.

Torres, M., Hall, F., and O'Neill, K. (1993). Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein kinases. *J. Immunol.* **150,** 1563-1578.

Van der Burght, A., Clijsters, P., Horbach, G., Andersson, P., Tysklind, M., and van den Berg, M. (1999). Structure-dependent induction of CYP1A by polychlorinated biphenyls in hepatocytes of cynomolgus monkeys (*Macaca fascicularis*). *Toxicol. Appl. Pharmacol.* **155,** 13-23.

Vandonselaar, M., Hickie, R., Quail, W., and Delbaere, L. (1994). Trifluoperazine-induced conformational change in Ca^{2+}-calmodulin. *Nat. Struct. Biol.* **1,** 795-801.

Vertessy, B., Bocskei, Z., Harmath, V., Naray-Szabo, G., and Ovadi, J. (1997). Crystallization and preliminary diffraction analysis of Ca^{2+}-calmodulin-drug and apocalmodulin-drug complexes. *Proteins.* **28,** 131-134.

Vertessy, B., Harmat, V., Bocskei, Z., Naray, G., Orosz, F., and Ovadi, J. (1998). Simultaneous binding of drugs with different chemical structures to Ca^{2+} calmodulin: crystallographic and spectroscopic studies. *Biochemistry.* **37,** 15300-15310.

Vervoordeldonk, M., van Rossum, G., Sanchez, R., Neys, F., and van den Bosch, H. (1997). Half-life of interleukin-1 β-induced group II phospholipase A_2 in rat mesangial cells. *Biochim. Biophys. Acta.* **1355,** 315-322.

Ville, P., Roch, P., Cooper, E., Masson, P., and Narbonne, J. (1995). PCBs increase molecular-related activities (lysozyme, antibacterial, hemolysis, proteases) but inhibit macrophage-related functions (phagocytosis, wound healing) in earthworms. *J. Invertebr. Pathol.* **65,** 217-224.

Voie, O., and Fonnum, F. (1998). *Ortho* substituted polychlorinated biphenyls elevate intracellular [Ca^{2+}] in human granulocytes. *Environ. Toxicol. Pharmacol.* **5.** 105-112.

Voie, O., Wiik, P., and Fonnum, F. (1998). *Ortho*-substituted polychlorinated biphenyls activate respiratory burst measured as luminol-amplified chemiluminiscence in human granulocytes. *Toxicol. Appl. Pharmacol.* **150,** 369-375.

Vos, J., and Van Loveren, H. (1998). Experimental studies on immunosuppression: how do they predict for man? *Toxicology.* **129,** 13-26.

Waddick, K., and Uckun, F. (1998). Innovative treatment programs against cancer. *Biochem. Pharmacol.* **56,** 1411-1426.

Waid, J. (1987). PCBs and the environment. Volumes I to III. CRC Press. Boca Raton, Florida.

Wanikiat, P., Woodward, D., and Armstrong, R. (1997). Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils. *Br. J. Pharmacol.* **122,** 1135-1145.

Weisdorf, D., and Thayer, M. (1989). Occult intracellular calcium pools: Relevance to neutrophil oxidant production. *J. Lab. Clin. Med.* **114,** 260-265.

Weiss, S., Lampert, M., and Test, S. (1983). Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science.* **222,** 625-628.

Wheeler, C., Abu, R., Cospedal, R., Houliston, R., Martin, J., and Zachary, I. (1997). Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A_2 in endothelial cells via p42/p44 mitogen-activated protein kinase. *FEBS Lett.* **420,** 28-32.

Wilkinson, S., Parker, P., and Nixon, J. (1993). Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.* **294,** 335-337.

Williams, R. (1998). Calcium in health and disease. *Cell Calcium.* **24,** 233-237.

Wilsson, A., Lundqvist, H., Gustafsson, M., and Stendahl, O. (1996). Killing of phagocytosed Staphylococcus aureus by human neutrophils requires intracellular free calcium. *J. Leukoc. Biol.* **59,** 902-907.

Wolf, M., and Gross, R. (1996). The calcium-dependent association and functional coupling of calmodulin with myocardial phospholipase A_2. *J. Biol. Chem.* **271,** 20989-20992.

Wolff, M., Toniolo, P., Lee, E., Rivera, M., and Dubin, N. (1993). Blood levels of organochlorine residues and risk of breast cancer. *J. Natl. Cancer. Inst.* **85,** 648-652.

Wolfle, D. (1997-98). Interactions between 2,3,7,8-TCDD and PCB's as tumor promoters: limitations of TEFs. *Teratog. Carcinog. Mutagen.* **17(4-5),** 217-24.

Wong, P., and Pessah, I. (1997). Non-coplanar PCB 95 alters microsomal calcium transport by an immunophilin FKBP12-dependent mechanism. *Mol. Pharmacol.* **51,** 693-702.

Wong, P., and Pessah, I. (1996). Ortho-substituted polychlorinated biphenyls alter calcium regulation by a ryanodine receptor-mediated mechanism: structural specificity toward skeletal- and cardiac-type microsomal calcium release channels. *Mol. Pharmacol.* **49**, 740-751.

Wong, P., Brackney, W., and Pessah, I. (1997a). *Ortho*-substituted polychlorinated biphenyls alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. *J. Biol. Chem.* **272,** 15145-15153.

Wong, P., Joy, R., Alberston, T., Schantz, S., and Pessah, I. (1997b). *Ortho*-substituted 2,2'3,5',6-pentachlorobiphenyl (PCB 95) alters rat hippocampal ryanodine receptors and neuroplasticity *in vitro*: evidence for altered hippocampal function. *Neurotoxicology.* **18, 443-456.**

Wu, P., Greeley, E., Hansen, L., and Segre, M. (1999). Immunological, hematological, and biochemical responses in immature white-footed mice following maternal Aroclor 1254 exposure: A possible bioindicator. *Arch. Environ. Contam. Toxicol.* **36,** 469-476.

Yang, H., Mosior, M., Johnson, C., Chen, Y., and Dennis, E. (1999). Group-specific assays that distinguish between the four major types of mammalian phospholipase A_2. *Anal. Biochem.* **269,** 278-288.

Zallen, G., Moore, E., Johnson, J., Tamura, D., Barkin, M., Stockinger, H., and Silliman, C. (1998). New mechanisms by which secretory phospholipase A_2 stimulates neutrophils to provoke the release of cytotoxic agents. *Arch. Surg.* **133,** 1229-1233.

Zhang, H., Garlichs, C., Mugge, A., and Daniel, W. (1998). Involvement of tyrosine kinases, Ca^{2+} and PKC in activation of mitogen-activated protein (MAP) kinase in human polymorphonuclear neutrophils. *J. Physiol. (Lond).* **513,** 359-367.

Zhang, Y., Palmblad, J., and Fredholm, B. (1996). Biphasic effect of ATP on neutrophil functions mediated by P_2U and adenosine A_{2A} receptors. *Biochem. Pharmacol.* **51,** 957-965.

Zhao, F., Mayura, K., Kocurek, N., Edwards, J., Kubena, L., Safe, S., and Phillips, T. (1997). Inhibition of 3,3',4,4',5-pentachlorobiphenyl-induced chicken embryotoxicity by 2,2',4,4',5,5'-hexachlorobiphenyl. *Fund. Appl. Tox.* **35,** 1-8.

Zu, Y., Qi, J., Gilchrist, A., Fernandez, G., Vazquez, D., Kreutzer, D., Huang, C., and Sha'afi, R. (1998). p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or fMLP stimulation. *J. Immunol.* **160,** 1982-1989.