Alterations in T cell function and activation during exposure to the herbicide 3,4 dichloropropionanilide (DCPA) and its metabolites

Tricia L. Lewis
West Virginia University

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Alterations in T cell function and activation during exposure to the herbicide 3,4 dichloropropionanilide (DCPA) and its metabolites

Tricia L. Lewis

Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in
Immunology and Microbial Pathogenesis

John B. Barnett, Ph.D., Chair
Peter M. Gannett, Ph.D.
Michael I. Luster, Ph.D.
Karen H. Martin, Ph.D.
Vazhaikkurichi M. Rajendran, Ph.D.

Department of Microbiology, Immunology and Cell Biology
Morgantown, West Virginia
2010

Keywords: 3,4-dichloropropionanilide, DCPA, propanil, 3,4-dichloroaniline, herbicide, calcium, T cells, Stim1, metabolites
ABSTRACT

Alterations in T cell function and activation during exposure to the herbicide 3,4 dichloropropionanilide (DCPA) and its metabolites

Tricia L. Lewis

Approximately 5.3 billion pounds of pesticides are applied annually across the United States and 15 of the top 25 most used pesticides are herbicides. Total herbicide use in the United States represents 28% of all worldwide herbicide use. DCPA (3,4 dichloropropionanilide, common name propanil) is a post-emergent herbicide that is used extensively for control against several broadleaf plants and grasses. It is the 17th most common herbicide in the United States and 6-9 million pounds are applied annually to 2 million acres of rice fields. In mammals, DCPA is metabolized in the liver and produces 3,4-dichloroaniline (DCA) and propionic acid as its major metabolites. DCA is further biotransformed leading to the production of 2 hydroxylated metabolites: 6-hydroxy-3,4-dichloroaniline (6OH-DCA) and N-hydroxy-3,4-dichloroaniline (NOH-DCA). The immunomodulatory effects of DCPA are well documented but only limited data is available on the effects of its metabolites. Previous studies have shown that DCPA alters transcription factors involved in the expression of IL-2 and decreases mRNA and IL-2 protein in human and mouse T cells. IL-2 is an early cytokine that is secreted by activated T cells and plays an important role in the activation, proliferation and differentiation of several immune cells. Expression of IL-2 relies on activation and influx of calcium through channels in the plasma membrane. This study was conducted to examine the effects of DCPA and its metabolites on T cell activation and function and to propose a mechanism for the observed effects. Human Jurkat T cells, a model cell line for T cell signaling, were exposed to increasing concentrations of DCPA or its metabolites and T cell function was assessed by measuring IL-2 secretion. DCPA and its metabolites all inhibit IL-2 secretion in a concentration-dependent manner, however, NOH-DCA is the most potent inhibitor, followed by DCPA. To better understand the mechanism by which they suppress IL-2, NFAT activity and calcium influx were investigated. Interestingly, DCPA and DCA inhibited IL-2 in a calcium-dependent manner whereas the hydroxylated metabolites inhibited IL-2 in a calcium-independent manner. The calcium-dependent alterations in IL-2, N FAT activity and calcium influx were influenced by the presence of chlorines, as substitution with fluorines abrogated all effects. Further studies investigating the role of DCPA in calcium release-activated calcium (CRAC) channels revealed that activation of a key protein, Stromal interaction molecule-1 (Stim1), is inhibited by DCPA. Collectively, this data supports the conclusion that DCPA suppresses IL-2 production by inhibiting Stim1. This mechanism describes a novel pathway for immunosuppression.
DEDICATION

I dedicate this dissertation to David Light and our children, Summer and Willem. This work is a testament to their love and patience. It is my hope that I may instill in my children a curiosity and love of learning that will guide and inspire them in all they do. I would also like to dedicate this dissertation in memory of Dr. George Wollner, my uncle, who understood the hard work and sacrifice required to achieve this goal.
ACKNOWLEDGEMENTS

The completion of this work would not have been possible without the encouragement, love and support of so many people. My sincere thanks to my advisor and mentor, John Barnett, whose limitless patience and thoughtful guidance through many obstacles made the completion of this dissertation possible. I also acknowledge my committee members Vazhaikkurichi Rajendran, Peter Gannett, Karen Martin and Michael Luster. I would especially like to thank Karen Martin for all her assistance and suggestions with several of my microscopy experiments and Peter Gannett for his help and insight with all things chemistry. I am also thankful for the valuable comments and suggestions of Rosana Schafer and David Klinke.

I would also like to acknowledge Miranda Hanson, a wonderful and strong friend who kept things in perspective and made me laugh. I am eternally grateful to my fellow grad students Siera Talbott, Laura Kelley and Janna Jackson for helping me maintain my sanity through energizing bike rides and great conversations, which often occurred at the same time. My thanks to Siera for her help with experiments and for a shoulder to laugh and cry on and to Laura for your critical eye and constructive and honest comments. I also want to thank Ron Fecek, Alex Rowe, Irina Ustyugova and Cheryl Walton for their friendship and help throughout my graduate studies.

My family provided endless love and support at times when I needed it most. I would like to thank my sister, Christine, who listened and unconditionally supported me during this long journey and to my Grandparents, Auntie and late Uncle George for their constant support and for encouraging me to reach my goals.

Lastly, I would like to thank my greatest supporter, David Light. Throughout this journey he has been steadfast through all the ups and downs and has sacrificed everything to see me through my graduate studies. No words can convey the deep gratitude and respect I have for his unwavering love and support. He made this achievement possible and I thank him with all my heart.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2-APB</td>
<td>2-Aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>6OH-DCA</td>
<td>6-hydroxy-3,4-dichloroaniline</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ASC</td>
<td>Antibody secreting colony</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BFU</td>
<td>Burst forming unit</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAD</td>
<td>CRAC-activating domain</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>con-A</td>
<td>Concanavalin-A</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release-activated calcium</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCA</td>
<td>3,4-dichloroaniline</td>
</tr>
<tr>
<td>DCPA</td>
<td>3,4-dichloropropionanilide</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DFA</td>
<td>3,4-difluoroaniline</td>
</tr>
<tr>
<td>DFPA</td>
<td>3,4-difluoropropionanilide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>I_{CRAC}</td>
<td>Calcium release-activated calcium current</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1, 4, 5 triphosphate</td>
</tr>
<tr>
<td>Ib</td>
<td>Pound</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose 50</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappaB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOH-DCA</td>
<td>N-hydroxy-3,4-dichloroaniline</td>
</tr>
<tr>
<td>PFC</td>
<td>Plaque forming colony</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca-ATPase</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile alpha motif</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic reticulum Ca-ATPase</td>
</tr>
<tr>
<td>SHD</td>
<td>Stim1 homerization domain</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>Stim</td>
<td>Stromal interaction molecule</td>
</tr>
<tr>
<td>TBAB</td>
<td>Tetra-n-butylammonium bromide</td>
</tr>
<tr>
<td>TCAB</td>
<td>3,3 ',4,4' tetrachloroazobenzene</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TOTP</td>
<td>Triorthotolyl phosphate</td>
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## T CELL ACTIVATION AND SIGNALING

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## T CELLS AND ION CHANNELS

- CRAC channels
- CRAC regulation
- CRAC inhibitors

## OTHER ION CHANNELS AND PUMPS

- SERCA
- PMCA
- RyR
- Na/Ca exchanger
- Ca uniporter
- Potassium (K+) channels
- Non-store-operated cation channels

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## CHAPTER 2


## ABSTRACT


## INTRODUCTION


## MATERIALS AND METHODS


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- Figure 1 DCPA decreases intracellular calcium
- Figure 1B
- Figure 1C and 1D
- Figure 2A. DCPA does not affect early IP3
- Figure 2B
- Figure 2C and 2D
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- Figure 4 DCPA does not alter the membrane potential
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CHAPTER 1
Literature Review

DCPA (3,4-dichloropropionanilide)

Background

DCPA (chemical name 3,4-dichloropropionanilide, common name propanil) is the active ingredient in a commercially available herbicide distributed under several trade names, including Chem-Rice, Herbax and Propanex. It is a widely used, post-emergent acetanilide contact herbicide and is registered for use against several broadleaf and grassy weeds, such as barnyard grass, crabgrass, pigweed, foxtail and smartweed (USEPA 2006). It has been registered for use as an herbicide in the United States (US) since the 1950s and has recently been required by the United States Environmental Protection Agency (USEPA) to be re-registered due to increased adverse ecological and toxicological effects (USEPA 2006). In the US, DCPA is applied predominantly on rice fields, with lesser use on small grain and wheat crops. Annual use of DCPA is estimated to be 6-9 million pounds per year and is distributed on approximately 2 million acres of crop land (USDA 2006). In the United States, 50-80% of total rice crops are treated with propanil, with major use in California, the Mississippi Delta and the Gulf Coast (USDA 2006). In addition to crop use, DCPA has also recently been registered for turf use on commercial sod farms (USEPA 2006). This new application of DCPA will further increase use and exposure.
**Methods and rates of application**

Ground boom sprayers and aerial equipment are the most common means of application with hand spraying also occurring in some areas. Commercial DCPA labels indicate that handlers should wear long sleeve shirts, long pants, socks, shoes, waterproof gloves and protective eyewear (USEPA 2006). In addition, in some situations, workers must use maximum protective controls, including closed mixing and loading systems and enclosed cockpits and trucks (USEPA 2006). Application of DCPA to rice fields typically occurs twice, once approximately 15-25 days after the rice is planted and again 10-15 days later. At this time, the rice fields are flooded for 30 days and a third application is possible during the flooding period. After the flooding period, crop fields are drained and the rice is subsequently harvested 140-150 days after planting. The maximum rate of application of propanil, set by the USEPA, on rice fields is 8 lb/acre/season with an average use of 6 lb/acre/season (USEPA 2006). Application of DCPA on turf in commercial sod farms has a maximum rate of 10 lb/acre (USEPA 2006).

**Routes of exposure and risk assessment**

Workers involved in manufacturing, handling or application of propanil are at greatest risk but there have also been reports of non-occupational exposure, including a child under the age of 6 (USEPA 2006). The routes of exposure include dermal, inhalation, ingestion and contact with the eyes. The USEPA’s Registration Eligibility Decision (RED) for propanil conducted a risk assessment which included determining a margin of exposure (MOE) for workers who are involved in mixing, loading and application activities (USEPA 2006). For propanil, MOE values of less than 300 are
considered a potential risk concern. Commercial propanil labels indicate that handlers should wear long sleeve shirts, long pants, socks and shoes, waterproof gloves and protective eyewear. Using this minimal and widely used level of protection, the EPA determined that the MOE of all workers (in rice fields and turf) was less than 300, with several handling methods having a MOE less than 100. In addition, in some situations, workers using maximum protective controls (closed mixing and loading systems and enclosed cockpit, cabs or trucks) still had a MOE of less than 300. Post occupational risk assessment, for workers entering treated areas 12 hours after application, was also conducted. Workers entering a field with maximum allowable application of propanil had a MOE of less than 300 while turf workers involved in transplantation, fertilizing or mechanical weeding or harvesting had a MOE less than 100. This assessment indicates that exposure to propanil is a risk concern for all workers, including those with maximal protection.

**Phytotoxicity**

In rice, as well as broadleaf plants and grasses, DCPA can translocate from the leaves to the growing shoots and then back to other leaves. The translocation and absorption of DCPA occurs at similar rates in both rice and plants targeted by the herbicide (Carey V. F. 1995). The phytotoxic effects of DCPA are influenced by the ability of the plant to produce the enzyme acylamidase (Adachi 1966; Still and Kuzirian 1967). Acylamidase cleaves the amide bond on DCPA and inactivates its herbicidal activity (Yih, M. McRae et al. 1968). Gaynor et al. determined that, in the rice plants (*Oryza sativa*), acylamidase is primarily localized to the outer membrane of the mitochondria (Gaynor and Still 1983). Rice leaves produce 60 times more acylamidase
than barnyard grasses and are able to degrade DCPA to avoid its phytotoxic effects. Inhibition of acylamidases can occur during co-application with DCPA and certain organophosphorus or carbamate insecticides rendering the rice plants susceptible to the phytotoxic effects of propanil (Matsunaka 1968; Chang, Smith et al. 1971). Plants susceptible to the herbicidal activity of DCPA express low levels of acylamidase and phytotoxic effects occur primarily through the inhibition of photosynthesis. Specifically, DCPA inhibits the photosynthetic electron transport system in chloroplasts (Matsunaka 1968). During photosynthesis, two distinct stages are required for the synthesis of sugars. In the light reaction, chlorophyll absorbs a photon of light and loses an electron. This electron gets passed through the electron chain transport resulting in the production of NADPH (nicotinamide adenine dinucleotide phosphate). In addition, the light reaction also converts light energy into chemical energy stored in the form of ATP (adenosine triphosphate). Both NADPH and ATP are required for the conversion of CO₂ to sugar that occurs in the dark reaction in the stroma of chloroplasts. Interruption of the electron chain transport prevents the production of sugars required for plant growth. Recently Yun et al reported that DCPA also inhibits the enzyme, 4-coumarate:CoA ligase (Yun, Chen et al. 2007). 4-Coumarate is found only in the plant kingdom and upon binding to the cofactor, CoA ligase, plays an important role in the phenylpropanoid pathway, a pathway important in plant growth, mechanical support and cell wall rigidity.

**Metabolism**

In soil, plants and mammals, metabolism of DCPA occurs by hydrolysis of the amide bond resulting in the production of 3, 4-dichloroaniline (DCA) and propionic acid (Still
1968). When McMillan et al. treated rat hepatic microsomes with radio-labeled DCPA they identified DCA as the major metabolite (McMillan, Freeman et al. 1990). Hydrolysis of the amide bond by hepatic acylamidases was supported when formation of DCA was inhibited with a acylamidase inhibitors. In addition, they identified 2 minor metabolites produced through oxidation of DCA; 6-hydroxy-3,4-dichloroaniline (6OH-DCA) and N-hydroxy-3,4-dichloroaniline (NOH-DCA) (McMillan, Le akey et al. 1990). Figure 1 outlines the metabolic pathway in mammals and the structures of DCPA and its major metabolites.

The half life of DCPA in soil is reported to be 2-3 days (Chisaka and Kearney 1970; Konstantinou, Zar kadis et al. 2001). Recovery of propanil, in sterile-treated soil, is almost 100% after incubation for 25 days (Chisaka and Kearney 1970). Analysis of soil samples from rice fields treated with DCPA reveals that most of the DCPA is metabolized within 15 days and DCA and propionic acid can be readily detectable. Microbial activity in the soil is thought to be responsible for the metabolic breakdown of propanil. Acylamidase activity in bacteria including Pseudomonas striata and Pseudomonas fluorescens as well as the fungus Vibrio fisheri can convert DCPA to DCA (Surovtseva and Funtikova 1978; Zabl otowicz, L ocke et al. 2001). Fusarium solani has also been reported to hydrolyze DCPA to produce DCA and propionic acid (Lanzilotta and Pramer 1970). The propionic acid can serve as a carbon source to F. solani to provide energy to the fungus. However, it does not appear to be able to further degrade DCA and an increasing accumulation of DCA can result in inhibited growth of the fungus (Lanzilotta and Pramer 1970). Microbial oxidation of propionic acid releases CO₂ and water within 4 days (Yih, McRae et al. 1968; Chisaka and Kearney 1970).
Soils treated with propanil, at near-field application rates (6lb/acre), reveal that 70-80% of the DCA produced is chemically bound to humic substances (Bartha and Pramer 1967; Bartha 1971). Degradation of the DCA-humic complex was estimated to take 5-10yrs. DCA can also complex with the plant protein, lignin, with no apparent detrimental effects to the plant (Yih, McRae et al. 1968). A small portion (10%) of
unbound DCA can also form complexes with sugars to produce N-(3,4-dichlorophenyl) glucosylamine and other minor sugar conjugates (Still and Kuzirian 1967).

There are also reports that two molecules of DCA, derived microbiologically from propanil, condense and form 3,3,’4,4’ tetrachloroazobenzene (TCAB) (Bartha 1971; Hill, Rollen et al. 1981). It has been suggested that microbial action, specifically peroxidase activity, is involved in the condensation of DCA to TCAB in soils (Bartha and Pramer 1967; Bordeleua 1972). TCAB has not been found in plant tissues treated with DCPA (Still, Balba et al. 1981). In a study examining 99 soil samples from the rice-growing states of Arkansas, California, Louisiana, Mississippi, and Texas, TCAB was detected in 6 samples (Carey, Yang et al. 1980). Production of TCAB is thought to occur primarily in loamy soil and is inhibited under dry soil conditions (Bartha 1971). Workers involved in the manufacturing and packaging of DCPA report several adverse exposure effects including dizziness, nausea, muscle weakness, fatigue, and chloracne. Only the effect of chloracne was directly related to the presence of TCAB (Kimbrough 1980).

**Ecological effects**

The effect of DCPA on microbial communities and aquatic animals and plants has been well documented. Contamination of waterways has been reported to occur following release of water from flooded rice fields with reported DCPA levels of 0.1µg/l (0.1ppb) in soil water slurry and irrigation water (Papadopoulou-Mourkidou, Karpouzas et al. 2004). A single application of DCPA (6 lb/ac), in a closed flooded rice field, resulted in a DCPA concentration of 200µg/l (200ppb) after 24 hrs and decreased to 50µg/l (50ppb) for the next 5 days (Papadopoulou-Mourkidou, Karpouzas et al. 2004).
After 10-15 days only metabolites were detectable. The half life of DCPA in water is reported to be approximately 2 days (Dahchour, Bitton et al. 1986).

Several studies have documented the effects of DCPA on aquatic life. Exposure of DCPA to fathead minnows (*Pimephales promelas*) has several deleterious effects with an LD$_{50}$ value of 3.4mg/l (3.4ppm) after 192 hours of exposure (Call, Brooke et al. 1983). Early life toxicity was observed at 3.8µg/l (3.8ppb) and resulted in significant decreases in egg hatch and increases in dead and deformed hatched minnows. By post-hatch day 54 the mortality rate was 100%. Significant decreases in survival were also seen at 1.2µg/l (1.2ppb), with only 17% surviving past post-hatch day 54. These levels are sufficiently high enough to affect fish populations in the rice paddy water or in the paddy water that is released near spawning or nursery grounds. Adverse effects to DCPA have also been observed in fish and frogs. The frog species, *Xenopus laevis*, has a reported LD$_{50}$ of 8.64mg/l (8.64ppm) 48 hours after exposure. The 96 hr LC$_{50}$ for the fish species, Rainbow trout (*Oncorhynchus mykiss*), Blue gill (*Lepomis macrochirus*), and the fingerling channel catfish (*Ictalurus punctatus*) exposed to DCPA ranged from 2.3 - 6mg/l (2.3-6ppm) (McCorkle, Chambers et al. 1977) (Moore, Pierce et al. 1998) (USEPA 2003). Exposure of the metabolite DCA on zebrafish, *Danio rerio*, results in alterations in early life stage development (Nagel, Breshe et al. 1991). Deformations in the spine were observed at 0.25mg/l DCA and an 11 day subchronic test (0.5mg/l) revealed decreases in locomotor activity and mortality. *Daphnia magna* are also very sensitive to DCA as the 48-hr LC$_{50}$ of 0.14 mg/L (0.14ppm) (Pereira, Antunes et al. 2009).
Microbial communities within rice fields are also altered during exposure to DCPA. Exposure of 20µg/ml (20ppm) DCPA to the cyanobacterium, *Anabaena MH*, resulted in a significant inhibition of growth (Habte and Alexander 1980) with in a loss of more than 85% of its chlorophyll with no recovery after 10 days of exposure. Application of 25µg/ml (25ppm) suppressed 98% of algae growth for over 30 days.

**DCPA metabolites**

**3,4-Dichloroaniline**

3,4-Dichloroaniline (DCA) is the major metabolite of DCPA as well as other herbicides, including diuron and linuron. In addition, DCA is used as an intermediate in the manufacturing of several pesticides, dyes and pharmaceuticals (ECB 2006). DCA is persistent in soil and water but can be readily photodegraded in the atmosphere. It has a half life of approximately 1000 days (ECB 2006). DCA is subject to chemical oxidation, reacts with the organic matter in soil and has high volatility. In areas treated with propanil, large amounts of DCA are bound to soil particle making it unavailable for further metabolism or plant uptake (Bartha 1971). However, DCA residues can be detected in most commercial rice (*Oryza sativa*) grain samples at a concentration of 1µg/g of rice (Still 1968; SCTEE 2001). Rats treated with an acute intraperitoneal (i.p.) injection of DCA reach maximum detection of DCA in the liver and plasma within 30-60 minutes with levels decreasing quickly within a few hours (Guilhermino, Soares et al. 1998). Elimination of DCA in rats occurs after 3 days and is primarily excreted in urine and feces. In humans, DCA has been detected in the urine of occupational and non-occupationally exposed subjects (Wittke, Hajimiragha et al. 2001; Turci, Barisano et al. 2001).
2006) with levels in the 0.01-6.2µg/l range. DCA-hemoglobin (Hb) adducts have also been detected in workers involved in the application of DCPA to rice fields (Pastorelli 1998). These adducts were still detectable 4 months after the last application of propanil.

**N-hydroxy-3,4-dichloroaniline and 6-hydroxy-3,4-dichloroaniline**

N-hydroxy-3,4-dichloroaniline (NOH-DCA) and 6-hydroxy-3,4-dichloroaniline (6OH-DCA) are the major mammalian metabolites of DCA. In preparations of rat hepatic microsomes, McMillan et al. detected 2 oxidative metabolites, NOH-DCA and 6OH-DCA (McMillan, Freeman et al. 1990). Further studies in chromium-51 (Cr-51) labeled erythrocytes treated with DCPA indicate that NOH-DCA can undergo rapid redox cycling resulting in the oxidation of oxyHb to metHb. Little is known about the effects of 6OH-DCA but it is also involved in the production of metHb, although the NOH-DCA metabolite is reported to be 10 times more potent (McMillan, Freeman et al. 1990). Methemoglobin (metHb) is a form of hemoglobin that does not bind or transport oxygen. Increases in metHb leads to a serious medical disorder known as methemoglobinemia.

**TOXICITY**

**General toxicity**

Using metHb levels as a marker for toxicity, the USEPA has assessed a toxicity category for DCPA based on routes of exposure (USEPA 2003). Inhalation and dermal routes, the most common routes of exposure, were placed in Toxicity Category IV. This indicates that caution should be used but little toxicity has been shown. An oral route of
exposure is signed a Toxicity Category II, with caution indicating slight toxicity. Toxicity Category II, with a hazard warning for moderate toxicity, was indicated as studies demonstrated that DCPA is an eye irritant.

**Mutagenicity and carcinogenicity**

Conflicting studies are available that assess the possible mutagenic effects of exposure to DCPA and its metabolites. Using the Salmonella typhimurium reversion assay, chemical-induced base changes or frameshift mutations in the genome of this organism can be measured (McMillan, Shaddock et al. 1988). Over a range of concentrations, propanil, DCA and NOH-DCA demonstrated no increase in mutations (McMillan, Shaddock et al. 1988). The ability of propanil, DCA and NOH-DCA to induce mutations in Chinese hamster ovary (CHO) cells was also assessed. Although exposure of all 3 chemicals decreased CHO cell viability over a wide range of concentrations, no changes in mutation rates were observed (McMillan, Shaddock et al. 1988). Similarly, no change in DNA damage in rat hepatocytes was observed. However, in more recent studies using the more sensitive Drosophila wing spot assay, larvae were treated for 3 days with varying concentrations of DCPA. The wing spot assay can detect a loss of heterozygosity in suitable recessive markers that leads to the formation of mutant clones of cells that are then expressed as spots on the wings. In these assays, DCPA does induce genotoxic effects (Kaya, Creus et al. 2000). A cytogenic assay on DCA indicated that exposure of DCA disturbs spindle formation but with no changes in chromosome formation (Bauchinger, Kulka et al. 1989).
The USEPA has assessed DCPA for carcinogenic potential and has determined that there is “suggestive evidence of carcinogenic potential by all routes of exposure but not sufficient to assess human carcinogenic potential.” {USEPA, 2003 #555}

**ACUTE TOXICITY**

*General toxicity*

Early studies on the acute toxicological effects of DCPA established LD\(_{50}\) values for oral exposure in rats and dogs (Ambrose, Larson et al. 1972). An acute oral LD\(_{50}\) of 1384mg/kg for rats and 1217mg/kg for dogs was reported. Oral exposure to DCPA over a 7 day period resulted in toxicity characterized by central nervous system depression within 12 hours of exposure. In studies in Wistar rats, oral doses between 39-170mg/kg of DCA can alter body, spleen, liver and testis weight (Zhang and Lin 2009). Inhalation studies set an LD\(_{50}\) concentration of 1.1mg/kg for a 4hr exposure but LD\(_{50}\) concentrations of more than 5000mg/kg were reported for dermal exposure in rabbits (Stevens 1991). Acute studies in male mice were conducted using a single i.p injection of DCPA at a dose of 400mg/kg (Singleton and Murphy 1973). Signs of toxicity included central nervous depression, loss of righting reflex, and cyanosis. At higher doses, 600 and 800 mg/kg, deaths occurred within 6 to 24 hours after injection. Rats treated with 300mg/kg DCPA and triorthotolyl phosphate (TOTP), an enzyme that inhibits amidases, displayed no signs of cyanosis but the loss of righting reflex, CNS depression or mortality remained (Singleton and Murphy 1973). This data suggests that cleavage of the amide bond is necessary for cyanosis.
Erythrocyte toxicity

Due to the nature of exposure there are limited studies on the effects of DCPA on humans. However there are cases of occupational exposure and DCPA self-poisoning and that help characterize the toxicity of DCPA. DCPA self-poisoning has been reported in Cuba and Japan, but in Sri Lanka, DCPA is the second most lethal herbicide used in deliberate self-poisoning cases (Eddleston, Rajapakshe et al. 2002). Between 2002 and 2007 over 400 patients were identified in self-poisoning cases (Roberts, Heilmair et al. 2009). Symptoms of DCPA poisoning included nausea, vomiting, dizziness, increased heart rate, tissue hypoxia, cyanosis, methemoglobinemia and depression of CNS and respiratory system (Morse, Baker et al. 1979; Kimbrough 1980). Methemoglobinemia is a well-characterized effect of DCPA exposure in humans and animal models. Methemoglobin (metHb) is a form of hemoglobin that does not bind or transport oxygen. MetHb levels greater than 20% can result in decreased consciousness, convulsions, shock and, metHb above 70% results in death. Approximately 10% of all patients in Sri Lanka died despite active treatment (Roberts, Heilmair et al. 2009). The reported half life of DCPA, in the human body, was 3.2 hours but the concentration on DCA was detectable and more persistent than that of DCPA (Roberts, Heilmair et al. 2009).

The mechanism for DCPA toxicity on erythrocytes is due to the formation of the hydroxylated metabolites, N OH-DCA and 6OH-DCA (Singleton and Murphy 1973; Guilhermino, Soares et al. 1998). In vitro studies in erythrocytes from Sprague-Dawley rat exposed to DCA revealed no increase in metHb levels (McMillan, McRae et al. 1990). However, exposure to NOH-DCA and 6OH-DCA produced increased levels of
methHb with maximum methHb levels by 90 minutes. Sprague-Dawley rats injected with DCPA (100mg/kg) had detectable levels of DCA and NOH-DCA, in the blood, which reached peak concentration after 20mins and 4.5 hrs, respectively (McMillan, McRae et al. 1990). During methemoglobinemia, oxygen carrying the ferrous ion (Fe²⁺) of the heme group of the hemoglobin molecule is oxidized by the oxidative metabolites of DCPA to the ferric state (Fe³⁺). This converts hemoglobin to methemoglobin, a non-oxygen binding form of hemoglobin that binds a water molecule instead of oxygen. Although both NOH-DCA and 6OH-DCA are known to form metHb, the NOH-DCA metabolite is reported to be 10 times more potent (McMillan, Freeman et al. 1990).

In addition to methemoglobinemia, hemolytic anemia has been observed as a result of exposure to propanil. *In vivo* and *in vitro* studies in Sprague-Dawley rats demonstrated that NOH-DCA induced hemolytic anemia, but DCPA and DCA were not direct-acting hemolytic agents (McMillan, Bradshaw et al. 1991). In addition, although 6OH-DCA can induce methemoglobinemia, it does induce hemolytic anemia (Chow and Murphy 1975).

**CHRONIC TOXICITY**

Chronic toxicity studies were conducted in rats and dogs over a 2 year period and various parameters were measured (Ambrose, Larson et al. 1972). In rats, significant decreases in body weight (from onset), mortality (after 20 months) and Hb levels (after 3 months) were observed at 1600ppm. In addition, higher organ-to-body weight ratios were also reported in the spleen (both sexes), liver (females only) and testes at the 1600ppm dose. Studies in dogs revealed no changes in mortality, hematologic values, or organ-to-weight ratios over a 2 year period. Reproduction studies...
studies in rats revealed no adverse effects in weight, reproductive performance, litter size or litter mortality and no histopathological changes in weaned pups. During a 3 month study of subchronic oral exposures in rats, survival was only affected at the highest concentration (50,000 ppm) (Ambrose, Larson et al. 1972). Decreases in Hb levels were observed at all doses above 1000 ppm indicating hemolytic anemia. Several increases in organ-to-body weight ratios were also observed. At a dose of 3300 ppm increases in kidney and liver ratios in only the females were observed. Increases in spleen ratio were observed in only the females at the 1000 ppm dose. At the higher dose (10000 ppm) increased testes ratio in the males and increased heart ratios in both the males and females were noted. A similar 4-week study was conducted in dogs with decreased food consumption and weight observed only the two highest doses (10000 and 50000 ppm) (Ambrose, Larson et al. 1972).

**Reproductive Toxicity**

In studies with Wistar rats, oral doses between 39-170 mg/kg of DCA decreased testis weight. In addition, alterations in several testicular enzymes suggests modulatory effects of spermatogenesis, testicular degeneration, and possible deterioration of the germinal epithelium (Zhang, Pan et al. 2009). DCA has also been shown to weakly bind the androgen receptor and is suspected as acting as an endocrine disruptor (Bauer, Meyer et al. 1998; USEPA 2006).

**Nephrotoxicity**

There are various reports on the toxicity of NOH-DCA and DCA in the kidney. Exposure to DCPA, DCA and NOH-DCA has been reported to result in renal cytotoxicity in Fisher 344 rats (Valentovic, Yahia et al. 1997; Valentovic, Ball et al. 2001). In these
ex vivo experiments, renal cortical slices were exposed to varying concentrations of DCPA and its metabolite and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) levels. Exposure to 500µM NOH-DCA resulted in significant cytotoxicity but higher concentrations were required for similar results in renal cortical slices exposed to DCPA (1mM), DCA (2mM) and propionic acid (5mM). Decreases in glutathione levels were observed and cytotoxicity was reversed by addition of glutathione in NOH-DCA exposed samples, indicating an increase in the oxidation of glutathione in order to neutralize the intermediate NOH-DCA.

IMMUNOTOXICITY

The immune system is a complex system comprised of innate and adaptive responses that require surveillance and balance to ensure an optimal response to foreign attacks. In humans, the development of immune cells begins in the bone marrow. Pluripotent stem cells can differentiate down 2 pathways: myeloid or lymphoid. Differentiation down the myeloid pathway results in the production of colony forming units-spleen (CFU-S) progenitor cells that give rise to erythrocytes, platelets, granulocytes and monocytes. In a C57/B6 mouse model of acute exposure to DCPA, there was a dose dependent decrease in CFU-S cells as well erythroid burst forming units (BFU-E), progenitor cells for erythrocytes (Blyler, Landreth et al. 1994). Similar results are also reported in human blood cord progenitor cells but exposure to DCA appears less toxic (Malerba, Castoldi et al. 2002).

Early toxicological studies revealed alterations in both the spleen and the thymus. The spleen-to-body weight ratio was increased in albino rats exposed to oral doses of 3300ppm of DCPA for 3 months and continued in chronic studies conducted over a 2
year per iod (Ambrose, Lar son et al. 1972). In C57/B6 mice an i.p. injection of 
200mg/kg of propanil, or a molar-equivalent dose of DCA (150mg/kg) resulted in an 
increased spleen weight and size (Barnett and Gandy 1989; Barnett, Gandy et al. 
1992). Decreases in thymic weight were also observed in DCPA treated mice but not 
DCA treated mice (Barnett, Gandy et al. 1992; Zhao, Schafer et al. 1995). In addition to 
thymic atrophy, several thymocyte subpopulations were also decreased. Significant 
concentration dependent decreases in thymic CD3+CD4+CD8+ and CD3+CD4+CD8- 
populations were seen at all doses (100-200mg/kg) but no changes were observed in 
the spleen or mesenteric lymph nodes (Zhao, Schafer et al. 1995).

INNATE AND ADAPTIVE IMMUNITY

Innate and adaptive immune responses are the two main components of an 
active immune response. Innate immunity is a mechanism of protection that does not 
require specific recognition of antigens and is important in the early detection of 
pathogens. Adaptive immunity is an antigen-specific response that includes both 
humoral and cell-mediated immunity. Alterations in both innate and adaptive responses 
have been reported after exposure to propanil (reviewed in (Salazar, Ustyugova et al. 
2008)).

Innate Immunity

Adverse effects of exposure of DCPA to macrophages have been well documented. In vivo studies with peritoneal macrophages from C57/B6 mice exposed 
to 200mg/kg DCPA resulted in the decreased production and secretion of IL-6 and TNF- 
α. In both oral and i.p. routes of exposure, DCPA (400mg/kg) significantly decreased
IL-6 and TNF-α production in LPS-stimulated macrophages (Xie, Schafer et al. 1997). Ex vivo experiments with peritoneal macrophages resulted in similar decreases (Xie, Schafer et al. 1997). In addition, the ability of LPS-stimulated peritoneal macrophages to phagocytose was also decreased with concomitant decrease in reactive oxygen species (ROS), reactive nitrogen species (RNS) and inducible nitric oxide synthase (iNOS) (Ustyugova, Frost et al. 2007). In the human monocytic cell line, THP-1, TNF-α secretion and phagocytosis was also inhibited (Ustyugova, Frost et al. 2007). The mechanisms for these decreases may be due to a decrease in the p65 subunit of NF-κB and to alterations in intracellular calcium (Ca) homeostasis (Xie, Schafer et al. 1997; Frost, Neeley et al. 2001). In addition to macrophages, natural killer (NK) cells appear to be sensitive to the effects of DCPA and DCA. Exposure of DCPA and its major metabolite, DCA, results in decreased NK lytic activity. C57/B6 mice treated with 75-200mg/kg DCPA (or the molar equivalent of DCA) resulted in dose-dependent decreases in the ability of NK cells to lyse target cells (Barnett, Gandy et al. 1992).

**Adaptive Immunity**

**B cell response**

Recognition of foreign antigens and the production of antigen-specific antibodies are important functions for B cells. Immature B cells are produced in the bone marrow and develop from a progenitor B cell to a Pro-B cell to a pre-B cell and finally reach an immature B cell stage, where it expresses IgM, and migrates to the spleen for differentiation into a mature B cell. Several studies indicate that the humoral immune response is altered by DCPA and its metabolite, DCA. Mice treated with DCPA or DCA,
have increased spleen size and weight but the mechanism for this observation is unknown. Decreased pre-B and IgM populations in the bone marrow of C57/B6 mice exposed DCPA were observed 7 days post exposure but return to normal 24 days post-exposure (de la Rosa, Barnett et al. 2003). Exposure of C57/B6 mice to 400mg/kg of DCPA also reduced the proliferation of LPS-stimulated B cells (Barnett and Gandy 1989). Initial toxicological studies investigating the effects of DCPA on the immune response revealed that i.p. exposure of DCPA or DCA, in C57/B6 mice, leads to decreased in T cell-dependent and T cell-independent antibody responses (Barnett and Gandy 1989; Barnett, Gandy et al. 1992). Interestingly, C57/B6 mice immunized with heat-killed *Streptococcus pneumoniae* and exposed to DCPA have a dose-dependent increase in the number of phosphocholine (PC)-specific IgM, IgG2b and IgG3 antibody secreting B cells (ASC) in the spleen (Salazar, de la Rosa et al. 2005). At 7 days post-exposure, the number of ASC had increased 4-6 fold with no increases in the number of ASC in the bone marrow or serum. The reason for this increase has not been elucidated but the mechanism appears to be ovary-dependent, estrogen and progesterone-independent but requires a functioning steroid synthesis pathway (Salazar, Miller et al. 2006). Male mice produce a smaller increase in PC-specific ASC compared to females when exposed to DCPA but this increase is testes-independent (Salazar, Miller et al. 2006).

**T cell response**

T cells play an important role in cell-mediated immunity and involve specific recognition of foreign antigens leading to the production of antigen-specific antibodies through CD4+ T cells or the targeted destruction of infected cells (CD8+ T cells). Although there are several types of T cells, studies on the effects of DCPA are limited
to CD4+ helper T cells and CD8+ cytotoxic T cells. As mentioned above, exposure to DCPA results in a significant decrease in the CD3+CD4+CD8+ and CD3+CD4+CD8-thymic subpopulations (Zhao, Schafer et al. 1995). Mixed lymphocyte reactions (MLR) and delayed-type hypersensitivity (DTH) reactions were both reduced in C57/B6 mice exposed to high doses of DCPA (400mg/kg) (Barnett and Gandy 1989). In addition, concavalin-A (con-A)-stimulated splenocytes, from mice treated with DCPA, resulted in decreased proliferation as well as decreased IL-2 and IL-6 production (Barnett and Gandy 1989; Zhao, Schafer et al. 1998). Further studies, using murine (EL-4) and human (Jurkat) T cell lines, demonstrated a dose-dependent decrease in IL-2 production, at low concentrations of DCPA (25-100µM) when stimulated with PMA or PMA/ionomycin (Io), respectively (Zhao, Schafer et al. 1999; Brundage, Schafer et al. 2004). In the EL-4 cell line, transcription and stability of the IL-2 mRNA was inhibited and in Jurkat cells, decreases in the amount and phosphorylation of c-jun, one of two proteins that make up the transcription factor AP-1, resulted in the decreased DNA binding ability of AP-1.

Cytotoxic CD8+ T cells are responsible for identifying infected cells and secreting lytic proteins, such as perforin, granzymes and granulysin, in order to kill infected cells. Investigations of thymic subpopulations demonstrated that CD3+CD4-CD8+ T cells were decreased (Zhao, Schafer et al. 1995). However, studies investigating the effects of DCPA and DCA, demonstrated that neither the parent compound nor its metabolite impaired cytotoxic T cell activity (Barnett, Gandy et al. 1992). More recently, it has been reported that although primary stimulation of CD8+ T cells does not impair lytic
function, secondary stimulation of mice CD8+ T cells, in the absence of further DCPA exposure, abrogated the lytic function (Sheil, Frankenberry et al. 2006).
T CELL ACTIVATION AND SIGNALING

T cell recognition of a foreign peptide presented on a major histocompatibility complex (MHC) on the surface of an antigen presenting cells (APC), along with co-stimulation of CD28, sets into motion a complex series of signaling events that culminates in the activation of a T cell (reviewed in (Smith-Garvin, Koretzky et al. 2009). The TCR is comprised of a series γε, δε, and ξξ dimers that associate with a single αβ heteromer. These dimers contain specific immunoreceptor tyrosine-based activation motifs (ITAMs) consisting of 2 tyrosines that flank an amino acid sequence that contains key leucine and isoleucines. Ligation of the TCR results in the recruitment of cytosolic Src family protein tyrosine kinases (Lyn and Lck) that phosphorylate tyrosines associated with ITAMs and thereby provide a docking site for Zap-70. Zap-70 is a 70kDa phosphoprotein belonging to the Syk kinase family. Recruitment of Zap-70 leads to a cascade of phosphorylation events important in the activation of downstream pathways. Two key targets of Zap-70 are the adapter proteins, linker of the activation of T cells (LAT) and Src homology-2 (SH2) domain-containing leukocyte phosphoprotein (SLP-76). These 2 proteins help to stabilize the TCR complex and organize effectors proteins to allow for activation of multiple pathways. LAT proteins can bind the SH2 domain of PLC-γ and as well as adapter proteins GRB2 (growth factor receptor bound protein-2) and Gads (GRB2-related adapter downstream of Shc). SLP-76 interacts with the SH2 domains and activates adapter proteins Vav1, Nck and Itk (IL-2-induced tyrosine kinase). All these proteins help to increase the stability of the complex and interact to activate PLCγ-dependent pathways, including calcium (Ca) and DAG-
mediated pathways, cytoskeletal rearrangements and integrin activation (Smith-Garvin, Koretzky et al. 2009).

Early signaling events, dependent on LAT and SLP-76 activation, result in the recruitment of PLC-γ₁ to the TCR complex. Once activated, PLC-γ₁ hydrolyzes the membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP₂) resulting in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG remains in the membrane where it plays a role in the activation of the Ras and PKCθ pathways. Ras-GRP (Ras guanyl nucleotide-releasing protein) contains a DAG-binding domain that recruits it to the membrane where it is phosphorylated by PKCθ and can then convert RAS-GDP to the active Ras-GTP. Ras-GTP activates Raf-1, a Ser/Thr kinase, and phosphorylates and activates MAPK (mitogen-associated protein kinase) pathway. Activation of the MAPK pathway leads to activation of Elk, a transcription factor important for the expression of c-fos. Dimerization of c-fos and c-jun form AP-1, an important transcription factor required for early gene expression. In addition to activation of the MAPK pathway, DAG also recruits PKCθ to the membrane through its DAG-specific lipid-binding domain and activates the NF-κB pathway (Quest, Ghosh et al. 1997). Activation of NF-κB occurs when its inhibitory molecule, IκB, is phosphorylation by the IκB kinase (IKK) complex (Wanda and Lenardo 2010). Phosphorylation of IκB marks it for ubiquitination and degradation and allows NF-κB to translocate into the nucleus where it acts as a transcription factor for genes involved in T cell function. Figure 2 outlines a general schematic for T cell activation.
Figure 2 Schematic of T cell signaling

Adapted from Baine et al Immunol Rev 2009
PLC-γ hydrolysis of PIP2 also results in the production of IP3. IP3 is released into the cytosol where it binds to its receptor, IP3-R on the ER membrane resulting in the release of Ca from the ER stores. Upon depletion of the ER stores, Stim1, a transmembrane protein on the ER, aggregates and initiates clustering of Orai1, a plasma membrane protein, leading to the activation of CRAC channels and the influx of Ca into the cell (described in detail in Chapter 5) (Barr, Bernot et al. 2008). This increase in cytosolic Ca, through the CRAC channels, triggers several signaling events that are critical for T cell function. NFAT is a transcription factor that is strictly regulated by Ca and cooperatively binds to DNA to control the expression of genes important in T cell activation and function (Jain, Loh et al. 1995; Rooney, Sun et al. 1995). In resting T cells, NFAT is phosphorylated at several Ser residues and resides in the cytosol but upon stimulation, is dephosphorylated by calcineurin and can freely translocate into the nucleus. The influx of Ca through CRAC channels following activation of T cells is essential for the activation of NFAT. Binding of Ca, to the 4 ion-binding sites on calmodulin (CaM), leads to the activation of calcineurin (Cn). Cn is a calmodulin-dependent serine/threonine phosphatase with 2 subunits; a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B) (Feske 2007). The Ca-CaM complex binds to the regulatory subunit of Cn and thereby activating its catalytic activity. Once activated, Cn de-phosphorylates NFAT and exposes a nuclear localization sequence (NLS) which leads to binding of importins and translocation of NFAT into the nucleus. In the nucleus, NFAT acts in a coordinate manner with other transcription factors to activate specific genes required for T cell activation, function and differentiation (Gwack, Feske et al. 2007). NFAT-driven gene expression is highly
dependent on sustained Ca influx and calcineurin activity. A decrease in intracellular Ca levels or treatment with the calcineurin inhibitor cyclosporin A results in the immediate export of N FAT from nucleus by N FAT kinases and alterations in gene expression (Gwack, Feske et al. 2007).

T CELLS AND ION CHANNELS

CRAC channels

In T cells, influx of Ca is regulated through Ca release-activated Ca (CRAC) channels. Activation of the IP3 receptors on the surface of the ER releases Ca from internal ER stores resulting in the aggregation of the ER transmembrane protein, Stim1. Stim1 is a single transmembrane protein that exists as a monomer when ER Ca stores are full (Manji, Parker et al. 2000). During T cell activation, ER stores are depleted and Ca is released from the EF-hand domain, located in the lumen of the ER. Stim1 dimerization occurs and aggregates, or puncta, form in areas within 10-25µm of the plasma membrane initiating clustering of Orai1 proteins on the plasma membrane and activation of the CRAC channel (Liou, Kim et al. 2005; Wu, Buchanan et al. 2006). Orai1 is a 4 transmembrane spanning protein with both the N- and C-terminus located in the cytoplasm (Yeromin, Zhang et al. 2006). Clustering of 4 Orai1 subunits forms the pore forming unit of CRAC channels (Xu, Lu et al. 2006). The complete CRAC channel complex has not been elucidated but expression of Stim1 and Orai1 are sufficient to produce a CRAC current (ICRAC) (Li, Lu et al. 2007; Salido, Sage et al. 2009). CRAC channels were originally described using electrophysiological methods but the recent identification of Stim1 and Orai1 have allowed for a more detailed characterization.
CRAC channels are characterized by activation through ER store depletion, an extreme selectivity for Ca and a low conductance rate, or the rate of ion travel through the channel (Parekh 2006). Cells are small in size, 5-10 µm, and, at rest, the intracellular Ca concentration is around 50 nM (estimated to be about 10,000 free Ca ions) so a very small, Ca selective current, on the scale of picoamps, is capable of evoking a substantial rise in the concentration of cytosolic Ca (Cahalan and Handy 2009). The selectivity of Ca through CRAC channels is such that monovalent ions are excluded by ion-pore and ion-ion interactions (McNally, Yamashita et al. 2009). Absence of extracellular divalent ions allows the flow of monovalent ions but addition of very low concentrations of Ca blocks monovalent permeation through high affinity Ca binding to the channels. The presence of one Ca ion is suggested to be sufficient to block monovalent flow through the channel (Prakriya 2009).

**CRAC regulation**

CRAC channels can be regulated by several mechanisms (DeHaven, Smyth et al. 2007; Hogan and Rao 2007). Ca-dependent potentiation (CDP) enhances CRAC activity through increases in extracellular Ca. The reversal of this, depotentiation, can decrease CRAC activity when extracellular Ca is removed. The mechanism for this is unknown but the degree of potentiation is dependent on the pore occupancy by divalent ions, whereby ions with higher permeability support greater potentiation. CRAC channels are also regulated through fast inactivation where ICRAC decreases in hyperpolarizing environments. Lastly, it has been proposed that high intracellular Ca surrounding CRAC channels inhibits CRAC activity and thereby produces an inhibitory feedback. The nature of inactivation and the binding sites are unknown. Recently it has
been reported that CRAC channels may be regulated by the refilling of the ER stores and the reversal of Stim1 puncta formation. It is hypothesized that as ER Ca stores are refilled, Stim1 dimers disassemble and monomeric Stim1 can bind Ca through its EF-hand domain leading to a disruption of the Stim1/Orai1 assembly (Smyth, Dehaven et al. 2008; Fahrner, Muik et al. 2009).

**CRAC inhibitors**

CRAC channels can be differentially regulated by 2-APB (2-aminoethyl diphenyl borate). At low concentrations (1-5µM), 2-APB can increase I_{CRAC} 2-5 fold but at higher concentrations (>10µM) it strongly inhibits I_{CRAC} (Peppiatt, Collins et al. 2003; Peinelt, Lis et al. 2008). At inhibitory concentrations of 2-APB, reversal of Stim1 puncta can be observed in HEK293 cells over-expressing Stim1 (DeHaven, Smyth et al. 2008; Peinelt, Lis et al. 2008). However, when Orai1 is overexpressed no effect is observed indicating that disruption of Stim1-Orai1 is not the mechanism of action (Navarro-Borelly, Somasundaram et al. 2008). Enhancement of CRAC activity at low 2-APB concentrations has been proposed to be mediated through recruitment of CRAC channels that facilitate the association between Stim1 and Orai1 (Navarro-Borelly, Somasundaram et al. 2008; Wang, Deng et al. 2009). Recently analogs of 2-APB have been reported to interrupt Stim1 puncta formation but the specificity of these analogs are unknown (Goto, Suzuki et al.). In T cells, inhibition of CRAC channels decreases Ca influx and inhibits T cell function, which in humans, leads to a SCID-like syndrome characterized by an inability to activate T cells (Feske, Gwack et al. 2006; Thompson, Mignen et al. 2009).
OTHER ION CHANNELS AND PUMPS

**SERCA**

T cells also express other channels that modulate cytosolic Ca. Sarco/Endoplasmic reticulum Ca-ATPase (SERCA) pumps are located on the ER and function to pump Ca out of the cell. Thapsigargin (Tg) is a commonly used inhibitor that irreversibly inhibits the SERCA pump and its used to activate CRAC channels.

**PMCA**

Plasma membrane Ca-ATPase (PMCA) pumps are located on the plasma membrane of T cells. At rest, PMCA are autoinhibited and when cytosolic Ca increases pump activity is initially slow. Over tens of seconds the Ca-Cam complex binds the C terminus of PMCA and displaces the inhibitory domain. The PMCA pump plays a role in modulating Ca signaling and also to prevent the accumulation of Ca in the cytosol that can lead to apoptosis.

**RyR**

Ryanodine receptors (RyR) are located on the ER and are activated by changes in Ca, as well as through binding of cADP ribose and NADP. RyR facilitate the movement of Ca from ER stores to the cytosol and are important in later Ca signaling events.

**Na/Ca exchanger**

Sodium/calcium (Na/Ca) exchangers are located on the plasma membrane and the mitochondria. They function to prevent high Ca levels in the cytosol by sequestering
it in the mitochondria or exporting it out of the cell. Movement of Ca in one direction occurs with the concomitant movement of Na in the other direction.

**Ca uniporter**

Ca uniporters are located on the mitochondria and are activated by high Ca levels in the cytosol. They are involved in sequestering Ca inside the mitochondria to prevent accumulation of Ca inside the cell.

**Potassium (K+) channels**

T cells express 3 types of K+ channels; Kv, KCa, and TRPM. Kv and TRPM4 and TRPM5 are activated by the depolarization of the plasma membrane (an increase of positive charge inside the cell). Kv channels function to increase the Ca driving potential by exporting K+ ions. TRPM channels have been reported to inhibit the Ca driving force but their function in T cells is controversial.

**Non-store-operated cation channels**

There have been several reports of other cation channels that are not activated by ER Ca depletion. TRPV6(CaT1) channels are constitutively active, non-selective cation channels on the plasma membrane that are inhibited by increases in cytosolic Ca. TRPM2(LTPC7) channels are also plasma membrane bound and are activated by pyrimidine nucleotides, ADPR (adenosine 5'-diphosphoribose) and NAD (nicotiamide adenine dinucleotide) and produce a nonselective cation channel. TRPM7(MIC) channels are also nonselective cation channels that are activated by intracellular Mg (magnesium) levels.
REFERENCES


CHAPTER 2

3,4-dichloropropionanilide (DCPA) inhibits T cell activation by altering the intracellular calcium concentration following store depletion.\(^1\)

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Tricia L. Lewis\(^{†\dagger}\), Kathleen M. Brundage\(^{†}\), Rodney A. Brundage\(^{‡,2}\), and John B. Barnett\(^{†\dagger1}\).

\(^{†}\)Department of Microbiology, Immunology and Cell Biology, \(^{‡}\)Center for Immunopathology and Microbial Pathogenesis, West Virginia University School of Medicine, Morgantown, WV 26506. \(^{‡\dagger}\)National Institute for Occupational Health and Safety, Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, Morgantown, WV 26506

Running Title: DCPA alters calcium homeostasis in T cells

\(^1\)Address correspondence to:
John B. Barnett, Ph.D.
Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine
PO Box 9177, Morgantown, WV. 26506-9177
Tel. 304-293-4029; Fax 304-293-7823
E-Mail: jbarnett@hsc.wvu.edu

\(^2\)Present address: Department of Microbiology, Immunology and Cell Biology, West Virginia University School of Medicine, Morgantown, WV 26506.

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ABSTRACT
Stimulation of T cells through the T cell receptor (TCR) results in the activation of a series of signaling pathways that leads to the secretion of IL-2 and cell proliferation. Influx of calcium (Ca\(^{2+}\)) from the extracellular environment, following internal Ca\(^{2+}\) store depletion, provides the elevated and sustained intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) critical for optimal T cell activation. Our laboratory has documented that exposure to the herbicide 3, 4-dichloropropionanilide (DCPA) inhibits intracellular signaling events that have one or more Ca\(^{2+}\) dependent steps. Herein we report that DCPA attenuates the normal elevated and sustained [Ca\(^{2+}\)]\(_i\) that follows internal store depletion in the human leukemic T cell line, Jurkat cells, and primary BALB/c mouse T cells. DCPA did not alter the depletion of internal Ca\(^{2+}\) stores when stimulated by anti-CD3 or thapsigargin demonstrating that early IP\(_3\)-mediated signaling and depletion of Ca\(^{2+}\) stores were unaffected. 2-aminoethyldiphenol borate (2-APB) is known to alter the store-operated Ca\(^{2+}\) (SOC) influx that follows Ca\(^{2+}\) store depletion. Exposure of Jurkat cells to either DCPA or 50 µM 2-APB at attenuated the increase in [Ca\(^{2+}\)]\(_i\) following thapsigargin or anti-CD3 induced store depletion in a similar manner. At low concentrations, 2-APB enhances SOC influx but this enhancement is abrogated in the presence of D CPA. This a iteration of nuclear NFAT levels and IL-2 secretion without altering the plasma membrane polarization profile. Taken together, these data indicate that D CPA inhibits T cell activation by altering Ca\(^{2+}\) homeostasis following store depletion.
INTRODUCTION

The herbicide DCPA, commonly referred to as propanil, is widely used and applied several times throughout the growing season. It is applied predominantly on rice fields for control against several broadleaf and grassy weeds but, was recently registered for use on turf. Annual use of DCPA is estimated to be 7 million pounds per year and represents use on 50-70% of all rice crops in the United States. The broad application and heavy use of this herbicide under scores the importance of investigating its immunotoxic effects.

The effects of DCPA on mammalian immune cells have been well documented (Xie, Schafer et al. 1997; Zhao, Schafer et al. 1998; Brundage, Schafer et al. 2004; Salazar, Miller et al. 2006; Sheil, Fr ankenberry et al. 2006; C orsini, Codeca et al. 2007; U styugova, Fr ost et al. 2007). *In vivo* administration of DCPA to mice results in decreased *ex vivo* cytokine production by macrophages (IL-1β, IL-6 and TNF-α) and T cells (IL-2 and IFN-γ)(Barnett 1992; Zhao, Schafer et al. 1998). Previous studies using LPS-stimulated macrophages demonstrated a decrease in [Ca^{2+}]i after exposure to DCPA(Xie, Schafer et al. 1997). Using the murine EL-4 T cell line and human Jurkat T cell lines, we have demonstrated that exposure to DCPA decreased IL-2 production and IL-2 mRNA levels (Zhao, Schafer et al. 1999; Brundage, Schafer et al. 2004). In addition, Jurkat T cells exposed to DCPA demonstrate decreased DNA binding ability of the transcription factor, activating protein-1 (AP-1), and decreased c-Jun protein (Brundage, Schafer et al. 2004). The production of IL-2, an important early cytokine, requires the coordinate activation of transcription factors, AP-1, NF-κB, and NFAT, which all depend, to varying degrees, on the [Ca^{2+}]i (Garrity, Chen et al. 1994).
Activation of T cells is initiated through recognition of a peptide presented on the surface of an antigen-presenting cell, along with co-stimulatory interactions, which triggers a series of events including phosphorylation of the TCR complex and recruitment of kinases and adapter proteins to the plasma membrane (PM). Subsequent activation of phospholipase-Cγ1 (PLC-γ1) results in the hydrolysis of phosphatidylinositol 4, 5 bisphosphate (PIP2) and the production of two second messengers, diacylglycerol (DAG) and inositol 1, 4, 5 triphosphate (IP3) (Feske, Okamura et al. 2003; Panyi, Varga et al. 2004). DAG remains in the PM and activates proteins such as protein kinase C (PKC). IP3 is released from the PM and interacts with the IP3-receptor (IP3-R) on the surface of the endoplasmic reticulum (ER) resulting in the release of Ca^{2+} from the ER. Depletion of ER Ca^{2+} stores activates SOC channels located on the PM allowing for the influx of Ca^{2+} from the extracellular environment. This results in an increased and sustained [Ca^{2+}], and activation, via calmodulin, of calcineurin which leads to the dephosphorylation of NFAT and its translocation into the nucleus. Once in the nucleus NFAT acts as a transcription factor for the production of key cytokines (Feske, Okamura et al. 2003). Ca^{2+} also plays a role in the activation of the transcription factors N F-κB, and AP-1 (Lewis 2001; Quintana, Griesemer et al. 2005). Cooperative binding of these three transcription factors is required for optimal transcription and production of IL-2, an essential early cytokine required for T cell proliferation and differentiation (Garrity, Chen et al. 1994).

The importance of calcium as a second messenger in T cell activation has been well established (Lewis 2001; Quintana, Griesemer et al. 2005; Feske 2007). Two coupled mechanisms exist to provide the necessary [Ca^{2+}], required for optimal T cell activation.
The first mechanism involves an initial increase in \([\text{Ca}^{2+}]_i\) through the IP$_3$-mediated depletion of ER Ca$^{2+}$ stores. This transient increase is necessary but not sufficient for optimal T cell activation (Feske, Gwack et al. 2006). Emptying of the ER Ca$^{2+}$ stores is coupled to the activation of Ca$^{2+}$-release activated Ca$^{2+}$ (CRAC) channels on the PM. Upon ER store depletion STIM-1 (stromal interaction molecule-1) redistributes into puncta on the ER and accumulates close to the PM (Zhang, Yu et al. 2005; Wu, Buchanan et al. 2006; Xu, Lu et al. 2006). Orai1 (also known as CRACM1) has recently been described as an essential pore subunit of the CRAC channel (Prakriya, Feske et al. 2006; Vig, Peinelt et al. 2006). During T cell activation, aggregation of STIM-1 on the ER induces clustering of Orai1 on the PM resulting in an influx of Ca$^{2+}$ through the CRAC channel (Xu, Lu et al. 2006). This increase in [Ca$^{2+}]_i$ through the CRAC channels is essential for the activation of transcription factors necessary for cytokines production. Defects in the CRAC channel have been reported in patients with severe combined immunodeficiency (SCID) syndrome, under scoring the importance of Ca$^{2+}$ in T cell activation (Feske, Draeger et al. 2000; Feske, Giltnane et al. 2001; Feske, Prakriya et al. 2005).

Due to the importance of Ca$^{2+}$ homeostasis in T cell activation and function, we investigated the effect of DCPA on [Ca$^{2+}]_i$ in T cells, using primary BALB/c mice T cells and the Jurkat human T cell leukemia cell line. The human Jurkat T cell line, has been used as a model for human T cell signaling for over 2 decades (Abraham and Weiss 2004). When investigating the immunotoxic effects of DCPA, Jurkat cells are a valuable tool that allows elucidation of mechanisms involved in exposure to DCPA. Since our previous research has demonstrated alterations in T cell signaling we wanted to define
more clearly the immunosuppressive role of DCPA on T cells. Our experiments indicate
that DCPA inhibits T cell activation by attenuating increases in [Ca\(^{2+}\)], following the
depletion of internal Ca\(^{2+}\) stores.
MATERIALS AND METHODS

Cell lines
Experiments were performed using the human T cell leukemia cell line, Jurkat clone E6-1, obtained from the A TCC (American Tissue Culture Collection, Manassas, VA). Jurkat cells were maintained in incomplete RPMI (Mediatech Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (v/v) (FBS) (Hyclone Inc. Logan, UT), 100 units/ml penicillin (BioWhittaker), 100 µg/ml streptomycin (BioWhittaker), 20 mM glutamine (BioWhittaker) and 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO). The cultures were kept at 37°C in 5% CO₂.

Mice
Female BALB/c mice (8-10 weeks old) were purchased from Charles River Labs (Wilmington DE). Mice were housed in the vivarium at West Virginia University Health Sciences Center under the care of a full-time veterinarian and professional staff. They were given access to food and water ad libitum and allowed to acclimate to the facility for at least 1-week prior to use. Experiments were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Isolation of Mouse T cells
Spleens from BALB/c mice were removed aseptically, pooled and made into a single cell suspension. Red blood cells were lysed using Tris-NH₄Cl and resuspended in sorting buffer (PBS, 0.5% bovine serum albumin and 2 mM EDTA) at 2.5 x 10⁶ cells/ml. An enriched (>90% by flow cytometry) population of T cells was isolated through negative selection using the Pan T cell isolation kit (Miltenyi Biotec, Auburn, CA).
Briefly, spleen cells were incubated at 4°C for 10 min with a cocktail of biotin-conjugated monoclonal antibodies specific for CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A, as described in the manufacturer’s protocol (Miltenyi Biotec). Next, monoclonal anti-biotin antibody-conjugated magnetic microbeads were added to the sample and incubated for 15 min at 4°C. Cells were washed with sorting buffer and resuspended at 1 × 10^8 cells in 500 µl of cold buffer. Cells were then loaded onto autoMACS columns (Miltenyi Biotec) and purified T cells were collected through a negative selection process. Purified T cells were stimulated using anti-CD3 (BD Bioscience) and goat anti-Armenian hamster antibody (Jackson ImmunoResearch, West Grove PA) to crosslink the anti-CD3 as previously described (Kubo, Born et al. 1989).

**Fluorescence Measurement of [Ca^2+]i:**

Jurkat cells or splenic mouse T cells were loaded with the calcium-indicator dye fluo-3 AM (Invitrogen, Carlsbad, CA) as previously described (Grynkiewicz, POenie et al. 1985). Briefly, cells were harvested and resuspended to a concentration of 5 × 10^6 cells/ml and incubated for 30 min (37°C in 5% CO2) in complete RPMI media (1.5% FBS, v/v) containing 0.1 µM fluo-3 AM in the presence of 0.02% pluronic F-127 (Invitrogen) and 2.5 mM probenecid (Sigma, St. Louis, MO). Cells were then washed twice in Ca^2+- and Mg^2+-free Hank's Balanced Salt Solution (HBSS) (Mediatech, Inc, Herndon, VA) containing 10 mM HEPES, pH 7.4, 2% FBS and 2.5 mM probenecid, resuspended to a concentration of 1 × 10^6 cells/ml and incubated 30 min at room temperature. Since the addition of 2% FBS is essential for cell viability the media contains a nominal concentration of Ca^2+ (2.5 µM). Samples were kept at room temperature.
temperature and protected from light until ready for analysis. 2 × 10^6 cells were placed in a quartz cuvette and the fluorescence was measured using a PTIQM-2000-4 spectrofluorometer (Photon Technology International (PTI), Birmingham, N J) with constant stirring. The fluorescence of the fluo-3 dye was measured with excitation at 490 nm and emission at 525 nm. Stock solutions of DCPA (ChemServices, West Chester, PA) and 2-APB (CalBioChem, San Diego, CA) were diluted in absolute ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY). Vehicle samples were treated with an equivalent concentration (0.1%v/v) of ethanol. The fluorescence was measured and digitized at 1 Hz using the software program Felix 1.42b (PTI). In Jurkat cell, approximately 45 s after starting data collection, 2.0 µl of DCPA, 2-APB or vehicle was added to the cuvette, followed immediately by mouse anti-human CD3 antibody at a final concentration of 5.0 µg/ml (UCHT1) (BD Biosciences, San Diego, CA). Splenic mouse T cells were stimulated with anti-CD3 (BD Bioscience) and goat anti-Armenian hamster antibody (Jackson ImmunoResearch, West Grove PA) to crosslink the anti-CD3 as previously described (Kubo, Born et al. 1989). When the fluorescence returned to background levels, CaCl₂ (Fluka, Switzerland) was added (final concentration 2.5 mM) to the media to provide an external source of Ca²⁺. Addition of ionomycin (final concentration 200 µM) (Sigma, St. Louis, MO) provided evidence that the cells were loaded evenly and that the dye remained in the cytosol. Cell membranes were lysed with 0.1%, v/v Triton X-100 (Fisher Scientific, Hampton, NH) to measure the maximum fluorescence \( F_{\text{max}} \) parameter for calculation of \([\text{Ca}^{2+}]_i\), to monitor compartmentalization of the dye and ensure the amount of dye was not a limiting factor. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA) (Sigma) was
added to a final concentration of 50 mM (pH 7.5) to lower the free Ca$^{2+}$ to a nominally Ca$^{2+}$-free level ($F_{min}$).

Fluorescence values were converted to $[Ca^{2+}]_i$ using the following equation:

$$\text{free}[Ca^{2+}]_i = K_D \frac{(F-F_{min})}{(F_{max}-F)},$$

where $K_D$ (360 nM) is the dissociation constant of the Fluo-3/Ca$^{2+}$ complex, $F$ is the measured fluorescence intensity, $F_{min}$ is the minimum fluorescence at very low $[Ca^{2+}]_i$ (fluorescence after the addition of 50 mM EGTA) and $F_{max}$ is the fluorescence measured at high $[Ca^{2+}]_i$ (fluorescence after the addition of Triton X-100) (Grynkiewicz, Poenie et al. 1985). The background fluorescence obtained from unloaded cells over a 3 minute time period was subtracted from all data points before $[Ca^{2+}]_i$ was calculated.

Measurement of IL-2 Production

Jurkat cells were cultured in complete RPMI media or RPMI media without Ca$^{2+}$ at 5 × 10$^5$ cells/well in 48-well plates (Costar, Corning, NY) coated with mouse anti-human CD3 antibody (10 µg/ml)(BD Sciences). Cells were treated with 25, 50, 100, or 200 µM DCPA, or vehicle control. DCPA solutions were diluted in absolute ethanol and vehicle control samples were given an equivalent amount of absolute ethanol (0.1% v/v). Cells were also simultaneously stimulated with anti-CD28 antibody (2 µg/ml) (BD PharMingen, San Diego, CA). Cells were incubated at 37°C in 5% CO$_2$ for 48 h after which supernatants were collected and placed at -20°C. IL-2 production was determined using the sandwich ELISA method following the manufacturer’s protocol (BD PharMingen). All cultures and ELISA analyses were performed in triplicate and the experiment was repeated three times.
**Nuclear Extracts**

Jurkat cells were cultured to 1 x $10^6$ cells/ml in complete RPMI and stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml A23187 (Sigma-Aldrich). Cells were treated with 0.1% v/v ethanol (vehicle control), 100 µM DCPA or received no treatment. After a 1, 2, 4, and 6 h exposure, cells were harvested and centrifuged at 1200 rpm for 8 min at 4°C. Nuclear extracts were prepared as previously described (Schreiber, Matthias et al. 1989). Briefly, cells were resuspended in 1ml PBS, pelleted, resuspended in cold buffer A (10mM Hepes pH 7.9, 10mM KCl, 0.1mM EDTA (disodium ethylenediamine tetraacetate), 0.1mM EGTA, 1mM DTT (dithiothreitol) and 0.5 mM PMSF (phenylmethanesulfonyl fluoride)) and incubated on ice for 15 min. After incubation, 25 µl of a 10% solution of Nonidet NP-40 was added, vortexed and centrifuged for 30 s at 14,000 rpm. Supernatants, containing the cytoplasmic fraction of the cells, were frozen at –70°C. The nuclear pellet was resuspended in cold buffer C (20mM Hepes pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and 1mM PMSF) and incubated on ice for 15 min with vortexing every 5 min. Samples were centrifuged for 5 min at 14,000 rpm and the supernatants containing the nuclear fraction were stored at –70°C. The protein concentrations of the nuclear extracts were determined using Coomassie plus protein assay reagent kit following the manufacturer’s protocol (Pierce, Rockford, IL).

**Western Blots**

A 30 µg aliquot of each nuclear extract was boiled for 5 min to denature the proteins and electrophoresed through an 8% Tris polyacrylamide gel with a 4% stacking gel at 25 mAmps for 18 h. Proteins were transferred onto Hybond-P membranes (Amersham Pharmacia, Piscataway, NJ) at 0.1 amps for 20 h. Blots were washed in TBS for 5 min.
at room temperature, blocked for 1 h in TBS + 0.1% Tween 20 (TBS/T) plus 5% dry milk at room temperature and then washed three times in TB S/T. Blots were incubated overnight at 4°C with primary antibodies specific for total N FATc2 (Santa Cruz Santa Cruz, CA) or β actin (Santa Cruz) in TBS/T plus 5% BSA. The next day, blots were washed three times in TB S/T, incubated for 1 h at room temperature with anti-Biotin (Cell Signaling Tecnology, I nc., D anvers, M A) and either a goat ant i-mouse IgG-horseradish per oxidase (HRP) (Santa C ruz) o r a rabbit anti-goat IgG-HRP ( Sigma-Aldrich). Finally, the blots were washed three times in TB S/T and developed using Phototope-HRP detection kit for western blots (Cell Signaling Technology, I nc) and bands were visualized on X-Ray film (BioMax M R, E astman K odak C ompany). Densitometric analysis was performed using Optimus software (Media C ybernetics, S ilver S pring, M D) and nuclear N FAT protein levels were normalized to actin protein levels for each sample.

**Measurement of membrane potential**

The membrane potential of Jurkat cells was measured using the membrane potential-sensitive DiB AC₄(3) dye (Invitrogen). This bis-oxonol dye produces an excitation maximum at approximately 490 nm. As the cell depolarizes increasing amounts of dye enters the cell while intracellular proteins or membranes and exhibit enhanced fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. DiBAC₄(3) is excluded from mitochondria because of its overall negative charge, allowing measurement of the plasma membrane potential (Wolff, Fuks et al. 2003). Membrane potential experiments were carried out in a time course similar to the fluo-3 experiments. Briefly, 1 x 10⁶ cells/ml of Jurkat cells were suspended Ca²⁺ and
Mg$^{2+}$-free Hanks Balanced Salt Solution (HBSS) (Mediatech, Inc.) containing 10 mM HEPES, pH 7.4 and 2% FBS containing 20nM DiBAC$_4$(3). Samples were kept at room temperature and protected from light until ready for analysis. A 1.0 ml ($1 \times 10^6$ cells) aliquot of loaded cells was placed in a FACS tube and the fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickson, Franklin Lakes, NJ). Each recorded time point consisted of 10,000 cells. At t=0 a background fluorescence was recorded after which DCPA, or vehicle control was added, followed immediately by anti-CD3 antibody at a final concentration of 5.0 µg/ml (BD Biosciences). Fluorescence measurements were recorded immediately following treatment and anti-CD3 addition (t=1 min) and again at t=3 min and t=5 min. External Ca$^{2+}$, in the form of CaCl$_2$, (Fluka, Switzerland) was subsequently added (final concentration 2.5 mM) to the cells to provide an external source of Ca$^{2+}$. Fluorescence measurements were collected at 3 time points following addition of external Ca$^{2+}$ (t= 5.5, 6.5, and 9.5 min). Addition of 40 µl KCl (final concentration 100 mM) completely depolarized the cell and fluorescence values were recorded at t=10, 12 and 14 min. Background fluorescence of unloaded cells was subtracted from all data points before the net change in fluorescence was calculated. The fluorescence was represented as the net change in the fluorescence signal as a percent of the initial background fluorescence (F$_o$) of the T cells at rest as described by $\Delta F/F_o=(F_t-F_o)*100/F_o$ (where $F_t$ is the fluorescence at each time point) (Wolff, Fuks et al. 2003).

**Statistical Analysis**

All data were analyzed using MS Excel 2003 (Redmond, WA) and Sigma Stat 3.1 (Port Richmond, CA). The area under the curve (AUC) is an indirect measurement of the
increase of [Ca\textsuperscript{2+}], over a selected time period. The AUC includes all data points from the time the external Ca\textsuperscript{2+} was added until the [Ca\textsuperscript{2+}] reached a plateau and was calculated using Sigma Stat 3.1. The peak [Ca\textsuperscript{2+}] was calculated by determining the highest [Ca\textsuperscript{2+}] between addition of CaCl\textsubscript{2} and addition of ionomycin. Several curves were used for each sample and the mean ± S.D. is reported. A t-test was used to determine statistical significance for all Ca\textsuperscript{2+} fluorescence and membrane potential experiments. ANOVA was used to determine statistical significance in IL-2 production. An alpha value of <0.05 was considered significant.
RESULTS

Effect of DCPA on \([Ca^{2+}]_i\) in Anti-CD3 Stimulated Jurkat Cells

T cell activation and proliferation requires a cascade of signaling events mediated by two \(Ca^{2+}\) sensitive mechanisms. The first involves the IP\(_3\)-mediated depletion of \(Ca^{2+}\) stores in the ER and is coupled to the second mechanism, the activation of CRAC channels and the influx of \(Ca^{2+}\). To determine the effect of DCPA on \([Ca^{2+}]_i\), Jurkat cells, loaded with fluo-3 in a nominally \(Ca^{2+}\)-free (2.5 µM) HBSS solution, were treated with 25, 50, 100, 200 µM DCPA or vehicle (ethanol) control and stimulated with anti-CD3. We determined that DCPA and ethanol, hereafter referred to as the vehicle, did not interfere with the fluorescence of the fluo-3 dye (data not shown). There was no significant difference between cells stimulated with anti-CD3 or cells that were exposed to vehicle and stimulated with anti-CD3 (data not shown). As shown in Figure 1, addition of DCPA or vehicle followed by stimulation with anti-CD3, in a nominally \(Ca^{2+}\)-free environment, resulted in an equivalent small, transient increase in fluorescence. This increase in the \([Ca^{2+}]_i\) represents the depletion of IP\(_3\)-sensitive \(Ca^{2+}\) stores and its subsequent removal out of the cell by activated \(Ca^{2+}\)-ATPases or its sequestration within the cell (Fig. 1A). DCPA and vehicle treated cells exhibited similar changes in \([Ca^{2+}]_i\), indicating that DCPA does not affect the early IP\(_3\)-mediated signaling pathway or the depletion of internal \(Ca^{2+}\) stores.

In T cells, optimal activation of several transcription factors requires a sustained and elevated \([Ca^{2+}]_i\), that is maintained by \(Ca^{2+}\) influx through CRAC channels following internal \(Ca^{2+}\) store depletion. To determine if DCPA alters \([Ca^{2+}]_i\) following store depletion we added \(Ca^{2+}\) to the media following anti-CD3 induced store depletion (Fig. 1A). Figure 1B is an expanded view of the change in \([Ca^{2+}]_i\) following store depletion.
and addition of external Ca\(^{2+}\), where the fluorescence intensity, seen in Figure 1A, was converted to an \([\text{Ca}^{2+}]_i\). A decrease in the \([\text{Ca}^{2+}]_i\), following store depletion was observed in Jurkat cells exposed to all four DCPA concentrations, when compared to the vehicle control (Fig. 1A and B). Cells were further treated with Triton X-100 to monitor compartmentalization and ensure that the amount of available dye was not a limiting factor. In addition, Triton X-100 and EGTA provided maximum and minimum fluorescence values, respectively, to calculate the \([\text{Ca}^{2+}]_i\). (See Material and Methods). Similar fluorescent levels after addition of ionomycin, Triton X-100 and EGTA were seen independent of treatment (Fig. 1A).

To further quantify the attenuation of the \([\text{Ca}^{2+}]_i\) following store depletion the area under the curve (AUC), peak \([\text{Ca}^{2+}]_i\), and time to peak, were calculated. The AUC was calculated for each sample to determine the relative amount of free Ca\(^{2+}\) in the cytosol over a 103 s time period (t = 337-440 s). The AUC and peak \([\text{Ca}^{2+}]_i\) data for cells exposed to increasing concentrations of DCPA and stimulated with anti-CD3 are shown in Figure 1C and D. Exposure of cells to 200 \(\mu\)M DCPA resulted in a significant decrease (79%) in the AUC and the peak \([\text{Ca}^{2+}]_i\) (78%). A 56% decrease in the AUC and a 52% decrease in the peak \([\text{Ca}^{2+}]_i\), was observed in cells treated with 100 \(\mu\)M. Cells treated with 25 and 50 \(\mu\)M DCPA also resulted in decreases in AUC and peak \([\text{Ca}^{2+}]_i\). Although the decreases observed in the presence of 25 and 50 \(\mu\)M were not statistically significant they highlight the trend in the concentration dependent effect of DCPA. In addition, the time to reach peak \([\text{Ca}^{2+}]_i\), following addition of external Ca\(^{2+}\), increased with increasing concentration of DCPA exposure. Vehicle control cells reached a peak \([\text{Ca}^{2+}]_i\) after approximately 65 s. Treatment of cells with 25, 50, 100,
and 200 µM D CPA resulted in a peak $[\text{Ca}^{2+}]_i$ at approximately 75, 81, 93 and 108 s, respectively. Taken together, these results demonstrate that exposure to D CPA decreases $[\text{Ca}^{2+}]_i$ following anti-CD3 induced store depletion in a concentration dependent manner.
Figure 1. DCPA decreases intracellular calcium in a concentration-dependent manner.

Jurkat cells were loaded with fluo-3 and at 50s, anti-CD3 was added simultaneously with 25, 50, 100, 200 µM DCPA or vehicle control and changes in (Ca^{2+}) were recorded with a spectrofluorometer. At 335 s, the external [Ca^{2+}] concentration was increased to 2.5 mM with CaCl_2. Starting at 442 s, ionomycin, Triton X-100 and EGTA were sequentially added to the cell media at the times indicated. A representative experiment of the complete fluorescence curve in the absence (solid line) and presence of DCPA (dashed lines) at increasing concentrations.
Figure 1B: DCPA decreases intracellular calcium in a concentration dependent manner.

Fluorescence intensity data from Figure 1A were used to calculate $[\text{Ca}^{2+}]_i$ for the time frame of interest ($t=320-442$ s). These time points represent the influx of Ca into the cell.
Figure 1C and 1D. DCPA decreases intracellular calcium in a concentration dependent manner.  

C, Statistical analysis of the area under the curve (AUC) for DCPA and vehicle control from 3 separate experiments.  
D, Statistical analysis of the peak [Ca^{2+}] for DCPA and vehicle control from 3 separate experiments.  
Error bars reflect the ± S.D. and asterisks (*) indicates statistically significant results, p< 0.05.  
Statistical analysis was performed using ANOVA.
The Effect of DCPA on Thapsigargin Stimulated Jurkat Cells

To determine if exposure to DCPA affected the early IP$_3$-induced signaling pathway, the SERCA pump inhibitor thapsigargin was used. Thapsigargin depletes ER Ca$^{2+}$ stores and prevents refilling, thereby activating Ca$^{2+}$ influx through C RAC channels independent of IP$_3$ production (Bergling, Dolmetsch et al. 1998). The addition of 100 µM DCPA or vehicle control followed by 2 µM thapsigargin resulted in an equivalent small, transient increase in fluorescence, similar to that observed with anti-CD3 stimulation (Fig 2A). However, the normal increase in [Ca$^{2+}$]$_i$, following internal Ca$^{2+}$ store depletion, is significantly decreased in DCPA-treated cells, compared to the vehicle control cells. (Fig. 2A and B). Cells treated with thapsigargin and exposed to 100 µM DCPA exhibited a significant decrease in the AUC and peak [Ca$^{2+}$]$_i$, similar to that observed with anti-CD3 stimulation (Figure 2C and D). During a 70s time period (t=360-430s) D CPA exposed cells exhibited an approximately 50% decrease in the AUC and an approximate 55% decrease in the peak [Ca$^{2+}$]$_i$. This data indicates that early IP$_3$-induced signaling events and depletion of ER Ca$^{2+}$ stores were not affected by the exposure of Jurkat cells to D CPA. However, the addition of extracellular Ca$^{2+}$ following store depletion resulted in an overall decrease in the available free cytosolic Ca$^{2+}$. 
**Figure 2A.** DCPA does not affect early IP3-mediated signaling or depletion of ER Ca\textsuperscript{2+} stores.

Jurkat cells were loaded with fluo-3 in nominally Ca\textsuperscript{2+}-free solution. At 50 s, thapsigargin was added simultaneously with 100µM DCPA or vehicle control and changes in (Ca\textsuperscript{2+})\textsubscript{i} were recorded with a spectrofluorometer. At 375 s, the external [Ca\textsuperscript{2+}] was raised to 2.5 mM with CaCl\textsubscript{2}. Starting at 430 s, ionomycin, Triton X-100 and EGTA were sequentially added to the cell media at the times indicated. **A**, a representative experiment of the complete fluorescence curve of vehicle control (dashed line) and 100 µM DCPA-treated cells (solid line).
Figure 2B. DCPA does not affect early IP$_3$-mediated signaling or depletion of ER Ca$^{2+}$ stores. Fluorescence intensity data from Figure 2A were used to calculate [Ca$^{2+}$], for the time frame of interest (t=360-430 s). These time points represent the influx of Ca into the cell.
Figure 2C and 2D. DCPA does not affect early IP$_3$-mediated signaling or depletion of ER Ca$^{2+}$ stores. C, statistical analysis of the AUC for DCPA and vehicle control from 3 separate experiments. D, statistical analysis of the peak [Ca$^{2+}$]$_i$ for DCPA and vehicle control from 3 separate experiments. Error bars reflect the ± S.D. and asterisks (*) indicates statistically significant results, p<0.05. Statistical analysis was performed using a t-test.
The Effect of DCPA and 2-APB on [Ca\textsuperscript{2+}]\textsubscript{i}

To further characterize the effect of DCPA on [Ca\textsuperscript{2+}]\textsubscript{i}, we compared DCPA-induced inhibition to that seen with 2-APB, a known inhibitor and enhancer of Ca\textsuperscript{2+} influx through CRAC channels (Prakriya and Lewis 2001). At low concentrations (<5 µM) 2-APB enhances Ca\textsuperscript{2+} influx through CRAC channels but at higher concentrations (>10 µM) it inhibits Ca\textsuperscript{2+} influx (Prakriya and Lewis 2001). Since 2-APB has been shown to alter Ca\textsuperscript{2+} influx following store depletion we compared the effects of 2-APB on [Ca\textsuperscript{2+}]\textsubscript{i} to that of DCPA.

Jurkat cells were stimulated with anti-CD3 and the change in [Ca\textsuperscript{2+}]\textsubscript{i} was measured in cells treated with vehicle control, 100 µM DCPA, 2.5 µM 2-APB, 50 µM 2-APB or a mixture of 100 µM DCPA and 2.5 µM 2-APB (Fig. 3A). As expected there was no change in the release of Ca\textsuperscript{2+} from internal stores regardless of treatment, except for the cells exposed to 50 µM 2-APB (data not shown). High concentrations of 2-APB have been reported to affect IP\textsubscript{3}-mediated signaling in addition to inhibiting Ca\textsuperscript{2+} influx but IP\textsubscript{3} signaling is not affected at lower enhancing concentrations of 2-APB (Prakriya and Lewis 2001). Figure 3A shows the changes in [Ca\textsuperscript{2+}]\textsubscript{i} following store depletion and subsequent addition of external Ca\textsuperscript{2+}. Cells treated with 2.5 µM 2-APB demonstrated a significant increase in [Ca\textsuperscript{2+}]\textsubscript{i} compared to the vehicle control whereas 50 µM 2-APB abrogates the increase in the [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 3A). When cells were simultaneously treated with 100 µM DCPA and 2.5 µM 2-APB the enhanced effect of 2-APB was abrogated.

Cells treated with DCPA or 50 µM 2-APB revealed significant decreases in the AUC and the peak [Ca\textsuperscript{2+}]\textsubscript{i}, as compared to the vehicle control (Fig. 3B and C). The AUC and peak [Ca\textsuperscript{2+}]\textsubscript{i} were significantly increased (30%) in cells treated with 2.5 µM 2-APB (Fig. 3B and C). Cells treated simultaneously with 100 µM DCPA and 2.5 µM 2-APB
exhibited a 30% decrease in the AUC and peak $[\text{Ca}^{2+}]_i$ when compared to the enhanced influx of 2.5µM 2-APB (Fig. 3B and C). Together these results provide further evidence that, following store-operated $\text{Ca}^{2+}$ depletion, DCPA-exposed cells decrease $[\text{Ca}^{2+}]_i$ in a similar manner as 2-APB and DCPA interferes with the ability of 2-APB to enhance $\text{Ca}^{2+}$ influx through CRAC channels.
Figure 3A. The effect of DCPA and 2-APB on $[\text{Ca}^{2+}]_i$.
Jurkat cells were loaded with fluo-3 in a nominally free Ca$^{2+}$ solution. Anti-CD3 was added simultaneously with 100 µM DCPA, 2.5 µM 2-APB, 50 µM 2-APB, a mixture of 100 µM DCPA and 2.5 µM or vehicle control (solid line). Fluorescence changes in ($\text{Ca}^{2+}$) was recorded with a spectrofluorometer and converted to a $[\text{Ca}^{2+}]_i$. A, a representative experiment of the conversion of the fluorescence intensity to a $[\text{Ca}^{2+}]_i$ from t=300-420 s for DCPA and vehicle control.
Figure 3B and 3C. The effect of DCPA and 2-APB on [Ca\textsuperscript{2+}]. B, statistical analysis of the AUC for DCPA, 2-APB (2.5 μM and 50 μM), 100 μM DCPA plus 2.5 μM 2-APB and vehicle control from 3 separate experiments. C, statistical analysis of the peak [Ca\textsuperscript{2+}] for DCPA, 2-APB (2.5 μM and 50 μM), 100 μM DCPA plus 2.5 μM 2-APB and vehicle control from 3 separate experiments. Error bars reflect the ± S.D. and asterisks (*) indicates statistically significant change compared to vehicle control, p<0.05. Statistical analysis was performed using a t-test.
**The Effect of DCPA on Membrane Potential**

It is well known that changes in the membrane potential of T cells can alter Ca\(^{2+}\) influx (Sarkadi, Tordai et al. 1990). To determine if the effect on [Ca\(^{2+}\)] seen in DCPA-exposed cells was due to alterations in the membrane potential, Jurkat cells were loaded with the membrane potential sensitive dye, DiBAC\(_4\)(3). As the cell depolarizes, increasing amounts of the DiBAC\(_4\)(3) dye enters the cell membrane resulting in an increased fluorescence signal whereas a decrease in the fluorescence signal indicates hyperpolarization. Jurkat cells were exposed to 100µM DCPA or vehicle control and stimulated with anti-CD3 in a nominally Ca\(^{2+}\)-free buffer. Changes in the fluorescence signal using flow cytometry are depicted in Figure 4. The background resting T cell fluorescence was recorded at t =0. Fluorescence measurements were taken immediately following the simultaneous addition of the treatment and anti-CD3 (t=1 min), then again at t =3 min and t =5 min. External Ca\(^{2+}\) was then added and fluorescence measurements were taken immediately at t=5.5 m, t=6.5 and t=9.5 m. Addition of 50 mM KCl at t=10 min depolarized the cell and demonstrated that the dye responds to this depolarization. Further fluorescence measurements were also taken at t=12 and 14 m in. This data demonstrates that the DCPA-induced attenuation of [Ca\(^{2+}\)] following store depletion is not due to changes in the membrane potential.
Figure 4  DCPA does not alter the membrane potential of anti-CD3 stimulated Jurkat cells. Jurkat cells were loaded with DiBAC$_4$(3) in a nominally Ca$^{2+}$ free solution and analyzed via flow cytometry. Open circles are DCPA-treated cells and closed triangles are vehicle control cells. At t =0 the background resting potential was recorded. Fluorescence measurements were taken immediately following addition of anti-CD3 and treatment (t=1 min), then again at t =3 min and t =5 min. External Ca$^{2+}$ (2.5mM CaCl$_2$) was then added and fluorescence measurements were recorded at t =5.5 min, t =6.5 and t =9.5 min. 50 mM KCl was added to depolarize the cells and fluorescence measurements were taken at t =10, 12 and 14 min. Error bars reflect the ±S.D. from 3 experiments. Statistical analysis was performed using a t-test.
Effect of DCPA on IL-2 Secretion in Jurkat cells

A reduction in the elevated [Ca^{2+}]_{i} following store depletion has direct downstream affects on Ca^{2+}-dependent pathways. In order to establish a functional consequence of exposure to DCPA we assessed its effect on IL-2 secretion. IL-2 is an essential early cytokine required for T cell proliferation and differentiation. The activation of transcription factors NF-κB, NFAT and AP-1, depend, to varying degrees, on the [Ca^{2+}]_{i} and are important in production of IL-2. Jurkat cells were stimulated with anti-CD3 and anti-CD28 and exposed to 25, 50, 100, or 200 µM DCPA or vehicle control. After 48 h in culture, IL-2 levels in the supernatant were assayed via a sandwich ELISA. The results of a representative experiment are shown in Table 1. In the presence of 2.5 mM extracellular Ca^{2+}, DCPA decreased IL-2 production in a concentration-dependent manner, with no detectable IL-2 production when exposed to 200 µM DCPA. Exposure of Jurkat cells to 100 µM DCPA inhibited IL-2 production by 72%. In experiments performed with the same DCPA and vehicle control concentrations but with nominal extracellular Ca^{2+} (≤2.5 µM), no detectable levels of IL-2 were measured (data not shown).
Table 1. Effects of DCPA on IL-2 production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (µg/ml ± SD)</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>3420±272</td>
<td>97.4</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>3510±564</td>
<td>100.0</td>
</tr>
<tr>
<td>25 µM DCPA</td>
<td>2720±169</td>
<td>77.5</td>
</tr>
<tr>
<td>50 µM DCPA</td>
<td>2262±312</td>
<td>64.4</td>
</tr>
<tr>
<td>100 µM DCPA</td>
<td>968±245</td>
<td>27.6</td>
</tr>
<tr>
<td>125 µM DCPA</td>
<td>1171±137</td>
<td>33.4</td>
</tr>
<tr>
<td>200 µM DCPA</td>
<td>&lt;32(^a)</td>
<td></td>
</tr>
</tbody>
</table>

Note: IL-2 is measured in µg/ml ± SD

\(^a\)below the level of detection
Effect of DCPA on Nuclear Translocation of NFAT

In order to establish a direct mechanistic consequence of DCPA on the inhibition of Ca\(^{2+}\)-dependent signaling events we examined the Ca\(^{2+}\)-dependent transcription factor NFAT. A sustained [Ca\(^{2+}\)]\(_i\) via Ca\(^{2+}\) influx results in the calmodulin-stimulated activation of the protein phosphatase, calcineurin (Lewis 2001; Feske, Okamura et al. 2003; Quintana, Riesemer et al. 2005). Calcineurin dephosphorylates cytoplasmic NFAT allowing its translocation into the nucleus where it acts as a transcription factor for many genes, including IL-2. To assess the effect of DCPA on NFAT we measured the levels of nuclear NFAT at various time points after T cell stimulation. Jurkat cells were treated with vehicle control, 100µM DCPA or no treatment and stimulated with PMA and A23187. After 1, 2, 4 or 6 h incubations nuclear extracts were made and the level of nuclear NFAT was determined by western blot. Cells exposed to 100 µM DCPA exhibited decreases in nuclear NFAT at all time points (Fig. 5A and 5B). Densitometric analysis of the decrease in nuclear NFAT in DCPA-treated Jurkat cells indicated that 100 µM DCPA decreased nuclear NFAT approximately 30% compared to the vehicle control (Fig. 5C). Since NFAT is a Ca\(^{2+}\)-dependent transcription factor, this data demonstrates a direct mechanism linking the decrease in [Ca\(^{2+}\)]\(_i\) observed in DCPA-treated cells to decreased IL-2 production.
Figure 5A. DCPA decreases nuclear NFAT levels. Nuclear extracts from Jurkat cells were stimulated and exposed to 100 µM DCPA at various time points. A, a representative blot indicating the bands that represent different forms of NFAT (top blot). The bands in the bottom blot are β-actin levels used as a loading and transfer control. B, the ratio of NFAT protein to β-actin protein using densitometry of the bands in part A.
Figure 5B and 5C. DCPA decreases nuclear NFAT levels. **B**, the ratio of NFAT protein to β-actin protein using densitometry of the bands in part A. **C**, the ratio the ratio of NFAT protein to β-actin protein expressed as a percent of the vehicle control.
**Effect of DCPA on Primary Mouse T Cells**

In order to establish that the DCPA-induced attenuation of \( \text{[Ca}^{2+}\text{]}_i \) was not unique to the Jurkat cell line, we assayed the effect of DCPA on \( \text{[Ca}^{2+}\text{]}_i \) levels in primary mouse T cells. Splenic T cells were isolated from female BALB/c mice by negative selection and magnetic sorting. The T cells were loaded with fluo-3 and the effect of 100 \( \mu\text{M} \) DCPA on \( \text{[Ca}^{2+}\text{]}_i \) was assayed as described previously. DCPA exposure did not affect the anti-CD3 induced release of \( \text{Ca}^{2+} \) from internal stores (data not shown). However, there was a significant decrease in \( \text{[Ca}^{2+}\text{]}_i \) following store depletion and addition of external \( \text{Ca}^{2+} \) (Fig. 6). There were not enough data points to assess the change in peak [Ca\(^{2+}\)], but the AUC (t=517-616 s) was significantly decreased (16%) in the DCPA treated cells (Fig. 6B). Altogether, these results demonstrate that the effect of DCPA on Ca\(^{2+}\) influx following store depletion is not limited to the human Jurkat T cells.
Figure 6. DCPA decreases [Ca2+]i in primary mouse T cells. Purified mouse splenic T cells were loaded with fluo-3. Anti-CD3 was added simultaneously with 100μM DCPA or vehicle control and Ca2+ fluorescence measured. At 567 s, the external [Ca2+] was raised to 2.5 mM with CaCl2. A, a representative experiment showing [Ca2+]i in the presence of vehicle control (solid line) or 100 μM DCPA (dashed line). B, statistical analysis of the AUC. Error bars reflect the ± S.D. from 2 experiments and analysis was performed using a student t-test, p<0.05.
DISCUSSION

DCPA is a widely used herbicide that is heavily used on rice crops. Its recent registration for use on turf will further increase not only its use but will increase the likelihood of human exposure to DCPA. Currently, workers involved in manufacturing, handling or applying DCPA are at greatest risk of exposure but there have also been reports of non-occupational exposure, including a child under the age of 6 (EPA 2006). The United States Environmental Protection Agency (EPA) conducted a risk assessment for workers involved in mixing, loading and application of DCPA. The results from this assessment indicate that workers using maximal protection, including closed mixing and loading systems and enclosed cockpit, cabs or trucks, are at risk for exposure to DCPA (EPA 2006). In addition, workers entering treated areas 12 hours after application were also at risk (EPA 2006). These conclusions emphasize the need to investigate the effects of human exposure to DCPA.

The effects of DCPA in the rodent model have been well established. In a mouse model, exposure to DCPA results in thymic atrophy, depressed NK and macrophage functions, altered cytotoxic T cell response, and decreased CD4+ T cells (Barnett and Gandy 1989; Zhao, Schafer et al. 1995; Sheil, Frankenberry et al. 2006; Ustyugova, Frost et al. 2007). The effect of DCPA on human immune cells has been limited. Human macrophages exposed to DCPA produce reduced levels of TNF-α, reactive oxygen species and reactive nitrogen species (Ustyugova, Frost et al. 2007). Human T cells exposed to DCPA exhibit decreased IL-2 production and decreases binding of transcription factor AP-1 to DNA (Brundage, Schafer et al. 2004). Recently Corsini et al. examined agricultural workers exposed to D CPA and reported alterations in leukocyte cytokine production (Corsini, Codeca et al. 2007).
Since the sustained and elevated influx of Ca\(^{2+}\), following store depletion, is necessary for T cell activation and proliferation, we examined the effects of DCPA on [Ca\(^{2+}\)]. Our experiments demonstrate that DCPA attenuates the increased [Ca\(^{2+}\)] following store depletion in a concentration-dependent manner. Anti-CD3 or thapsigargin-induced store depletion in DCPA-exposed Jurkat cells did not affect IP\(_3\)-mediated release of Ca\(^{2+}\) nor the depletion of ER Ca\(^{2+}\) stores. However, our experiments demonstrate that DCPA-exposed Jurkat T cells and mouse splenic T cells were able to attenuate the normal elevated [Ca\(^{2+}\)] observed following store depletion. As a consequence of this attenuation nuclear NFAT levels and IL-2 production were also decreased.

2-APB is widely used as both an inhibitor and enhancer of Ca\(^{2+}\) influx through CRAC channels (Prakriya and Lewis 2001; Prakriya and Lewis 2006). Low concentrations of 2-APB potentiate Ca\(^{2+}\) influx through CRAC channels following store depletion but do not affect IP\(_3\)-mediated release of Ca\(^{2+}\) from the ER (Prakriya and Lewis 2001). We used 2-APB to further elicit the inhibitory role of DCPA in Ca\(^{2+}\) signaling. At low concentrations of 2-APB (<5.0 µM) Ca\(^{2+}\) influx is enhanced through CRAC channels following store depletion. Jurkat cells exposed to a mixture of 100 µM DCPA and 2.5 µM 2-APB abrogated the enhanced Ca\(^{2+}\) influx seen with 2.5 µM 2-APB. This data suggests that T cells exposed to DCPA prevent the increased influx of Ca\(^{2+}\) seen at low concentrations of 2-APB by inhibiting events surrounding CRAC channel activation.

Both voltage-gated and Ca\(^{2+}\)-activated potassium (K\(^+\)) channels are PM channels involved in regulating the polarization state of T cells and modulating the rate of Ca\(^{2+}\)
influx through CRAC channels (Lewis 2001; Panyi, Varga et al. 2004; Quintana, Griesemer et al. 2005). Alterations in the K⁺ channel can change the polarization state and result in alterations of Ca²⁺ influx (Panyi, Varga et al. 2004). Depolarization of the cell can inhibit Ca²⁺ influx whereas hyperpolarization increases the driving force of Ca²⁺ into the cell. Our experiments indicate that DCPA does not alter the membrane potential of T cells (Fig.4) and therefore DCPA is unlikely to affect these channels.

In T cells, the plasma membrane Ca²⁺-ATPase (PMCA) pump is considered the primary Ca²⁺ extrusion mechanism. Modulation of PMCA activity can occur in response to an influx of Ca²⁺ through CRAC channels and results in long term stability of the Ca²⁺ signal (Feske, Prakriya et al. 2005). Since we have demonstrated that DCPA-treated cells attenuate the increase in [Ca²⁺], following store depletion it may be possible that DCPA enhances PMCA activity and thereby reduces the apparent amount of cytosolic Ca²⁺. However, this mechanism appears unlikely since PMCA activity would also result in a decrease [Ca²⁺] during the initial IP₃-mediated release of Ca²⁺ from internal stores. Figures 1A, 2A and 3A demonstrate no change in [Ca²⁺] during the initial IP₃-mediated release of Ca²⁺ from internal stores in DCPA-treated cells.

The mitochondria also play an important role in Ca²⁺ homeostasis (Parekh 2003; Quintana, Griesemer et al. 2005). Mitochondria can act as a Ca²⁺ sink and sequester large amounts of Ca²⁺ quickly and release it slowly after Ca²⁺ influx subsides (Parekh 2003). Expression of a uniporter in the mitochondrial inner membrane and a Na⁺/Ca²⁺ exchanger allow for Ca²⁺ uptake and release, respectively (Parekh 2003). Alterations in the activity of the Na⁺/Ca²⁺ exchanger or altered membrane potential of the
mitochondrial membrane could result in an apparent decrease in \( \text{Ca}^{2+} \) influx. Although we have not investigated the effects of DCPA on the mitochondria any increased function of \( \text{Ca}^{2+} \) uptake by the mitochondria would also result in an apparent decrease the initial IP3-mediated release of \( \text{Ca}^{2+} \) from internal stores, which was not observed in DCPA-exposed T cells.

Our data indicates that DCPA alters \( \text{Ca}^{2+} \) homeostasis in human and murine T cells. Specifically, DCPA is able to attenuate the increase in \( [\text{Ca}^{2+}]_i \) following store depletion. The ability of DCPA to abrogate the enhanced \( \text{Ca}^{2+} \) influx produced by 2.5 \( \mu \text{M} \) 2-APB through CRAC channels provides some evidence that DCPA is acting on mechanisms involved in the activation or regulation of CRAC channels. This decrease in \( [\text{Ca}^{2+}]_i \) has significant downstream consequences on nuclear NFAT levels and secretion of IL-2. Although the exact mechanism by which DCPA exerts its effect is unknown it is clear that the decrease in \( [\text{Ca}^{2+}]_i \) results in a functional consequence to human T cells. The data presented here indicates that exposure to DCPA alters \( [\text{Ca}^{2+}]_i \) in murine and human Jurkat T cells and may result in immunosuppression resulting in serious consequences on human health.
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CHAPTER 3

Differential effects of T cell function by metabolites of the herbicide, propanil.

Tricia L. Lewis* and John B. Barnett†

*Department of Microbiology, Immunology and Cell Biology, West Virginia University
School of Medicine, Morgantown, WV 26506

Running Title: Differential effects of propanil metabolites.

†Address correspondence to:
John B. Barnett, Ph.D.
Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine
PO Box 9177, Morgantown, WV 26506-9177
Tel. 304-293-4029; Fax 304-293-7823
E-Mail: jbarnett@hsc.wvu.edu

Key Words: T cells, 3,4-dichloropropionanilide, DCPA, propanil, metabolites, 3,4-dichloroaniline, N-hydroxy-3,4-dichloroaniline, DCA, T cells, fluorine substitution
ABSTRACT

Each year 2 billion pounds of herbicide are used worldwide to control the unwanted growth of plants. In the United States, over 0.5 billion pounds are used, representing 28% of worldwide use. Propanil (chemical name 3,4-dichloropropionanilide, DCPA) is the 17th most commonly used herbicide in the United States and 6-9 million pounds are applied annually to 2 million acres of rice fields. The immunomodulatory effects of DCPA have been well documented but no data is available on the effects of its metabolites. In mammals, hepatic enzymes metabolize DCPA into 3 major metabolites; 3,4-dichloroaniline (DCA), 6-hydroxy-3,4-dichloroaniline (6OH-DCA), and N-hydroxy-3,4-dichloroaniline (NOH-DCA). We report for the first time the immunotoxic effects of propanil metabolites on T cell function. Jurkat T cells were exposed to varying concentrations of DCPA and its metabolites and assayed for viability, IL-2 secretion, NFAT activity and calcium flux. In addition, the fluorine analogs of DCPA and DCA were investigated to determine the relative role of chlorine substituents on T cell immunotoxicity. As previously reported, Jurkat T cells exposed to DCPA decreased IL-2 secretion in a concentration and calcium-dependent manner. Here we report that exposure of Jurkat T cells to DCA also alters IL-2 secretion, NFAT activity and calcium influx, similar to DCPA. Interestingly, exposure to 6OH-DCA and NOH-DCA reduces IL-2 secretion and NFAT activity but has no effect on calcium influx. When both chlorines in DCPA and DCA were substituted with fluorines all effects were abrogated. Our data indicates that metabolites of DCPA have differential effects on T cell function and the presence of chlorines plays a critical role in eliciting these effects.
INTRODUCTION

Approximately 5.3 billion pounds of pesticides are applied annually across the United States and 15 of the top 25 most used pesticides are herbicides (Grube 2004). Propanil (chemical name 3,4-dichloropropionanilide, DCPA) is a post-emergent contact herbicide and is the 17th most used herbicide in the United States (USEPA 2006). Annual use of DCPA is estimated to be 6-9 million pounds per year and is distributed over 2 million acres of crop land, primarily rice fields. Rice plants are able to avoid the herbicidal effects of DCPA because they produce acylamidase, an enzyme that cleaves the amide bond, resulting in the production of 2 metabolites, 3,4-dichloroaniline (DCA) and propionic acid (Still 1968). In mammals, DCPA can also be metabolized to DCA and propionic acid via acylamidases in the liver (McMillan, Leakey et al. 1990). DCA can undergo further biotransformation in the liver to produce the oxidative metabolites, N-hydroxy-3,4-dichloroaniline (NOH-DCA) and 6-hydroxy-3,4-dichloroaniline (6OH-DCA) (Fig.A). Propionic acid is non-toxic and converted to CO₂ and water but limited data is available on the effects of DCA and its oxidative metabolites.

Workers involved in manufacturing, handling or application of DCPA are at greatest risk for exposure but there have also been reports of non-occupational exposure (Pastorelli 1998). Numerous in vitro studies have reported the immunomodulatory effects of exposure to DCPA (reviewed in (Salazar, Ustyugova et al. 2008)). Exposure to DCPA alters macrophage phagocytic activity and cytokine production as well as the lytic function of CD8+ T cells after secondary stimulation (Sheil, Frankenberry et al. 2006; Ustyugova, Frost et al. 2007; Salazar, Ustyugova et al. 2008). Other immunotoxic effects include decreased natural killer (NK) cell function, and an increase in antibody secreting cells (Barnett and Gandy 1989; Salazar, de Ia
Rosa et al. 2005). Recent studies from our lab demonstrate that exposure of Jurkat T cells to DCPA results in a concentration dependent decrease in IL-2 production that is mediated by alterations in NFAT translocation and calcium homeostasis (Lewis, Brundage et al. 2008).

Limited data is available on the toxic effects of DCA, NOH-DCA and 6OH-DCA. It has been reported that DCA and NOH-DCA are toxic to the bladder, liver and kidney of rats (Valentovic, Yahia et al. 1997). DCA can also alter the male reproductive system, binds weakly to the androgen receptor and may act as an endocrine disruptor (Bauer, Meyer et al. 1998; Zhang and Lin 2009). Mice exposed to molar equivalent concentrations of DCPA or DCA have decreased natural killer (NK) cell activity (Barnett and Gandy 1989). However, some studies suggest that DCPA may be more toxic than DCA (Zhao, Schafer et al. 1995; Malerba, Castoldi et al. 2002). Corsini et al reported that workers exposed to DCPA had several alterations in immune parameters (Corsini, Codeca et al. 2007). Agricultural workers exposed to DCPA had increased plasma IgG_{1} and IL-6 production in whole blood assays and decreased IL-10 and interferon-γ (IFN-γ) when compared to control subjects. In vitro assays demonstrated similar results and at molar equivalent concentrations, both DCPA and DCA inhibited IL-10 and IFN-γ production in enriched CD4+CD8+ samples. In addition, alterations in calcium homeostasis and cytokine production were observed in anti-CD3 and PHA-stimulated peripheral blood mononuclear cells, suggesting that human T cells may be sensitive to the effects of DCPA.

Using Jurkat T cells we investigated the effects of DCA, NOH-DCA, and 6OH-DCA and the role of halogen substitution on T cell function (Fig. 1A and 1B). Chlorine-
containing pesticides are a common class of pesticides and 45% of all pesticides introduced into the market after 1989 contain a chlorine-carbon bond (Jeschke 2004). The addition of chlorine to many chemical compounds can increase its activity with negative biological consequences. DDT is a well-known organochlorine pesticide whose effects are mediated through the presence and positioning of chlorines (Crinnion 2009). Although alterations in calcium-dependent signaling events have been reported in T cells exposed to DCPA, the effects of the metabolites produced in humans are unknown. We report that exposure to DCA alters IL-2 secretion in a calcium-dependent manner but at higher concentrations than that observed with DCPA. In addition, NOH-DCA and 6OH-DCA appear more toxic to T cells than DCPA and DCA and inhibit IL-2 secretion in a calcium-independent manner. Finally, the substitution of fluorines for chlorines results in an abrogation of all investigated effects in Jurkat T cells. This is the first reported study on the effects of DCPA metabolites on T cells and advances our knowledge on the metabolic and structural effects of DCPA.
Figure 1A DCPA metabolic pathway and structures and in mammals

A

Propanil
3,4- dichloropropionanilide (DCPA)

acylamidase

3,4- dichloroaniline (DCA)

N-hydroxy 3,4- dichloroaniline
(NOH-DCA)

6-hydroxy 3,4- dichloroaniline
(6OH-DCA)

Figure 1B Structure of fluorine analogs

B

3,4- difloropropionaniline (DFPA)

3,4- difloroaniline (DFA)
MATERIALS AND METHODS

Cell lines and reagents

Experiments were performed using the human T cell leukemia cell line, Jurkat clone E6-1, obtained from the ATCC (American Tissue Culture Collection, Manassas, VA). Jurkat cells were maintained in complete Roswell Park Memorial Institute (RPMI 1640) (Mediatech Inc., Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (v/v) (FBS) (Hyclone Inc. Logan, UT), 100 units/ml penicillin (BioWhittaker, Walkersville, MD), 100 µg/ml streptomycin (BioWhittaker), 20 mM glutamine (BioWhittaker) and 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO). The cultures were kept at 37°C in 5% CO₂.

Stock solutions of 3,4-dichloropropionanilide (DCPA) (ChemServices, West Chester, PA), 3,4-dichloroaniline (DCA) (Chem Services), 3,4-difluoropropionanilide (DFPA), and 3,4-difluoroaniline (DFA) (Sigma) were diluted in absolute ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY). Vehicle samples were treated with an equivalent concentration (0.1% v/v) of ethanol. Stock solutions of N-hydroxy-3,4-dichloroaniline (NOH-DCA) and 6-hydroxy-3,4-dichloroaniline (6OH-DCA) (a generous gift from G. Rankin) were diluted in dimethyl sulphoxide (DMSO) (Sigma) and vehicle samples were treated with an equivalent concentration (0.1% v/v) of DMSO.

Synthesis of DFPA and NOH-DCA

3,4-Difluoropropionanilide (DFPA) was synthesized from 3,4-difluoroaniline (DFA) (Sigma-Aldrich, Milwaukee, WI). Two grams of propanoic acid (Sigma-Aldrich) was added to 1g of DFA and heated to 100°C for 1 h followed by addition of 5 ml of water and continued heating at 100°C for an additional hour. The precipitate was
cooled to room temperature, filtered through a sintered glass funnel, washed with water and dried *in vacuo* resulting in 1.26g of D FPA. The crude D FPA was then re-crystallized from a 1:1 water and ethanol solution.

N-hydroxy-3,4-dichloroaniline (NOH-DCA) was synthesized by methods described by Lerman (Lerman, Weinstock-Rosin et al. 2004). In the presence of nitric acid, dichloromethane and tetra-n-butylammonium bromide (TBAB), 3,4-dichlorophenol was converted to 4,5-dichloro-2-nitrophenol, which was analyzed using NMR and mass spectroscopy and was found to be in agreement with others (Lerman, Weinstock-Rosin et al. 2004). Ethanol (100%) was added to 0.32 g 4,5-dichloro-2-nitrophenol and 20 mg platinum dioxide and hydrogenated (30-50 psi) on a Parr shaker for 1 h. The mixture was filtered through celite and concentrated *in vacuo* to yield NOH-DCA.

Production and purity of DFPA, NOH-DCA and the intermediate, 4,5-dichloro-2-nitrophenol were verified using NMR spectra from a Varian Unity-300 NMR spectrometer (Palo Alto, CA) and exact mass data was obtained using a Thermo-Fisher LTQ-FTICR and were in agreement with previously reported analytical data (Lok, Leone et al. 1996; Lerman, Weinstock-Rosin et al. 2004).

**Viability assays**

Viability assays were performed for DCPA, DCA, NOH-DCA, 6OH-DCA, DFPA, DFA using 7-AAD (7-amino-actinomycin) (BD Pharmingen, San Diego, CA) and following the manufacturer’s protocol. Briefly, 1.0 x 10^6 Jurkat cells were treated with or without varying concentrations of DCPA, DCA, NOH-DCA, 6OH-DCA, DFPA, DFA, and including ethanol and DMSO vehicle controls and incubated at 37°C in 5% CO₂ for 24 hours. Cells were then incubated in PBS with 5 µl (0.25 µg) 7-AAD and incubated on ice.
for 20 min in the dark. Cells were then washed and resuspended in 0.4% paraformaldehyde and analyzed by flow cytometry. Emission was detected in the FL-3 channel (>650nm) using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

**Calcium Fluorescence Measurements**

Jurkat cells were loaded with the calcium-indicator dye fluo-3 AM (Invitrogen, Carlsbad, CA) as previously described (Grynkiewicz, Poenie et al. 1985). Briefly, cells were harvested and re-suspended to a concentration of $5 \times 10^6$ cells/ml and incubated for 30 min (37°C in 5% CO₂) in complete RPMI media (1.5% FBS, v/v) containing 0.1 µM fluo-3 AM in the presence of 0.02% pluronic F-127 (Invitrogen) and 2.5 mM probenecid (Sigma). Cells were washed twice in Ca²⁺ and Mg²⁺-free Hanks Balanced Salt Solution (HBSS) (Mediatech Inc.) containing 10 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, 2% FBS and 2.5 mM probenecid, resuspended to a concentration of $1 \times 10^6$ cells/ml and incubated 30 min at room temperature. Since the addition of 2% FBS is essential for cell viability, the media contains a nominal concentration of Ca²⁺ (2.5 µM). Samples were kept at room temperature and protected from light until ready for analysis. For each sample, $2 \times 10^6$ cells were placed in a quartz cuvette and the fluorescence was measured using a PTI QM-2000-4 spectrofluorometer (Photon Technology International (PTI), Birmingham, NJ) with constant stirring. The fluorescence of the fluo-3 dye was measured with excitation at 490 nm and emission at 525 nm. The fluorescence was measured and digitized at 1 Hz using the software program Felix 1.42b (PTI). Data points were collected every second and cells were either treated with vehicle control, DCPA, or its...
metabolites or analogs and 2 µM hapsigargin (Sigma) or 2 µM hapsigargin alone. Reagents were added after baseline data was collected for 45 seconds. Following the return of fluorescence to background levels, 2.5 mM CaCl₂ (Sigma) was added to the media. Addition of 200 µM ionomycin (Sigma) ensured even loading of the cells. Cell membranes were lysed with 0.1% (v/v) Triton X-100 (Fisher Scientific, Hampton, NH) to measure the maximum fluorescence (Fₘₐₓ) parameter for calculation of [Ca²⁺]ᵢ and to monitor compartmentalization of the dye. To chelate the free Ca²⁺ to a nominally Ca²⁺-free level (Fₘᵢₙ), 50 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA), pH 7.5 (Sigma) was added to the cuvette.

Fluorescence values were converted to [Ca²⁺]ᵢ using the following equation:

$$\text{free}[\text{Ca}^{2+}] = K_D \frac{(F-F_{\text{min}})}{(F_{\text{max}}-F)},$$

where $K_D$ (360 nM) is the dissociation constant of the Fluo-3/Ca²⁺ complex, $F$ is the measured fluorescence intensity, $F_{\text{min}}$ is the minimum fluorescence at very low [Ca²⁺]ᵢ (fluorescence after the addition of 50 mM EGTA) and $F_{\text{max}}$ is the fluorescence measured at high [Ca²⁺]ᵢ (fluorescence after the addition of Triton X-100) (Grynkiewicz, Poenie et al. 1985). The background fluorescence obtained from unloaded cells over a 3 minute time period was subtracted from all data points before [Ca²⁺]ᵢ was calculated.

**Measurement of IL-2 Production**

Jurkat cells were cultured in complete RPMI media at $5 \times 10^5$ cells/well in 48-well plates (Costar, Corning, NY) coated with mouse anti-human CD3 antibody (10 µg/ml) (BD BioSciences, San Diego, CA). Cells were treated with varying concentrations of DCPA, DCA, DFPA, DFA, NOH-DCA, 6OH-DCA, ethanol or DMSO vehicle control. Cells were also simultaneously stimulated with anti-CD28 antibody (2 µg/ml) (BD BioSciences).
Cells were incubated at 37 °C in 5% CO₂ for 24 h after which supernatants were collected and placed at -20°C. IL-2 production was determined using the sandwich ELISA method and following the manufacturer’s protocol (BD PharMingen). All cultures and ELISA analyses were performed in triplicate and the experiment was repeated at minimum of three times.

**Transfections and Luciferase assay**

Jurkat cells were plated in RPMI media with 1.5% FBS at 6 x 10⁵ cells/well in a 6-well plate (Costar). For each well, 750 ng of pN FAT-luc (firefly luciferase plasmid) (Stratagene) and 10 ng pRL-TK (Renilla luciferase plasmid) (Promega) were transfected with 1 μl Lipofectamine 2000 (Invitrogen). Cells were incubated for 5 h at 37°C in 5% CO₂ and media was replaced with complete RPMI +10% FBS and incubated over night at 37°C in 5% CO₂. Following transfection, cells were treated with varying concentrations of DCPA, DCA, DFPA, DFA, NOH-DCA, 6OH-DCA, ethanol or DMSO or left untreated and stimulated with 10 ng/ml PMA and 1 μg/ml A23187. Cells were incubated for 4 h at 37°C in 5% CO₂, centrifuged, lysed and stored at -70°C until ready for analysis. NFAT activity was determined using the Dual Luciferase Assay Kit and following the manufacturer’s protocol (Promega). Briefly, 25 μl of sample was added to 100 μl of Luciferase Assay I and the firefly luciferase activity measured. Addition of Stop & Glo quenched the firefly luminescence and provides a substrate for the Renilla luciferase activity. Luminescence was detected using a Berthold Lumat LB 9507 (Oak Ridge, TN). NFAT-firefly luciferase transfection efficiency was normalized to the Renilla luciferase activity and the average fold change was reported. All luciferase assays were performed in triplicate and the experiment was repeated at least twice.
Statistical Analysis

All data were analyzed using MS Excel 2007 (Redmond, WA) and Sigma Stat 3.1 (Port Richmond, CA). A NOVA with a Student-Newman Keuls post hoc test was used to determine statistical significance with an alpha value of <0.05 considered significant.
RESULTS

Hydroxy metabolites of DCPA are more cytotoxic than its parent.

In mammals, metabolism of DCPA results in the hydrolysis of the amide side chain and production of the 3,4-DCA with further biotransformation resulting in the formation of 6OH-DCA and NOH-DCA. To assess the cytotoxicity of DCPA and its metabolites on Jurkat T cells, viability and proliferation assays were conducted over a range of concentrations. For cells exposed to DCPA, DCA, DFPA, and DFA, ethanol (0.1% v/v) was used as a vehicle control and DMSO was used as a vehicle control for 6OH-DCA and NOH-DCA. Jurkat T cells were treated or left untreated, loaded with 7-AAD and viability was assessed after 24 hours (Fig.2). The viability of cells treated with increasing concentrations of DCPA up to 100µM did not decrease viability (Fig.2A). Treatment with 200µM DCPA decreases viability by approximately 14.6% (Fig.2A) and subsequent studies were conducted using a maximum concentration of 100µM DCPA. DCA appeared to be less toxic and concentrations up to 200 µM were not cytotoxic (Fig.2B). However, Jurkat T cells were more sensitive to the hydroxylated DCA metabolites and cytotoxicity was observed at 100µM for both 6OH-DCA and NOH-DCA (Fig.2C and 2D). This concentration was excluded for both 6OH-DCA and NOH-DCA in all subsequent assays. Proliferation assays were also performed and proliferation of Jurkat T cells was inhibited only at those concentrations that were also cytotoxic (data not shown).
Figure 2A and 2B Cytotoxic effects of DCPA and DCA

Jurkat T cells were treated with or without varying concentrations of A) DCPA, B) DCA, or their vehicle control, ethanol for 24 h. Cells were stained with 7-AAD to determine viability. Results using flow cytometry are representative of 3 experiments each performed in triplicate. Error bars reflect ± SD.
Figure 2C and 2D Cytotoxic effects of 6OH-DCA and NOH-DCA

Jurkat T cells were treated with or without varying concentrations of, C) 6OH-DCA, D) NOH-DCA, or their vehicle control DMSO for 24 h. Cells were stained with 7-AAD to determine viability using flow cytometry. Results are representative of 3 experiments each performed in triplicate. Error bars reflect ± SD.
**DCPA and its metabolites decrease IL-2 secretion.**

We have previously reported that exposure to DCPA decreases IL-2 production in Jurkat T cells in a calcium-dependent manner (Lewis 2008). IL-2 is an important early cytokine produced by T cells and is critical for the activation and proliferation of several immune cells, including NK cells, T cells and B cells. To determine if exposure to metabolites of DCPA alter IL-2 production, Jurkat T cells were treated over range of concentrations with or without vehicle controls (DMSO for the hydroxylated metabolites and ethanol for all others) and stimulated with anti-CD3 and anti-CD28. After 24 h in culture, IL-2 secretion levels in the supernatant were assessed using an ELISA. DCPA decreased IL-2 secretion in a concentration-dependent manner with significant decreases observed at 25µM, 50µM, and 100µM DCPA and represented a 20%, 48% and 74% decrease in IL-2 production, respectively (Fig. 3A). This data is an agreement with previously reported decreases of IL-2 in response to DCPA treatment.

Metabolism of DCPA is reported to occur in the liver through the action of acylamidases which cleave the amide side chain resulting in the production of DCA (McMillan). Several studies have reported detectable levels of DCA in blood and urine in DCPA-exposed individual. When Jurkat cells are exposed to increasing concentrations of DCA (25-200µM), concentration-dependent decreases in IL-2 secretion are also observed (Fig. 3B). At a concentration of 50µM decreases are observed but statistically significant decreases are only observed at 100µM and 200µM DCA (Fig. 3B). Exposure to 50µM, 100µM and 200µM DCA resulted in a 10%, 45% and 78% decrease in IL-2, respectively. These results suggest that, in T cells, DCPA is more potent inhibitor of IL-2 than its metabolite, DCA.
In mammals, hydroxylation of DCA to 6 OH-DCA and NO H-DCA occurs in the liver and both oxidative metabolites are capable of converting oxy-hemoglobin (Hb) to met-Hb. In Jurkat T cells, NOH-DCA and 6O H-DCA are cytotoxic at 100 µM so to assess their effects on IL-2 secretion lower concentrations were evaluated. Both 6OH-DCA and NO H-DCA decreased IL-2 secretion however NOH-DCA is a more potent inhibitor of IL-2 secretion (Fig. 3C and 3D). 6O H-DCA decreased IL-2 production by 30% at the highest concentration (50 µM) whereas 25 µM and 50 µM NOH-DCA decreased IL-2 secretion by 51% and 90%, respectively. No change in IL-2 secretion was observed in cells treated with 5 µM NOH-DCA. This data indicates that Jurkat T cells are highly sensitive to exposure of NOH-DCA.
Figure 3A and 3B DCPA and DCA inhibit IL-2 secretion

Jurkat T cells were treated with or without varying concentrations of A) DCPA, B) DCA, and stimulated with anti-CD3 and anti-CD28 for 24h. Supernatants were analyzed by ELISA and results are representative of three separate experiments. Error bars reflect ± SD and asterisks (*) indicate statistically significant results, p<0.05 using ANOVA with a Student-Newman Keuls post hoc test.
Figure 3C and 3D NOH-DCA and 6OH-DCA inhibit IL-2 secretion
Jurkat T cells were treated with or without varying concentrations of C) 6OH-DCA, D) NOH-DCA and stimulated with anti-CD3 and anti-CD28 for 24 h. Supernatants were analyzed by ELISA and results are representative of three separate experiments. Error bars reflect ± SD and asterisks (*) indicate statistically significant results, p<0.05 using ANOVA with a Student-Newman Keuls post hoc test.
**DCPA and its metabolites alter NFAT activity**

In T cells, optimal transcription of the IL-2 gene requires coordinated binding of three transcription factors; NFAT (nuclear factor of activated T cells), NF-kB (nuclear factor kappaB) and AP-1 (activator protein-1). We have previously reported that DCPA-exposed Jurkat cells have decreased AP-1 DNA binding ability, decreased c-Jun phosphorylation and decreased nuclear NFAT levels. To further understand the effects of DCPA and its metabolites on IL-2 secretion Jurkat T cells were co-transfected with a NFAT luciferase plasmid (pNFAT-luc) and a Renilla luciferase plasmid (pRL-TK), to control for transfection efficiencies. In resting T cells, NFAT is localized to the cytoplasm in a phosphorylated state and upon stimulation becomes de-phosphorylated by the calcium-dependent phosphatase, calcineurin. Dephosphorylation of NFAT exposes a nuclear localization sequence facilitating its translocation into the nucleus and binding to the promoter region of the targeted gene. In this system, stimulation of Jurkat cells leads to the de-phosphorylation of endogenous NFAT that binds to the NFAT DNA binding sites on the pNFAT-luc plasmid which controls expression of the firefly luciferase. In cells exposed to 25-100µM DCPA there is a significant decrease in NFAT activity (Fig 4A), with a 37%, 63% and 87% decrease in NFAT activity in cells treated with 25µM, 50µM and 100µM DCPA, respectively. Similarly, cells treated with DCA also have decreased NFAT activity at those concentrations that resulted in decreased IL-2 secretion (Fig 3B). Cells exposed to 100 µM and 200µM DCA statistically decreased NFAT activity levels by 66 and 18%, respectively (Fig 4B). 6OH-DCA-treated cells had a 30% decrease in NFAT activity at 50µM but no change at 25µM (Fig.4C). NOH-DCA decreased NFAT activity by 33% and 47% at 25µM and
50µM, respectively (Fig.4D). Although 50µM NOH-DCA inhibited IL-2 secretion by 90% NFAT activity is only decreased by 47%, whereas 100µM DCPA decreased IL-2 by 74% with an 87% decrease in NFAT activity. This data suggests that NOH-DCA may inhibit IL-2 thorough an alternate mechanism than DCPA. In all experiments, cells treated with cyclosporin, an inhibitor of NFAT that prevents dephosphorylation, produced similar levels of NFAT activity as those in unstimulated cells.
Jurkat T cells were co-transfected with a firefly luciferase-NFAT reporter plasmid and a Renilla luciferase plasmid to control for tranfection efficiency. Cells were treated with or without varying concentrations of DCPA or vehicle control and stimulated with PMA and A23187 for 5 h. Unstimulated and cyclosporin treated cells were included as controls. Cell lysates were analyzed for luciferase activity and normalized to Renilla activity. Results are representative of at least 2 experiments each performed in triplicate. Asterisks (*) indicate statistically significant results, p <0.05 using ANOVA with a Student-Newman Keuls post hoc test.
Figure 4B DCA alters NFAT activity

Jurkat T cells were co-transfected with a firefly luciferase-NFAT reporter plasmid and a Renilla luciferase plasmid to control for tranfection efficiency. Cells were treated with or without varying concentrations of DCA or vehicle control and stimulated with PMA and A23187 for 5 h. Unstimulated and cyclosporin treated cells were included as controls. Cell lysates were analyzed for luciferase activity and normalized to Renilla activity. Results are representative of at least 2 experiments each performed in triplicate. Asterisks (*) indicate statistically significant results, p <0.05 using ANOVA with a Student-Newman Keuls post hoc test.
Figure 4C. 6OH-DCA alters NFAT activity.
Jurkat T cells were co-transfected with a firefly luciferase-NFAT reporter plasmid and a Renilla luciferase plasmid to control for transfection efficiency. Cells were treated with or without varying concentrations of 6OH-DCA or vehicle control and stimulated with PMA and A23187 for 5 h. Unstimulated and cyclosporin treated cells were included as controls. Cell lysates were analyzed for luciferase activity and normalized to Renilla activity. Results are representative of at least 2 experiments each performed in triplicate. Asterisks (*) indicate statistically significant results, p<0.05 using ANOVA with a Student-Newman Keuls post hoc test.
Figure 4D. NOH-DCA alters NFAT activity

Jurkat T cells were co-transfected with a firefly luciferase-NFAT reporter plasmid and a Renilla luciferase plasmid to control for tranfection efficiency. Cells were treated with or without varying concentrations of NOH-DCA or vehicle control and stimulated with PMA and A23187 for 5 h. Unstimulated and cyclosporin treated cells were included as controls. Cell lysates were analyzed for luciferase activity and normalized to Renilla activity. Results are representative of at least 2 experiments each performed in triplicate. Asterisks (*) indicate statistically significant results, p<0.05 using ANOVA with a Student-Newman Keuls post hoc test.
**DCPA and its metabolites have differential effects on Ca homeostasis.**

It has been previously reported that exposure of Jurkat T cells to DCPA results in decreased intracellular calcium influx following ER Ca store depletion. To examine the effect of DCPA metabolites on calcium homeostasis, Jurkat T cells were loaded with a calcium-sensitive dye and changes in intracellular calcium were monitored over time. At the start of these experiments, the extracellular media is essential Ca-free and depletion of ER Ca stores with thapsigargin, an inhibitor of the SERCA (Sarco/endoplasmic reticulum Ca ATPase) pump, results in a small, transient increase in intracellular Ca (Fig.5). Addition of 2mM Ca to the media results in a large and sustained increase in intracellular Ca that reflects store-operated calcium influx (Fig 5). Jurkat T cells exposed to DCPA decrease calcium influx following store depletion in a concentration dependent manner (Fig 5A). At concentrations of 100µM and 200µM DCA, decreases in intracellular calcium are also observed (Fig 5B). It should be noted that DCPA and DCA-treated cells do not alter the transient increase in intracellular calcium observed when ER calcium stores are depleted. Interestingly, cells treated with 6OH-DCA and NOH-DCA did not inhibit ER calcium store depletion or calcium influx (Fig 5C and 5D). This data supports the conclusion that hydroxylated metabolites alter IL-2 secretion through a different mechanism than that of DCPA and DCA.
Figure 5A DCPA alters calcium influx

Jurkat T cells were loaded with the calcium-sensitive dye, fluo-3, and treated with or without varying concentrations of D CPA or vehicle control. Immediately following addition of treatment, 2µM thapsigargin was added to deplete Ca stores. When fluorescence returned to baseline 2mM CaCl₂ was added and the effect on Ca influx was recorded. Results are representative of at least 3 experiments.
Figure 5B DCA alters calcium influx
Jurkat T cells were loaded with the calcium-sensitive dye, fluo-3, and treated with or without varying concentrations of DCA or vehicle control. Immediately following addition of treatment, 2µM thapsigargin was added to deplete Ca stores. When fluorescence returned to baseline 2mM CaCl₂ was added and the effect on Ca influx was recorded. Results are representative of at least 3 experiments.
Figure 5C 6OH-DCA does not alter intracellular calcium concentrations
Jurkat T cells were loaded with the calcium-sensitive dye, fluo-3, and treated with or without varying concentrations of 60H-DCA or vehicle control. Immediately following addition of treatment, 2µM thapsigargin was added to deplete Ca stores. When fluorescence returned to baseline 2mM CaCl$_2$ was added and the effect on Ca influx was recorded. Results are representative of at least 3 experiments.
Figure 5D NOH-DCA does not alter intracellular calcium concentrations

Jurkat T cells were loaded with the calcium-sensitive dye, fluo-3, and treated with or without varying concentrations of NOH-DCA or vehicle control. Immediately following addition of treatment, 2µM thapsigargin was added to deplete Ca stores. When fluorescence returned to baseline 2 mM CaCl₂ was added and the effect on Ca influx was recorded. Results are representative of at least 3 experiments.
Chlorine substituents play a role in the immunotoxic effects of DCPA

To assess the role that chlorines, in the 3 and 4 positions on DCPA, may have on the immunotoxic effects in T cells, we synthesized the fluorine analogs of DCPA, DFPA (see materials and methods) and used DFA, a commercially available fluorine analog of DCA. Concentrations up to 400µM of both DFPA and DFA were not cytotoxic to Jurkat T cells (Fig 6A and 6B). Proliferation assays were also conducted and no changes in proliferation after 24 hour were observed at concentrations up to 400 µM (data not shown). To determine the effect of DFPA and DFA on IL-2 secretion, Jurkat T cells were stimulated with anti-CD3 and anti-CD28 and IL-2 was measured as described above. No changes in IL-2 secretion were detected when Jurkat cells were exposed to DFPA or DFA with increasing concentrations up to 400µM (Fig 6C and 6D). These results indicate the presence of chlorine at the 3 and 4 positions play an important role in inhibiting IL-2 secretion in DCPA and DCA exposed T cells. To confirm the apparent inert effects of fluorine substitution, NFAT activity and Ca influx were also examined as described above. Consistent with the IL-2 secretion data, Jurkat T cells exposed to DFPA and DFA did not alter NFAT activity or Ca homeostasis (Fig 6E-6H). This data provides clear evidence that the immunotoxic effects of DCPA and its metabolites, on T cells, can be attributed, in large part, to the presence and location of chlorines at the 3 and 4 positions.
Figure 6A and 6B Cytotoxic effects of DFPA and DFA

Jurkat T cells were treated with or without varying concentrations of A) DFPA or B) DFA and vehicle control for 24h. Cells were stained with 7-AAD to determine viability and analyzed with flow cytometry. Results are representative of 3 experiments each performed in triplicate. Error bars reflect ± SD.
Figure 6C and 6D DFPA and DFA do not inhibit IL-2 secretion

Jurkat T cells were treated with or without varying concentrations of A) DFPA or B) DFA, and vehicle control and stimulated with anti-CD3 and anti-CD28 for 24 h. Supernatants were collected and analyzed by ELISA. Results are representative of three separate experiments. Error bars reflect ± SD.
Jurkat T cells were co-transfected with a firefly luciferase-NFAT reporter plasmid and a Renilla luciferase plasmid to control for tranfection efficiency. Cells were treated with or without varying concentrations of E) DFPA or F) DFA and vehicle control and stimulated with PMA and A23187 for 5 h. Unstimulated and cyclosporin treated cells were included as controls. Cell lysates were analyzed for luciferase activity and normalized to Renilla activity. Results are representative of at least 2 experiments each performed in triplicate.
Figure 6G and 6H DFPA and DFA do not alter intracellular calcium concentrations
Jurkat T cells were loaded with the calcium-sensitive dye, fluo-3, and treated with or without varying concentrations of G) DFPA, H) DFA and vehicle control. 2µM thapsigargin was added to deplete Ca stores. When fluorescence returned to baseline 2mM CaCl₂ was added and the effect on Ca influx was recorded. Results are representative of at least 3 experiments.
DISCUSSION

In the United States, agricultural use of herbicides represents 58% of total pesticide use and approximately 2 billion pounds are applied to crop fields every year (Grube 2004). DCPA is a commonly used herbicide in the United States and is used on 50-80% of all rice crops for control against broadleaf and grassy plants. The toxic effects of DCPA on the immune system have been well documented (reviewed in Salazar, Ustyugova et al. 2008). In mouse models, in vivo and in vitro exposure to DCPA decreases the phagocytic ability of macrophages and its ability to produce IL-6 and TNF-α when stimulated with LPS (Xie, Schafer et al. 1997; Xie, Schafer et al. 1997; Frost, Neeley et al. 2001; Ustyugova, Frost et al. 2007). Primary stimulation of CD8+ T cells exposed to DCPA is not affected but the secondary response, in the absence of further exposure to DCPA, abrogates lytic activity (Barnett and Gandy 1989; Heil, Frankenberry et al. 2006). In human Jurkat T cells, DCPA decreases IL-2 protein and mRNA levels as well as the binding ability of the transcription factor AP-1 (activator protein-1) and the protein levels and phosphorylation of c-jun (Zhao, Schafer et al. 1999; Brundage, Schafer et al. 2004). The decreases in IL-2 secretion observed in DCPA-treated T cells are mediated by alterations in NFAT translocation and calcium homeostasis (Lewis, Brundage et al. 2008). Although the immunotoxic effects of DCPA are well documented little is known about the effects of its metabolites.

In mammals, DCPA is metabolized in the liver by acylamidases resulting in the production of DCA which can be hydroxylated to form 6OH-DCA and NOH-DCA (Fig.1). Exposure to DCA can occur in several ways, as breakdown products of DCPA and other herbicides such as linuron and diuron and also through the manufacturing of several pesticides, dyes and pharmaceuticals (SCTEE 2001). In humans, DCPA has
an estimated half life of 3.2 hours and is quickly metabolized into DCA which can be detected in the urine and blood of occupationally and non-occupationally exposed humans (Wittke, Hajimiroga et al. 2001; Turci, Barisano et al. 2006; Roberts, Heilmair et al. 2009).

Limited data is available on the immunotoxic effects of DCA but Barnett et al reported that molar equivalent concentrations of DCA and DCPA decreased natural killer (NK) cell activity and increased spleen weight (Barnett, Gandy et al. 1992). NOH-DCA and 6OH-DCA are reactive metabolites that have been studied for their effects on erythrocytes (Singleton and Murphy 1973). Both NOH-DCA and 6OH-DCA can bind hemoglobin leading to methemoglobinemia, a serious medical condition that results in the formation of methemoglobin adducts that do not bind or transport oxygen. NOH-DCA has also been reported as a nephrotoxicant and can induce hemolytic anemia (McMillan, Bradshaw et al. 1991; Valentovic, Ball et al. 2001). No data exists on the immunotoxic effects of NOH-DCA and 6OH-DCA.

Studies on exposure and the effects of DCPA in human subjects are limited. Richards et al measured air levels of DCPA in farms adjacent to rice fields and determined that individuals living near rice fields are at risk for exposure to DCPA (Richards, McClure et al. 2001). Accompanying this study, McClure et al reported that individuals living in areas sampled by Richards et al did not appear to be at higher risk for altered immune functions (McClure, Helm et al. 2001). Although individuals surrounding rice fields may not be at risk, there is evidence to suggest that workers in the agricultural and manufacturing sectors are at risk. Corsini et al examined agricultural workers exposed before, during and after exposure to DCPA (Corsini,
DCA was readily detected in the urine of workers at concentrations up to 332ng/ml. Whole blood assays of the workers revealed that cells stimulated with PHA had decreased IL-10 and IFN-γ production. In addition, Pastorelli et al. reported that DCA-Hb adducts are detectable in agricultural workers exposed to DCPA for as long as 4 months following the last application of the herbicide (Pastorelli 1998). This suggests that metabolites of DCPA can remain in the body for an extended period of time.

We report here for the first time the immunotoxic effects of the DCPA metabolites, DCA, NOH-DCA and 6OH-DCA on human Jurkat T cell function. Jurkat T cells have been used for over 20 years as a model for human T cell signaling (Abraham 2004). Previous research has demonstrated that exposure of DCPA to human Jurkat T cells inhibits IL-2 secretion in a concentration and Ca-dependent manner. Similar to DCPA, DCA inhibits IL-2 secretion, NFAT activity and calcium homeostasis, although at higher concentrations than that of DCPA (Fig 3B, 4B, 5B). This is in agreement with others who have reported that immune parameters, including T cell dependent antibody production, myelotoxic effects, and IL-6 response in mouse T cells, were altered by DCPA but required higher concentrations of DCA to produce similar effects (Barnett, Gandy et al. 1992; Malerba, Castoldi et al. 2002)(Zhao unpubl data).

NOH-DCA appears more cytotoxic to T cells than both DCPA or DCA and inhibits IL-2 secretion more potently, with a 90% reduction in IL-2 at 50µM NOH-DCA whereas at 50µM DCPA IL-2 is decreased by only 50% (Fig 2A, 2C, 3A, 3C). However, a 2.5 fold decrease in NFAT activity in cells exposed to 50µM DCPA resulted in a 50% decrease in IL-2 but 50µM NOH-DCA decreased IL-2 by 90% with only a 2 fold change.
in N FAT activity (Fig. 4A and 4C). The effect of these metabolites on calcium homeostasis suggests that the mechanism by which NOH-DCA inhibits IL-2 secretion is different from that of DCPA (Fig 5A and 5C). NOH-DCA and 6OH-DCA appeared to inhibit IL-2 in a Ca-independent manner, whereas the effects of DCPA and DCA on T cell function are elicited in a Ca-dependent manner. There is no data available on the immunotoxic effects of 6OH-DCA, however, McMillan et al reported that although 6OH-DCA produced methHb adducts NOH-DCA was 10 times more potent (McMillan, Leakey et al. 1990). Our data supports a diminished effect of 6OH-DCA on T cell function. 6OH-DCA and NOH-DCA are equally cytotoxic but 6OH-DCA only decreased IL-2 secretion at 50µM compared to 5µM NOH-DCA (Fig 2C and 2D). Calcium homeostasis is also unaffected by treatment with 6OH-DCA. The mechanism for the decreased in IL-2 and NFAT activity in 6OH-DCA and NOH-DCA exposed cells is unknown.

Approximately 45% of all herbicides contain a carbon-chlorine bond and 1/6th of all organochlorines requires special safety precautions for use in the workplace (Naumann 2000). In many cases the biological activity of the compound is conferred by the presence of the chlorines (Naumann 2000). The best known example is DDT, as the removal of two specific chlorines renders this pesticide in active (Crinnion 2009). Other toxic organochlorine pesticides include atrazine and 2,4-D. In order to determine the role that chlorine substituents have in DCPA-exposed T cells, we substituted fluorines for both of the chlorines found in DCPA and DCA. In the last 20 years, the number of fluorinated chemicals has increased significantly and now 28% of all halogenated agrochemicals are fluorinated (Jeschke 2004). The position and number of fluorines in agrochemicals determines the activity of many pesticides (Jeschke 2004).
Unlike DCPA and DCA, DFPA and DFA are not cytotoxic (up to 400µM) and do not alter IL-2 secretion, NFAT activity or calcium homeostasis (Fig. 6). This indicates that chlorine substitution plays an important role in exerting immunotoxic effects in T cells. It has been reported that 3,4-DFA is toxic to the liver but at concentrations higher than that for 3,4-DCA, suggesting a negative biological role for chlorine at the 3 and 4 positions (Hong, Anastis et al. 2000). Several possibilities exist for the differential effects observed with chlorine and fluorine. First, fluorines are highly electronegative and act only as hydrogen acceptors whereas chlorine and other halogens act as both hydrogen acceptors and donors. Second, trifluoro-substitution can increase lipophilicity but mono- or difluoro-substitution has been reported to decrease it (Purser, Moore et al. 2008). It has recently been reported that, in Jurkat T cells, DCPA becomes localized to the cytosol, indicating that it can pass through the membrane (Hanson, Peer et al. 2010). Increases in electronegativity and lipophilicity could prevent binding to proteins targeted by DCPA. Lastly, fluorines are similar in size to hydrogen and may not produce the confirmation required to elicit the effects of DCPA. These specific properties of fluorine may be responsible for the apparent reversal of effects observed in DFPA and DFA treated cells.

In conclusion, metabolites of DCPA display differential effects on Jurkat T cells. Although DCA elicits similar Ca-dependent effects on IL-2 secretion, the parent compound, DCPA, is more toxic. However, DCPA is quickly metabolized to DCA and persists in the body possibly allowing DCA to accumulate to concentrations greater than that of DCPA. Hydroxylation of DCA can result in the production of NOH-DCA and 6OH-DCA which, in Jurkat T cells, can be cytotoxic and can alter IL-2 secretion in a Ca-

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independent manner at low concentration (5μM NOH-DCA). Further studies are required to determine the mechanism for the decreases in IL-2 observed in NOH-DCA-exposed T cells and to more fully understand the mechanism by which DCPA elicits its effects.
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CHAPTER 4

The inhibitory effects of 3,4-dichloropropionanilide (DCPA) on Stromal Interaction Molecule-1 (Stim1) puncta formation.

Tricia L. Lewis* and John B. Barnett*

* Department of Microbiology, Immunology and Cell Biology, West Virginia University School of Medicine, Morgantown, WV 26506

Running title: DCPA inhibits Stim1 puncta formation

Address correspondence to:

John B. Barnett, Ph.D.
Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine
P.O. Box 9177
Morgantown, West Virginia 26506-9177
Tel. 304-293-4029; Fax 304-293-7823
E-Mail: jbarnett@hsc.wvu.edu

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ABSTRACT

Calcium (Ca) is a ubiquitous second messenger that is required in almost all cells and at almost all stages of life. This widespread use of Ca in intracellular signaling pathways requires tight regulation. Calcium release-activated calcium (CRAC) channels are a type of store-operated (SOC) channel that have been identified and described in detail, especially in immune cells. Activation of CRAC channels results from the depletion of Ca located in endoplasmic reticulum (ER) stores. Depletion of the ER stores signals the ER protein, Stim1 to aggregate into punctate structures near the plasma membrane where it clusters with the plasma membrane protein, Orai1. Formation of the Stim1/Orai1 complex activates CRAC channels allowing the selective entry of Ca into the cell. Alterations in the activation and assembly of Stim1 and Orai1 have serious consequences on human health. DCPA is a post-emergent herbicide used in rice fields and numerous studies suggest that it has negative effects on the immune system. We have previously reported that DCPA inhibits Ca influx in Jurkat T cells with adverse effects on IL-2 production and NFAT activity. Here we examine the effects of DCPA on Stim1 puncta formation. Upon ER Ca store depletion, HEK293 cells expressing YFP-Stim1 formed puncta-rich regions near the plasma membrane of the cell. DCPA-treated cells formed few punctate structures and failed to form the puncta-rich regions when ER stores were depleted. Here we propose a mechanism and identify DCPA as an inhibitor of Stim1 puncta formation.
INTRODUCTION

Calcium signaling

Calcium (Ca) is a ubiquitous second messenger that is important in all aspects of life, from conception to death. Many signaling systems are used to regulate the flow of Ca into and out of a cell. Store-operated Ca entry (SOCE) is used by virtually all animal cells to increase intracellular Ca levels in response to a stimulus (Feske 2009). As the name implies, entry of Ca into the cell is regulated by the levels of free Ca stored in the lumen of the ER. SOCE is initiated by stimulation of receptors on the surface of a cell which leads to the production of IP3. IP3 binds to the IP3 receptor on the surface of the ER resulting in the release of Ca from the ER stores. Stim1, an ER protein with an EF-hand domain extending into the lumen of the ER, acts as a Ca sensor to detect changes in Ca. Multimerization of Stim1 initiates interactions with Orai1 proteins, located in the plasma membrane, and results in the activation of CRAC channels (Fig 2). The formation of Stim1-Orai1 clusters is essential for CRAC activation and translocation of Stim1 to areas within 10-25nm of the membrane occurs within 6-10 seconds prior to CRAC activation (Wu, Buchanan et al. 2006). Opening of CRAC channels enables the refilling of ER Ca stores and functions to produce sustained and elevated Ca signaling required for activation of various signaling pathways. In T cells, influx of Ca through CRAC channels and subsequent activation of the Ca-dependent transcription factor NFAT are critical for the expression of IL-2, an important early cytokine responsible for activation, proliferation and differentiation of several immune cells (Vig and Kinet 2009).
**Stromal Interaction Molecule-1 (Stim1)**

Stim1 is a single transmembrane protein that has been identified as an essential protein in SOC signaling in many cell types. Originally described in 1996 this protein, then designated SIM, was identified in a screening for proteins that bind B cell precursors (Oritani and Kincaide 1996). The gene for this protein was cloned from the human chromosome region 11p15.5 and is evolutionarily conserved from Drosophilia to mammals. Further studies revealed that Stim1 had tumor suppressor abilities in some cancer cell lines (Sabbioni, Barbanti-Brodano et al. 1997; Sabbioni, Veronese et al. 1999). The link between SOCE and Stim1 came in 2005 when Roos et al discovered that, in siRNA against Stim, in Drosophila, decreased Ca influx (Roos, DiGregorio et al. 2005). Simultaneously, Liou et al screened 2304 human genes using siRNA for each gene and looked for those altering Ca influx in HeLa cells (Liou, Kim et al. 2005). They identified 2 genes, Stim1 and Stim2. Stim1 and Stim2 are ubiquitously expressed in almost all mammalian tissue and, to date, the role of Stim1 in SOCE has been identified in numerous cell types including T cells, B cells, macrophages, mast cells, platelets, skeletal muscle, smooth muscle, vascular endothelium and neuronal cells (Soboloff, Spassova et al. 2006; Frischauf, Schindl et al. 2008).

Stim1 is characterized as a 685 amino acid, 90k Da, Type I transmembrane domain protein (Williams, Manji et al. 2001) (Fig 1). The N terminus resides in the lumen of the ER and the C-terminus in the cytosol. The N terminus is a highly conserved region that contains a single sterile alpha motif (SAM) domain that is modified by N-linked glycosylation at 2 sites (Asn131 and Asn171). The SAM domain is thought to mediate several hetero- and homotypic interactions. Deletion of the SAM
domain results in the failure of Stim1 to form puncta and CRAC channels are not activated (Baba, Hayashi et al. 2006). In addition, the presence of an EF-hand domain, in the N terminus acts as a Ca sensor for ER Ca stores. A single mutation (Asp76Ala) in the EF-hand domain of Stim1 results in prelocalized puncta formation in the absence of ER Ca store depletion along with constitutive activation of the CRAC current ($I_{CRAC}$) (Liou, Kim et al. 2005; Zhang, Yu et al. 2005).

The C-terminus of Stim1 is located in the cytosol and contains 2 highly conserved alpha helical coiled-coil regions, an ezrin-radixin-moesin (ERM) domain and Ser and Lys-rich regions (Stathopulos, Li et al. 2006). Within the ERM domain, a Stim/Orai-activating region (SOAR) and a Stim1 homomerization domain (SHD) have recently been identified (Muik, Fahrner et al. 2009; Yuan, Zeng et al. 2009). Deletion of the ERM domain disrupts Stim1 puncta formation and CRAC activation. Further mutational studies have identified a CRAC-activating domain (CAD), also called Orai-activating small fragment (OASF), within the SOAR domain (Muik, Fahrner et al. 2009; Park, Hoover et al. 2009). Expression of CAD alone is sufficient to activate Orai1 and deletion of CAD results in diffuse Stim1 expression when ER Ca stores are depleted (Park, Hoover et al. 2009). Stim1 also contains a CRAC modulatory domain (CMD), C terminal to the SHD, which is reported to regulate the degree of coupling to Orai1 as well as CRAC activation (Derler, Fahrner et al. 2009). The extreme C terminus of Stim1 contains a polybasic region which can bind calmodulin, in a calcium-dependent manner, and is critical for translocation to the plasma membrane (Bauer, O'Connell et al. 2008).
It is estimated that 20-30% of Stim1 is expressed on the plasma membrane but limited and conflicting data is available to determine to its function (Manji, Parker et al. 2000). Some studies have identified a role for plasma membrane bound Stim1 in platelet aggregation and vascular smooth muscle cell migration (Jardin, Lopez et al. 2009) (Li, Sukumar et al. 2008). In addition, a Stim1 antibody, applied externally, blocks $I_{CRAC}$ in Jurkat T cells and SOCE in HEK293 cells, revealing that Stim1 on the plasma membrane may be functionally important (Soboloff, Spassova et al. 2006; Spassova, Soboloff et al. 2006). However, Liou et al reported did not detect Stim1 on the plasma membrane of HeLa cells, indicating the role of Stim1 on the plasma membrane may be cell type specific (Liou, Fivaz et al. 2007).

**Knockdown and mutational studies of Stim1**

Numerous knockdown and mutational studies have contributed to the understanding of Stim1 in SOCE. Knockdown of Stim1 in several cell types, as well as in Drosophila, result in inhibition of $I_{CRAC}$ (Ong, Liu et al. 2007; Lyfenko and Dirksen...
Overexpression of Stim1 and Orai1, in HEK293 and Jurkat T cells, greatly increased $I_{\text{CRAC}}$ with a 10-60 fold increase in Ca influx but with no change in basal resting Ca levels (Soboloff, Spassova et al. 2006; Soboloff, Spassova et al. 2006). However, overexpression of Orai1 alone does not alter $I_{\text{CRAC}}$, but overexpression of Stim1 increased $I_{\text{CRAC}}$ 2 fold, indicating an important role for Stim1 in CRAC activation (Vig, Peinelt et al. 2006).

Stim1$^{-/-}$ mice die in utero or shortly after birth but studies in mice with conditionally targeted deletion of Stim1 have been reported (Oh-Hora, Yamashita et al. 2008). Conditional deletion of Stim1 in T cells and fibroblasts showed almost no Ca$^{2+}$ influx in response to depletion of ER Ca$^{2+}$ stores. In addition, TCR-stimulated production of IL-2, IFN-γ, and IL-4 production is significantly decreased in T cells from Stim1$^{-/-}$ mice (Oh-Hora, Yamashita et al. 2008).

Recently, three individuals from a single family were identified with a homozygous nonsense mutation in Stim1. These individuals have no mutations in Orai but have decreased SOCE and profound immunodeficiencies. Ex vivo studies demonstrated that SOCE could be partially rescued by introducing functional Stim1 but only partially rescued with Stim2 (Picard, McCarl et al. 2009).

**Stim2**

Stim2 was originally identified by Williams et al during a screening for Stim1-like sequences (Williams, Manji et al. 2001). Differential levels of phosphorylation account for two molecular mass isoforms (105kDa and 115kDa). Stim2 is ubiquitously expressed and although Stim1 and Stim2 have high sequence homology, there are several important differences that may account for differences in function (Fig 1).
(Williams, Manji et al. 2001). The N-termini of both proteins are almost identical, with the noted except that Stim1 has 2 N-linked glycosylation sites in the SAM domain and Stim2 only has one site. The greatest differences lie within the cytosolic C-terminal domain. As mentioned previously, Stim1 is expressed in the ER and plasma membrane, however, an ER retention sequence in the C-terminus of Stim2, but not on Stim1, confines its expression to the ER (Wang, Deng et al. 2009). In addition, the C-terminus of Stim2 contains phosphorylated Ser/His residues and a large Pro-rich domain.

In vitro and in vivo experiments have demonstrated that Stim2 can oligomerize with Stim1, indicating a possible functional interaction between Stim1 and Stim2 (Stathopoulos, Zheng et al. 2009; Wang, Deng et al. 2009). Overexpression of Stim2 does not result in puncta formation when ER stores are depleted but puncta formation does occur when both Stim1 and Stim2 are overexpressed (Soboloff, Spassova et al. 2006). In HEK 293 and Jurkat T cells, overexpression of Stim2 alone decreased Ca influx and $I_{\text{CRAC}}$ but knockdown of Stim2, using siRNA, has little effect on Ca influx or $I_{\text{CRAC}}$ (Dziadek and Johnstone 2007). Interestingly, overexpression of both Stim2 and Orai1 slightly increase baseline $I_{\text{CRAC}}$ when ER stores were filled and addition of 50µM 2-APB, normally inhibitory, increased $I_{\text{CRAC}}$ when Stim2 and Orai1 are overexpressed (Parvez, Beck et al. 2008). This data suggests that Stim2 acts as a regulator of SOC signaling. Further studies revealed that expression of the mutant Stim1(D76A) forms puncta when ER stores are full and produced constitutive Ca entry but when co-expressed with Stim2, puncta formation and constitutive Ca entry could be reversed (Brandman, Liou et al. 2007). This suggests that Stim2 can interfere with Stim1-
mediated SOC at a point downstream of puncta formation. It has been proposed that the effects of Stim2 are dependent on its ratio with Stim1 and that after store depletion, Stim1 and Stim2 aggregate and organize into puncta (Soboloff, Spassova et al. 2006). If the Stim1-Stim2 ratio is high, functional coupling to Orai1 occurs and SOCE proceeds but if the ratio is low, Stim2 interferes with coupling and inhibits SOCE.

There are also some studies that suggest that Stim2 is involved in stabilizing basal cytosolic and ER Ca levels (Brandman, Liou et al. 2007). Knockdown of Stim2 in HEK293 cells reduced basal cytosolic and ER Ca levels whereas overexpression increased basal Ca levels. Similar studies have reported that Stim2 responds to changes in ER Ca near resting levels, whereas Stim1 is activated only when ER Ca stores are depleted (Frischauf, Schindl et al. 2008).

**Orai / CRACM**

In 2006, Feske et al identified a protein in patients with a hereditary form of severe combined immunodeficiency (SCID) syndrome whose T cells failed to activate CRAC channels (Feske 2009). They named this protein Orai1, after the Greek mythological Orai, keepers of heaven’s gate. At the same time Vig et al identified the same protein through a siRNA screening of the Drosophila genome for proteins that altered Ca influx and designated the protein CRACM1 (Vig, Peinelt et al. 2006). Orai1 or CRACM1 is distributed diffusely in the plasma membrane at rest but aggregates and interacts with Stim1 in overlapping clusters after store depletion to activate CRAC channels (Fig 2) (Vig, Beck et al. 2006; Navarro-Borelly, Somasundaram et al. 2008). Forster resonance energy transfer (FRET) analysis confirmed that Stim1 and Orai1 co-immunoprecipitate and overlapping puncta occur in parallel with increases in FRET.
The Orai family consists of 3 members, Orai1, Orai2 and Orai3. A functional CRAC channel requires the formation of an Orai tetramer. The exact contribution from each family member has not been fully elucidated but appears to be dependent on cell type (Mignen, Thompson et al. 2008). Orai1/CRACM1 is the dominant form expressed in most mammalian cells. It is a 33kDa, 301 amino acid protein with 4 transmembrane (TM) spanning domains with both N and C termini located in the cytosol. Orai1 has been shown to form the essential pore-forming unit of the CRAC channel (Prakriya, Feske et al. 2006; Feske 2010). The N terminus of Orai1 contains a Pro/Arg-rich region (not found in Orai2 or Orai3) with a conserved polybasic (Arg/Lys) motif before TM1. The Pro/Arg-rich region is thought to play a role in Orai1 assembly. The C-terminus region contains a coiled-coil domain and protein interaction domains. Deletion of the complete N terminus in Orai1 abolishes Ca influx but co-clustering interaction with Stim1 is retained, indicating that the C-terminus contains regions important in the interaction with Stim1 (Li, Lu et al. 2007; Muik, Frischauf et al. 2008).
The transmembrane (TM) regions are important in forming the CRAC channel pore. Based on mutational analysis, TM1 and TM3 and the extracellular loop between TM1 and TM2 form the CRAC channel pore (Vig, Beck et al. 2006; Yeromin, Zhang et al. 2006). A Glu106Asp mutation in TM1 and a Glu190Gln mutation in TM3 both decrease the Ca selectivity of the pore through a proposed mechanism that increases the size of the pore (Yeromin, Zhang et al. 2006; Yamashita, Navarro-Borelly et al.).
As p to A la mutations i n am ino acids 11 0, 112, and 114 i n t he TM 1-TM2 extracellular loop also alters ion selectivity (Yeromin, Zhang et al. 2006).

Interaction with Stim1 is thought to be mediated through an amphipathic domain on the C terminus of Orai1 and also through two hydrophobic Lys residues. Isolation of a 107 am ino acid region, within the coiled-coil domain of the C-terminus of Stim1, can co-immunoprecipitate with Orai1 and activate CRAC channels independent of the E R Ca stores (Wang, Deng et al. 2009).

In HEK 293 and Jurkat T cells, overexpression of both Stim1 and Orai1 produce an e xtremely large I_{CRAC} but o verexpression of Orai1 alone produces no increase in I_{CRAC} (Mercer, Dehaven et al. 2006; Soboloff, Spassova et al. 2006; Vig, Beck et al. 2006). Knockdown of Orai1 abolishes Ca influx and CRAC activity (Vig, Beck et al. 2006). Expression of Orai1 and Stim2 increased cell proliferation of HEK 293 cells and endothelial cells, with no evident role for Stim1 (Potier, Gonzalez et al. 2009; El Boustany, Katsogianou et al. 2010). Silencing Orai1 or Stim2 abolished proliferation but silencing Stim1 had no effect.

**Orai2/CRACM2 and Orai3/CRACM3**

Orai1 is the dominant form of Orai expressed in most cell lines, however, Orai2 and Orai3 can also produce I_{CRAC} upon co-expression with Stim1 (Lis, Peinelt et al. 2007). In HEK 293 cells, overexpression of both Stim1 and Orai2 increased Ca influx and I_{CRAC} but to a lesser degr ee t han i n c ells ov erexpressing both Stim1 and Orai1 (Soboloff, Spassova et al. 2006). I n addition, overexpression of both Stim1 and Orai2 has no effect i n T c ells f rom S CID pat ients (Feske 2010). When Stim1 and Orai3 are overexpressed, Ca influx did not increase (Lis, Peinelt et al. 2007). Chimeric constructs
of Orai2 with the Orai1 C-terminus increases $I_{\text{CRAC}}$, and deletion of part of N-term of Orai1 results in a diminished Ca influx, comparable to that of wild type Orai2 and Orai3 (Li, Lu et al. 2007; Yuan, Zeng et al. 2009). In addition, four Orai1 proteins are thought to homodimerize to form an active CRAC channel but, based on co-immunoprecipitation experiments, heteromultimerization can also occur between Orai2 and Orai3 (Penna, Demuro et al. 2008; Maruyama, Ogura et al. 2009). These studies suggest multiple functions for Orai proteins which may be cell-type specific.
MATERIALS AND METHODS

Cell lines
Experiments were performed using human embryonic kidney-293 (HEK-293) cells (a generous gift from J. Soboloff). HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 1 00 units/ml penicillin (BioWhittaker, Walkersville, MD), 100 µg/ml streptomycin (BioWhittaker), 2.0 mM glutamine (BioWhittaker). The cultures were kept at 37°C in 5% CO₂.

Reagents
Stock solutions of DCPA (ChemServices, West Chester, PA) were diluted in absolute ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY). Vehicle samples were treated with an equivalent concentration (0.1%v/v) of ethanol.

Transfections
The day prior to transfection, 0.7 x 10⁵ HEK 293 cells were added to Delta T culture dishes (Bioptechs, Butler, PA) in DMEM supplemented with 10% FBS, without antibiotics and incubated overnight at 37°C in 5% CO₂. The day of transfection, HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Briefly, for each plate 1µl of Lipofectamine 2000 was placed in 50µl of OptiMem (Invitrogen) and incubated at room temperature for 5 minutes. The transfection solution was added to a tube with 50µl OptiMem and 0.5µg YFP-Stim1 plasmid (a generous gift from J. Soboloff) and incubated at room temperature for 20 minutes. The DNA-transfection complex was added to the HEK 293 cells and incubated at 37°C in 5% CO₂ for 5 hours, after which the media was replaced.
with fresh, complete DMEM and incubate for 24 hours at 37°C in 5% CO₂. On the day of the experiment the cells were placed in buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 15 mM Hepes, 0.1% BSA and 2 mM CaCl₂. Cells were kept at 37°C throughout the experiment and reagents added to the plate as indicated. YFP- Stim1 images were acquired using a 60x/1.49 Apo TIRF objective and 1x1 binning on a Nikon Eclipse TE2000-E inverted fluorescent microscope equipped with the Perfect Focus system and a Photometrics CoolSNAP HQ CCD camera. A z-stack of fluorescent images was collected at 1 µm intervals every 20 seconds for 10 minutes. Cells were maintained at 37°C with a Biotech Delta T dish heater. Images were acquired and analyzed using the NIS-Elements software package. Images shown are from a single focal plane.
RESULTS

In T cells exposure to DCPA leads to decreased IL-2 production, NFAT activity and calcium influx. Since Ca influx in T cells is regulated by CRAC channels we sought to determine the effects of DCPA on Stim1 puncta formation. Characterization of the role of Stim1 in CRAC channel activation has been thoroughly investigated in both Jurkat and HEK 293 cells (Parekh 2006; Feske 2007; Prakriya 2009). In both cell types, Stim1 puncta formation, its interaction with Orai1 and activation of CRAC channels all occur in a similar manner. HEK 293 cells are adherent, easy to transfect cells that have a relatively large cytoplasmic space that allows easy identification of puncta formation. These cells were used to determine the effects of DCPA on Stim1 puncta formation. HEK 293 cells were transfected with a YFP-Stim1 plasmid and ER Ca stores were depleted with 2µM thapsigargin approximately 70-80 s after data collection began (Fig 3A). Cells treated with DCPA received 100µM DCPA just prior to the addition of 2µM thapsigargin (Tg). Images were collected over a 16 minute period. Prior to treatment, all cells displayed a diffuse distribution of YFP-Stim1. Upon depletion of ER Ca stores with Tg in control cells, puncta formation was observed after approximately 7.5 minutes and continued to form over time (white arrows indicate areas of puncta formation). Puncta-rich regions are apparent around the membranes of the cells. Upon ER store depletion, cells treated with DCPA appeared to form some puncta but fail to form the puncta-rich regions observed in control cells (Fig.3B). These results suggest that DCPA can block Ca influx through CRAC channels by inhibiting Stim1 puncta formation.
Figure 3A Formation of Stim1 puncta-rich regions in control cells

HEK 293 cells were transfected with YFP-Stim1 and store-operated Ca influx was initiated by depletion of ER Ca stores with 2µM Tg. Tg was added to control cells at 70 sec. The cells presented here represent an average response of 30-40 cells.
Figure 3B DCPA inhibits formation of Stim1 puncta-rich regions
HEK 293 cells were transfected with YFP-Stim1 and store-operated Ca influx was initiated by depletion of ER Ca stores with 2µM Tg. 100µM DCPA was added prior to Tg at t=67 sec. The cells presented here represent an average response of 30-40 cells.
Regulation of intracellular calcium levels is essential for almost all aspects of cell function, and alterations in calcium homeostasis can have profound effects on cell function and viability. Many cells, including HEK 293 and Jurkat T cells, use SOC channels to regulate Ca influx (Peinelt, Vig et al. 2006; Vig and Kinet 2009). SOCE is a form of Ca influx that is dependent on the depletion of its ER Ca stores in order to activate Ca channels on the plasma membrane. CRAC channels are the most widely known and described SOC channel and were identified over 20 years ago (Vig and Kinet 2007; Prakriya 2009; Vig and Kinet 2009). Until recently, CRAC channels could only be characterized by electrophysiological methods. The mechanism by which CRAC channels function has become clearer with the identification of two essential proteins, Stim1 and Orai1. Stim1 is a single transmembrane protein that predominantly resides in the ER, with some studies indicating a role for Stim1 in the plasma membrane (Baba and Kurosaki 2009). It contains an EF-hand domain that acts as a Ca sensor in the lumen of the ER. In unstimulated cells, both Stim1 and Orai1 proteins are diffusely distributed in the ER and plasma membrane, respectively (Liou, Kim et al. 2005). Depletion of ER Ca stores releases Ca from the Stim1 EF-hand and induces a conformational change that facilitates aggregation of Stim1 and results in the formation of overlapping clusters with Orai1, in areas close to the plasma membrane (Luik, Wang et al. 2008). This Stim1/Orai1 complex activates CRAC channels and allows Ca to flow into the cell (Fahrner, Muik et al. 2009). Once inside the cell, Ca plays an important in activating multiple signaling pathways, including the NFAT (Gwack, Feske et al. 2007).
Alterations in Stim1 can have profound effects on cell function and, in T cells, can result in severe immunodeficiencies (Feske 2009). Recently three individuals, from a single family, were identified with a homozygous nonsense mutation in Stim1 but with no mutations in Orai1 (Picard, McCarl et al. 2009). These patients have profound immunodeficiencies and limited T cell SOCE. In addition, several other in vitro and in vivo studies support the important role for Stim1 in SOCE and calcium signaling (Feske, Prakriya et al. 2005; Feske 2010). Knockdown of Stim1 in several cell types, including Hek 293 and Jurkat T cells, resulted in inhibition of I_{CRAC} whereas overexpression of Stim1 increased I_{CRAC} 2 fold (Liou, Kim et al. 2005). In vivo experiments, with a conditional knockdown of Stim1 in mice, revealed almost no Ca influx in response to depletion of Ca stores (Oh-Hora, Yamashita et al. 2008). TCR-stimulated CD4+ T cells from these mice also produce significantly less IL-2, IFN-γ, and IL-4.

Inhibitors of SOCE have been described but most have proven non-specific for CRAC channels (DeHaven, Smyth et al. 2008; Nam, Shin et al. 2009; Salido, Sage et al. 2009). A widely used modulator of CRAC channels is 2-APB (Bootman, Collins et al. 2002). It is a bimodal modulator that activates CRAC at low concentrations (<5µM) and inhibits CRAC at higher concentrations (>10µM) (DeHaven, Smyth et al. 2008). We have previously reported that DCPA inhibits Ca influx in Jurkat T cells and attenuates the enhanced Ca influx observed with low concentrations of 2-APB (Lewis, Brundage et al. 2008). Here we more clearly define the mechanism by which DCPA alters Ca influx. HEK 293 cells have a diffuse expression of YFP-Stim1 when cells are at rest and aggregate into punctate structures when ER Ca stores are depleted. DCPA decreased the puncta-rich regions observed in control cells (Fig 3B). Some puncta do appear to
form in DCPA-treated cells but the dense puncta-rich regions observed in control cells were never observed. We conclude that DCPA inhibits Stim1 puncta formation in HEK 293 cells and extend the results here to conclude that DCPA also inhibits Stim1 puncta formation in Jurkat T cells. Both cells lines are known to express CRAC channels and overexpression and mutational studies in both cells lines respond in a similar manner. Our data, in Jurkat T cells, supports a mechanism whereby inhibition of Stim1 results in decreased Ca influx, NFAT activity and IL-2 production. In addition, CRAC channels are the only Ca channels expressed on T cells that respond to ER store depletion. A recent study examining the localization of DPCA in Jurkat T cells concluded that, after stimulation, DCPA is localized to the cytosol and not found in the membrane or subcellular fractions (Hanson, Peer et al. 2010). This data supports a role for a mechanism in which DCPA interact with Stim1 in the cytosol to inhibit puncta formation. An analog of 2-APB was recently identified as a potential inhibitor of Stim1 but the specificity of its actions are unknown (Goto, Suzuki et al. 2010). No other known inhibitors of Stim1 have been reported. DCPA may serve as a novel inhibitor of Stim1 with the potential for use as a tool to better understand Stim1/Orai1 interactions and CRAC channels.
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CHAPTER 5

GENERAL DISCUSSION

The overall goal of this dissertation was to determine the effects that DCPA and its metabolites have on T cell activation and function and to propose a possible mechanism to explain the effects. Numerous studies have been conducted on the immunotoxic effects of DCPA (reviewed in Salazar, Ustyugova et al. 2008). Earlier studies in mouse models and human cell lines, laid the foundation for a more detailed investigation into the effects of DCPA on T cells. In addition, it is known that DCPA is metabolized quickly in the liver of mammals, but little immunotoxic data is available on the effects of its metabolites (McMillan, Freeman et al. 1990). The first goal was to determine the effects of DCPA and its metabolites on human T cell function and activation by examining immune parameters and signaling events. Once this was determined, a possible mechanism for these effects was then investigated. The results of these experiments shed light on the importance of investigating metabolic products when evaluating the immunotoxicity of chemicals and reveal that the effects of DCPA on T cells may provide a novel mechanism for immunosuppression.

In T cells, IL-2 is one of the first cytokines to be expressed and its transcription is dependent on the cooperative DNA binding of AP-1, NFAT and NF-κB (Jain, Loh et al. 1995; Serfling, Berberich-Siebelt et al. 2007). NFAT is a transcription factor that is strictly calcium-dependent. In T cells, activation of NFAT occurs through a series of complex signaling events that begin with the recognition of a foreign peptide, and co-stimulation of the CD28 receptor. ITAMs, located on the intracellular portion of the TCR, act as a staging area for the phosphorylation and recruitment of several kinases.
and adapt proteins that form the immunological synapse. One important consequence of this is the recruitment of PLC-γ which hydrolyzes the membrane lipid PIP2 resulting in the production of IP3 and DAG. DAG remains in the membrane where it plays a role in the activation of Ras and PKCθ pathways. IP3 is released into the cytosol and binds to the IP3-R on the ER allowing the release of Ca from the internal ER Ca stores. Depletion of the ER stores releases Ca from the EF-hand domain on Stim1 allowing aggregation of Stim1 and interaction with Orai1 near the plasma membrane (Luik, Wang et al. 2008). Clustering of Stim1 and Orai1 activate CRAC channels and allows the selective entry of Ca into the cell. This increase in intracellular Ca enables it to bind to sites on calmodulin (CaM). The Ca-CaM complex binds to the regulatory subunit of calcineurin, thereby activating its phosphatase activity. Dephosphorylation of NFAT, by calcineurin, exposes a nuclear localization sequence which leads to translocation of NFAT into the nucleus. NFAT-driven gene expression is highly dependent on a sustained and elevated Ca influx (Rao 2009). Patients with T cells that have altered CRAC function also display changes in NFAT activity and IL-2 production that results in a SCID-like syndrome (Feske, Gwack et al. 2006). In addition, IL-2 is important in T cell homeostasis and tolerance and deficiencies can impair T cell activation, proliferation and lead to age-dependent tolerance and anergy (Smith-Garvin, Koretzky et al. 2009).

Immunotoxic studies indicate that exposure of DCPA to C57/B6 mice resulted in thymic atrophy, decreased thymic weight and alterations in several thymocyte populations (Barnett and Gandy 1989; Blyler, Landreth et al. 1994). Mice treated with DCPA also have enlarged spleens and decreased IL-2 and IL-6 production in
Splenocytes stimulated with Con-A, a T cell mitogen (Zhao, Schafer et al. 1998). Similar decreases in IL-2 production, as well as mRNA levels, were reported in mouse (EL-4) and human T (Jurkat) cell lines (Zhao, Schafer et al. 1999; Brundage, Schafer et al. 2004). Additional studies confirmed that human Jurkat T cells exposed to DCPA altered c-jun protein levels as well as phosphorylation levels (Brundage, Schafer et al. 2004). AP-1 is a transcription factor that is important in T cells for the expression of IL-2 and is comprised of c-jun and c-fos. Reductions in c-jun resulted in the decreased DNA binding ability of AP-1 with downstream decreases in IL-2. Transcription of IL-2 is dependent on the cooperative binding of 3 transcription factors; AP-1, NFAT and NF-kB (Jain, Loh et al. 1995). Alterations in the timing and binding of these transcription factors can have adverse effects on T cell function (Jain, Loh et al. 1995; Hogan, Chen et al. 2003).

Although immunological studies indicate that DCPA can alter T cell function, there is sparse data on the immunotoxic effects of its metabolites. In humans, DCPA is metabolized by hepatic acylamidases to produce DCA which can undergo further biotransformation to produce NOH-DCA and 6OH-DCA (McMillan, Leakey et al. 1990). Roberts et al examined DCPA self-poisoning patients and reported that the estimated half-life of DCPA in the body is 3.2 hours and that DCA was detectable and more persistent than DCPA (Roberts, Heilmair et al. 2009). No data is available on the half life of DCA in humans, but in rats, elimination of DCA is reported to occur after 3 days. In humans, DCA is detectable in both occupationally and non-occupationally exposed individual and DCA-Hb adducts are detectable in agricultural workers four months after the last application of DCPA (Pastorelli 1998; Wittke, Hajimiragha et al. 2001; Turci,
Barisano et al. 2006). This indicates that exposure to DCPA may result in an accumulation of DCA in the body long after DCPA had been metabolized. Toxicity studies on DCA are limited but have been reports that DCA can alter reproductive, endocrine, and liver functions (Valentovic, Yahia et al. 1997; Bauer, Meyer et al. 1998; Zhang and Lin 2009). Limited immunotoxicity studies indicate that DCA may be as toxic as DCPA, as molar equivalent doses of DCPA and DCA increased spleen weight and size and decreased NK cell function (Barnett, Gandy et al. 1992). In contrast, myleotoxic effects are observed in mice exposed to DCPA, but not when exposed to DCA (Malerba, Castoldi et al. 2002). In vitro studies, using a murine T cell line, resulted in decreased IL-6 production when exposed to 50µM DCPA but IL-6 was only inhibited by DCA at higher concentration (300µM) (Zhang unpublished data).

NOH-DCA and 6OH-DCA are metabolites that have been documented in their ability to form met-Hb adducts (McMillan, McRae et al. 1990). Both of these oxidative metabolites can convert oxy-Hb to met-Hb, a form of Hb that does not bind or transport oxygen. Formation of met-Hb can lead to a serious medical condition known as methemoglobinemia. NOH-DCA is also nephrotoxic and can induce hemolytic anemia (McMillan, Bradshaw et al. 1991; Valentovic, Ball et al. 2001). 6 OH-DCA is also nephrotoxic but does not induce hemolytic anemia. In addition, no immunotoxicity studies on either NOH-DCA or 6OH-DCA have been conducted.

The immunotoxic effects of DCPA and its metabolites on T cells are reported here for the first time. As a test for overall T cell function, IL-2 secretion was measured in Jurkat T cells exposed to DCPA and its metabolites. DCPA decreased IL-2
secretion, up to 78%, in a concentration dependent manner. Exposure of Jurkat T cells to DCA also decreased IL-2 secretion in a concentration dependent manner but only at higher concentrations (100µM and 200µM). A 78% decrease in IL-2 production observed with 100µM DCPA required 200µM DCA to achieve the same inhibition. This suggests that D CPA is a more potent inhibitor of IL-2 secretion. Metabolic transformation can increase the toxicity of a chemical and, in NOH-DCA treated Jurkat T cells, IL-2 levels are decreased significantly more than that of DCPA. Although 50µM NOH-DCA decreased IL-2 by 90%, 50µM DCPA only inhibited IL-2 by 50%, indicating that metabolism of DCPA produces a metabolite more toxic than its parent. In addition, NFAT activity is also inhibited by D CPA and its metabolites, although to varying degrees. Interestingly, although a concentration of 50µM NOH-DCA inhibited IL-2 secretion by 90%, NFAT activity only reduced 2 fold. In contrast, 50µM DCPA inhibited IL-2 secretion by almost 50% but NFAT activity was decreased 2.5 fold. In addition, 100µM DCPA decreased IL-2 secretion by 78% with an 8-fold decrease in NFAT activity. These data suggests that NOH-DCA and DCPA may inhibit IL-2 production through different mechanisms. To support this hypothesis, DCPA and DCA were also shown to inhibit Ca influx in a concentration-dependent manner but again, DCPA appears to be a more potent inhibitor. However, even at the highest concentrations of 6OH-DCA (50µM) and NOH-DCA (50µM) Ca influx was not altered. The mechanism by which NOH-DCA and 6OH-DCA inhibit IL-2 production is currently unknown, however, measurements of NFAT activity and IL-2 secretion require incubation times of 5hrs and 24hrs, respectively, and calcium flux was measured immediately after addition of the treatment. It is possible that NOH-DCA alters Ca
homeostasis at later time points and that may account for the decreases in NFAT and IL-2. It is also possible that NOH-DCA and 6OH-DCA alter T cell function through a different mechanism that DCPA and DCA. Further studies are required to determine the mechanism for these differential effects.

Several mechanisms could account for the Ca-dependent changes observed in Jurkat T cells exposed to DCPA and DCA. T cells express 2 plasma membrane potassium (K) channels, Kv1.3 and KCa, which are involved in regulation of Ca flux in T cells. Kv1.3 channels are voltage-gated and activated when the membrane potential is depolarized, whereas KCa channels are activated by increases in intracellular Ca. Both channels function to move K ions out of the cell to increase the driving force of Ca. Alterations that prevent the efflux of K ions reduce the Ca driving force resulting in a decrease in intracellular Ca. Using a membrane potential dye we determined that DCPA-induced decreases in Ca influx were not mediated by alterations in the membrane potential.

In T cells, CRAC channels are responsible for the Ca influx observed in activated cells. In T cells from patients with SCID syndrome, Feske et al reported that these T cells retained only 1-2% of the normal Ca influx observed in stimulated cells (Feske 2009). Using electrophysiological and molecular biological methods they determined that this defect was due solely to the absence of functional CRAC channels. 2-APB is a widely used modulator of CRAC channels. Low concentrations (<5 µM) of 2-APB enhance Ca influx through CRAC channels following store depletion without altering IP3-mediated release of Ca from the ER (Prakriya and Lewis 2001). High concentrations of 2-APB (>10µM) inhibit CRAC channels. We used 2-APB to further
understand the effects of DCPA in Ca signaling. We demonstrate that, at CRAC-enhancing concentrations of 2-APB (2.5 µM), we could inhibit Ca influx with DCPA. This combined data supports our hypothesis that DCPA alters Ca influx through inhibition of CRAC channels.

The toxicity of some chlorine compounds has been well documented and toxicity is dependent on the number, structure and location of the chlorine(s) (Naumann 2000). Vinyl chloride, 1,2 dichloroethane, polychlorinated biphenyls, DDT and heptachlor are common chlorine products with known toxicities. DCPA is a small lipophilic compound with 2 chlorines at the 3 and 4 positions on the benzene ring. Notably, although DCPA is lipophilic, it has recently been reported that it becomes localized in the cytosolic fraction of T cells and heptocytes (Hanson, Peer et al. 2010). The effect of the chlorines on its toxicity is unknown. We report that, substitution of the 2 chlorines, in DCPA and DCA, with fluorines, to produce DFPA and DFA, respectively, resulted in a complete reversal of all effects. This clearly establishes a mechanism whereby the presence of the chlorines alters T cell function. Several possibilities exist for the differential effects observed with chlorine and fluorine. First, fluorines are highly electronegative and act only as hydrogen acceptors whereas chlorine and other halogens act as both hydrogen acceptors and donors (Purser, Moore et al. 2008). This increase in electronegativity could alter the distribution of charge so that the fluorine analogs do not interact with its target in the same manner as DCPA and DCA. Second, trifluoro-substitution has been reported to increase lipophilicity but mono- or difluoro-substitution can decrease it (Purser, Moore et al. 2008). Changes in the lipophilicity may also alter interactions with the target of DCPA. Since DCPA is
targeted to the cytosol, increases in lipophilicity may prevent access into the cytosol. Lastly, fluorines are similar in size to hydrogen and may not produce the 3-D confirmation required to elicit the effects of DCPA. Further studies are required to determine the mechanism of DCPA, in particular, how the position and location of chlorines alters T cell function.

Based on the data collected we hypothesized that the inhibition of Ca influx observed in T cells exposed to DCPA was due to alterations in CRAC channel activity. Only in recent years has it become possible to assess changes in CRAC activity by methods other than electrophysiology. The discovery of two critical proteins in CRAC activation, Stim1 and Orai1, allows for a more detailed investigation into possible inhibitors of CRAC channels. Stim1 is an ER transmembrane protein that aggregates into puncta when ER stores are depleted and interacts with Orai1 proteins in the plasma membrane to activate CRAC channels. Puncta formation can be visualized in cells that express YFP-tagged Stim1 and disruption of the puncta formation results in loss of CRAC activity and Ca influx (Liou, Fivaz et al. 2007; Hewavitharana, Deng et al. 2008). In the absence of Stim1, mouse CD4+ T cells produce little Ca influx when ER stores are depleted and TCR-stimulated T cells produce significantly less IL-2 (Oh-Hora, Yamashita et al. 2008). Knockdown of Stim1 in HEK 293 cells also results in decreased Ca influx and CRAC activity (Roos, DiGregorio et al. 2005). Here we report that HEK 293 cells, treated with DCPA, failed to produce significant puncta formation upon ER store depletion. Some puncta formation does occur in the DCPA-treated cells but the puncta rich regions observed in control cells are not observed. HEK 293 cells are adherent, easy to transfect cells and have a relatively large cytosolic space to
identify puncta formation so these were chosen for our studies. Characterization of the role of Stim1 and Orai1 proteins in CRAC channel activation has been thoroughly investigated in both HEK 293 and Jurkat T cells. In both cell types, mutational and overexpression studies reported that Stim1 puncta formation, its interaction with Orai1 and activation of CRAC channels all occur in a similar manner. We extend our results in HEK 293 cells and conclude that DCPA inhibits IL-2, NFAT and Ca influx by inhibiting Stim1 puncta formation in Jurkat T cells. Localization of DCPA to the cytosolic fraction in Jurkat T cells supports the possibility that DCPA can interact directly with the cytosolic domain of Stim1 to block puncta formation. In the cytosol, the C terminus of Stim1 contains a CRAC-activating domain (CAD) that, when deleted, does not form Stim1 puncta (Park, Hoover et al. 2009). Although little is known about this domain, it is possible that DCPA could alter this interaction leading to the inhibition of Stim1 puncta. However, there are several other possibilities that may also explain these results. A reduction in the mobility of the plasma membrane hydrocarbon chains, in stimulated Jurkat T cells exposed to DCPA, has been reported and it may be possible for this to occur on the ER membrane and possibly inhibit Stim1 dimerization (Brundage, Barnett et al. 2003). Interaction with cytoskeletal proteins could also prevent Stim1 puncta formation. Some studies indicate a role for the cytoskeleton in Stim1 aggregation. Stim1 can co-localize with alpha-tubulin and disruption of microtubules with nocodazole resulted in a diffuse distribution of Stim1 (Smyth, DeHaven et al. 2007). Since DCPA inhibits Stim1 puncta formation, it seems unlikely that DCPA directly alters Orai1 as aggregation of Stim1 is independent of Orai1 clustering (Xu, Lu et al. 2006). In fact, Stim1 puncta formation has been shown to
occur before Orai1 aggregation. Further studies are required to determine the exact nature of DCPA inhibition of Stim1.

In conclusion, exposure of Jurkat T cells to DCPA and its metabolites alters T cell function through inhibition of IL-2 and NFAT. The parent compound, DCPA, and its metabolite, DCA, both inhibit T cell function by decreasing IL-2 secretion in a NFAT and Ca-dependent manner. D CPA appears to be a more potent inhibitor of IL-2 secretion as greater decreases in IL-2 are observed at lower concentrations of DCPA than that of DCA. N OH-DCA almost eliminated IL-2 production but in a Ca-independent manner. The positioning and presence of chlorines on DCPA and DCA are critical in eliciting these toxic effects. Finally, DCPA appears to be a novel inhibitor of CRAC channels and its use as such may have widespread implications for immunotherapy.
REFERENCES


